α,β-METHYLENE-ADENOSINE-5'-TRIPHOSPHATE - EFFECTS OF A COMPETITIVE INHIBITOR OF ADENYLATE CYCLASE ON CYCLIC AMP ACCUMULATION AND LIPOLYSIS IN ISOLATED FAT CELLS

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Received December 27, 1972

SUMMARY. The rapid, transient rise in the intracellular concentration of cyclic AMP which follows addition of L-epinephrine to isolated fat cells is completely prevented by an ATP analog, α,β-methylene-adenosine-5'-triphosphate [Ap(CH_2)pp], a competitive inhibitor of adenylate cyclase activity in liver and fat cell membrane preparations. The concentration of cyclic AMP falls distinctly below that in the basal state after incubating fat cells for seven minutes in the presence of Ap(CH_2)pp. The results are consistent with the view that the ATP analog is also an effective in vivo inhibitor of adenylate cyclase activity, and that intracellular cyclic AMP levels are normally delicately balanced by very rapid processes of synthesis and degradation. Epinephrine-induced lipolysis in fat cells is not inhibited but is instead enhanced by Ap(CH_2)pp. This is probably explained by the ability of the analog to act (like ATP) as a high-energy phosphate donor, an effect which is independent of its inhibition of adenylate cyclase activity. The predominant effect of this compound on glucose oxidation by fat cells also appears to be the result of this property since its effects are mimicked by ATP.

The phosphonic acid analog of ATP, α,β-methylene-adenosine-5'-triphosphate [Ap(CH_2)pp], inhibits adenylate cyclase activity in isolated liver and fat cell membranes (1). The inhibition by Ap(CH_2)pp is competitive with respect to ATP and is independent of the manner by or extent to which the enzyme is activated.

Addition of epinephrine to isolated fat cells results in a very rapid but transient rise in the intracellular content of cyclic AMP (2-5). In the present studies the effect of Ap(CH_2)pp on the basal as well as the epinephrine-stimulated changes in the levels of cyclic AMP in fat cells are examined. Furthermore, the effects of the analog on lipolytic processes, which are generally accepted as being mediated by cyclic AMP, are also measured.

The availability of an effective inhibitor of adenylate cyclase suggested a means of testing the possibility (2, 3, 6-8) that many if not all of the metabolic effects of insulin may be mediated by inhibition of adenylate cyclase

activity. The effects of ATP analogs on glucose oxidation are therefore also presented.

MATERIALS AND METHODS. α,β-Methylene-adenosine-5'-triphosphate [Ap(CH_2)pp] and β,γ-methylene-adenosine-5'-triphosphate [App(CH_2)p] were obtained from P-L Biochemicals. Ap(CH_2)pp was more than 95% pure by thin layer chromatography (isobutyric acid:H_2O:NH_4OH, 66:33:1).

Fat cells (9) were prepared from Sprague-Dawley rats (80 to 140 g). The cells were incubated at 37° in 2 ml of Krebs-Ringer-bicarbonate buffer (KRB), 1% (w/v) albumin, 1 mM of theophylline. One ml of buffer was added per 0.4 gm of original fat pad. The reaction was initiated by the addition of L-epinephrine (5×10^{-7} to 10^{-5} M) and 5 mM Ap(CH_2)pp and terminated by cooling in ice. [^{3}H]-Cyclic AMP was added (24 Ci per mmole; 50,000 cpm per sample) to determine the recovery of cyclic AMP. The cells were sonicated for 3 seconds and the supernatant proteins were precipitated with cold 5% trichloroacetic acid after removal of the fat layer by low speed centrifugation. The supernatant was extracted with ether and neutralized with Tris base after bubbling nitrogen through the sample. Saturated barium hydroxide (0.2 ml) and 7.7% zinc sulfate (0.2 ml) were added to the 3.5-ml sample (10). The recovery of [^{3}H]-cyclic AMP was about 40%.

