Lipolytic Activity of Ribonucleotide and Deoxyribonucleotide-3′,5′-Cyclic Monophosphates in Isolated Rat Fat Cells (34186)

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Evidence has accumulated during the past few years that 3′5′-AMP[1] serves as a critical intracellular signal mediating the action of a large number of hormones (1, 2). One of the basic lines of evidence in support of this concept is the demonstration that effects of these hormones in target tissues can be mimicked by exogenous 3′5′-AMP (or its acylated derivatives). For the most part, however, the specificity of these hormone-like effects of 3′5′-AMP have not been adequately evaluated. For example, cyclic nucleotide monophosphates with bases other than adenine, but with similar charge, have not been systematically examined.

In order to investigate the specificity of 3′,5′-AMP action to mimic hormone responses, we have selected the isolated rat fat cell preparation. In this cell type, where 3′,5′-AMP serves as a coupling intermediary in the action of lipolytic hormones, the cyclic nucleotide generated by these hormones, appears to activate a hormone-sensitive triglyceride lipase (1, 2). The lipolytic activity of 3′,5′-AMP in adipose tissue and isolated fat cells was first clearly established using the dibutyryl derivative; 3′,5′-AMP was ineffective when tested upon fat cells or adipose tissue incubated in Krebs–Ringer phosphate or bicarbonate buffer. Mosinger and Vaughan (3) showed that 3′,5′-AMP readily stimulates lipolysis, if fat cells are studied in an incomplete Krebs–Ringer phosphate buffer, where divalent cations (Ca^{2+} and Mg^{2+}) are omitted. Removal of Ca^{2+} and Mg^{2+} (and in a lesser extent, K^+) from the incubation medium apparently modified fat cells so that sufficient amounts of 3′,5′-AMP penetrate into the cells to stimulate lipolysis.

In the present study, we have compared the lipolytic activity of a series of ribonucleotide and deoxyribonucleotide-3′,5′-cyclic monophosphates in fat cells. Information concerning the specificity and the structural requirements for lipolytic activity of this nucleotide series is presented in this paper. This study has additional significance in view of suggestions that cyclic nucleotides, other than 3′,5′-AMP, may also serve as intracellular coupling signals, in the action of certain hormones (2, 4).

Materials and Methods. Sprague-Dawley rats weighing 110–160 g, fed a standard Purina laboratory chow diet *ad libitum* and maintained at 22 ± 0.5° were used. Fat cells were prepared incubating rat epididymal fat pads (5) with collagenase in a saline–phosphate medium containing 140 m*M* of NaCl, 9.1 m*M* of Na_2HPO_4, 50 mg/100 ml of glucose and 2.5% BSA pH 7.4. After tissue disintegration, the cells were washed five times with saline–phosphate medium containing 1% BSA.

Lipolysis was determined by measuring the glycerol formed in fat cells incubated for 90 min. In each experiment, aliquots from a

[1] Abbreviations: 3′,5′-AMP = adenosine-3′,5′-cyclic monophosphate; 3′,5′-GMP = guanosine-3′,5′-cyclic monophosphate; 3′,5′-IMP = inosine-3′,5′-cyclic monophosphate; 3′,5′-UMP = uridin-3′,5′ cyclic monophosphate; 3′,5′-CMP = cytosine-3′,5′-cyclic monophosphate; 3′,5′-TMP = thymidine-3′,5′-cyclic monophosphate; 3′,5′-dAMP = deoxyadenosine-3′,5′-cyclic monophosphate; 3′,5′-dGMP = deoxyguanosine-3′,5′-cyclic monophosphate; 3′,5′-dUMP = deoxyuridine-3′,5′-cyclic monophosphate; DB-3′,5′-AMP = N^6,$O^{2'}$-dibutyryl-adenosine-3′,5′-cyclic monophosphate; DB-3′,5′-GMP = N^2,$O^{2'}$-dibutyryl-guanosine-3′,5′-cyclic monophosphate; BSA = bovine serum albumin; EDTA = ethylendiaminetetraacetate; MED = minimal effective dose.

single pool of fat cells were incubated in plastic vials in a total volume of 1 ml of saline–phosphate medium containing 2.5% BSA in a Dubnoff shaking water bath at a temperature of 37°. Incubations were stopped by the addition of 1 ml of 8% tricholoroacetic acid. Glycerol was determined enzymatically according to Wieland (6). The results were calculated per gram of fat cell total lipids.

