INSULIN INHIBITION OF MEMBRANE-BOUND ADENYLATE CYCLASE IN *NEUROSPORA CRASSA*

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1. Introduction

The molecular basis of insulin action is one of the most exciting problems in the well developed field of hormonal control of metabolism. Since the report that insulin increases cell permeability for sugars [1], a lot of work has been carried out searching for a unitary hypothesis on the mechanisms of insulin action [2–4]. Very early it became apparent that not all the cell responses to insulin could be explained on the basis of selective increase in cell permeability [5]. The pioneer work of Sutherland's group on cyclic 3′,5′-AMP and its role in the amplification of hormonal signals at the cell membrane level marked a crucial step in the elucidation of the mechanisms of hormonal action [6, 7]. It seemed that under certain conditions, insulin is able to decrease intracellular cyclic 3′,5′-AMP levels [8, 9].

The fact that after cell disruption most of the insulin effects are lost, was a great obstacle in the elucidation of the problem. However, some results obtained with cell-free preparations indicated that insulin would act by decreasing intracellular cyclic 3′,5′-AMP levels through the activation of a specific membrane-bound phosphodiesterase [10, 11]. In the course of our studies on the control of cyclic 3′,5′-AMP metabolism in *Neurospora crassa* the occurrence of a plasma membrane-bound adenylate cyclase was detected [12, 13]. The substrate for this cyclase is the Mn^{2+}–ATP complex, and free Mn^{2+} ions activate the enzyme [14]. On the other hand, as reported elsewhere, this enzyme is stimulated by glucagon [15].

In this paper evidence is presented that the membrane-bound adenylate cyclase in *Neurospora crassa* is also under insulin control.

2. Materials and methods

Most of the methods employed in this work were described in the preceding papers [13–16].

The slime mutant of *N. crassa* (strain F_z:O_s-1-N1118-FGSC) was used throughout this work. This mutant, devoid of a cellular wall, grows as protoplasts surrounded by a plasma membrane. The organism was grown in Vogel's liquid minimal medium supplemented with 2% sucrose, 0.75% nutrient broth, and 0.75% yeast extract. The cells obtained from a 24 hr liquid culture were collected by centrifugation at 900 *g* for 7 min. The supernatant fluid was decanted and the cellular pellet was resuspended in 1 mM $NaHCO_3$ (one tenth of the culture volume). The suspension was left in the cold for 30 min and centrifuged for 20 min at 15,000 *g*. The supernate thus obtained was spun down 120 min at 105,000 *g*. After this step, more than 80% of the adenylate cyclase activity was recovered in the precipitate. The pellet thus obtained was resuspended in 1 mM $NaHCO_3$ and used as enzyme. Unless otherwise indicated the standard incubation mixture for adenylate cyclase assay contained 100 mM piperazine-*N*,*N*′-bis-2-ethane-sulphonic acid-NaOH buffer, pH 6.35; 0.50 mM $MnCl_2$; 0.5 mM α-^{32}P-labelled ATP (25–50 μCi); and enzyme (0.1 to 0.2 mg protein). The total volume was 0.1 ml. Incubations were carried out at 37° for the indicated periods. Reactions were stopped and cyclic adenylate was isolated as de-

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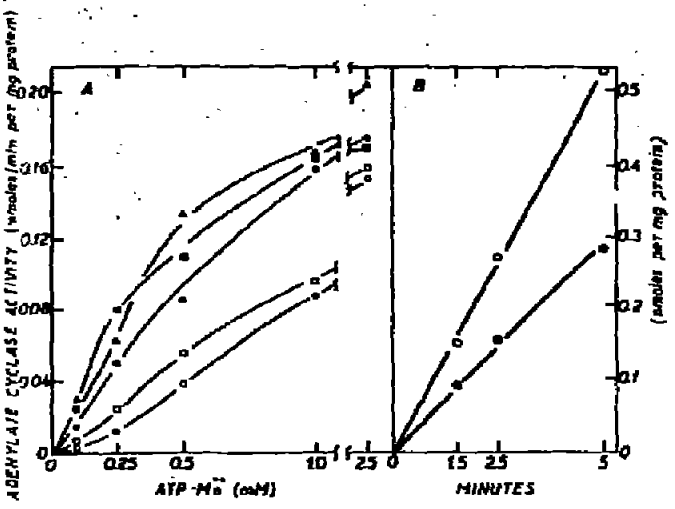

Fig. 1. A) Adenylate cyclase activity as a function of Mn^{2+}–ATP concentration. The mixture for the assay of adenylate cyclase contained the concentration of ATP and $MnCl_2$ indicated on the abscissa, plus the following additions: (●–●–●) none; (○–○–○) 100 μunits/ml insulin (pork, recrystallized); (■–■–■) 10^{-6} M glucagon; (□–□–□) 100 μunits/ml insulin plus 10^{-6} M glucagon; and (△–△–△) 2 mM $MnCl_2$. The incubation time was 2.5 min. B) Time course of the adenylate cyclase reaction carried out in the absence (○–○–○) or presence (●–●–●) of insulin (amorphous porcine, 100 μunits/ml). Other conditions were as described under Materials and methods.

scribed elsewhere [13].

