ANTAGONISTIC ACTIONS BETWEEN DIBUTYRYL ADENOSINE-3', 5'-CYCLIC MONOPHOSPHATE AND INSULIN ON THE METABOLISM OF THE SURVIVING RAT DIAPHRAGM[*].

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Received December 19, 1968

The ubiquitous occurence of cyclic-3', 5'-adenosine monophosphate (cyclic AMP) in biological systems and its participation in the mechanism of numerous hormonal stimulations elicited recently a series of experiments attempting to correlate the intracellular level of this nucleotide and various hormonal actions of insulin on isolated tissues or cells. An insulin induced decrease of the level of cyclic AMP after epinephrine stimulation has been reported by Butcher et al. (1966) on the rat epididymal fat pad; in vivo, the level of cyclic AMP seems to be modulated in the liver under the influence of glucagon, insulin and insulin antibodies (Jefferson et al., 1968). Inhibition of adenyl-cyclase activity in the isolated epididymal fat pad has been reported under the action of insulin (Jungas, 1966). Recently, an antagonistic action between exogeneous cyclic AMP and insulin has been described on the turnover of phosphorylated compounds in isolated adipose tissue cells (Hepp et al., 1968). It must be emphasized however that the issue is still controversial: all effects of insulin do not seem to be antagonized by all compounds known to raise cyclic AMP levels (Rodbell, 1967), and in the muscle no variations of this level under the action of insulin has been detected (Larner, 1966).

The present work aims to study the antagonism between insulin and N^6-2'-O-dibutyryl adenosine-3', 5'-cyclic monophosphate (DB-cyclic AMP) on various parameters of the metabolism of the isolated rat diaphragm known to

[*] The present work is part of a Doctoral Thesis to be submitted by one of the authors (A. M. Chambaut).

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respond independently one from another to the hormonal stimulations (Clauser et al., 1962; Eboué-Bonis et al., 1963; 1967).

Experimental.

Crossed hemidiaphragms from female Wistar Rats (homogeneous lots weighing from 100 to 120 g) have been used.

Cofactors, hormones and radioactive compounds are the following: adenosine-3', 5'-cyclic phosphate (Sigma).
N^6-2'-O-dibutyryl adenosine-3', 5'-cyclic phosphate (Calbiochem); crystalline insulin (Choay, Paris); theophylline (Merck); D-galactose 1-^{14}C, D-glucosamine 1-^{14}C hydrochloride, 2-aminoisobutyric acid 1-^{14}C (The Radiochemical Centre, Amersham); D-glucose (U)^{14}C, L-valine (U)^{14}C, L-methionine ^{14}C, $NaH_2{}^{32}PO_4$ (C. E. A., France).
^{14}C sugar uptake and incorporation of labelled precursors into glycogen are determined according to procedures described elsewhere (Eboué-Bonis et al., 1967). The incorporation of aminoacids into protein material is assayed according to Manchester (1961). The incubation procedure and the determination of the turnover of phosphorylated compounds have been described previously (Clauser et al., 1962). Cyclic AMP, DB-cyclic AMP, theophylline, when present have been preincubated with the diaphragm during a 15 minutes period prior to the addition of labelled substrates. Insulin when present is added with the labelled substrates.

Results.

Table I shows that cyclic AMP exhibits various and rather inconsistent effects on glucose uptake and glycogen biosynthesis from labelled glucose in the rat diaphragm. These two events seem to be slightly stimulated in agreement with the results of Edelman et al. (1966) who reported an insulin-like effect of exogeneous cyclic AMP; when, however the dibutyryl derivative of cyclic AMP was used, consistent and concentration dependent inhibitions of glucose uptake and glycogen biosynthesis appear. Hence in subsequent experiments, the dibutyryl derivative alone has been systematically used. It is not clear from the results of Table I if the inhibitory effect of DB-cyclic AMP operates both on the facilitated diffusion of glucose through the membrane (Morgan et al., 1961) and on the glycogen-synthetase system (Villar-Palasi et al., 1961) both of which have been reported to be stimulated separately

Table I: Effect of adenosine-3', 5'-cyclic phosphate and dibutyryl adenosine-3', 5'-cyclic monophosphate on glucose uptake and glycogen biosynthesis in the isolated rat diaphragm in the presence and in the absence of insulin.

Glucose (U)^{14}C: 0.08 μc/ml ; glucose: 2 mg/ml ; incubation time: 20 minutes.

