Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 22, Issue of November 25, pp. 11424-11427, 1981
Printed in U.S.A.

Persistent Activation of Steroidogenesis in Adrenocortical Cells by Photoaffinity Labeling of Corticotropin Receptors*

(Received for publication, August 17, 1981)

J. Ramachandran, James Hagman, and Koji Muramoto

From the Hormone Research Laboratory, University of California, San Francisco, California 94143

Photolysis of rat adrenocortical cells in the presence of the photoreactive derivative [(2-nitro-5-azidophenylsulfenyl)Trp^9]-adrenocorticotropic hormone (2,5-NAPS-ACTH) at 24 °C resulted in persistent activation of corticosterone production. The basal rate of steroidogenesis became maximal when photolysis was performed at 24 °C but remained the same as that of control cells when irradiation was performed at 0 °C. No increase in basal rate was observed with dark controls or cells photolyzed with [(2,4-dinitrophenylsulfenyl)Trp^9]ACTH, a photoresistant analog of the hormone. Prephotolyzed 2,5-NAPS-ACTH failed to induce persistent activation. Both ACTH and 2,4-(dinitrophenylsulfenyl)Trp^9-ACTH blocked the photo-induced activation of steroidogenesis elicited by 2,5-NAPS-ACTH. Under photolysis conditions which caused the basal rate of steroidogenesis to become maximal, a 3-fold increase in the basal rate of cAMP formation was observed.

Photoaffinity labeling is a promising new approach which is being used increasingly in studies of polypeptide hormone receptors (1, 2). Until recently, photoaffinity-labeling studies have been mainly concerned with demonstrating covalent attachment of the radioactive ligand to macromolecular component(s) of the target cell membrane and specific inhibition of labeling by nonradioactive ligand. The functional consequences of the covalent attachment of hormones to target tissues are just beginning to be investigated (3-5).

We have recently utilized the technique of photoaffinity labeling for the covalent attachment of the pituitary hormone, corticotropin, to functional adrenocortical cells (6). The photoreactive derivative, [(2-nitro-5-azidophenylsulfenyl)Trp^9]ACTH,[1] stimulates corticosterone synthesis in isolated rat adrenocortical cells to 60% of the maximal rate induced by ACTH. However, when adrenocortical cells were photolyzed in the presence of 2,5-NAPS-ACTH at 0 °C, the responsiveness of the cells to subsequent stimulation with ACTH was decreased (6). Covalent attachment of the photoreactive ACTH derivative with agonist properties would be expected to result in irreversible activation of the response. In order to elucidate this further, we have investigated the effect of temperature on the covalent attachment of 2,5-NAPS-ACTH to rat adrenocortical cells by photoaffinity labeling. In this communication, we present evidence that persistent activation of steroidogenesis can be accomplished by performing the photolysis at room temperature.

* This work was supported by Grant CA-16417 awarded by the National Cancer Institute, Department of Health, Education, and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: ACTH, adrenocorticotropin; 2,5-NAPS-ACTH, [(2-nitro-5-azidophenylsulfenyl)Trp^9]ACTH; 2,4-DNPS-ACTH, [(2,4-dinitrophenylsulfenyl)Trp^9]ACTH; BSA, bovine serum albumin.

MATERIALS AND METHODS

The photoreactive ACTH derivative, 2,5-NAPS-ACTH, was prepared as previously described (7). Adrenocortical cells were isolated from decapsulated adrenal glands of adult male Sprague-Dawley rats by digestion with collagenase and DNase as described (8). Cells were used fresh after isolation or in monolayer cultures 5 days after plating. Cell culture conditions have been described in detail elsewhere (9, 10).

