Studies of Adenosine and Dibutyryl Adenosine 3′,5′-Monophosphoric Acid on the Isolated Fat Cell

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SUMMARY

The action of adenosine 3′,5′-monophosphate (c-AMP) and N^6, $O^{2'}$-dibutyryl adenosine 3′,5′-monophosphate (dc-AMP) was studied in isolated rat adipocytes using parameters of glucose oxidation and lipolysis. Whereas c-AMP stimulated the conversion of glucose-U-C-14 or glucose-1-C-14 to C-14-O_2, dc-AMP inhibited C-14-O_2 production below the baseline in each case. Both N^6-monobutyryl adenosine 3′,5′-monophosphate (N^6 mbc-AMP) and $O^{2'}$-monobutyryl-adenosine 3′,5′-monophosphate ($O^{2'}$-mbc-AMP) stimulated C-14-O_2 production from glucose-U-C-14. Only N^6-mbc-AMP, however, was significantly lipolytic. When glucose-6-C-14 was utilized as the substrate, both c-AMP and dc-AMP stimulated its conversion to C-14-O_2.

Dibutyryl cyclic AMP antagonized the action of insulin and proinsulin on glucose-U-C-14 oxidation in the isolated fat cell by an apparent competitive mechanism. Paradoxically, insulin potentiated C-14-O_2 production from glucose-6-C-14 in the presence of 1.0 mM dc-AMP. C-AMP exhibited no such response.

It is concluded that under the experimental conditions described, (1) c-AMP and dc-AMP exhibit divergent effects with regard to glucose oxidation; (2) by the use of glucose-U-C-14 and glucose-1-C-14, dc-AMP competitively inhibits the action of insulin or proinsulin on glucose oxidation; (3) insulin synergistically stimulates C-14-O_2 production from glucose-6-C-14, in the presence of dc-AMP; and (4) both N^6- and $O^{2'}$-butyrate moieties are necessary for the inhibitory effect on glucose oxidation observed with dc-AMP. DIABETES *21*:1027-34, October, 1972.

The compound, adenosine 3′,5′ (cyclic) monophosphate (c-AMP), has been implicated as a mediator substance in the mechanism of action of hormones on sensitive tissue[1] and in the mediation of hormonal release.[2] N^6, $O^{2'}$-dibutyryl-adenosine 3′,5′ (cyclic) monophosphate (dc-AMP) was thought to represent a physiologically active analogue of c-AMP with a more nonpolar nature, and hence, an easier permeability of the cell membrane.[3] The latter properties presumably accounted for its greater biologic potency. Recent investigations from these[4,5] and other laboratories,[6,7] however, have established that dc-AMP and c-AMP differ markedly in their ability to stimulate glucose uptake in vitro. Because of this difference, further studies of c-AMP and dc-AMP on the isolated fat cell[4] were undertaken.

In this paper we present data obtained using the isolated fat cell on the effect of c-AMP and dc-AMP on conversion of C-14-U, C-14-1, and C-14-6-labeled glucose to C-14-O_2 and lipid. Additional studies were also carried out, with both N^6 and $O^{2'}$ monobutyryl derivatives of c-AMP. The results obtained suggest that dc-AMP, but not c-AMP (or the monobutyryl derivatives), inhibits glucose oxidation and exhibits a potentiating effect with insulin on oxidation of glucose-6-C-14.

MATERIALS AND METHODS

Animals

Male Holtzman rats weighing 90 to 140 gm. were used in the studies of the conversion of labeled glucose to C-14-O_2. Rats of the same strain, weighing 140 to 180 gm., were used in the studies involving lipolysis. The rats were maintained on Purina Laboratory Chow ad libitum until the time of sacrifice.

Chemicals

Bovine plasma albumin fraction V (Lot 30308) was obtained from Armour Pharmaceutical Company, Chicago, Illinois. Crude bacterial collagenase (Lot CLS

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9AA) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. Glucose U-C-14, glucose-1-C-14, and glucose-6-C-14 were obtained from the Nuclear Chicago Corporation, Chicago, Illinois. Glucagon-free pork insulin (Lot PJ 5589) and pork proinsulin (Lot 615-1039B-45-C) were gifts of Dr. R. Chance of the Eli Lilly Company, Indianapolis, Indiana. Adenosine 3′,5′ (cyclic) monophosphoric acid (Lots 109B-7320 and 109B-7163), N^6, $O^{2'}$-dibutyryl-adenosine 3′,5′ (cyclic) monophosphoric acid (Lots 109B-7070, 119B-7223, and 20C-7540), and $O^{2'}$-monobutyryl-adenosine 3′,5′ (cyclic) monophosphate (Lot 80C-7090) were obtained from Sigma Chemical Company, St. Louis, Missouri. N^6-monobutyryl-adenosine 3′,5′ (cyclic) monophosphoric acid (Lot 7510-207) was obtained from Boehringer-Mannheim Company, New York, New York. β-1-24 corticotropin (Cortrosyn) (Lot 60-10-14/C) was obtained from Organon, Incorporated, West Orange, New Jersey. N-Butyric acid was obtained from Eastman Organic Chemicals, Rochester, New York.

