Effect of Antilipolytic Compounds on Cyclic 3′, 5′-Adenosine Monophosphate Activation of Adipose Tissue Lipolysis

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SUMMARY

The lipolytic effect of cyclic 3′,5′-adenosine monophosphate (cAMP) is inhibited by the beta-adrenergic blocker propranolol. This is demonstrated on both isolated fat cells and intact epididymal fat pad preparations. A cell-free extract of either rat or human adipose tissue contains a lipase system which can be activated by cAMP. This activation is also prevented by propranolol as well as the alpha-adrenergic blocker, phentolamine, in a dose-related fashion. Because of this nonspecific action, pharmacologic differentiation of alpha- or beta-adrenergic receptor sites should be based upon direct measurements of cyclic nucleotide, adenyl cyclase or phosphodiesterase, and not target enzyme activity. DIABETES *20*:146-50, March, 1971.

Adipose tissue lipolysis is mediated by cyclic 3′, 5′-adenosine monophosphate (cAMP).[1] A variety of antilipolytic agents have been presumed to inhibit lipolysis by lowering intracellular cAMP, either by decreasing the adenyl cyclase or by increasing phosphodiesterase activity. The beta-adrenergic blocker, propranolol, is known to prevent the stimulation of adenyl cyclase[2] by isoproterenol and has, therefore, been presumed to inhibit lipolysis by simply preventing a rise in adipocyte cAMP. Recent evidence suggests, however, that antilipolytic agents may also act at a more direct site, i.e., activation of tissue lipase by cAMP.[3,4] Our work was designed to investigate the nature of this latter effect.

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This work was presented in part at the Sixty-first Annual Meeting of the American Society for Clinical Investigation.[11]

MATERIALS AND METHODS

Isolated Fat Cells: Epididymal fat pads were obtained from unanesthetized Charles River rats weighing between 150 and 230 gm. The animals were fed laboratory chow until sixteen hours prior to sacrifice by decapitation. A modification of the Rodbell method[5] was used for isolated adipocyte preparations. The distal two-thirds of each pad was digested for one hour at 37° C. by crude Worthington collagenase (Lot No. CLS 6596-6), 2 mg./ml. in 3 gm. per cent Armour albumin (Lot No. D 27712) in Krebs-Ringers phosphate buffer containing one half the recommended Ca^{2+} concentration. The isolated cells were then washed carefully at least three times with warm buffer. No proteolytic activity was detectable in the final washed cell suspension.[6] All incubations were performed in glucose-free buffer with 3 per cent albumin for three hours in a 37° C. metabolic shaker with oscillations of seventy-five cycles per minute. Quantitation of lipid content was done gravimetrically. Dispensing of fat cell suspensions was reproducible to 95 per cent accuracy.

Intact Epididymal Fat Pads: Paired fat pads from a single animal were rinsed with buffer, blotted dry, divided into four approximately equal portions and weighed before incubation. The incubation medium was identical to that used for the isolated cell experiments. Free fatty acids (FFA) were determined[7] on aliquots of incubation medium at 0 and 3 hours. Results are expressed as μEq free fatty acids released per gram wet weight in three hours.

Adipose Tissue Extracts: Fat pads were pooled from several fed rats. Approximately 10 gm. of wet tissues were placed in 20 ml. distilled water and disrupted by sonication in an ice bath. Large connective tissue fragments were then discarded and the remaining emulsion was

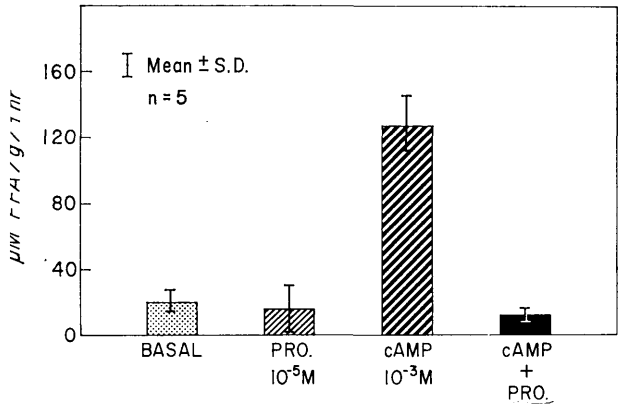


FIG. 1. Effect of cyclic 3', 5'-adenosine monophosphate (cAMP) and propranolol (PRO) alone or in combination, on free fatty acid (FFA) release from isolated rat adipocytes. Activity is expressed as μMoles FFA released per gram triglyceride per hour.