Cells were incubated with 2,5-NAPS-ACTH (50 nM) in Medium 199 containing 0.5% BSA purified as described (11). After a 30-min incubation in the dark, the cell suspension was irradiated using a Blak-Ray UV lamp emitting principal radiation at 366 nm. The cell suspensions were kept stirring throughout the preincubation with photolysis at the same temperature (0 or 24 °C) 10-15 cm from the lamp. Photolysis was generally conducted for 4 min, 2 times, with a 2-min pause between exposure to irradiation. At the end of the photolysis, the cells were washed by centrifugation with Medium 199-0.5% BSA (twice) and then incubated in fresh medium at 37 °C for 30 min to facilitate dissociation of noncovalently bound peptide. The cells were washed again twice and reincubated in medium 199-0.5% BSA at 37 °C for 1 h in the absence and presence of different concentrations of ACTH. At the end of this incubation, the cells were kept in boiling water for 5 min and stored. Corticosterone (12) and cAMP (13) were measured by specific radioimmunoassays as described previously (14).

Cells in monolayer culture were subjected to multiple cycles of photolysis with fresh 2,5-NAPS-ACTH each time. After a 1- or 2-min photolysis as described above, the cells were washed twice with medium 199-0.5% BSA and reincubated with fresh 2,5-NAPS-ACTH (50 nM) and the cycle of photolysis and washing was repeated 3 times. At the end of four cycles of photolysis, the cells were washed twice, incubated with fresh medium at 37 °C for 30 min, washed twice again, and finally incubated with and without ACTH for 1 h at 37 °C to assess the steroidogenic capacity and cAMP production. Control cells were irradiated under identical conditions in the absence of 2,5-NAPS-ACTH. Dark controls were exposed to the same concentration of 2,5-NAPS-ACTH under the same conditions but were covered with foil to prevent photolysis.

RESULTS

Irradiation of adrenocortical cells at 24 °C for periods up to 10 min had no significant effect on the ability of the cells to respond to subsequent stimulation with ACTH. However, continuous irradiation tended to increase the temperature. In order to maintain the temperature, irradiation was performed for 4 min at a time with a 2-min pause between irradiations. The results in Fig. 1 show that when rat adrenocortical cells were incubated with 50 nM 2,5-NAPS-ACTH in the dark, there was no covalent attachment of the peptide. The peptide was readily washed off and the basal production of corticosterone remained low. Addition of ACTH to these cells resulted in the normal stimulation of steroidogenesis. When the adrenocortical cells were photolyzed in the presence of 50 nM 2,5-NAPS-ACTH and washed, the basal rate of steroidogenesis became maximal (Fig. 1). Addition of ACTH to these persistently activated cells did not cause a further stimulation of corticosterone production.