Preparation of adipose tissue

Isolated fat cells were prepared by the modified[8] methods of Rodbell[9] and Gliemann.[10]

Method of fat cell bioassay

The incorporation of radioactive carbon from glucose-U-C-14, glucose-1-C-14, or glucose-6-C-14 into C-14-O_2 and lipids was determined by previously published methods.[5,8] Counting of radioactive components was performed in a Mark II Nuclear Chicago Liquid Scintillation Spectrometer. Results are reported as nanoatoms of glucose carbon incorporated into CO_2 or lipid per 100,000 fat cells per two hours of incubation. Studies involving lipolysis utilized the standard technics reported.[11] Where indicated, lipolysis was initiated with 0.1 μg. of β-1-24 corticotropin per milliliter of incubation mixture. In the lipolysis experiments, following the one-hour incubation, separation of the cells and incubation mixture was carried out and the glycerol content of the incubation medium measured, utilizing the method of Chernick.[12] Glycerol release was expressed as nanomoles of glycerol released per 100,000 cells per one hour.

Statistical analyses were done by conventional technics. Lineweaver-Burke plots were computed and drawn by the method of least squares fit.

RESULTS

Biologic activity of c-AMP and dc-AMP in the presence or absence of insulin

Studies of glucose-U-C-14 oxidation. In order to further study the nature of inhibition of glucose-U-C-14 conversion to C-14-O_2 by dc-AMP, the latter compound was studied in the presence and absence of various concentrations of insulin and proinsulin. The data are presented in a double reciprocal fashion in figure 1. As can be seen from figure 1, the kinetics suggest possible competitive inhibition between dc-AMP and insulin or proinsulin. Similar plots (data not shown) were obtained with shorter incubation time (15, 30 and 60 minutes), thus precluding the possibility of dc-AMP toxicity on the isolated fat cell.

Because of the possibility of the biologic effects being produced by conversion of cyclic to noncyclic nucleotides, studies shown in table 1 were carried out. Data shown here illustrate the biologic activity of the noncyclic nucleotides of adenosine compared to c-AMP on C-14-O_2 release from uniformly labeled glucose in the isolated fat cell. Only adenosine monophosphate (AMP) and c-AMP showed stimulatory capacity. Adenosine, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) all showed a significant inhibitory effect on C-14-O_2 release in the isolated fat cell.

Studies of glucose incorporation into C-14-O_2 and lipids utilizing glucose-1-C-14, glucose-U-C-14, and glucose-6-C-14. We studied the effects of c-AMP and dc-AMP on glucose oxidation and incorporation into lipid in the isolated fat cell utilizing three different types of glucose, uniformly labeled, 1-C-14-labeled, and 6-C-14-labeled. Figure 2 illustrates the dose response curves measuring both C-14-O_2 production and incorporation into lipid for each of these labeled glucoses. The divergent action of c-AMP and dc-AMP in the isolated fat cell is apparent in the studies utilizing uniformly labeled or 1-C-14-labeled glucose. In the experiments utilizing 6-C-14-labeled glucose, however, lower concentrations of dc-AMP (0.1 to 2.5 mM) stimulated, whereas higher concentrations (5 and 10 mM) inhibited glucose oxidation. Despite the stimulation of C-14-O_2 release from glucose-6-C-14 at low dc-AMP concentrations, glucose-6-C-14 incorporation into Dole-extractable lipid was decreased and followed the same pattern seen with glucose-C-14 and glucose-U-C-14. The stimulatory effect of dc-AMP at low concentrations is consistent with the findings of Bray,[6] but not with those of Blecher,[13] who reported a paradoxical effect of dc-AMP on C-14-O_2 production using glucose-U-C-14. Similarly, paradoxical effects have been observed with different size fat cells.[21] Because of the significant difference between the oxidation of glucose-

