Activation of Membrane-Bound Adenylate Cyclase by Glucagon in *Neurospora crassa*

(glycogen synthetase/glycogen phosphorylase)

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ABSTRACT Membrane-bound adenylate cyclase in *Neurospora crassa* is activated by glucagon. Half-maximal effect is observed at hormone concentrations of about 10 nM. After solubilization of the enzyme with Lubrol-PX, the glucagon effect is lost. Incubation of *Neurospora* cells with glucagon leads to a decrease in the activity of glycogen synthetase (EC 2.4.1.11) and to an increase in the activity of glycogen phosphorylase (EC 2.4.1.1) and in the rate of glycogenolysis.

Adenylate cyclase, the enzyme responsible for the synthesis of adenosine 3':5' cyclic monophosphate (cyclic AMP) from ATP (1), is associated with membrane fractions of animal cells from two different phyla: *echinodermata* (2) and *chordata* (for reviews see refs. 3 and 4). In animal cells adenylate cyclase is stimulated by different hormones, showing a wide range of specificity, and seems to be associated with the plasma membrane (5–7) where the receptors for some hormones are localized (8–10).

Recent evidence indicates that, in the ascomycete fungus *Neurospora crassa*, some of the mechanisms designed for the

amplification of environmental signals seem to be well evolved at the level of glycogen metabolism. Glycogen synthetase (EC 2.4.1.11) and phosphorylase (EC 2.4.1.1) have two interconvertible forms, and the extent of activation of the phosphorylase is under the control of cyclic AMP (11, 12). In addition, adenylate cyclase has been found in particulate fractions from *Neurospora*. This fraction is enriched with a cellular component showing the structure of plasma membrane. An account of the properties of this adenylate cyclase system will be published elsewhere (13).

This paper reports evidence indicating that glucagon activates adenylate cyclase in *Neurospora crassa* membranes and increases the rate of glycogenolysis in fungal cells.

EXPERIMENTAL PROCEDURE

Cell cultures

The slime mutant of *Neurospora crassa* (strain Fz:Os-1:N1118-FGSC) (14) was used throughout this work; it grows as isolated protoplasts surrounded by a plasma membrane; the cellular wall characteristic of the mycelial strain is absent. *Neurospora* was grown in Vogel's liquid minimal medium (15) supplemented with 2% sucrose, 0.75% yeast extract, and 0.75% nutrient broth (16), in 1000-ml erlenmeyer flasks containing 200 ml of medium. 10^7 cells from a solid medium (the same medium but containing 1.5% agar) culture were inoculated into flasks and incubated for 18 hr at 32° in a rotatory shaker (120 cycles/min).

Enzyme preparations

The cells obtained from a liquid culture were collected by centrifugation at 900 × *g* for 7 min at 4°. The supernatant was decanted and the cellular precipitates were suspended in 1 mM NaHCO₃ (0.1 culture volume). The suspension was left at 2° for 30 min, then centrifuged for 20 min at 15,000 × *g*. Lubrol-PX was added to a portion of the supernatant, to a final concentration of 1.3% (v/v). The mixture was centrifuged for 120 min at 105,000 × *g*. After this step, more than 80% of the cyclase activity was recovered in the precipitate ("membrane-bound" enzyme) from the preparation without Lubrol; more than 80% of the adenylate cyclase activity remained in the supernatant fluid from the preparation with Lubrol ("solubilized" enzyme) (13).

Assays of glycogen synthetase and phosphorylase were performed on enzyme preparations as follows: aliquots of 20 ml from the liquid cultures were cooled in ice. The cells were then
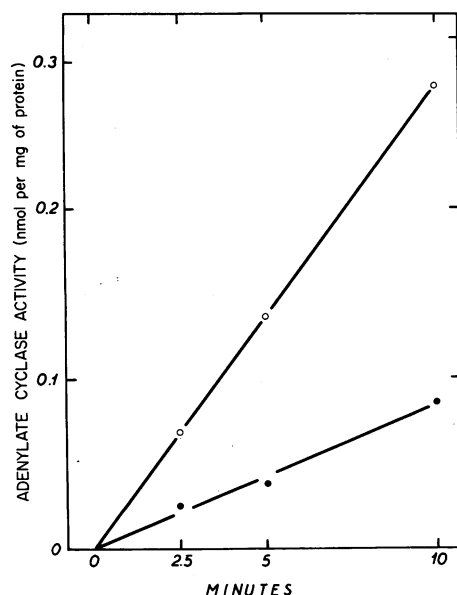