Cyclic AMP or DB-cyclic AMP	0	0	10^{-4}M		10^{-3}M		2×10^{-3}M	
Insulin (10 μg/ml)	0	+	0	+	0	+	0	+
1) Cyclic AMP								
Glucose uptake *	131.1	233.6	121.5	234	163.2	264	102.3	232
Glycogen biosynthesis *	71.5	170	62.6	243	94.1	208	54.8	186
Glycogen specific radioactivity (cpm/μg)	50.0	87.6	18.1	51.7	34.6	72.8	35.8	68.2
2) Dibutyryl cyclic AMP								
Glucose uptake *	131.1	233.6	87.6	230	102.5	158	68.8	112
Glycogen biosynthesis *	71.5	170	20.4	107.4	34.4	74.6	8.8	33.0
Glycogen specific radioactivity (cpm/μg)	50.0	87.6	14.5	59.2	25.7	46.6	10.6	35.0

* μg/100 mg fresh weight .

Table II: Effect of dibutyryl adenosine-3', 5'-cyclic monophosphate on the uptake of galactose 1-^{14}C and of glucosamine 1-^{14}C and the biosynthesis of glycogen from these substrates in the isolated rat diaphragm in the presence and in the absence of insulin.

Galactose 1-^{14}C:1.4 μci/ml; incubation time: 20 minutes; Glucosamine 1-^{14}C:0.08 μci/ml; incubation time: 60 minutes.

DB-cyclic AMP (10^{-4}M)	0	0	0	0	+	+
Theophylline (10^{-3}M)	0	0	+	+	+	+
Insulin (0.1 μg/ml)	0	+	0	+	0	+
Galactose uptake *	19,070	30,060	19,050	30,550	19,175	21,675
Glycogen biosynthesis from galactose 1-^{14}C *	630	1,112	412	615	185	342
Glucosamine uptake *	18,560	38,900			17,700	27,300
Glycogen biosynthesis from glucosamine 1-^{14}C *	6,900	21,400			1,710	6,960

* cpm/100 mg fresh weight.

(Eboué-Bonis et al., 1967) by insulin. These parameters can be studied specifically by using galactose 1-^{14}C, the diffusion of which is stimulated by insulin (Levine et al., 1955) but which is not metabolized extensively by the muscle tissue, and glucosamine 1-^{14}C which permeates the membrane by passive diffusion (Harbon et al., 1967) but may participate in the biosynthesis of glycogen (Maley et al., 1966).

Table II shows that the insulin induced increase of the galactose distribution space in the isolated muscle is almost completely abolished by DB-cyclic AMP. There is no effect on the uptake of galactose in the absence of insulin, a result which might have been expected as within the incubation time used galactose distribution between the extracellular and intracellular phases has reached its equilibrium. There is a slight but significant biosynthesis of glycogen from labelled galactose and it has been controlled that the radioactive sugar incorporated into glycogen from galactose 1-^{14}C is glucose ^{14}C.

When glucosamine 1-^{14}C is used as a radioactive substrate, extensive incorporation of glucose ^{14}C occurs into the glycogen pool of the muscle and this incorporation is stimulated by insulin and inhibited by DB-cyclic AMP and theophylline. However in this case the relative increase under the influence of insulin remains the same, whether DB-cyclic AMP and theophylline are present or not. In a series of experiments, which are not reported in the present paper, it has been controlled that glucosamine penetration into the intracellular space at zero time is a concentration independent, passive diffusion which is not stimulated by insulin.

Hence the data obtained with galactose 1-^{14}C and glucosamine 1-^{14}C seem to indicate that DB-cyclic AMP antagonizes separately both parameters studied: insulin stimulated sugar penetration into the intracellular space and glycogen biosynthesis.

Table III shows that DB-cyclic AMP counteracts both the uptake of labelled aminoacids (methionine ^{14}C and 2-aminoisobutyric 1-^{14}C) and their incorporation into proteins (valine (U)^{14}C and methionine ^{14}C). It is well known that all natural aminoacids exhibit insulin-sensitive incorporation kinetics into proteins (Manchester, 1961) whereas only a few of them (as L-methionine) exhibits insulin-sensitive penetration kinetics into the cell (Akedo et al., 1962). As with glycogen biosynthesis, the relative stimulatory

Table III: Effect of dibutyryl adenosine-3',5'-cyclic monophosphate on the uptake of labelled aminoacids and their incorporation into proteins in the isolated rat diaphragm in the presence and in the absence of insulin.

L-valine(U)^{14}C:0,08 μci/ml; L-methionine^{14}C:0.25 μci/ml; 2-aminoisobutyric acid 1-^{14}C:0.06 μci/ml; incubation time: 60 minutes.

Insulin (0.1 μg/ml)	0	+	0	+	0	+
DB-cyclic AMP (10^{-4}M) + theophylline (10^{-3}M)	0	0	+	+	0	0
DB-cyclic AMP (2. 10^{-4}M) + theophylline (10^{-3}M)	0	0	0	0	+	+
Valine uptake *	1591	1605	1623	1505	1690	1415
Valine incorporated into proteins **	149	235	104.8	145	88.2	132.5
Methionine uptake *	11180	12712	9730	10280		
Methionine incorporated into proteins **	327	549	172.5	253		
2-aminoisobutyric acid uptake *	2260	3892	2026	2992		
2-aminoisobutyric acid incorporated into proteins **	0.7	0.6	2	1.4		

* cpm/100 mg tissue (extracellular space deduced) ; ** cpm/mg protein.