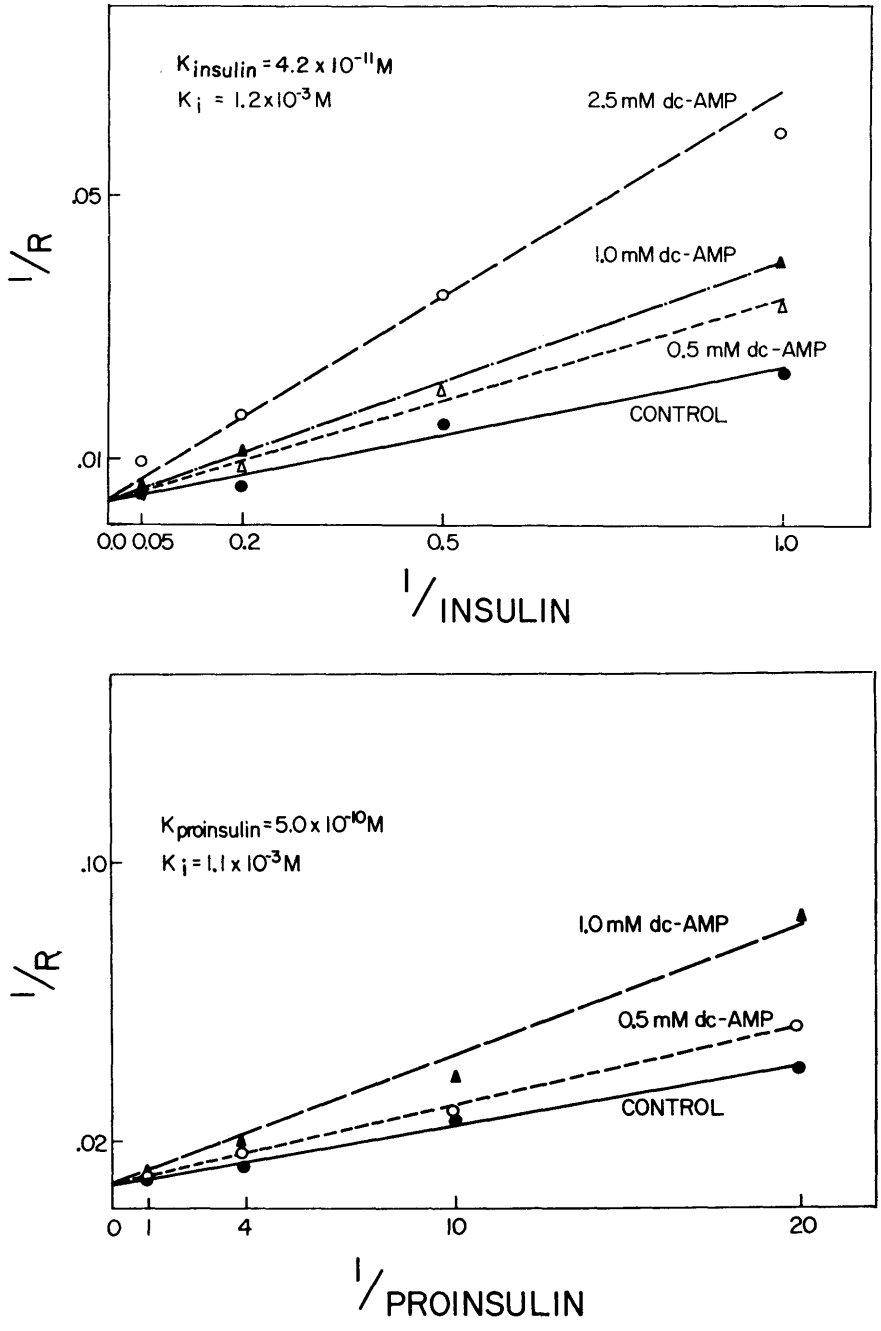


FIGURE 1

Kinetics of inhibition of insulin and proinsulin-induced glucose uptake by various concentrations of dc-AMP. The ordinate value is plotted as 1/response and has been measured by the conversion of glucose-U-C-14 to C-14-O_2 in the absence or presence of dc-AMP, and insulin or proinsulin. Values along the abscissa are plotted as 1/concentration of insulin or proinsulin. All of the points shown are the average of four replicate determinations. K_m and K_i values are shown for each set of data.