Fig. 1. Rate of the adenylate cyclase-catalyzed reaction with "membrane-bound" enzyme. Reactions were performed in the presence (O——O) or absence (●——●) of 0.1 μM glucagon. Each assay mixture contained 0.05 mg of enzyme protein.

TABLE 1. *Effect of different concentrations of ATP and Mn^{++} on the glucagon activation of membrane-bound adenylate cyclase*

ATP (mM)	$MnCl_2$ (mM)	Enzyme activity	
		Without glucagon	With glucagon
		pmol per min per mg of protein	
0.25	0	0	0
0.25	0.25	5	15
2.50	2.50	108	115
0.25	0.75	38	40

The incubation time was 2.5 min. The concentration of glucagon when added, was 0.1 μM. Other conditions were as described in *Experimental Procedure*.

collected by centrifugation for 5 min at 10,000 × g, and the pellets were suspended in 1 ml of a cold buffer solution containing 50 mM Tris·HCl (pH 7.4)–10 mM 2-mercaptoethanol–50 mM NaF–20 mM EDTA. The suspensions were homogenized with a glass–Teflon homogenizer until the cells were disrupted. Afterwards, the homogenates were filtered through Sephadex G-25 columns equilibrated with buffer solution (11).

Enzyme assay

Adenylate Cyclase. Unless otherwise indicated, the incubation mixture for assay of adenylate cyclase contained: 100 mM piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (pH 6.35)–0.25 mM $MnCl_2$–0.25 mM [α-^{32}P] ATP (25 μCi)–enzyme (0.1–0.2 mg of protein) and other additions, as specified. Total volume was 0.1 ml. Incubations were at 37° for the indicated periods. Reactions were stopped (6) and the cyclic adenylate was isolated (17).

Glycogen Synthetase was assayed in the absence or presence of 2 mM glucose 6-phosphate (11). The activity measured in the absence, as compared to that in the presence, of glucose 6-phosphate is the ratio of independence.

Glycogen Phosphorylase was measured in the absence of added 5'-AMP (12).

Isolation of glycogen

2-ml Aliquots of the liquid cultures were centrifuged for 15 min at 12,000 × g, and the pellets were taken up in 33% KOH and heated for 15 min at 100°. Glycogen was isolated from these alkali-treated samples by ethanol precipitation (18); radioactivity was determined in Bray's scintillation fluid (19).

Materials

Glucagon was generously supplied by Dr. Lutz Birnbaumer (National Institutes of Health, Bethesda, Md.). Lubrol-PX was obtained from Duperial-Imperial Chemical Industries (Buenos Aires) and D-[U-^{14}C]glucose was obtained from the Radiochemical Center (Amersham, England). [α-^{32}P]ATP was prepared by enzymatic phosphorylation of labeled 5'-AMP. 5'-[^{32}P]AMP was synthesized by a reaction between isopropylidene adenosine and ^{32}Pi in the presence of trichloroacetonitrile and triethylamine by a modification (13) of the method of Greenless and Symons (20).
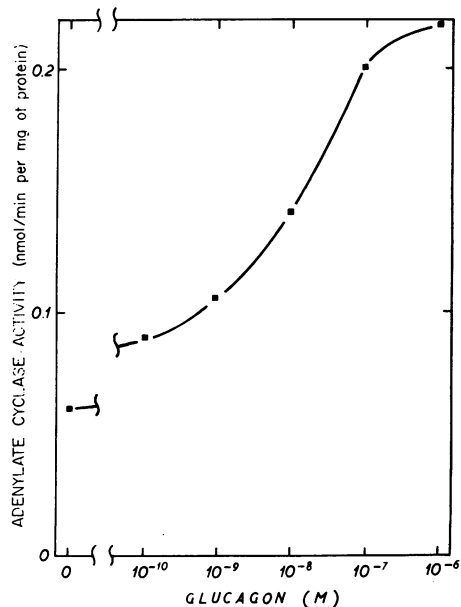

FIG. 2. "Membrane-bound" adenylate cyclase activity as a function of glucagon concentration. The incubation time was 2.5 min.

