Proc. Nat. Acad. Sci. USA
Vol. 68, No. 7, pp. 1631–1633, July 1971

Hormone-Controlled Synthesis of Endoplasmic Reticulum in Barley Aleurone Cells

(gibberellic acid/abscisic acid)

WARREN H. EVINS* AND J. E. VARNER

Department of Biochemistry and MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Mich. 48823

Communicated by Anton Lang, May 14, 1971

ABSTRACT **The rate of synthesis of the endoplasmic reticulum in barley aleurone cells after treatment with gibberellic acid was determined by measurement of [^{14}C]-choline incorporation into acid-insoluble material in a semipurified fraction containing the endoplasmic reticulum. 94% of the ^{14}C incorporated into this fraction is extractable by lipid solvents and only 9% is removed by procedures for nucleic acid extraction. Gibberellic acid increases the rate of synthesis of the endoplasmic reticulum 4- to 8-fold, starting about 4 hr after addition of the hormone (at about the same time as polysome formation). Abscisic acid inhibits this gibberellic acid-enhanced increase in the rate of synthesis of endoplasmic reticulum.**

Isolated barley aleurone layers respond to exogenous gibberellic acid by synthesizing α-amylase and protease after an 8- to 10-hr lag period (1–3); abscisic acid prevents these characteristic responses to gibberellic acid (4, 5). During the lag period, gibberellic acid causes an increase in the number and proportion of ribosomes that can be isolated as polysomes in the hormone-treated cells, starting 3-4 hr after hormone addition (Evins, manuscript in preparation). The polysomes are, *in vivo*, probably bound to the endoplasmic reticulum (ER), because electron microscopy studies show extensive development of the rough ER in the aleurone cells of germinating barley (6) as well as changes in the rough ER of isolated barley aleurone layers during both the lag and synthesis phases of α-amylase production (7, 8, and personal communication from E. L. Vigil and M. Ruddat, University of Chicago). However, the quantitative determination of differences in the rate of ER synthesis between hormone-treated and control tissues and the time of the onset of ER synthesis after hormone addition are difficult to establish by electron microscopy. We now report the rate of ER synthesis estimated by the measurement of [^{14}C]choline incorporation into a semipurified ER fraction (method of Nagley and Hallinan, ref. 9). With this method it can be shown that gibberellic acid increases the rate of ER synthesis 4- to 8-fold. This increase starts at about the same time as polysome formation. Abscisic acid inhibits the gibberellic acid-enhanced increase in the rate of ER synthesis within 2 hr after addition.

METHODS

For each sample, 10–40 aleurone layers were prepared by the methods of Chrispeels and Varner (1) from barley seeds (*Hordeum vulgare* L. cv. Himalaya) and incubated for the times specified in 5 ml of 1 mM Na acetate buffer (pH 4.8)–20 mM $CaCl_2$–1 μM gibberellin A_3 on a Dubnoff metabolic shaker at 25°C.

The method of Nagley and Hallinan (9) was used to measure the rate of synthesis of ER. At the end of the incubation the aleurone layers were transferred for 30 min to a medium containing 5 μCi of [*methyl*-^{14}C]choline chloride (New England Nuclear Corp., 8.25 Ci/mol) and either 10 mg of neutralized casein acid hydrolysate (per 40 layers) or 1 mM L-serine and 1 mM L-methionine (A grade, Calbiochem). Trichloroacetic acid-precipitable membrane material in a semipurified ER fraction was collected on a Millipore filter and dried at 70°C, and its radioactivity was determined in 10 ml of scintillation fluid A (4 g of PPO + 100 mg of POPOP per liter of toluene).

