PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 471–474 (1978)

Effect of Hypothalamic Extracts on the Incorporation of [^{3}H]-Thymidine by Pituitary Cells in Culture (40228)

ASHOK KHAR, LUCIANO DEBELJUK AND MARIAN JUTISZ

Laboratoire des Hormones Polypeptidiques, C.N.R.S. 91190 Gif-sur-Yvette, France

Different hormones or factors have been shown to stimulate the synthesis of DNA. Partially purified preparations of thyroid-stimulating hormone (TSH) caused the initiation of DNA synthesis in 3T3 cells (1). Similarly, Armelin (2), found an increase in the total cell count and in the incorporation of radioactive thymidine into DNA when 3T3 fibroblasts were exposed to partially purified bovine luteinizing hormone (LH). Clark showed that bovine LH markedly increases the activity of the enzyme ornithine decarboxylase and stimulates cell division in the growing 3T3 mouse fibroblasts (3). Further, a contaminant of LH was isolated from bovine pituitary gland which had a mitogenic effect on the ovarian cell line; this contaminant was named ovarian growth factor (4). Gospodarowicz (5) isolated from pituitary gland a potent mitogenic agent which initiated DNA synthesis in resting cultures of 3T3 cells which he referred to as fibroblast growth factor (FGF). FGF was found to be a peptide possessing a mol wt 13,400, and has been shown to be mitogenic for primary cultures of myoblasts (6). Stratmann *et al.* (7) have reported an increase in the incorporation of tritiated thymidine into pituitary cell nuclei by thyrotropin-releasing homone (TRH). TRH also stimulated the mitotic activity of rat adenohypophysial cells *in vivo* and *in vitro* (8).

Recently Miller *et al.* (9) demonstrated vasopressin to be the factor responsible for the increased [^{3}H]-thymidine uptake when fetal rat chondrocytes were grown in tissue culture. Richman *et al.* (10) found stimulation of DNA synthesis in cultured hepatocytes from partially hepatectomized rats, by insulin, epidermal growth factor and glucagon. Growth hormone and triiodothyronine had no significant effect on DNA synthesis, whereas dexamethasone was found to have an inhibitory action on DNA synthesis. Hollenberg and Cuatrecasas (11) have also shown a stimulation of thymidine incorporation by cultured human fibroblasts in the presence of insulin and epidermal growth factor.

We considered it of interest to determine the effect of known synthetic hypothalamic hormones, a crude rat hypothalamic extract and an extract of cerebral cortex on the incorporation of [^{3}H]-thymidine by monolayer cultures of rat anterior pituitary cells.

Materials and methods. Anterior pituitary glands from 50 adult female rats (Wistar strain) were dispersed with collagenase and viokase as described previously (12). The dispersed cells (1–1.5 × 10^6 cells per dish) were incubated at 37 C in F-10 medium (Gibco, Bio-Cult), to which was added 15% rat serum, 2.5% fetal calf serum (Gibco, Bio-Cult), 25 m*M* *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES, Sigma) and a mixture of three antibiotics (penicillin, streptomycin and fungizone). The incubation was continued up to the 7th day of the culture with a change of medium on the 4th day. On the 7th day, the spent medium was replaced by fresh medium as described above, containing a total of 5 μCi of [^{3}H]-thymidine per Petri dish, and the desired concentration of test substance.

Rat hypothalami or fragments of cerebral cortex of equal weights were extracted with 0.1 *M* acetic acid and centrifuged. The clear supernatants were lyophilized and the resultant powders were dissolved in phosphate buffer-saline, pH 7.35, so that 10 μl of the final solutions were equivalent to one hypothalamus. To test the effect of heating and dialysis, one portion of hypothalamic extract was heated for 5 min in a boiling water bath and the other portion was dialyzed in cellulose tubing (Pore size 24 Angstrom), for 48 hr against 0.05 M phosphate buffer, pH 6.5.