RESULTS AND DISCUSSION

Glucagon increases the rate of the reaction catalyzed by cyclase. At low concentrations of membrane protein, the rate of this reaction is roughly linear, in either the presence or absence of glucagon (Fig. 1). On the other hand, the activation does not require prior incubation of the membranes with
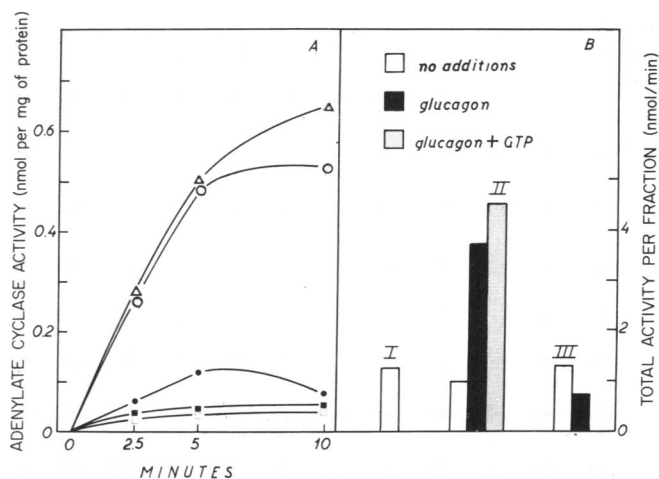

FIG. 3. Effect of glucagon on the "membrane-bound" and "solubilized" preparations of *Neurospora crassa* adenylate cyclase. (*A*) Rate of the reaction. Assays of the "membrane-bound" enzyme: no additions (●——●); glucagon (○——○); glucagon plus GTP (△——△). Assays of the "solubilized" preparation: No addition (■——■); glucagon (□——□). (*B*) Total activity of the fractions assayed under different conditions. *I*, 15,000 × g supernatant; *II*, "membrane-bound" enzyme; *III*, "solubilized" enzyme. Assays were performed for 2.5 min in the presence of the additions indicated. The amount of enzyme protein per assay was 0.2, 0.5, and 0.1 mg for the 15,000 × g supernatant, "membrane-bound," and "solubilized" preparations, respectively. When added, the concentration of glucagon and GTP was 1 μM and 25 μM, respectively.
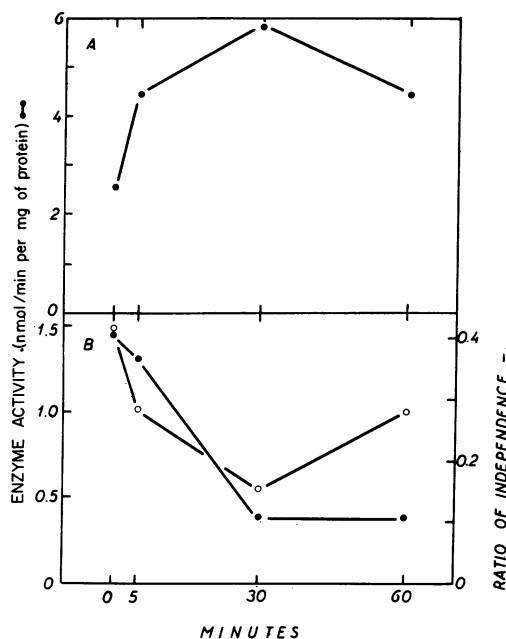
FIG. 4. Effect of glucagon on glycogen phosphorylase and synthetase in the slime strain of *N. crassa*. The cells were grown in a liquid medium (200 ml) for 18 hr. After that, an aliquot (20 ml) was taken from the culture and, at time zero, glucagon was added to the culture, to a final concentration of 2.5 nM. At the indicated times, 20-ml aliquots were taken; glycogen phosphorylase (12) (*A*) and synthetase (11) (*B*) were assayed.

glucagon. In fact, this treatment tends to diminish the effect of the hormone.