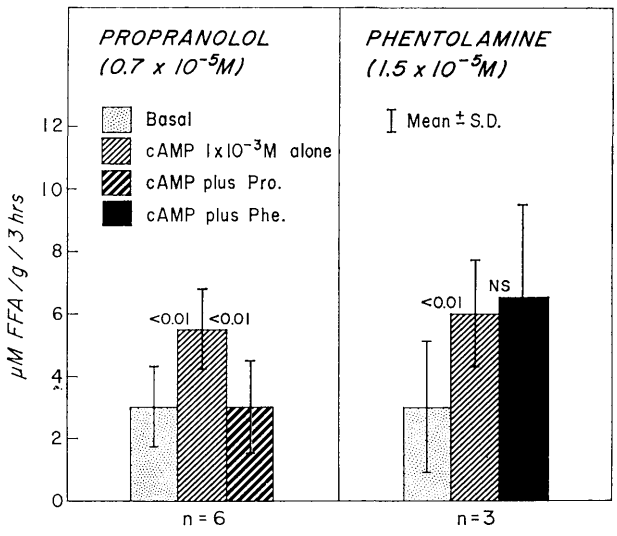


FIG. 2. Effect of propranolol or phentolamine on cAMP - activated lipolysis of intact rat epididymal fat pads. Activity is expressed as μMoles FFA per gram wet tissue per three hours.

centrifuged at 40,000 rpm for one hour in a Model L Spinco ultracentrifuge using a No. 40 fixed angle rotor. The clear. infranatant separating the bottom pellet from the upper fat cake layer was then passed through Sephadex-25 gel. The rapidly-moving protein fraction was saved for lipase assay. Protein content was determined by the method of Lowry.[8] In separate experiments, C-14-cyclic 3', 5'-adenosine monophosphate was preincubated with the tissue extract before Sephadex chromatography. Results of the radioisotope recovery are shown in figure 4.

The substrate employed for this lipase assay consisted of a commercial coconut oil emulsion (Ediol-Calbiochem)* containing additives of glyceryl monostearate and Tween 60, both of which are hydrolyzed by monoglyceridase.[9] Fatty acid-poor albumin (Pentex) in Krebs-Ringers phosphate buffer at pH 7.40 in a concentration of 10 gm. per cent was used as a fatty acid-acceptor. The final incubation volume was 3.2 ml. with 1 ml. Ediol, 2 ml. albumin-buffer, 0.1 ml. of aqueous tissue extract and 0.1 ml. additions. One ml. aliquots were removed at 0, 1 and 2 hours for free fatty acid determinations. The reaction rate was linear for two hours.

In a separate experiment, cyclic 3', 5'-adenosine monophosphate (cAMP)† was preincubated in albumin-buffer with propranolol at 37° C. for one hour in the absence of tissue extract. The mixture was then chromatographed by the descending paper technic in albumin-barbital buffer at pH 8.0. Similar chromatograms were prepared with cAMP and propranolol* alone. Results are shown in figure 3. Propranolol was identified by I_2 vapor staining and cAMP by fluorescence under ultraviolet light.

Chemicals: All reagents and hormone solutions were prepared fresh daily.

RESULTS

Propranolol (10^{-5} M) had no significant effect on basal lipolysis of isolated fat cells. The addition of 1 mM cyclic 3', 5'-adenosine monophosphate (cAMP) produced a marked stimulation of FFA release which could be completely prevented by propranolol (figure 1).

To determine whether this effect was peculiar to collagenase-treated fat cells, the same agents were tested with intact epididymal fat pads. Again, the cAMP-stimulation of lipolysis was blocked by propranolol (figure 2). Phentolamine† in a concentration of .015 mM had no significant effect. Theophylline‡ 1 mM, a known inhibitor of adipose tissue phosphodiesterase[2] showed the expected stimulation of lipolysis by intact fat pads. Propranolol inhibited this response to theophylline (table 1).