The cyclic AMP content was determined by an isotope dilution assay (10, 11). The incubation medium consisted of 0.1 ml of 0.05 M sodium phosphate buffer, pH 6.5, containing 300 μg protein kinase (12), 5 picomoles of [^{3}H]-cyclic AMP (24 Ci per mmole), and 20 μl of the diluted cell extract or of the buffer containing cyclic AMP. After incubating for 30 minutes at 30°C the samples were diluted with 2 ml of ice-cold 0.05 M sodium phosphate buffer, pH 6.5. The samples were filtered on Millipore membranes (HAWP 2500) and washed with 10 ml of ice-cold buffer. The filters were incubated for 30 minutes in 1 ml of 10% sodium dodecyl sulfate before counting. The cyclic AMP content was calculated according to the [^{3}H]-cyclic AMP bound in the presence of known amounts of unlabeled cyclic AMP. Ap(CH_2)pp in the incubation medium did not affect the binding of cyclic AMP to protein kinase.

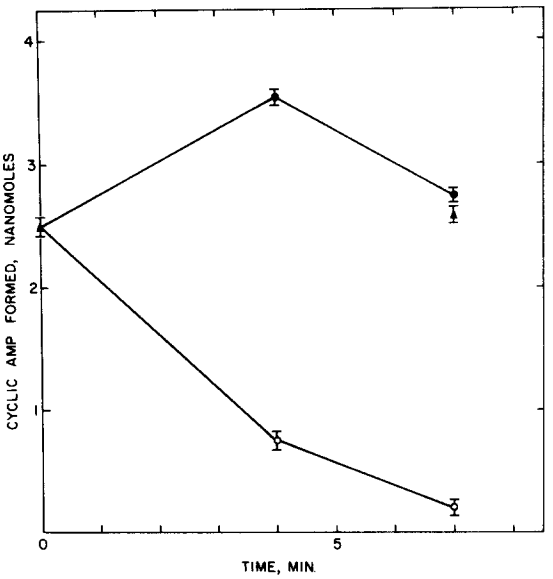

Figure 1 - Effect of $Ap(CH_2)pp$ on cyclic AMP levels of isolated fat cells. Rat adipocytes were suspended in KRB buffer, 1% (w/v) albumin (1 ml per 0.4 g of original fat pad). Samples (2 ml) of rat adipocytes were incubated in the absence (●) and presence (o) of 5 mM $Ap(CH_2)pp$ at 37° C in KRB buffer, 1% (w/v) albumin, 1.0 μM L-epinephrine. The reaction was started by the simultaneous addition of epinephrine and inhibitor and terminated by cooling to 4°. The cyclic AMP content of the samples containing no inhibitor or epinephrine are also indicated for the zero and 7 minute measurements (▲). The data is expressed as nmoles of cyclic AMP per sample. Samples containing 10^{-7} M cyclic AMP in KRB buffer containing 1% (w/v) albumin and 1 mM theophylline in the presence and absence of 5 mM $Ap(CH_2)pp$ were subjected to the purification procedure used for the extraction of cyclic AMP from fat cells. No significant differences in the apparent cyclic AMP content could be detected in these samples.

Adipose tissue protein kinase was prepared by homogenizing 800 g of sheep omental fat with a PT30 Polytron (Brinkmann) after addition of 800 ml of 0.05 M sodium phosphate buffer, pH 6.5. The congealed fat was removed, the suspension was centrifuged (40,000 x g, 30 minutes), and the protein was precipitated at 4° with $(NH_4)_2SO_4$ (60%). The pellet (50,000 x g, 30 minutes) was dissolved in 30 ml of 0.05 M sodium phosphate buffer, pH 6.5, and dialyzed at 4° for 48 hours. Insoluble material was removed by centrifugation (40,000 x g, 30 minutes).