Activation of the adenylate cyclase by glucagon is observed at low equimolar concentrations of ATP and Mn^{++} (0.25 mM). When the concentrations of both components are increased up to 2.5 mM each, or when Mn^{++} concentration is three times that of ATP, the effect on the rate of the reaction is negligible (Table 1).

Fig. 2 shows a plot of the reaction rate as a function of glucagon concentration. Half-maximum activation was obtained at 10 nM glucagon, and a significant response was observed at 0.1 nM glucagon. The response curve of the *Neurospora* adenylate cyclase is strikingly similar to that of liver-cell membranes (7), which exhibit greater sensitivity to glucagon—about 10-fold higher—than do fat-cell membranes (8).

As reported above, treatment of the membranes with the nonionic detergent-Lubrol-PX-solubilizes the enzyme after centrifugation for 120 min at 105,000 × *g* (13). This "solubilized" cyclase has similar kinetic properties to those of the membrane-bound cyclase; however, as can be seen in Fig. 3, the solubilized cyclase is not activated by the hormone. This result, and similar observations with animal enzymes (7, 8, 21) would confirm the supposition that the hormone receptor is not a component of the cyclase molecule.

On the other hand, recent evidence showed that guanine nucleotides increase the glucagon stimulation of adenylate cyclase activity in liver membranes (22). As is shown in Fig. 3, GTP slightly enhances the stimulation of cyclase activity by the hormone.

The results shown here have some interesting implications regarding the evolution of hormone receptors. It seems that
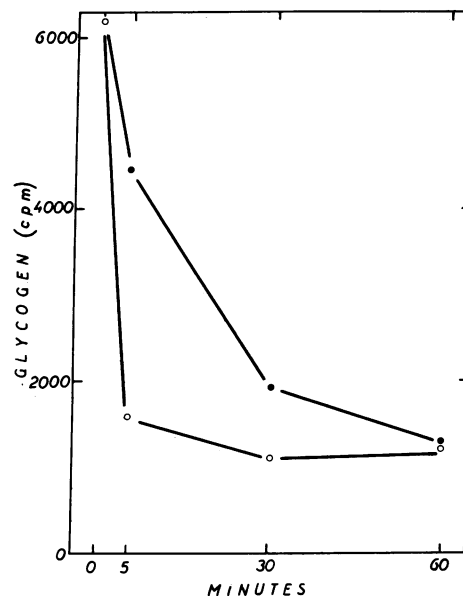

FIG. 5. Effect of glucagon on the rate of glycogenolysis in the slime strain of *N. crassa*. A cell culture was grown for 18 hr as described in Fig. 4, and centrifuged (5 min at 9000 × *g*). The pellet was suspended in 200 ml of Vogel's minimal medium (15) containing 0.75% yeast extract, 0.75% nutrient broth, and [^{14}C]glucose (10 μCi; 261 Ci/mol). The culture was incubated for 60 min and centrifuged. Labeled cells were suspended in 20 ml of Vogel's medium containing yeast extract and nutrient broth, and the suspension was equally distributed in two 125-ml Erlenmeyer flasks. A 2-ml aliquot was taken from each flask, and, at time zero, glucagon was added to one of the suspensions, to a final concentration of 20 nM. The flasks were further incubated and, at the indicated times, 2-ml aliquots were taken. Glycogen was isolated from these aliquots and counted for radioactivity. ●——●, control; O——O, glucagon.

similar hormone receptors are present in animal and fungal cells, suggesting that certain proteins of these cells evolved from a common ancestral gene.

A definitive proof of the similarities between control mechanisms of *Neurospora crassa* and animal cells would be obtained by the demonstration of a glycogenolytic response of the entire cells to glucagon. Previous evidence indicated that glycogenolysis may be under the control of cyclic AMP (12). The experiments shown in Figs. 4 and 5 corroborate this presumptive conclusion, since addition of the hormone leads to the following changes: (*a*) Increase in the rate of glycogenolysis; (*b*) increase in the activity of glycogen phosphorylase; and (*c*) decrease in the activity of glycogen synthetase.

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