INHIBITION OF THE GLUCAGON STIMULATED ADENYLATE CYCLASE ACTIVITY BY INSULIN

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1. Introduction

The mechanism of insulin action on such diverse cellular activities as membrane transport, lipolysis, glycolysis and protein metabolism is essentially unknown. Since the early observations that insulin can decrease the rise of the cyclic AMP level in adipose tissue [1–2] and in liver cells [3–4] due to sub-maximal concentrations of glucagon, there has been some effort to show a direct effect of insulin on the adenylate cyclase system.

It has been reported from different laboratories that insulin inhibited the glucagon stimulated adenylate cyclase activity of liver cell membranes [5–6]. This finding, however, could not be confirmed in several other laboratories [7–10] thus leaving the issue controversial.

The present work was aimed at reinvestigating the effect of insulin on the adenylate cyclase system of rat liver plasma membrane.

2. Materials and methods

2.1. *Materials*

Crystallized insulin and glucagon as well as ATP, GTP, Gpp(NH)p, isoproterenol, creatine phosphate and creatine phosphokinase were purchased from Sigma (St. Louis, Mo.). [α-^{32}P]ATP (20 Ci/mmol), [8-^{14}C]ATP (0.1 Ci/mmol) and cyclic [U-^{14}C]AMP (15 Ci/mmol) were obtained from the Radiochemical Centre (Amersham). Neutral aluminiumoxid was bought from Merck.

2.2. *Methods*

Liver plasma membranes were prepared from female PVG/c rats (6–8 weeks old) that had been starved overnight, according to the method of Neville [11].

In typical experiments, the assay mixture for the measurement of adenylate cyclase activity contained 0.5–1.0 mM [α-^{32}P]ATP (10–25 μCi/μmol), 4 mM $MgCl_2$, 1 mM cyclic AMP, 25 mM creatine phosphate, 60 μg creatine phosphokinase, 50 mM Tris–HCl pH 7.5 and 50–100 μg protein of liver plasma membrane in a final volume of 70 μl. Incubations were terminated according to the method of White [12] and cyclic AMP was separated by column chromatography on neutral alumina [12]. Under these conditions, the formation of cyclic AMP was linear with time up to 10 min.

Cyclic AMP dependent phosphodiesterase activity was measured in the above medium except that cyclic [U-^{14}C]AMP was substituted for [α-^{32}P]ATP. To measure the rate of ATP degradation (under the conditions of adenylate cyclase assay), [8-^{14}C]ATP was used.

ATP and its derivatives, including adenosine, were separated on polyethyleneimine impregnated cellulose plates (Merck) using 0.1 M LiCl as solvent.

Protein was determined according to the method of Lowry et al. [13] using bovine serum albumin as standard.

3. Results and discussion

Under the conditions described in the legend to

Table 1
Effect of insulin on adenylate cyclase activity of rat liver plasma membrane

Effector	pmol cyclic AMP/mg protein/10 min				
	Insulin				
	None	10^{-11} M	10^{-10} M	10^{-9} M	10^{-7} M
None	196 ± 19	202 ± 14	189 ± 12	209 ± 12	219 ± 14
Fluoride 15 mM	1581 ± 87	1612 ± 17	1636 ± 76	1603 ± 95	1696 ± 78
GTP 10^{-5} M	542 ± 18	–	514 ± 23	–	–
Gpp(NH)p 10^{-4} M	1349 ± 17	1336 ± 14	1344 ± 56	1346 ± 29	1276 ± 78
Isoproterenol 10^{-5} M	244 ± 5	–	216 ± 23	–	–
Isoproterenol 10^{-5} M + GTP 10^{-5} M	731 ± 23	702 ± 7	648 ± 27	633 ± 11	628 ± 9
Glucagon 10^{-7} M	613 ± 26	579 ± 16	485 ± 11	517 ± 13	532 ± 19
Glucagon 10^{-6} M	985 ± 17	951 ± 13	980 ± 28	926 ± 14	910 ± 29

Assay mixture in a final volume of 70 μl contained 1 mM [α-^{32}P]ATP (10 μCi/μmol), 4 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5, 1 mM cyclic AMP, 25 mM creatine phosphate, 60 μg creatine phosphokinase, 0.1% albumin, 76–81 μg of liver plasma membrane protein and other additions as indicated. Incubations were performed at 33°C for 10 min. Results are the mean ± SEM of two identically performed separate experiments, each assayed in triplicate

table 1, insulin at low concentrations (10^{-9}–10^{-10} M) inhibited the glucagon stimulated adenylate cyclase activity by about 20% if the concentration of the latter hormone was not higher than 10^{-7} M. In the presence of other activators, the activity of adenylate cyclase was less (with isoproterenol + GTP) or not (with fluoride, GTP or Gpp(NH)p) inhibited by insulin.

The reason for the absence of insulin effect at 10^{-6} M of glucagon, and for the lower inhibitory effect of 10^{-7} M of insulin is not clear. These aspects of insulin effect were observed by others too [5,6].