6-C-14 and the other two labeled glucoses, the effect of dc-AMP was further investigated in the presence of various concentrations of insulin and proinsulin. Since the data for proinsulin and insulin are identical, only the results of the insulin studies are presented (figure 3). As can be seen, insulin at low (2 μU./ml.) and intermediate (20 μU./ml.) concentrations potentiated the effect of dc-AMP on glucose oxidation from glucose-6-C-14 in the fat cell.

This potentiation occurred at all three dc-AMP concentrations shown, and contrasts with studies of combinations of insulin and dc-AMP in the presence of glucose-U-C-14 (figure 1) or glucose-1-C-14 (data not shown), in which dc-AMP inhibited the stimulatory effect of insulin. C-AMP, in contrast to dc-AMP, exhibited no potentiating effect when studied in the presence of either of the three labeled glucoses (data not shown).

TABLE 1

Biologic activity of the noncyclic nucleotides of adenosine as measured by conversion of glucose-U-C-14 to U-C-14-O_2 in the isolated fat cell

Additions	Concentration	Nanoatoms C-14-O_2/10^5 cells/2 hr.
Basal	0	72.4 ± 9.0
Adenosine	5.0 mM	45.8 ± 6.6
AMP	5.0 mM	88.8 ± 16.3
ADP	5.0 mM	51.1 ± 6.0
ATP	5.0 mM	49.9 ± 7.7
c-AMP	5.0 mM	110.0 ± 17.1

Each result represents the average ± S.E.M. of four replicate determinations.

Biologic activity of butyric acid: Studies of glucose oxidation

Since dc-AMP, in contrast to c-AMP, has two butyrate moieties, it is possible that inhibition of glucose oxidation could have been due to products resulting from hydrolysis of these side chains. The extent of hydrolysis, however, has been studied recently and is shown not to exceed 4 per cent.[14] Nevertheless, in order to rule out butyrate as a possible inhibitor of glucose oxidation, the experiment as depicted in table 2 was performed. Butyric acid tested at multiple concentrations in fat cells failed to alter the production of C-14-O_2 from labeled glucose, thus confirming an earlier report on the lack of effect of butyrate on glucose oxidation.[13] The divergent effect of c-AMP and dc-AMP on glucose-U-C-14 oxidation, therefore, does not appear to be related to the butyrate moiety as a result of hydrolysis of dc-AMP.

Biologic activity of N^6 and $O^{2'}$-monobutyryl cyclic AMP: Studies of glucose oxidation and lipolysis

Because the side chain configuration undoubtedly plays some role in the divergent effect of dc-AMP in adipose tissue, N^6-mbc-AMP and $O^{2'}$-mbc-AMP were studied for their ability to stimulate glucose oxidation and lipolysis in the isolated fat cell. Data shown in figure 4 illustrate that $O^{2'}$-mbc-AMP and N^6-mbc-AMP, similar to c-AMP itself, stimulate glucose-U-C-14 conversion to C-14-O_2. Therefore, it would appear that the presence of butyrate moieties, at both N^6 and $O^{2'}$ positions, is essential to produce the inhibition of glucose oxidation seen with dc-AMP.

The lipolytic activity of these compounds was studied, alone or in combination with another lipolytic agent, corticotropin. As can be seen from table 3, although N^6-mbc-AMP exhibits considerably more lipolytic activity than either c-AMP or $O^{2'}$-mbc-AMP, the presence of two butyrate moieties at N^6 and $O^{2'}$ positions confer upon the c-AMP molecule significantly greater lipolytic properties which are enhanced by corticotropin.