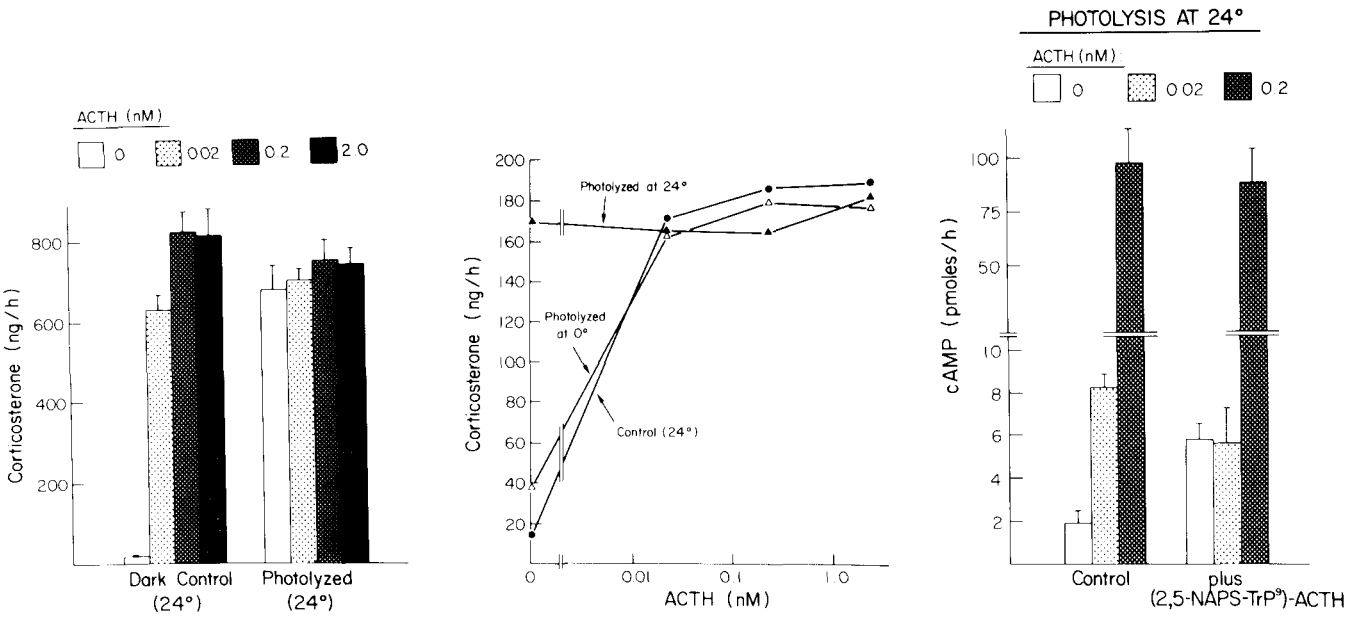

FIG. 1 (*left*). **Persistent activation of corticosterone production by photolysis.** Freshly isolated rat adrenocortical cells were incubated with 2,5-NAPS-ACTH (50 nM) at 24 °C for 30 min and then irradiated twice for 4 min with a 2-min pause. The cells were washed as described in the text and aliquots (10^5 cells/ml) were reincubated with or without ACTH for 1 h at 37 °C. Corticosterone was assayed by radioimmunoassay. Dark controls were treated in the same manner except that the tubes were covered with foil during irradiation. The *bars* represent mean values ± S.E. of triplicate incubations.

FIG. 2 (*center*). **Effects of photolysis of adrenocortical cells at different temperatures.** Adrenocortical cells in primary culture (10^5 cells/well) were incubated without or with 2,5-NAPS-ACTH (50 nM) for 30 min at 24 °C or 1 h at 0 °C. Cells were photolyzed 1 min and washed twice and photolysis was repeated with fresh 2,5-NAPS-ACTH (50 nM). After 4 cycles of photolysis and washing, the cells were washed as described in the text and incubated with or without ACTH for 1 h at 37 °C. Corticosterone was measured by radioimmunoassay. Values are mean ± S.E. for triplicate cultures. Control cells were photolyzed in the absence of 2,5-NAPS-ACTH.

FIG. 3 (*right*). **Persistent activation of cAMP production by photoaffinity labeling.** Adrenocortical cells in culture (10^5 cells/well) were incubated with or without 2,5-NAPS-ACTH (50 nM) for 30 min in the dark at 24 °C. Photolysis was performed for 1 min, the cells were washed twice and incubated with fresh medium containing 2,5-NAPS-ACTH (50 nM), and the photolysis was repeated. After 4 cycles of photolysis, the cells were washed as described under "Materials and Methods" and incubated with or without ACTH for 1 h at 37 °C. cAMP in the medium was measured by radioimmunoassay. Values are the mean ± S.E. for triplicate cultures.

Previous studies have shown that photolysis of rat adrenocortical cells at 0 °C for 15 min in the presence of 2,5-NAPS-ACTH did not result in persistent activation of steroidogenesis (6). In order to examine the effect of temperature on the persistent activation of response, cells were photolyzed in the presence and absence of 2,5-NAPS-ACTH at 24 and 0 °C in the same experiment. The results in Fig. 2 show that cells exposed to light in the absence of 2,5-NAPS-ACTH had a low basal rate of steroidogenesis and were maximally stimulated by 20 pM ACTH. The basal rate became maximal when the cells were photolyzed at 24 °C in the presence of 50 nM 2,5-NAPS-ACTH. In contrast, photolysis of the adrenocortical cells in the presence of 50 nM 2,5-NAPS-ACTH at 0 °C resulted in only a slight increase in the basal rate of corticosterone production. These cells also responded to exogenous ACTH. Persistent activation of steroidogenesis was observed in every experiment when the cells were photolyzed in the presence of 2,5-NAPS-ACTH at 24 °C (4/4 experiments with cells in suspension and 5/5 experiments with cells in culture).