Porcine amorphous (zinc-free) insulin (21 units/mg; lot 192-2358-188) and recrystallized trypsin-treated (glucagon-free) pork insulin (24 units/mg; lot P.J. 5589) were obtained from Eli Lilly and Co. (Indianapolis, USA) through the courtesy of Dr. O.K. Behrens. Standard bovine crystalline insulin (25 units/mg) was obtained from Eli Lilly (Buenos Aires, Argentina). Glucagon samples were a gift of Dr. L. Birnbaumer (N.I.H., Bethesda) and Dr. Anderson (Lilly, Argentina). Vasopresin was from Parke-Davis, and bovine growth hormone was a gift of Dr. Dellacha and Dr. Santome (Facultad de Farmacia y Bioquimica, Buenos Aires). The preparation of insulin A and B chains was carried from a sulfitolyzed bovine insulin according to the procedure of Meek and Bolinger [17].

3. Results and discussion

3.1. *Insulin effect on Neurospora adenylate cyclase*

Table 1
Effect of different substances on the activity of Neurospora adenylate cyclase.

Additions	Adenylate cyclase activity (nmoles/min per mg protein)
None	0.076
100 μU/ml insulin (4 ng/ml)	0.033
1 mM cyclic AMP	0.072
1 mM cyclic AMP plus 100 μU/ml insulin	0.030
10 mM theophylline	0.069
10 mM theophylline plus 100 μU/ml insulin	0.034
1 mM cyclic AMP plus 10 mM theophylline	0.069
1 mM cyclic AMP, 10 mM theophylline plus 100 μU/ml insulin	0.036
10^{-4} M $ZnCl_2$	0.080
10^{-5} M $ZnCl_2$	0.067
A chain (4 ng/ml)	0.090
A chain (40 ng/ml)	0.078
A chain (400 ng/ml)	0.078
B chain (4 ng/ml)	0.067
B chain (40 ng/ml)	0.072
B chain (400 ng/ml)	0.072
A chain plus B chain (4 ng/ml)	0.067
A chain plus B chain (40 ng/ml)	0.071
A chain plus B chain (400 ng/ml)	0.071
7×10^{-8} M Vasopresin	0.080
7×10^{-8} M Vasopresin plus 100 μU/ml insulin	0.030
2.5×10^{-10} M Growth hormone	0.085
2.5×10^{-10} M Growth hormone plus 100 μU/ml insulin	0.032

Conditions were as those indicated under Materials and methods. The incubation time was 2.5 min. Bovine crystalline insulin was used.

As can be see in fig. 1b, insulin decreases the rate of the cyclase catalyzed reaction. The following evidence indicates that this effect is not essentially a biochemical "artifact" of limited or trivial significance:

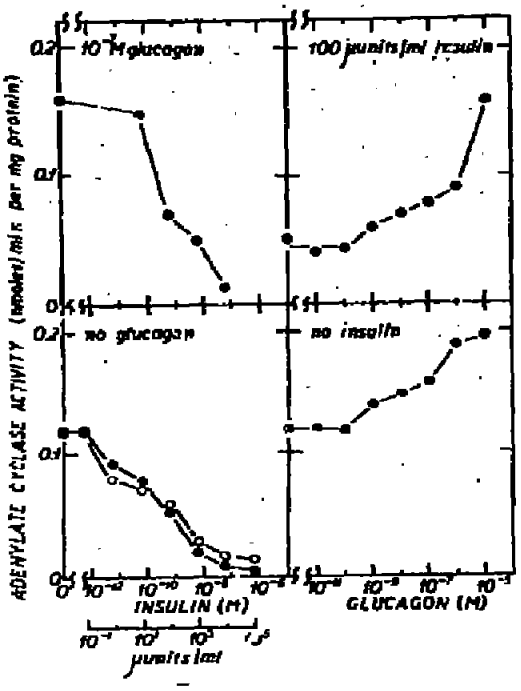

Fig. 2. Left: Adenylate cyclase activity as a function of insulin concentration in the absence (lower) or in the presence (upper) of 10^{-7} M glucagon. The experiment was carried out with two types of insulin: porcine, crystalline (glucagon-free) (●–●–●) or porcine amorphous (zinc-free) (○–○–○). Right: Adenylate cyclase activity as a function of glucagon concentration in the absence (lower) or in the presence (upper) of 100 μunits/ml porcine, crystalline (glucagon-free) insulin.