The 3′, 5′-AMP hydrolysis was assayed as follows: Phosphodiesterase was prepared by homogenizing isolated fat cells in 0.25 *M* sucrose, containing 1 mM EDTA, according to Weiss *et al.* (7). The assay components (in a final volume of 0.05 ml) consisted of Tris–HCl buffer (25 m*M*, pH 7.5), $MgSO_4$ (2 m*M*), EDTA (0.1 mM), 3′,5′-AMP-8-^{3}H (0.5 m*M*; 2 × 10_4 cpm), enzyme fraction (0.25 mg of protein/ml) and cyclic nucleotides (1 mM). Incubations were carried out for 60 min at a temperature of 37°. Hydrolysis of 3′,5′-AMP-8-$_3$H was evaluated by paper chromatography, using ethanol: 1 *M* ammonium acetate (7:3, v/v) as solvent system to separate 3′,5′-AMP from 5′-AMP and adenosine plus inosine applied as carriers. The spots were visualized by UV light, cut out, placed into scintillation vials and counted in a nuclear Chicago liquid scintillation counter.

Chemicals and Nucleotides. The BSA (Armour, Fraction V) was purified by charcoal treatment according to Chen (8), glycerol-1-phosphate dehydrogenase and glycerokinase were obtained from Boehringer. The 3′,5′-AMP was obtained from Sigma and DB-3′,5′-AMP from Boehringer. The 3′,5′-CMP 3′,5′-GMP 3′,5′-dAMP and 3′,-5′-dUMP were synthesized according to the method of Smith *et al.* (9). Samples of 3′,-5′-IMP 3′,5′-UMP, 3′,5′-TMP, 3′,5′-dGMP and DB-3′,5′-GMP were generously supplied by Drs. M. Nelbock and G. Weimann, Boehringer-Mannheim, Tutzig, Germany. The nucleotides studied were homogenous upon chromatography in two solvent systems: (isopropanol:ammonia:H_2O, 7:1:2 (v/v) and isobutyric acid:ammonia:H_2O, 66:1:33, v/v).

Results. In fat cells isolated and incubated in a medium from which Ca^{2+}, Mg^{2+}, and K^+ was omitted, the lipolytic activity of 3′,5′-AMP and its dibutyryl derivative was qualitatively and quantitatively very similar. Figure 1 (curves A and B) shows a typical experiment which illustrates that dose response curves obtained with 3′,5′-AMP and DB-3′,5′-AMP are approximately the same. A somewhat greater maximal stimulation, by about 12%, is obtained with DB-3′,5′-AMP relative to 3′,5′-AMP. Contrary to the report of Blecher *et al.* (10), the lipolytic response to 3′,5′-AMP has been found qualitatively consistent. Figure 2 illustrates the results of four separate experiments in which the lipolytic effect of 3′,5′-AMP was dose-dependent, although the magnitude of response was variable.

Figure 3 shows the relative lipolytic potencies of cyclic nucleotides of the ribose and deoxyribose series quantitated relative to 3′,-5′-AMP as a standard. In each experiment, aliquots of fat cells were incubated with increasing equimolar concentrations of two nucleotides and with 3′,5′-AMP; the maximum response produced by 3′,5′-AMP, taken as

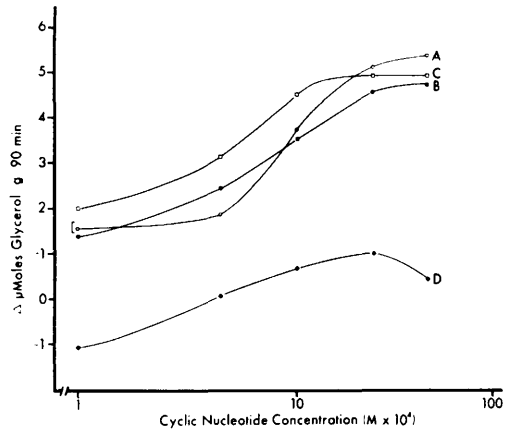

FIG. 1. Expt. I: Lipolytic effect of DB-3′,5′-AMP and 3′,5′-AMP (curves A and B) on isolated fat cells. Expt. II: Lipolytic effect of DB-3′,5′-AMP and DB-3′,5′-GMP (curves C and D). Results are expressed as net increase of glycerol over control during 90-min incubation. Glycerol production in the absence of nucleotide which was 3.05 ± 0.06 (Expt. I) and 2.05 ± 0.12 μmoles/g (Expt. II) has been subtracted. For Figs 1–3: each point represents the mean value of 3–4 incubations; vertical bars indicate ± SE of mean (not plotted where range has been less than 10% of the mean).