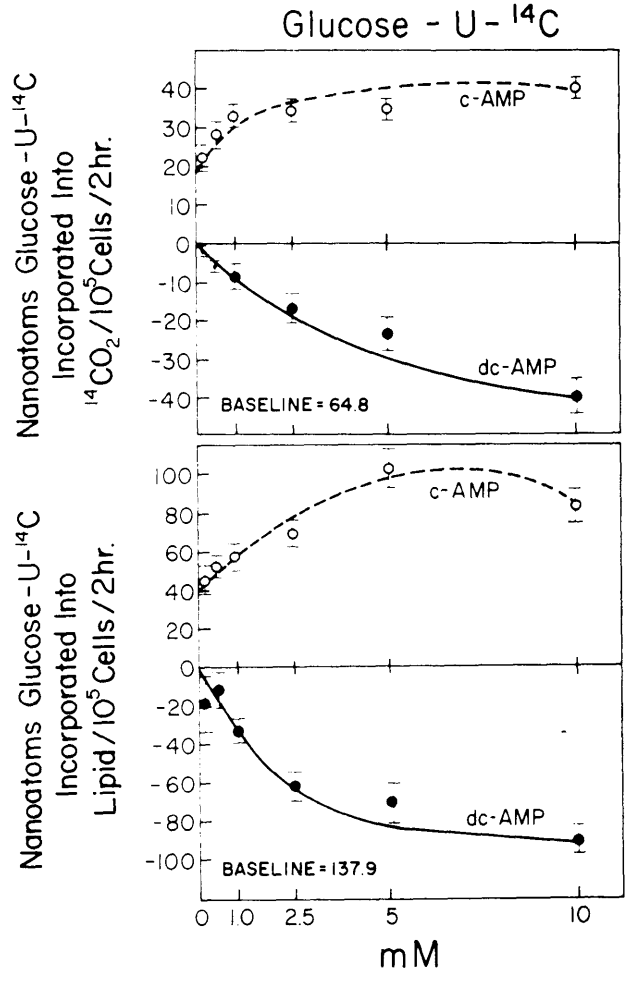
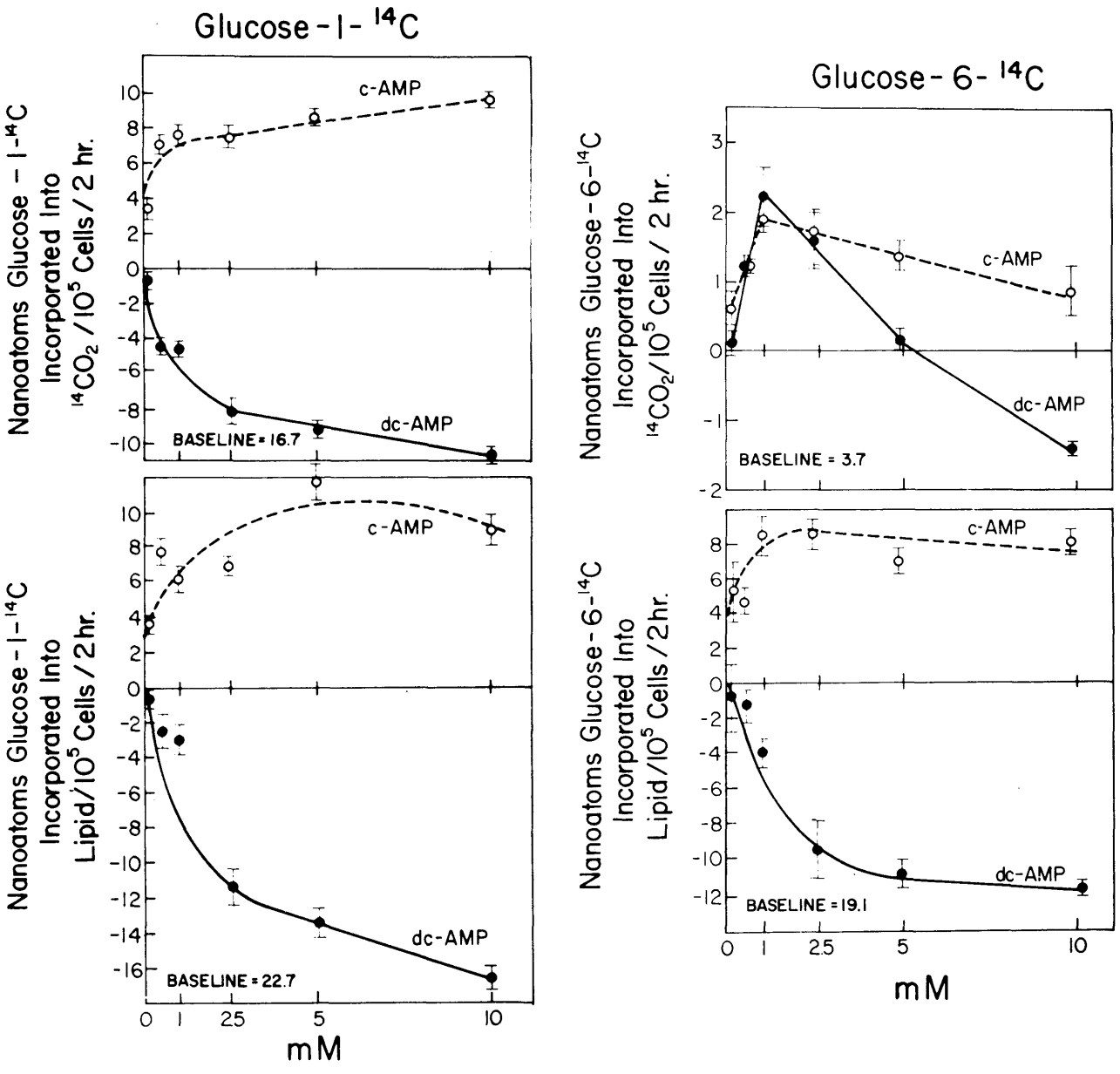