i) The effect does not seem to be due to an increased degradation of the cyclic AMP formed since the inhibitory effect is not modified by the addition of 3',5'-AMP or theophylline to the assay mixture (table 1). ii) The inhibition cannot be due to the Zn^{2+} usually present in insulin preparations; in fact one of the preparations used in these experiments was an amorphous zinc-free sample (see fig. 2). On the other hand concentrations of this cation of about 10^{-4} M had no effect on *Neurospora* cyclase (table 1). iii) The effect was not observed when insulin was replaced by other polypeptide hormone carrying disulfide bridges such as vasopresin or growth hormone (table 1). iv) Isolated insulin A and B chains did not modify the activity of *Neurospora* adenylate cyclase (table 1). v) Half maximum inhibition was observed at insulin concentrations between 30 and 60 μunits/ml ($1-2 \times 10^{-10}$ M) but a significant effect occurs in the range between 1 and 10 μunits/ml (7×10^{-12} to 7×10^{-11} M) (fig. 2). These low hormone levels that inhibit the cyclase catalyzed reaction are in the range considered as physiological in animal cells [18, 19].

In fact the curves of *Neurospora* adenylate cyclase activity as a function of insulin concentration are similar to those reported for insulin inhibition of glycerol release from adipose tissue [20] or stimulation of amino acid incorporation by isolated fat cells [21]. On the other hand, the insulin response curves are extended on a range covering at least four orders of magnitude. This would suggest that more than one kinetic component is formed as a consequence of the interaction between *Neurospora* membranes and insulin. vi) The insulin effect is partially reverted by glucagon, a well known metabolic antagonist of insulin action (fig. 2). vii) The opposite is also true; insulin counteracts the cyclase activation by glucagon (fig. 2). At an insulin concentration of 100 μunits/ml or higher the requirement for glucagon to give half maximum effect increases more than ten-fold.

As a first approach to the study of the kinetic basis of hormone effects on *Neurospora* adenylate cyclase, the reaction was studied as a function of Mn^{2+}–ATP concentration (fig. 1a). From the results summarized in table 2, the following conclusions are evident:

1) Insulin decreases adenylate cyclase activity at low but not at high concentrations of Mn^{2+}–ATP. Kinetically the insulin behaves as a "pseudo competitive inhibitor"; that is, it increases the $L_{0.5}$ value (from 0.64 to 1.4) but does not affect the slope of Hill plots.

2) Glucagon increases cyclase activity at low but not at high concentration of Mn^{2+}–ATP. The value for n in the Hill plots decreases from 1.67 in the absence of glucagon to 1.2 in its presence. Under these conditions the substrate concentration giving half maximum activity ($L_{0.5}$) decreases slightly (from 0.64 to 0.47 mM). Therefore, it seems that glucagon elicits on the enzyme the same kinetic effects of Mn^{2+}.

3) When both hormones act together, the effect obtained is a compromise between the extreme cases in which the action of each hormone is tested individually.

It is concluded that the effect of insulin on intracellular cyclic 3',5'-AMP levels could be explained in terms of an inhibition of the synthesis of this nucleotide. The possibility exists that in simple eukaryotic cells, membrane-bound enzyme systems are more

Table 2
Kinetic parameters of adenylate cyclase catalyzed reaction by *Neurospora crassa* enzyme.

Ligand	Effector			$L_{0.5}$ (mM)	n
	Mn^{2+}	Glucagon	Insulin		
Mn^{2+}–ATP	—	—	—	0.64	1.67
	2 mM	—	—	0.48	1.25
	—	10^{-6} M	—	0.47	1.2
	—	—	100 μU/ml	1.40	1.67
	—	10^{-6} M	100 μU/ml	1.25	1.40

resistant to cell disruption, and for that reason they maintain unaffected some properties such as those required for insulin action.

In addition other aspects of metabolic regulation in *Neurospora* are similar to those of animal cells [12, 22], confirming previous observations carried out in this laboratory [23, 24]. Moreover, results not shown here indicated that insulin increases cell permeability for glucose and glycogen deposition in slime cells.

Recent evidence coming from other laboratories indicated that insulin partially counteracts the activation of adenylate cyclase of liver or fat cell membranes by glucagon or epinephrine [25, 26].

The results reported in this paper showed that at least in *Neurospora crassa* insulin is able to modulate adenylate cyclase activity in the absence of other hormones through a mechanism different from that reported by other authors [10, 11], namely, stimulating a specific phosphodiesterase.

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