To rule out the possibility that propranolol was

*Ediol (Lipostrate-CB) A grade, Calbiochem—Lot 71596.

†Cyclic 3',5'-adenosine monophosphate A grade—Calbiochem—Lot 60046.

*Propranolol—powder form, Ayerst—Lot 64043.

†Phentolamine (Regitine HCl)—Ciba—Lot 74876.

‡Theophylline—USP, Nutritional Biochemicals Corporation—Lot 9403.

TABLE 1

Intact epididymal fat pad lipolysis
(μMoles FFA/gm. wet weight/3 hrs.)

Basal	Theophylline 10^{-4} M	Theophylline and Propranolol 10^{-5} M
0.23	4.76	0.58
0.36	3.76	0.55
0.52	4.50	0.40

chemically altering or binding the cAMP independent of tissue proteins, the two compounds were mixed in buffer alone for 30 min. at 37° C. Paper chromatography of this mixture revealed no difference in R_f value for the cAMP incubated with or without propranolol (figure 3).

The possibility that propranolol was in some other way preventing the cyclic nucleotide from entering the adipocyte was eliminated by examining lipolytic effects of cAMP on a cell-free extract of adipose tissue. Because workers have demonstrated an "activation" of lipase by simple mechanical disruption of adipose tissue, presumably through an increased availability of cAMP,[10] we removed free cAMP by Sephadex gel chromatography (figure 4). The rapidly eluting protein fraction contained most of the lipase activity, and no broken cell fragments could be seen by light microscopy. Cyclic 3′, 5′-adenosine monophosphate increased the lipolysis of an artificial substrate by over twofold at a picomolar concentration. However, in other still preliminary experiments, this effect proved to be biphasic with actual inhibition at higher concentrations. There was also a potentiating effect from adenosine triphosphate.[10,11] This activation by cAMP of a lipase extract could be completely abolished by propranolol, and, unlike the findings in intact systems, also by phentolamine (figure 5). In a separate experiment utilizing a similarly extracted lipase fraction from human subcutaneous adipose tissue, this inhibition by propranolol or phentolamine was shown to be dose-related (table

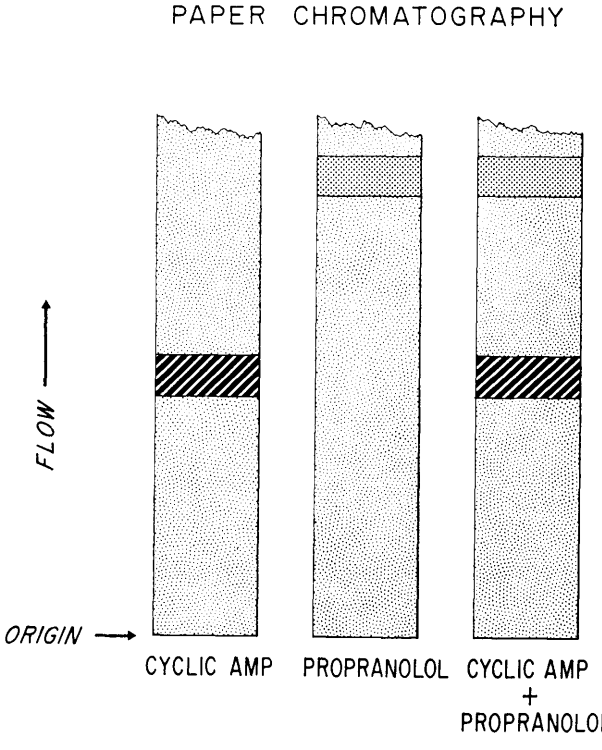


FIG. 3. Hydrodynamic flow paper chromatography of cyclic 3', 5'-adenosine monophosphate and propranolol, alone or in combination.