FIG. 2A. Dose response curves for c-AMP and dc-AMP using three different labeled glucoses. Studies of glucose conversion to C-14-O_2 utilizing glucose-U-C-14, glucose-1-C-14 and glucose-6-C-14 are shown. The comparative effects of c-AMP and dc-AMP with dose response curves above and below the baseline value are demonstrated. The upper set of curves represents C-14-O_2 release and the lower set lipid incorporation. The results are expressed as the average ± S.E.M. of nine replicate determinations.

DISCUSSION

Previous preliminary studies from this laboratory have demonstrated a difference between c-AMP and dc-AMP with regard to lipolytic activity and C-14-O_2



FIGS. 2B (left) and 2C (right). Dose response curves for c-AMP and dc-AMP using three different labeled glucoses. Studies of glucose conversion to C-14-O_2 utilizing glucose-U-C-14, glucose-1-C-14 and glucose-6-C-14 are shown. The comparative effects of c-AMP and dc-AMP with dose response curves above and below the baseline value are demonstrated. The upper set of curves represents C-14-O_2 release and the lower set lipid incorporation. The results are expressed as the average ± S.E.M. of nine replicate determinations.

production from labeled glucose.[4,5] Additional data presented here indicate that for both C-14-O_2 production and lipid incorporation from uniformly or C-1-labeled glucose, c-AMP and dc-AMP produce opposite effects in the isolated fat cell. Whereas dc-AMP inhibits glucose incorporation into C-14-O_2 or lipid, c-AMP stimulates these parameters. Furthermore, kinetic studies (figure 1) of glucose-U-C-14 conversion to C-14-O_2 in the presence of insulin and dc-AMP, suggest that dc-AMP inhibits glucose oxidation in a manner consistent with

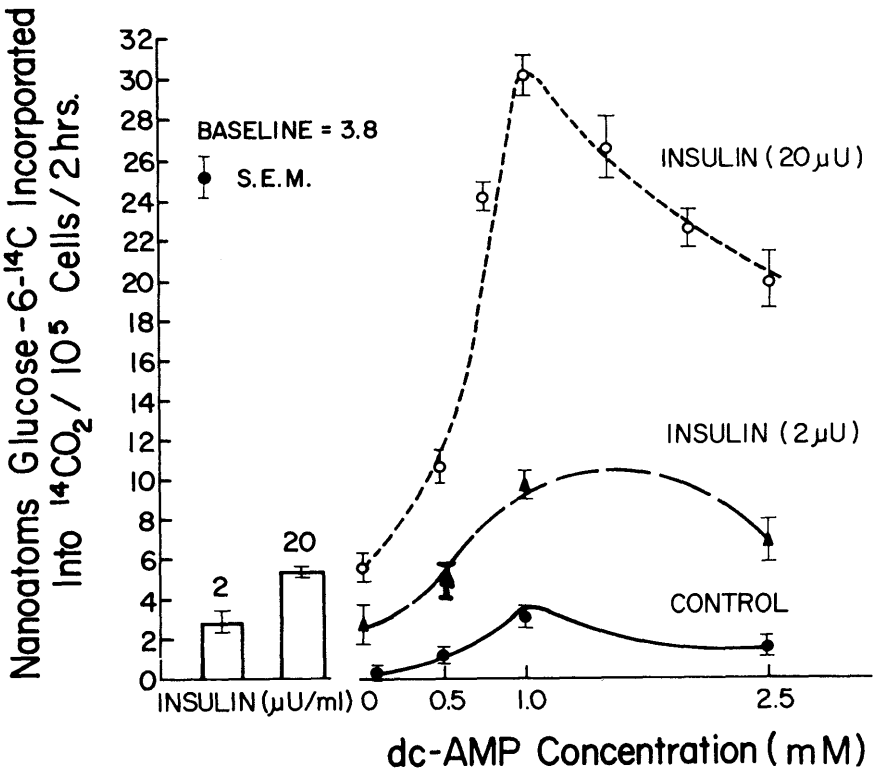


FIGURE 3

Effect of dc-AMP and various concentrations of insulin on glucose-6-C-14 conversion to C-14-O_2 in adipocytes. Net C-14-O_2 release is shown. Values for each point are the average ± S.E.M. of nine replicate determinations. The bar graphs shown correspond to the respective zero points on each of the curves.