The effect of photolysis in the presence of 2,5-NAPS-ACTH on cAMP production in rat adrenocortical cells was also examined. Photolysis at 24 °C in the presence of 50 nM 2,5-NAPS-ACTH resulted in a 3-fold increase in the basal rate of cAMP accumulation in the medium (Fig. 3). Cells photolyzed at 24 °C in the presence 2,5-NAPS-ACTH were as responsive to ACTH as control cells. No increase in basal cAMP production was observed with dark controls or when photolysis was conducted at 0 °C in the presence of 50 nM 2,5-NAPS-ACTH.

To further confirm that the persistent activation of response observed was due to the specific covalent attachment of the

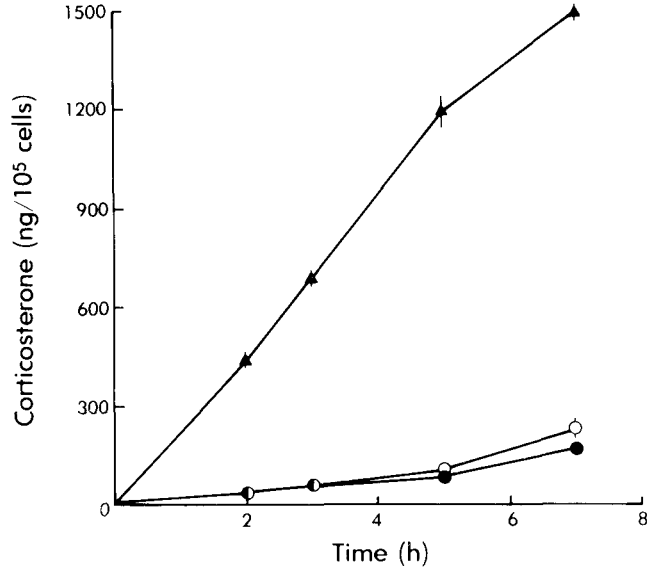

FIG. 4. **Effect of photolysis on the basal steroidogenesis.** Adrenocortical cells in suspension were incubated with or without 2,5-NAPS-ACTH (50 nM) or 2,4-DNPS-ACTH (50 nM) in the dark for 30 min at 24 °C. Photolysis was conducted twice for 4 min with a 2-min pause between irradiations. Cells were washed as described under "Materials and Methods" and incubated at 37 °C in the presence of 95% O_2:5% CO_2. Aliquots were removed at intervals and corticosterone was assayed by radioimmunoassay. Values are the mean ± S.E. of triplicate incubations. Control cells (●); cells photolyzed with 2,4-DNPS-ACTH (○); cells photolyzed with 2,5-NAPS-ACTH (▲).

TABLE I

Effect of ACTH and 2,4-DNPS-ACTH on persisent activation of steroidogenesis by 2,5-NAPS-ACTH

Adrenocortical cells were incubated without or with the various additions shown in the first column for 30 min at 24 °C and then photolyzed twice for 4 min with a 2-min pause. Cells were washed as described in the text and aliquots (3×10^4 cells/ml) were reincubated in the absence (basal) or presence of 2.2 nM ACTH for 1 h at 37 °C. Corticosterone was assayed by radioimmunoassay. Values are the mean ± S.E. of triplicate incubations. The prephotolyzed 2,5-NAPS-ACTH was prepared by irradiating the peptide for 30 min at 24 °C in the absence of cells.