The ER fraction was prepared after homogenization of the cells by a modified procedure (Evins, manuscript in preparation) of Wettstein *et al.* (10) and Staehelin *et al.* (11). After homogenization, a 10-min centrifugation at 4000 × *g* (first pellet), and a 15-min centrifugation at 10,000 × *g* (second pellet), the supernatant was layered onto a discontinuous sucrose density gradient and centrifuged for 12 hr at 50,000 rpm in a Beckman 65 or Ti50 rotor. The discontinuous gradient was composed of a bottom layer of 3.5 ml of 1.6 M sucrose buffer, containing ribonuclease-free sucrose and 50 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.55, with 25 mM K^+–2 mM Mg acetate–7 mM 2-mercaptoethanol (0.5 μl/ml), a middle layer of 0.6 M sucrose buffer, and in the top layer, the 10,000 × *g* supernatant fraction in 0.45 M sucrose. The pellet (thrid) was resuspended in 10% trichloroacetic acid (w/v), filtered on a Millipore filter, and washed with 50 ml of 5% trichloroacetic acid containing carrier choline chloride.

Phospholipids were extracted by the method of Bieber *et al.* (12, 13), modified as follows. The resuspended pellet (third) was extracted twice with 15 volumes of chloroform–methanol 1:1 containing 0.02% of butylhydroxytoluene (an antioxidant), and the organic layer was collected with a disposable Pasteur pipet after centrifugation. The extraction was repeated twice with chloroform–methanol 2:1. The four extracts were combined, washed twice with 0.2 volumes of 0.1% $MgCl_2$–0.8% NaCl, evaporated to dryness in an air stream, and counted in scintillation fluid A. Protein was measured by the method of Lowry *et al.* (14). When necessary for the estimation of protein concentration, 2-mercaptoethanol was removed by dialysis.

Abbreviation: ER, endoplasmic reticulum.

* Present address: The Rockefeller University, New York, N.Y. 10021

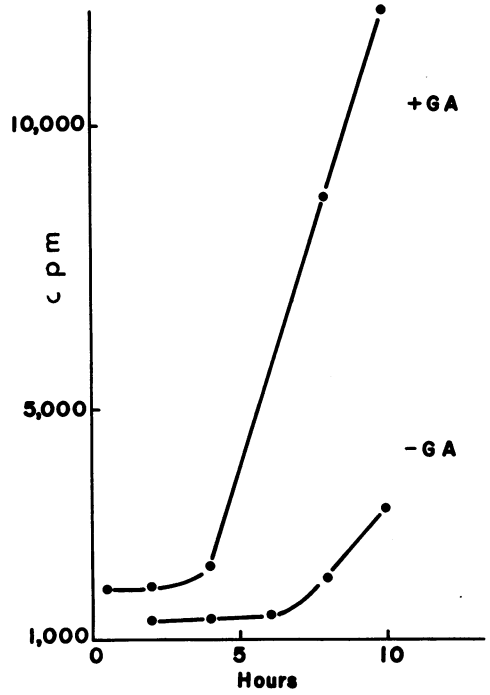

FIG. 1. Stimulating effect of gibberellic acid (GA) on the rate of endoplasmic reticulum synthesis in barley aleurone layers. 40 barley aleurone layers were incubated at 25°C for various times in 1 mM acetate buffer (pH 4.8)–20 mM $CaCl_2$ with (+GA) or without (−GA) 1 μM gibberellic acid (gibberellin A_3). The aleurone layers were then transferred to a medium containing 5 μCi of [*methyl*-^{14}C]choline and casein acid hydrolysate for the last 30 min of incubation. A semipurified ER fraction was prepared as described in *Methods*. Trichloroacetic acid-precipitable membrane material in this fraction was collected on a Millipore filter and counted. Each point is the average of duplicate samples. Similar results were obtained in three experiments.

RESULTS

After a 30-min labeling period, at different times after gibberellic acid treatment, the specific radioactivity of the trichloroacetic acid-insoluble protein of the microsomal fraction (referred to as the semipurified ER fraction) was 10 times that of other subcellular fractions (Table 1). Longer periods of labeling lead to a greater proportion of activity in other subcellular fractions; this is also true in rat liver (15). The counts in the microsomal fraction are soluble in lipid solvents and not removable by extraction procedures for nucleic acid (Table 2).