competitive inhibition. The isolated fat cell is sensitive to as little as 0.01 mM of c-AMP in stimulating glucose oxidation. In contrast, levels of dc-AMP required to block significant amounts of glucose oxidation were fifty to 100 times higher. The data demonstrating the stimulatory capacity of the noncyclic nucleotides of adenosine support the concept that c-AMP does not exhibit its biologic activity by conversion to 5′ AMP alone. Furthermore, studies with butyric acid preclude the possibility that the inhibitory effect of dc-AMP on glucose oxidation is the result of hydrolysis of the butyrate side chain.

TABLE 2

Biologic activity of insulin and butyric acid in the isolated fat cell as measured by C-14-O_2 production from glucose-U-C-14, glucose-1-C-14 and glucose-6-C-14

	Nanoatoms of C-14-O_2/10^5 cells/2 hr.		
Additions	glucose-U-C-14	glucose-1-C-14	glucose-6-C-14
Basal	64.2 ± 4.3	13.5 ± 1.3	5.9 ± 0.4
Insulin, 20 μU./ml.	272.4 ± 3.7	78.6 ± 3.5	12.7 ± 1.8
dc-AMP, 2.5 mM	51.0 ± 3.5	7.8 ± 1.3	7.8 ± 0.5
Butyric Acid,			
0.05 mM	65.8 ± 9.0		
0.1 mM	65.9 ± 4.2		
0.5 mM	62.1 ± 4.6	12.9 ± 0.9	6.2 ± 0.3
1.0 mM	58.9 ± 4.2		
2.5 mM	66.9 ± 2.5		

Each result represents the average ± S.E.M. of four replicate determinations.

The data in figure 2 show a concentration-dependent paradoxical response for dc-AMP on C-14-O_2 release from glucose-6-C-14. These results are consistent with the observations of Bray[6] who found an increase in C-14-O_2 production from C-6-labeled glucose with selective dc-AMP concentrations. Furthermore, additional studies demonstrate a synergistic effect of insulin with 1.0 mM dc-AMP. If the assumption is correct that glucose-U-C-14 oxidation to C-14-O_2 in adipose tissue represents a combination of pathways, among which the hexose monophosphate shunt (HMP) predominates, and glucose-6-C-14 oxidation to C-14-O_2 is achieved primarily via the nonshunt pathways, glycolytic and citric acid cycle, then from our studies, it would appear that dc-AMP inhibits glucose metabolism via the HMP shunt.[15] In addition, it would appear that in the presence of an inhibited shunt, insulin promotes glucose utilization and conversion to C-14-O_2, probably via the citric acid cycle.

Our studies of N^6-mbc-AMP and $O^{2'}$-mbc-AMP suggest that lipolytic activity is regulated by different structural entities than those controlling glucose uptake. Both N^6 and $O^{2'}$-butyrates are essential for the inhibition of glucose uptake, but lipolysis is significantly