Photolysis conditions	Corticosterone	
	Basal	ACTH (2.2 nM)
	ng/h	
No addition	1.1 ± 0.04	30.3 ± 1.1
2,5-NAPS-ACTH (50 nM)	16.0 ± 0.7	29.1 ± 3.7
Prephotolyzed 2,5-NAPS-ACTH (50 nM)	1.6 ± 0.5	19.2 ± 0.7
2,5-NAPS-ACTH (50 nM + ACTH (50 nM)	5.0 ± 1.0	24.5 ± 2.2
2,5-NAPS-ACTH (50 nM) + 2,4-DNPS-ACTH (100 nM)	1.3 ± 0.5	23.7 ± 0.5

hormone derivative to the cells, another control experiment was performed. [(2,4-Dinitrophenylsulfenyl)Trp^9]-ACTH is an analog closely related to the photoreactive 2,5-NAPS-ACTH but is not converted to a reactive nitrene by photolysis since it lacks the azido group. Cells were incubated with no addition, 2,4-DNPS-ACTH, or 2,5-NAPS-ACTH and photolyzed at 24 °C. The basal steroidogenesis was followed as a function of time (Fig. 4). It is clear that the basal production of corticosterone in cells photolyzed in the presence of 50 nM 2,4-DNPS-ACTH at 24 °C was not significantly different from the basal steroidogenic rate in control cells. On the other hand, cells photolyzed with 2,5-NAPS-ACTH at 24 °C were maximally stimulated and continued steroid production for several hours (Fig. 4).

The results in Table I show that no persistent activation of steroidogenesis resulted if the 2,5-NAPS-ACTH was prephotolyzed prior to photolysis in the presence of cells. The activation of basal steroidogenesis induced by photolysis in the presence of 2,5-NAPS-ACTH was effectively prevented when the photolysis was conducted in the presence of ACTH or 2,4-DNPS-ACTH (Table I).

In view of the differences in the degrees of persistent activation of the steroidogenic and cAMP responses, the time courses of persistent activation of the two responses were examined. Basal rate of steroidogenesis became nearly maximal after a 1-min photolysis and remained maximal with increasing times of photolysis. Basal cAMP production, on the other hand, increased 3-fold only after photolysis for 4 min.

DISCUSSION

These results show that persistent activation of steroidogenesis can be achieved by photoaffinity labeling of ACTH receptors on rat adrenocortical cells. Similar results were obtained with freshly isolated cells and cells maintained in primary culture for a week, although the steroidogenic capacity of the freshly isolated cells was higher. The steroidogenic enzymes are known to decay when cells are maintained in the absence of ACTH for several days (9). The control experiments clearly show that persistent activation of steroidogenesis occurred only when the cells were irradiated in the presence of hormone containing a photoreactive group which could be activated to form a covalent link with the receptor. Thus, both 2,4-DNPS-ACTH which lacks the photoreactive azido group and prephotolyzed 2,5-NAPS-ACTH were unable to induce persistent activation. Both ACTH and 2,4-DNPS-ACTH were able to block the persistent activation induced by 2,5-NAPS-ACTH upon photolysis, showing that persistent steroidogenesis results from specific activation of ACTH receptors.

The temperature at which photolysis is conducted appears to be a very important parameter for achieving persistent activation of function. It is clear that cells irradiated at 0 °C in the presence of 2,5-NAPS-ACTH do not exhibit elevated levels of steroidogenesis whereas cells irradiated at 24 °C under identical conditions are irreversibly activated. The failure to achieve persistent activation by photolysis at 0 °C suggests that either the efficiency of covalent attachment is inadequate at 0 °C or the link formed between hormone and receptor at 0 °C is different from that formed at 24 °C. This could arise if the receptor exists in an active (at 24 °C) and inactive (at 0 °C) conformation. Once the hormone becomes attached covalently to the inactive conformation by photolysis at 0 °C, subsequent incubation at 37 °C is not sufficient to generate the active form of the receptor-hormone complex which leads to persistent activation.