More [^{14}C]choline is incorporated into the microsomal fraction isolated from aleurone layers treated with gibberellic acid than into that from control layers (Fig. 1). The ratio between the slopes of the two curves (*with* versus *without* gibberellic acid) varied in different experiments between 3 and 8, as did the ratios of the amount of α-amylase produced later. The increase in ^{14}C incorporated into ER isolated from cells treated with gibberellic acid starts about 4 hr after hormone addition (an increase was seen at 2 hr in some experiments). The increase in [^{14}C]choline incorporation into control tissues begins later and is much slower.

The gibberellic acid-controlled increase in the rate of ER syntheis is inhibited by abscisic acid (Table 3). Abscisic acid added at the same time as gibberellic acid causes a complete

TABLE 1. [^{14}C]*Choline incorporation into acid-insoluble material in various cell fractions*

	Specific activity (cpm/mg protein)	Relative specific activity (% of microsomal fraction)
Microsomal fraction	2500	100.0
Supernatant fraction	234	9.5
First pellet (40,000 *g*-min)	65	2.6
Second pellet (150,000 *g*-min)	305	12.2

Forty aleurone layers were labeled for 30 min with [*methyl*-^{14}C]choline after 8 hr of incubation at 25°C. Results are the averages of triplicate samples.

inhibition of the increased ER synthesis after 6 hr of incubation. However, when abscisic acid is added during the latter part of the incubation period with gibberellic acid, there is a complete inhibition of the increased ER synthesis within 2 hr or less. Choline uptake into the aleurone cells was determined by measuring the amount of ^{14}C present in the acid-soluble supernatant fraction. The addition of abscisic acid to the incubation medium containing gibberellic acid did not affect choline uptake at any time. However, in the absence of both hormones, a 20% increase in choline uptake by the aleurone cells is observed.

DISCUSSION

In general, proteins to be secreted are synthesized on membrane-bound polysomes (16, 17). Many secretory cells, including aleurone cells, endocrine gland cells (anterior pituitary, pancreatic islets, and secretory neurons), and exocrine gland cells (salivary, Brunner's gland cells, pancreatic acinar cells, goblet cells of the intestinal mucosa, mucus and chief cells of the gastric glands) have large amounts of rough ER (18). Proliferation of ER frequency accompanies hormone-induced changes in growth and development (19, 20).

In the aleurone layers ER synthesis, as estimated by [^{14}C]-choline incorporation, starts about 4 hr after the addition of gibberellic acid, at about the same time as polysome formation. This increase in ER synthesis precedes and is very probably required for the gibberellic acid-induced synthesis

TABLE 2. *Removal of acid-insoluble radioactivity from the choline-labeled microsomal fraction by extraction procedures for lipids and nucleic acids*

	cpm remaining	% remaining	cpm removed	% removed
Microsomes	2890	100.0	—	—
In lipid extract	2710	93.9	176	6.1
Microsomes after nucleic acid extraction*	2620	90.7	269	9.3

Forty aleurone layers were incubated for 5 hr with 1 μM gibberellic acid at 25°C. Results are averages of triplicate samples. Similar results were obtained in two experiments.

* 5% trichloroacetic acid, 95°C, 30 min.

TABLE 3. *Effect of abscisic acid (ABA) on the rate of ER synthesis*

Treatment	cpm choline incorporated	% inhibition
−GA	3,160	—
+GA	10,200	—
+GA + ABA (2 hr)	3,200	100
+GA + ABA (6 hr)	2,800	112

Thirty aleurone layers were labeled for 30 min with [*methyl*-^{14}C]-choline after 6 hr of incubation at 25°C with (+GA) or without (−GA) 1 μM gibberellic acid (GA) as described in *Methods*. 0.25 μM abscisic acid (ABA) was present from the start (6 hr) or was added 4 hr after the start (2 hr) of incubation with gibberellic acid. The amount of trichloroacetic acid-insoluble radioactivity incorporated into the choline-labeled microsomal fraction was measured and the percent inhibition of the amount of ^{14}C incorporated was calculated. Results are averages of duplicate samples. Similar results were obtained in three experiments.

and release of hydrolytic enzymes. Actinomycin D and cycloheximide prevent the increase in the rate of ER synthesis at concentrations that prevent the synthesis of hydrolytic enzymes (unpublished experiments).