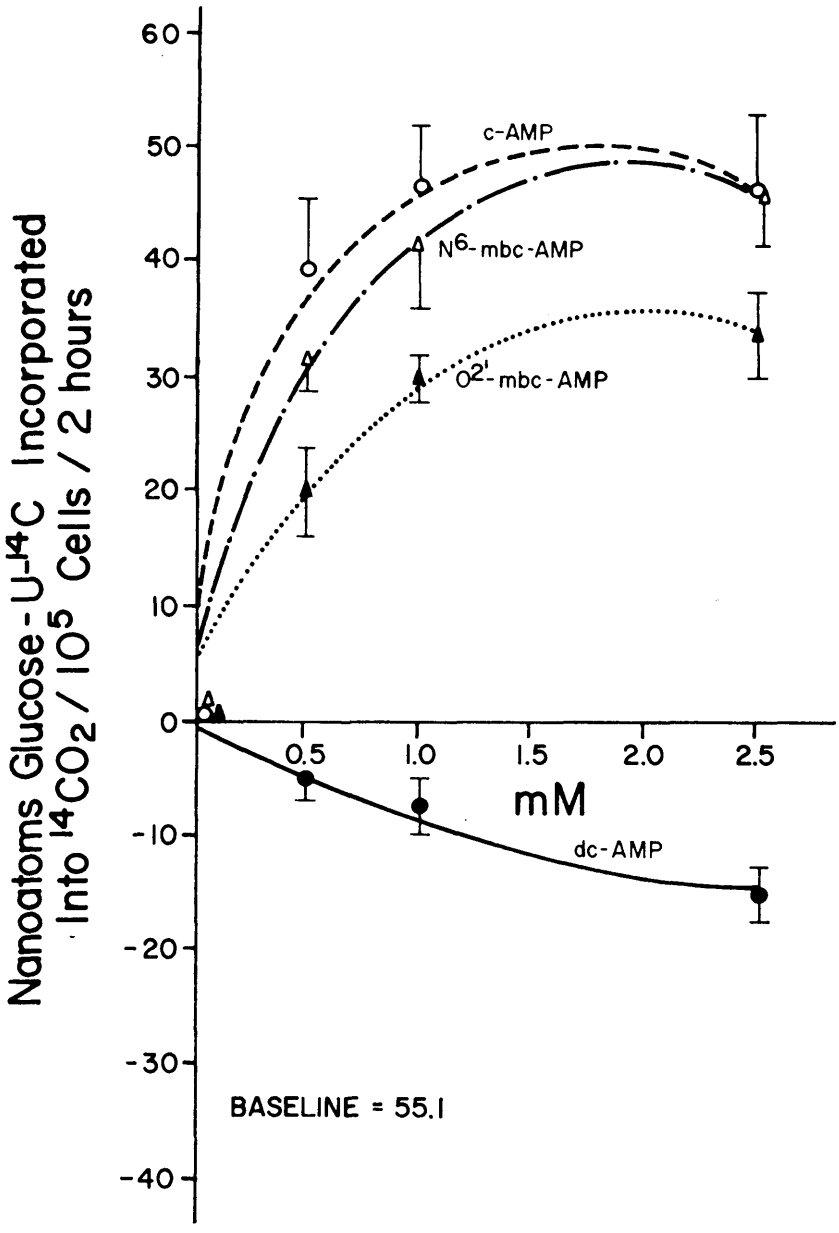


FIGURE 4

Dose response curves for c-AMP, dc-AMP, N^6-mbc-AMP and $O^{2'}$-mbc-AMP using glucose-U-C-14 in the isolated fat cell. Comparative effects are shown above or below the baseline value. The results are expressed as the average ± S.E.M. of six replicate determinations.

stimulated by the presence of N^6-butyrate alone. Apparently, structural interaction of N^6- and $O^{2'}$-substituted groups are essential for inhibition of glucose uptake, but not for lipolysis.

Recently, our observation of a different action of dc-AMP and c-AMP has been confirmed on glycogen synthesis in HeLa cells by Hilz and Tarnowski,[16] and in *E. coli* by Yokota and Gots.[17] This divergent effect has also been reported in the elaboration of growth hormone from the anterior pituitary in vitro[18] as well as in in vivo studies of antidiuretic hormone in human subjects.[19] In other systems, however, such as the isolated adrenal cells, no divergent action between dc-AMP and c-AMP is as yet demonstrable.[20]

NOTE

Using pyruvate-3-C-14 and acetate-2-C-14, confirmatory evidence for dc-AMP inhibition of the hexose monophosphate shunt and stimulation of the tricarboxylic acid cycle has been obtained in the isolated fat cell. Furthermore, additional data from Jarett et al.[22] on differences in protein synthesis and new data from our laboratory[23] showing competitive inhibition of cyclic nucleotide phosphodiesterase by dc-AMP, but not c-

TABLE 3

Lipolytic activity of c-AMP, dc-AMP, N^6-mbc-AMP and $O^{2'}$-mbc-AMP in the isolated fat cell

Additions	Nanomoles of glycerol released/10^5 cells/1 hr.
Baseline	24.4 ± 1.0
1.0 mM c-AMP	28.7 ± 3.3
1.0 mM N^6-mbc-AMP	95.3 ± 17.5
1.0 mM $O^{2'}$-mbc-AMP	23.0 ± 1.3
1.0 mM dc-AMP	252.7 ± 34.1
0.1 μg. β-1-24 corticotropin	301.1 ± 15.9
+ c-AMP	175.7 ± 13.1
+ N^6-mbc-AMP	438.1 ± 52.7
+ $O^{2'}$-mbc-AMP	232.3 ± 22.0
+ dc-AMP	552.3 ± 35.9

Each result represents the average ± S.E.M. of nine replicate determinations.

AMP, extend the divergent effect between c-AMP and dc-AMP beyond carbohydrate and lipid metabolism.

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