RESULTS. Effects on intracellular content of cyclic AMP - In agreement with earlier reports (2-4) exposure of fat cells to epinephrine results in a rapid but moderate increase in cell cyclic AMP which then falls nearly to basal values

TABLE I. Effect of $Ap(CH_2)pp$, $App(CH_2)p$ and ATP on basal and epinephrine-stimulated lipolysis in isolated fat cells

Isolated fat cells were incubated for 2 hours at 37° in 1 ml of KRB buffer, 3% (w/v) albumin, and, as indicated, L-epinephrine (0.1 μg per ml), $Ap(CH_2)pp$, and ATP. Glycerol was determined by the method of Ryley (14). The β-γ-CH_2-ATP analog, $App(CH_2)p$, in concentrations ranging from 0.1 mM to 2 mM do not significantly alter the basal rate of lipolysis; slight inhibition of epinephrine-stimulated lipolysis is observed with this compound although the same degree of inhibition (about 20%) is observed with concentrations varying between 0.1 mM to 5 mM.

Additions	Glycerol released[a]
None	7.5 ± 0.6
Epinephrine	21.0 ± 1.2
$Ap(CH_2)pp$, 0.1 mM	8.6 ± 0.8
1.0 mM	19.0 ± 1.0
5.0 mM	29.0 ± 1.4
Epinephrine + $Ap(CH_2)pp$, 0.1 mM	22.9 ± 0.8
1.0 mM	27.0 ± 1.2
5.0 mM	30.4 ± 1.5
ATP, 0.5 mM	13.0 ± 0.7
1.0 mM	16.8 ± 0.9
5.0 mM	23.4 ± 1.4

[a] μMoles of glycerol released per mmole of triglyceride; average value ± standard error of the mean of three replications.

in less than 10 minutes (Fig. 1). If $Ap(CH_2)pp$ is added simultaneously with epinephrine the elevation of cyclic AMP is abolished. Furthermore, in the presence of the inhibitor the level of cyclic AMP falls quite rapidly to values lower than those found in the basal state. By seven minutes the concentration of cyclic AMP falls to about 15% of the basal value found in unstimulated cells. These results suggest that $Ap(CH_2)pp$ is capable of effectively inhibiting adenylate cyclase activity *in vivo*.

Effects on lipolysis - No inhibition of lipolysis could be demonstrated using a wide range of inhibitor concentration (Table 1). To the contrary, it was found that $Ap(CH_2)pp$ actually enhanced epinephrine-induced lipolysis and that it had lipolytic properties when added in the absence of epinephrine.

Since the terminal γ-phosphate of $Ap(CH_2)pp$ can potentially serve as a high energy phosphate donor, the possibility exists that this compound can modify met-

abolic effects independently of its inhibition of adenylate cyclase. The similarity of the lipolytic effects produced by $Ap(CH_2)pp$ and by ATP (Table 1) suggests that this may be the case. The compound may possibly be acting in vivo as a substrate for protein kinases, which could in principle explain the occurrence of brisk lipolysis in the relative absence of cyclic AMP. $Ap(CH_2)pp$ is probably a good substrate for protein kinases since it can effectively decrease the kinase-catalyzed transfer of $[^{32}P]$ from $[^{32}P](\gamma)$-ATP to histone and casein (13).

Effects on glucose oxidation - Unlike the effect of insulin, the rate of glucose oxidation in fat cells is not enhanced by $Ap(CH_2)pp$ (Table 2). Glucose oxidation is actually inhibited by very low concentrations of this ATP analog, both in the absence and presence of insulin. As observed in the studies of lipolysis, $Ap(CH_2)pp$ may also have other important effects on glucose oxidation other than those which might result from inhibition of adenylate cyclase activity. ATP itself has effects which are very similar to those observed with $Ap(CH_2)pp$ (Table 2). The inability of the β-γ-methylene analog, $App(CH_2)p$, to mimic the effects of ATP or of $Ap(CH_2)pp$ on glucose oxidation indicates that the dominant metabolic effects of the latter nucleotides may result from alterations in processes which are dependent on the transfer or hydrolysis of the γ-phosphate group of the nucleotides. It is thus not possible under these circumstances to test the hypothesis which relates the action of insulin to changes in cyclic AMP since the other effects of $Ap(CH_2)pp$ could, in principle, serve to bypass or overcome the relative lack of the cyclic nucleotide.