The relatively rapid effect of abscisic acid in preventing the gibberellic acid-enhanced choline incorporation suggests a fairly direct role of abscisic acid in membrane biosynthesis and (or) assembly. Abscisic acid prevents the synthesis of α-amylase in the barley aleurone cells, but does not alter the rate of respiration or total RNA or protein synthesis (5), although it may inhibit specific RNA synthesis (21, 22). Abscisic acid also prevents the gibberellic acid-enhanced increase in ER-bound polysomes (Evins, manuscript in preparation).

These results suggest a role for both gibberellic and abscisic acids in the regulation of ER synthesis. How this occurs is at present being studied. In further experiments on the effect of the two hormones on membrane synthesis, we have found that ^{32}Pi incorporation into phospholipids during a 30-min incubation in ^{32}Pi is a more versatile measure of the rate of synthesis of phospholipids (Koehler and Varner, unpublished).

This research was supported by the U.S. Atomic Energy Commission (Contract AT(11-1)-1338) and a grant from the National Science Foundation (GB-8774). This paper is based on a dissertation submitted by W. H. E. in partial fulfillment of the requirements for a Ph.D. degree, Department of Biochemistry, Michigan State University. It is Michigan Agricultural Experiment Station journal article no. 5477.

1. Chrispeels, M. J., and J. E. Varner, *Plant Physiol.*, **42**, 398 (1967).
2. Filner, P., and J. E. Varner, *Proc. Nat. Acad. Sci. USA*, **58**, 1520 (1967).
3. Jacobsen, J. V., and J. E. Varner, *Plant Physiol.*, **42**, 1596 (1967).
4. Chrispeels, M. J., and J. E. Varner, *Nature*, **212**, 1066 (1966).
5. Chrispeels, M. J., and J. E. Varner, *Plant Physiol.*, **42**, 1008 (1967).
6. Eb, A. A. van der, and P. J. Nieuwdorp, *Acta Bot. Neer.*, **15**, 690 (1967).
7. Jones, R. L., *Planta*, **87**, 119 (1969).
8. Jones, R. L., *Planta*, **88**, 73 (1969).
9. Nagley, P., and T. Hallinan, *Biochim. Biophys. Acta*, **163**, 218 (1968).
10. Wettstein, F. O., T. Staehelin, and H. Noll, *Nature*, **197**, 430 (1963).
11. Staehelin, T., F. O. Wettstein, H. Oura, and H. Noll, *Nature*, **201**, 264 (1964).
12. Bieber, L. L., E. Hodgson, V. H. Cheldelin, V. J. Brookes, and R. W. Newburg, *J. Biol. Chem.*, **236**, 2590 (1961).
13. Bieber, L. L., L. G. Sellers, and S. S. Kumar, *J. Biol. Chem.*, **244**, 630 (1969).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15. Hallinan, T., T. Duffy, S. Waddington, and H. N. Munro, *Quart. J. Exp. Physiol.*, **51**, 142 (1966).
16. Siekevitz, P., and G. E. Palade, *J. Biophys. Biochem. Cytol.* (now *J. Cell Biol.*), **7**, 619 (1960).
17. Campbell, P. N., *FEBS Lett.*, **7**, 1 (1970).
18. Fawcett, D. W., *An atlas of fine structure: The cell, its organelles and inclusions* (W. B. Saunders Co., Philadelphia, 1966).
19. Tata, J. R., *Nature*, **219**, 331 (1968).
20. Tata, J. R., in *Biochemical Actions of Hormones*, ed. Litwack, G., (Academic Press, New York, 1970), p. 91.
21. Khan, A. A., and C. C. Anojulu, *Biochem. Biophys. Res. Commun.*, **38**, 1069 (1970).
22. Khan, A. A., and C. E. Heit, *Biochem. J.*, **113**, 707 (1969).