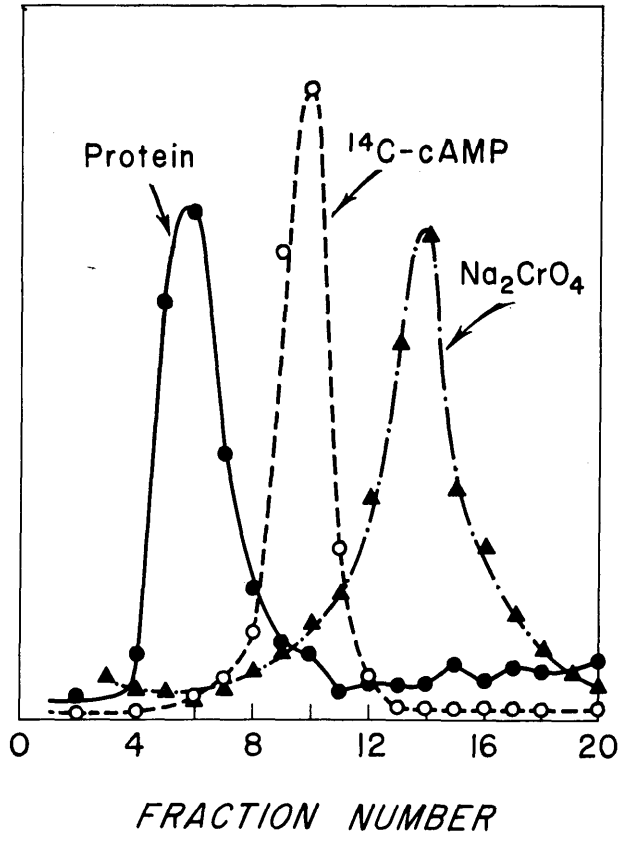
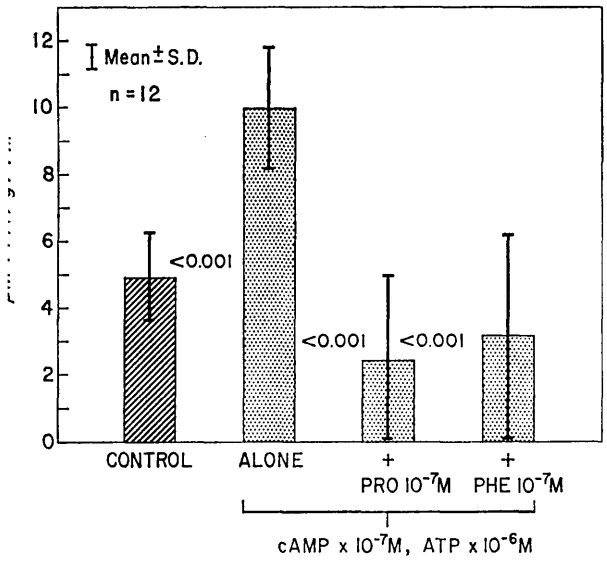


FIG. 4. Sephadex-25 gel chromatography of aqueous adipose tissue extract. The solid circles represent protein fractions corresponding to elution of a blue dextran 2000 marker. Open circles represent the recovery of isotopically-labelled cyclic 3', 5'-adenosine monophosphate. Closed triangles indicate the sodium chromate peak corresponding to slow-moving low molecular weight compounds.



IG. 5. Adipose tissue lipase extract activity against an artificial substrate. Enzyme activity is expressed as μMoles FFA produced per gram protein per hour. Cyclic 3', 5'-adenosine monophosphate plus adenosine triphosphate significantly increased activity in this cell-free system. Propranolol (PRO) or phentolamine (PHE) prevented this activation. The p values compare differences between cAMP alone and control, and between cAMP alone and cAMP plus propranolol or phentolamine.

). Also given in this table is the lack of effect from ATP plus isoproterenol, the respective substrate and timulator of membrane-bound adenyl cyclase. This data s taken as biochemical evidence in support of the microcopic observations that adipocyte membranes are not resent in the lipase extract.

When cAMP was preincubated for sixty minutes vith the lipase extract, propranolol still completely inibited the activation.