Table IV: Effect of dibutyryl adenosine-3',5'-cyclic monophosphate on the level of phosphocreatine and nucleotides and on the incorporation of ^{32}P-phosphate into these compounds in the presence and in the absence of insulin.

Incubation time: 10 minutes.

DB-cyclic AMP (10^{-4}M)		0	0	+	+	0	0	+	+
Theophylline (10^{-3}M)		0	0	+	+	0	0	+	+
Insulin (0.1 μg/ml)		0	+	0	+	0	+	0	+
		μg P/100 mg fresh weight				Specific radioactivity (cpm/μg P)			
Exp. 1	P. i. *	28.0	30.7	37.2	35.3	1360	1090	1178	944
	Phosphocreatine	18.8	20.2	14.8	15.9	640	706	571	685
	ATP	25.5	23.4	23.2	21.0	860	1010	634	806
	ADP	4.8	5.3	5.7	4.8	289	435	252	347
	U + G **	1.4	1.5	1.2	1.2	630	690	550	694
Exp. 2	P. i. *	27.2	27.7	32.7	33.5	2395	1820	1710	1830
	Phosphocreatine	26.7	25.9	20.4	22.9	925	1190	760	1060
	ATP	29.7	25.6	27.2	22.6	1110	1495	795	1295
	ADP	5.4	4.8	5.3	6.1	346	453	334	436
	U + G **	1.7	1.2	1.6	-	445	1400	780	-

* P. i. : inorganic intracellular phosphate; ** U + G: urydyl and guanosyl nucleotides.

effect of insulin on the incorporation of natural aminoacids into protein material is not abolished by DB-cyclic AMP and theophylline; in contrast with these findings, the insulin-induced increase of 2-aminoisobutyric acid uptake (Kipnis et al., 1958) is almost abolished in the presence of these agents. This result agrees with the abovementioned experiments concerning galactose, another compound which is not extensively metabolized in the muscle cell.

The last parameter of diaphragm metabolism which has been examined concerns the insulin increased turnover of phosphorylated compounds (phosphocreatine, adenyl-nucleotides) which has been previously reported in the muscle (Volfin et al., 1961) and in isolated adipose tissue cells (Hepp et al., 1968).

Table IV shows that DB-cyclic AMP and theophylline depress the incorporation of ^{32}P-phosphate into phosphorylated compounds and that this phenomenon is independent from the penetration of inorganic phosphate into the cell. As previously shown for other metabolic events, the stimulatory effects of insulin on the turnover of these compounds is not affected.

Discussion.

It appears that with the exception of galactose and 2-aminoisobutyric acid uptakes, the stimulatory effects of insulin do not depend on the presence of DB-cyclic AMP and theophylline. However, all the parameters examined so far are sensitive to DB-cyclic AMP and theophylline and the effects of these compounds are allways opposed to the effects of insulin. This situation suggests the existence of a competitive phenomenon although it seems difficult to ascertain true competition at the enzymatic level on metabolic functions as complex and integrated as those studied. However, it must be emphasized that this conclusion essentially agrees with those reached independently by Hepp et al. (1968) on the isolated adipose tissue cells. The two exceptions mentioned above (viz. 2-aminoisobutyric acid and galactose uptakes) concern pure peripheral facilitated diffusion phenomena and may not represent the true kinetic interrelation between insulin and cyclic AMP, as the measures have been performed under equilibrium conditions (galactose) or very near equilibrium conditions (2-amino-isobutyric acid). A detailed kinetic study seems necessary in order to reach a definite conclusion on this point.

The antagonism between insulin and cyclic-AMP may be discussed according to the three main working hypotheses which have been recently under

investigation in several laboratories. It does not seem easy to visualize how an inhibition of the adenyl-cyclase system by insulin, as postulated by Jungas (1966), could fit with the data obtained. Stimulation by insulin of the specific cyclic AMP-phosphodiesterase (Butcher et al., 1966) could agree with these data, as far as the rate of penetration of DB-cyclic AMP into the cell (and possibly also of its debutyrylation (Henion et al., 1967)) do not exceed the maximal rate of hydrolysis of the cyclic compound by the total amount of enzyme present in the cell. The third hypothesis concerns the biosynthesis under the influence of insulin of a transducer different from cyclic AMP which could competitively antagonize the action of the latter compound on all the enzymatic systems involved in insulin action. This hypothesis would agree with must of the data presently available but no such system has been detected so far.

We gratefully acknowledge the cooperation of Miss L. Bénichou during this work.

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