DISCUSSION. It is notable that $Ap(CH_2)pp$, in addition to inhibiting competitively the activity of adenylate cyclase in membrane preparations, also appears to inhibit the activity of the enzyme in intact cells. The very rapid fall (Fig. 1) in the level of cyclic AMP which occurs in fat cells exposed to $Ap(CH_2)pp$ suggests that cyclic AMP is normally degraded very rapidly and that even under basal conditions the intracellular concentration of the nucleotide is delicately balanced by brisk processes of synthesis and degradation. If a separate, relatively stable pool of intracellular cyclic AMP exists which is not in rapid equilibrium with the bulk

TABLE II. Effect of $Ap(CH_2)pp$, $App(CH_2)p$ and ATP on the basal and insulin-stimulated rates of glucose oxidation in isolated fat cells

Fat cells were incubated (15) at 37° for 90 minutes in 1.25 ml of KRB buffer containing 1% (w/v) albumin, 0.2 mM [^{14}C] glucose (8 μCi per μmole), and, where indicated, insulin (100 μunits per ml) and the analogs of ATP. Values represent averages of duplicate replications.

Addition		Conversion of [^{14}C] glucose to $^{14}CO_2$
		cpm per 2 hours
None		23,200
Insulin		127,400
$Ap(CH_2)pp$,	8 μM	19,300
	50 μM	17,200
	0.12 mM	15,100
	0.6 mM	14,600
	1.2 mM	12,100
	3 mM	12,200
Insulin + $Ap(CH_2)pp$,	0.12 mM	110,900
	1.2 mM	71,400
	3 mM	64,300
ATP,	50 μM	16,800
	0.2 mM	15,100
	1.0 mM	13,200
	3.0 mM	12,300
$App(CH_2)p$,	1 mM	22,100
	3 mM	20,600

of the cyclic AMP of the cell, it must be of such magnitude that it represents only a small fraction of that which is measurable under basal conditions.

$Ap(CH_2)pp$ has certain important shortcomings in studies designed to determine the effect of inhibiting adenylate cyclase activity on complex metabolic processes. In addition to its potential for inhibiting other cellular enzymes, $Ap(CH_2)pp$ can act as a high-energy phosphate donor in many enzymic reactions, as it appears to do in protein kinase reactions. Nevertheless, this ATP analog may be useful in other kinds of studies in which it is desired to measure the effects of the terminal pyrophosphate moiety of ATP without introducing complicating hydrolytic byproducts such as adenosine, AMP, ADP, and inorganic pyrophosphate. For example, this compound may simplify studies of the mechanism by which low concentrations of exogenous ATP modify lipolysis and glucose oxidation. By com-

bining the observations made with both the α,β- and the β,γ-methylene derivatives additional information can be gained. This kind of information, for example, implicates hydrolysis or transfer of the γ-phosphate group in the interesting effects of $Ap(CH_2)pp$ or ATP itself on lipolysis and glucose transport.

Adenylate cyclase inhibitors are in principle potentially useful tools for studying the role of cyclic AMP in metabolic processes provided that structural analogs with adequate selectivity can be found. On the basis of these and other (1) studies, it is suggested that the dimethylene analog of ATP, $Ap(CH_2)p(CH_2)p$, may be a much more selective and useful inhibitor of adenylate cyclase since both of its phosphonium groups should be resistant to hydrolysis.

Acknowledgements: F. Krug is a recipient of a Postdoctoral Fellowship from Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, West Germany. G. Illiano is on leave from Istituto di Chimica Biologica, Universita di Napoli, Italy. P. Cuatrecasas is a U.S.P.H.S. Research Career Development Awardee (AM31464). This work was supported by Grant AM14956 from NIAMD and grants from The American Cancer Society (BC-63), The American Diabetes Association, and The Kroc Foundation, Santa Ynez, California.

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