DISCUSSION

Several laboratories have independently observed an nhibition of cAMP or dibutyryl cAMP effects by a ariety of compounds. Ouabain,[12] dihydroergotamine,[13] nsulin,[14] sodium salicylate,[15] N-ethyl maleimide,[16] oxiative phosphorylation inhibitors,[17] pronethalol,[3] phenolamine[4] and KO 592, a beta-adrenolytic agent, have een shown to prevent cyclic nucleotide-activated lipolsis in adipose tissue. Chlorpropamide inhibits cAMPediated permeability changes in toad bladder.[18] Insuin antagonizes the dibutyryl cAMP-inhibited glucose ptake and glycogen synthesis in rat diaphragm.[19] Dihydroergotamine blocks cAMP-activated glycogenolysis n isolated perfused rat liver[20] and MJ 1999, a betadrenergic blocker, eliminates the augmented insulin

TABLE 2

Activation of human adipose tissue lipase extract

Additions		μM FFA/ gm./hr.
	n = 3	
Basal		1.2
cAMP 10^{-7} M and ATP 10^{-5} M		7.9
cAMP and ATP and Propranolol —	8 x 10^{-9} M	6.1
	8 x 10^{-8} M	2.8
	8 x 10^{-7} M	0.0
cAMP and ATP and Phentolamine —	12 x 10^{-9} M	2.9
	12 x 10^{-8} M	1.4
	12 x 10^{-7} M	0.0
	n = 2	
None		7.7
cAMP 10^{-7} M		12.2
ATP 10^{-5} M + Isoproterenol 1 μg./ml.		8.0

secretion after intravenous dibutyryl cAMP administration in mice.[21]

Our findings with human and rat adipose tissue lipolysis suggest that this type of inhibition is independent of membranes and is not a simple binding of cyclic nucleotide. In the case of propranolol, it can still be seen after preincubation of cAMP with the lipase. Other studies performed in our laboratory[11] have identified a monoglyceride system in cell-free extracts of adipose tissue which is activated by cAMP. This cAMP-activation of monoglyceridase can be prevented by several compounds including propranolol, phentolamine, nicotinic acid and prostaglandin E_1. This effect is, therefore, nonspecific for a variety of antilipolytic agents.

The diversity of enzyme systems activated by cAMP[1] suggests a common factor which mediates the effect. Krebs et al.[22,23] have elucidated a cAMP-dependent protein kinase from skeletal and cardiac muscle which is instrumental in phosphorylase activation. A similar protein kinase has also been found in brain tissue which can phosphorylate histones.[24] Such a protein kinase would be a candidate for this common factor which mediates cAMP effects on a wide variety of target enzyme systems. This hypothesis would help explain the direct inhibition of cAMP-activated lipase by antilipolytic agents having alpha- or beta-adrenolytic properties. Such agents may share a similar inhibition of cAMP-dependent protein kinase while affecting actual intracellular cAMP levels in different fashions. The observation that propranolol may inhibit the action of epinephrine but not ACTH or growth hormone on adipose tissue lipolysis[25] may be explained by dose-related fac-

tors in these intact preparations. The beta-blocker may affect membrane-bound adenyl cyclase at low concentrations but also act on the cAMP-activation of the soluble inactive lipase system at higher concentrations. Paton[26] has recently discussed the adrenergic receptor sites and emphasized their obscure chemical nature. He questions whether the beta-adrenergic receptor is always associated with the cell membrane or at an intracellular locus and points out that in the case of piperoxan antagonism of noradrenaline on isolated rabbit stomach strips, the former may be acting at several sites including the metabolizing enzymes. Because of these considerations, caution must be taken before designating a drug as an alpha- or beta-blocker on the basis of target enzyme action. In the case of adipose tissue, adrenergic activity should be investigated by measuring adenyl cyclase, phosphodiesterase and intracellular cAMP levels and not solely by lipolytic effects.

ACKNOWLEDGMENT

This study was supported by Research Grant AM-11176 and Training Grant 2-A-5060 from the National Institute of Arthritis and Metabolic Diseases, USPHS, and Grant FR-76 from the Division of Research Facilities and Resources.

The excellent technical assistance of Mrs. Millard Alexander and Miss Sandra Carlson is gratefully acknowledged.

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