Since 2,5-NAPS-ACTH is a partial agonist in stimulating steroidogenesis and an antagonist of ACTH-stimulated cAMP accumulation in rat adrenocortical cells (6), it is suprising that covalent attachment of 2,5-NAPS-ACTH results in maximal stimulation of steroid synthesis and partial stimulation of cAMP accumulation. This result suggests that the mode of noncovalent interaction of 2,5-NAPS-ACTH with the receptor is different from that of ACTH, but, upon covalent attachment to the receptor, the peptide is able to stimulate the cell in a manner analogous to that of ACTH. The adrenocortical cell receptor for ACTH appears to be exquisitely sensitive to modifications of the single tryptophan residue in the hormone. Thus, although 2,5-NAPS-ACTH acts as partial agonist in stimulating steroid synthesis, the isomeric derivative 2,4-NAPS-ACTH is a full agonist (data not shown).

Although steroidogenesis was stimulated maximally by brief photolysis with 2,5-NAPS-ACTH at 24 °C, cAMP production was increased only slightly under the same conditions. This can be explained readily by the spare receptor concept (15, 16). Photolysis with 2,5-NAPS-ACTH causes the covalent attachment of the peptide to a small fraction of the receptors which results in the small but significant increase in cAMP accumulation observed. However, activation of a small fraction of the receptors is sufficient to stimulate maximal steroidogenesis. In accord with this, we have observed an increase in the basal rate of cAMP production with increasing time of photolysis whereas the basal rate of steroidogenesis became maximal after brief photolysis and remained maximal with increasing time of photolysis.

Persistent activation of discharge of secretory proteins by dispersed guinea pig pancreatic acini preparations has been reported following six cycles of photolysis with a photoreactive derivative of cholecystokinin octapeptide (3). Photo-induced covalent binding of a photoreactive insulin derivative to rat adipocytes caused a persistent increase in lipogenesis (4). An irreversible and specific stimulation of melanophores was observed upon photolysis of *Xenopus* skins in the presence of *p*-azidophenylalanine^{13}-α-melanocyte-stimulating hormone (5). In all three cases, photolysis was performed at temperatures between 20 and 37 °C. Together with results presented in this article, these findings clearly show that the formation of the hormone-receptor complex is the primary event responsible for triggering the physiological response. These results are not in accord with the hypothesis that the response is

triggered by the dissociation of the hormone from the receptor.

Acknowledgment—We thank Professor C. H. Li for his interest.

REFERENCES

1. Bayley, H., and Knowles, J. R. (1977) *Methods Enzymol.* **46,** 69–114
2. Chaudry, V., and Westheimer, F. H. (1979) *Annu. Rev. Biochem.* **48,** 293–325
3. Galardy, R. E., Hull, B. E., and Jamieson, J. D. (1980) *J. Biol. Chem.* **255,** 3148–3155
4. Brandenburg, D., Diaconescu, D., Saunders, D., and Thamm, P. (1980) *Nature* **286,** 821–822
5. DeGraan, P. N. E., and Eberle, A. (1980) *FEBS Lett.* **116,** 111–115
6. Ramachandran, J., Muramoto, K., Kenez-Keri, M., Keri, G., and Buckley, D. I. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 3967–3970
7. Muramoto, K., and Ramachandran, J. (1980) *Biochemistry* **19,** 3280–3286
8. Ramachandran, J., and Suyama, A. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 113–117
9. Liles, S., and Ramachandran, J. (1977) *Biochem. Biophys. Res. Commun.* **79,** 226–233
10. McPherson, M., and Ramachandran, J. (1980) *J. Cell Biol.* **86,** 129–134
11. Ramachandran, J., Lee, V., and Li, C. H. (1972) *Biochem. Biophys. Res. Commun.* **48,** 274–280
12. Rao, A. J., Long, J. A., and Ramachandran, J. (1978) *Endocrinology* **102,** 371–378
13. Harper, J. F., and Brooker, G. (1975) *J. Cyclic Nucleotide Res.* **1,** 207–218
14. Lee, C-Y., McPherson, M., Licko, V., and Ramachandran, J. (1980) *Arch. Biochem. Biophys.* **201,** 411–419
15. Stephenson, R. P. (1956) *Br. J. Pharmacol. Chemother.* **11,** 379–393
16. Ramachandran, J., Moyle, W. R., and Kong, Y-C. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed) pp. 613–616, Ann Arbor Science Publications, Ann Arbor, MI