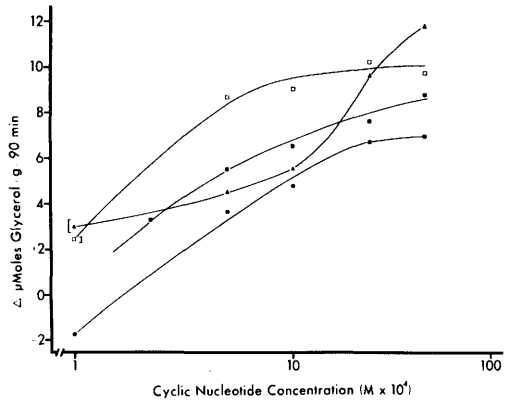

FIG. 2. Reproducibility of the lipolytic effect of 3′,5′-AMP. The results of four separate experiments are illustrated.

100%, was the standard reference point. As shown, 3′,5′-AMP is the most potent nucleotide in terms of the magnitude of lipolytic response and with respect to MED. Although the other ribonucleotide–3′,5′-cyclic monophosphates also stimulate lipolysis, the lipolytic effects produced by saturating concentrations are considerably less than with 3′,5′-AMP and the MED's are higher. The relative lipolytic potencies of the ribonucleotide–3′,5′-cyclic monophosphate series is in the order: 3′,5′-AMP>>3′,5′-UMP>3′,5′-IMP~3′,5′-CMP>3′,5′-GMP. In the deoxyribonucleotide series, only 3′,5′-dAMP possesses lipolytic activity (relative potency about 35% of 3′,5′-AMP), 3′,5′-TMP is essentially inactive; 3′,5′-dUMP and 3′,5′-dGMP, if anything, reduced the formation of glycerol below the basal level.

Having established that ribonucleotide–3′,5′-cyclic monophosphates other than 3′,5′-AMP possess lipolytic activity, the possibility arose whether differences in lipolytic potency may be related to differences in penetration, and/ or to differences in resistence to enzymatic degradation. To examine this possibility, the lipolytic activity of DB-3′,5′-GMP, which is liposoluble and, perhaps, more resistant to phosphodiesterase, was compared with that of DB-3′,5′-AMP. Figure 1 (curves C and D) shows that the lipolytic activity of DB-3′,5′-GMP is considerably lower than that of the DB-3′,5′-AMP; the maximal effect is about 20% of that produced by DB-3′,5′-AMP. Whether 3′,5′-AMP and 3′,5′-GMP are compared or their dibutyryl derivatives, a similar differential in potency is observed.

It is also unlikely that the lipolytic effects of ribonucleotide–3′,5′-cyclic monophosphates and 3′,5′-dAMP are due to an increase of 3′,5′-AMP levels, by interfering with the endogenous hydrolysis of 3′,5′-AMP. The results illustrated in Table I show that there is no straightforward relationship between the lipolytic potency of a cyclic nucleotide and its ability to alter the rate of 3′,5′-AMP hydrolysis. Thus, 3′,5′-TMP, 3′,5′-dUMP and 3′,5′-dGMP which do not possess lipolytic activity decrease the rate of 3′,5′-AMP breakdown as effectively as 3′,5′-CMP and 3′,5′-GMP which stimulate lipolysis.