To determine the reproducibility of the results shown in table 1, several experiments were performed under varied conditions. As a rule it was found that after 5 min of incubation, insulin inhibited the (10^{-7} M) glucagon-stimulated adenylate cyclase activity only if the concentration of ATP was at least 1 mM and the amount of membrane protein was around 100 μg (data not shown). At lower concentrations of ATP and membrane protein significantly longer incubation times than 5 min were required to observe significant insulin effect. These suggested some indirect effects of insulin that could be: (a) stimulation of ATP degradation; (b) stimulation of adenosine formation that in turn would inhibit adenylate cyclase [14]; or (c) stimulation of cyclic AMP dependent phosphodiesterase. Neither of these processes were however influenced by 10^{-9}–10^{-10} M of insulin. These observations suggested that the presence of an ATP metabolite was obligatory for the inhibitory effect of insulin. Adenosine was a possible candidate for such a role because it represented more than 95% of the total ATP loss: during 10 min of incubation about 15% of ATP was metabolized, corresponding to more than 0.1 mM of adenosine if 1 mM ATP and 100 μg membane protein was used. Therefore the combined effects of insulin and adenosine were studied subsequently at relatively low concentrations of ATP and membrane protein. Adenosine, at the concentration of 10^{-4} M, itself inhibited the (10^{-7} M) glucagon-stimulated adenylate cyclase (table 2). In the presence of 10^{-9} M of insulin, however, the inhibition was significantly higher ($P<0.01$) than by adenosine alone (table 2). In these experiments the concentration of adenosine, formed from ATP, was 2×10^{-5} M at the end of 5-min incubation. The absence of effect of insulin when adenosine was not added externally indicates that adenosine at lower concentrations is not effective to promote the inhibitory action of insulin. It should be mentioned that at present it is not possible to clearly

Table 2
Combined effects of insulin and adenosine on the glucagon stimulated adenylate cyclase activity

Additions	pmol cyclic AMP/mg protein/5 min		
	None	Insulin (10^{-9} M)	*P*
Glucagon 10^{-7} M	647 ± 23	615 ± 29	n.s.
Glucagon 10^{-7} M + adenosine 10^{-4} M	515 ± 13	381 ± 36	<0 01

Assay mixture (70 μl) contained 0.4 mM [α-^{32}P]ATP (25 μCi/μmol), 4 mM $MgCl_2$, 1 mM cyclic AMP, 25 mM creatine phosphate, 60 μg creatine phosphokinase, 0.1% albumin, 50 mM Tris–HCl, pH 7.5, 58–66 μg of plasma membrane protein and other additions as indicated. Incubations were performed at 33°C for 5 min. Results are the mean ± SEM of five separate experiments (with the same plasma membrane preparation) each assayed in triplicate. The plasma membrane preparation was different from that used for table 1; n.s., not significant by Student's *t* test

distinguish whether the inhibition by adenosine is dissociated from its promoting effect on insulin action, or insulin merely sensitizes the cyclase system for adenosine.

While adenosine inhibited all the stimulated forms of adenylate cyclase activity (table 3), the combined inhibitory effect of adenosine and insulin could be consistently observed only in the case of 10^{-7} M glucagon stimulated cyclase. The data presented in table 1 and table 3 are highly comparable in that the inhibitory effect of insulin both alone (table 1) and in combination with adenosine (table 3) was the highest in the case of 10^{-7} M glucagon-stimulated adenylate cyclase. This provides evidence that adenosine is implicated in the inhibition of the glucagon-stimulated adenylate cyclase system by insulin.

On the other hand, comparison of data in table 1 and in table 3 indicates that adenosine may not play a role in the inhibition of (isoproterenol + GTP)-activated adenylate cyclase activity by insulin. The

Table 3
Combined effects of insulin and adenosine on different activated forms of adenylate cyclase

Additions	pmol cyclic AMP/mg protein/5 min			
	None	Insulin (10^{-9} M)	Adenosine (10^{-4} M)	Insulin (10^{-9} M) + adenosine (10^{-4} M)
None	174 ± 11	170 ± 16	161 ± 13	157 ± 11
Fluoride 15 mM	1638 ± 86	1664 ± 64	1312 ± 27	1235 ± 38
Gpp(NH)p 10^{-4} M	1176 ± 78	1167 ± 53	918 ± 38	916 ± 8
Isoproterenol 10^{-5} M + GTP 10^{-5} M	634 ± 14	662 ± 13	520 ± 14	478 ± 24
Glucagon 10^{-7} M	665 ± 4	649 ± 11	577 ± 31	409 ± 29
Glucagon 10^{-6} M	1260 ± 53	1204 ± 20	982 ± 61	840 ± 55

Assay mixture in a final volume of 70 μl contained 0.4 mM [α-^{32}P]ATP (25 μCi/μmol), 4 mM $MgCl_2$, 1 mM cyclic AMP, 25 mM creatine phosphate, 60 μg creatine phosphokinase, 0.1% albumin, 50 mM Tris–HCl, pH 7.5, 63 μg of plasma membrane protein and other additions as indicated. Incubations were performed at 33°C for 5 min. Results are the mean ± SEM of four incubations in one representative experiment. Plasma membrane preparation was the same that used for the experiment of table 2

small inhibitory effect of adenosine + insulin, seen in the presence of isoproterenol + GTP (table 3), did not prove to be significant in three other experiments ($P>0.2$). Thus it seems that time is the major factor in the inhibition of the (isoproterenol + GTP)-stimulated adenylate cyclase activity by insulin.

The dependence of insulin effect on the presence of divalent cations was also tested. The combined inhibitory effect of adenosine and insulin on the (10^{-7} M) glucagon-stimulated cyclase activity was not significantly influenced by changing the concentration of Mg^{2+} between 2–10 mM. On the other hand, when the plasma membranes were preincubated with EGTA for 5 min at room temperature, the inhibitory effect of 10^{-4} M of adenosine was of the same extent as in previous experiments, however, insulin did not cause further inhibition (data not shown). This indicates that Ca^{2+}, bound to the plasma membrane, also plays role in the inhibitory effect of insulin.

In summary, present results confirm those data [5–6] that demonstrated the inhibitory effect of insulin on the glucagon stimulated adenylate cyclase activity. Such inhibition proved to require the presence of both adenosine and Ca^{2+}. These new findings can be important for further studies on the structural relationship between the insulin-receptor complex and the adenylate cyclase system.

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