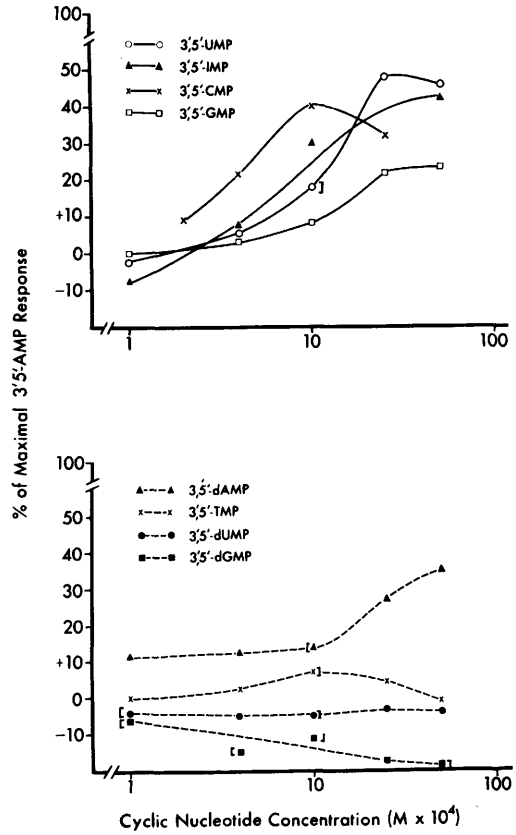

FIG. 3. Relative lipolytic potencies of ribonucleotide and deoxyribonucleotide–3′,5′-cyclic monophosphates as compared to 3′,5′-AMP the maximum response to 3′,5′-AMP was taken as 100%.

TABLE I. Effect of Cyclic Nucleotides on 3′,5′-AMP-8-^{3}H Hydrolysis.

Additions	3′,5′-AMP hydrolysis (nmoles/mg of protein/min)	Relative rates (%)
—	17.6[a]	100
3′,5′-GMP	15.8	90
3′,5′-IMP	13.0	73
3′,5′-CMP	15.8	90
3′,5′-TMP	15.5	88
3′,5′-dAMP	13.2	74
3′,5′-dUMP	15.8	90

[a] Mean value of two closely agreeing assays.

Discussion. The present results show that all of the ribonucleotide–3′,5′-cyclic monophosphates tested stimulate lipolysis in isolated fat cells. In the deoxyribonucleotide series, only 3′,5′-dAMP was lipolytically active, 3′,5′-dUMP, 3′,5′-dGMP and 3′,5′-TMP being inactive. Accordingly, the *in vitro* lipolytic effect of exogenous 3′,5′-AMP is not specific. Despite qualitative similarity, there are marked quantitative differences between 3′,5′-AMP and all other cyclic nucleotides in terms of both magnitude of lipolytic response and MED, which differ by one order of magnitude.

The results obtained serve to illustrate the importance of structural features of 3′,5′-AMP for lipolytic activity. Substitution of the adenine moiety by the purines, guanine and hypoxanthine, or the pyrimidines, cytosine and uracil, is uniformly associated with a considerable diminution of lipolytic activity. The difference in activity between pairs of ribo- and deoxyribonucleotides demonstrate the importance of the 2′-hydroxyl group of ribose moiety for lipolytic activity. In connection with this 2′-hydroxyl position it is pertinent to recall the observations of Posternak *et al.* (11) that the activity of derivatives of 3′,5′-AMP acylated at N^6- and $O^{2'}$-positions, is largely dependent upon the presence of cellular enzymes which remove the substituent acyl groups.

The present investigation on one aspect of fat cell metabolism does not answer the question whether cyclic nucleotides other than 3′,5′-AMP serve a physiological role as intermediary coupling signals. The possibility that these cyclic nucleotides may exhibit significant biological activity in other aspects of fat cell metabolism, or in other target tissues and cells, merits investigation.

Summary. The lipolytic activity of a series of ribonucleotide and deoxyribonucleotide–3′,5′-cyclic monophosphates was investigated in fat cells incubated in a saline–phosphate medium, from which Ca^{2+}, Mg^{2+}, and K^+ was omitted. It was found that the effect of 3′,5′-AMP to stimulate lipolysis is not specific; 3′,5′-UMP, 3′,5′-IMP, 3′,5′-CMP, 3′,5′-GMP, and 3′,5′-dAMP also exhibit lipolytic activity. Quantitatively, however, the lipolytic potency of 3′,5′-AMP is considerably greater than all of the other nucleotides tested. The results indicate the importance of the nucleotide base and the 2′-hydroxyl group of the ribose moiety for lipolytic activity.

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