In the first series of experiments, the effect of the incubation of pituitary cells with the following substances was tested: LHRH (Hoffmann La Roche, Basel) at a single dose

0037-9727/78/1583-0471$01.00/0
Copyright © 1978 by the Society for Experimental Biology and Medicine
All rights reserved.

of 0.02 nM, TRH (Hoffmann La Roche) 11 nM dibutyryl 3′–5′ cyclic AMP (dbcAMP, Sigma), 1 mM, rat hypothalamic extract, at a dose equivalent to one hypothalamus per dish, and hydroxyurea (Sigma) an inhibitor of DNA synthesis, 25 mM. The effect of these substances was tested at 9 hr and 24 hr from the start of the incubation. In the second series of experiments, the following substances were tested: a superactive agonistic analog of LHRH (D-glutamine [cyclohexyl]^{6}-Des-Gly^{10}-Pro^{9}-LHRH ethylamide, Hoechst, AG) 0.01 nM, somatostatin (Hoechst, AG), 5 nM, rat hypothalamic extract and cerebral cortex extract at a dose equivalent to one hypothalamus (10 μl), 17-β estradiol (Sigma), 4 nM and hydroxyurea 25 mM. The effect of these substances was also tested after 9 hr, 24 hr and 48 hr of incubation. In the third series of experiments, two different concentrations of cerebral cortical extract (5 μl and 15 μl/dish), a higher dose of TRH (33 nM), a higher dose of somatostatin (15 nM) and two doses of estradiol (0.4 nM and 40 nM) were tested. On the other hand, the influence of heating and of dialysis on the activity of hypothalamic extract was studied at 24 hr and 48 hr incubation periods. Finally, the effects of different doses of hypothalamic extract were tested after 24 hr of incubation.

After different time intervals of incubation, the medium was discarded and the attached cells were washed thrice with cold 10% trichloroacetic acid (TCA). Cells were detached mechanically from the Petri dishes and DNA was extracted by the phenol extraction method of Morley and Kingdon (13). Aliquots of the DNA extracts were counted in Bray's scintillation fluid. The significance of the differences among groups was tested by means of the Duncan's new multiple range test (14).

Results and discussion. Table I demonstrates that rat hypothalamic extract significantly increased the incorporation of [^{3}H]-thymidine into DNA by cultured rat anterior pituitary cells after 24 hr and 48 hr. A dose-response curve was obtained with different concentrations of hypothalamic extracts after 24 hr incubation time (Fig. 1). An optimal incorporation of [^{3}H]-thymidine was observed under our experimental conditions when the equivalent of one hypothalamus per Petri dish was used. An extract of cerebral cortex also significantly stimulated the incorporation of labeled thymidine by pituitary cells, but this stimulation was significantly lower than that obtained with the equivalent weight of hypothalamic extract. However, up to 9 hr of incubation time no significant increase in the uptake of the tritiated nucleotide in the presence of hypothalamic or cerebral cortical extract was noted (Table I A, B).

The various doses of synthetic hypothalamic hormones such as TRH, LHRH, somatostatin and the potent LHRH agonist, each had no significant effect on the incorporation of [^{3}H]-thymidine by pituitary cells (Table I), although Stratmann *et al.* (7) reported an increase in the uptake of tritiated thymidine into pituitary cell nuclei in the presence of TRH. These results are also at variance with those of Pawlikowski *et al.* (8) who stated that TRH stimulated the mitotic activity of rat adenohypophysial cells *in vivo* and *in vitro.* These latter authors did not observe any significant influence of LHRH on adenohypophysial cell proliferation. On the other hand, Vale *et al.* (15) observed a decrease in the incorporation of [^{3}H]-thymidine into DNA of an established cell line, GH3 cells, in the presence of TRH.

The failure of the three known hypothalamic peptides to stimulate the synthesis of DNA, raised the question of the nature of the active factor(s) in crude hypothalamic extracts. The existence of another hypothalamic hypophysiotropic hormone(s) with such activity cannot be ruled out. On the other hand, the presence in the hypothalamic extracts of neuropeptides, catecholamines, indolamines, acetylcholine and other neurotransmitters is well known. Some of these compounds could have a direct or indirect action on the synthesis of DNA in the pituitary.

In an attempt to characterize this factor, hypothalamic extracts were either heated or dialyzed and their stimulatory activity determined. As shown in Table IC, heating or dialysis significantly decreased the stimulatory effect of hypothalamic extracts. These preliminary data could mean that the active factor or factors are relatively small molecule(s), which are heat labile.

To determine if dbcAMP could affect the

TABLE I. EFFECT OF HYPOTHALAMIC AND CEREBRAL CORTICAL EXTRACTS, HYPOTHALAMIC HORMONES, dbcAMP, ESTRADIOL-17β AND HYDROXYUREA ON THE INCORPORATION OF [^{3}H]-THYMIDINE INTO DNA OF ANTERIOR PITUITARY CELLS IN CULTURE.[a]

Group	Concentration per ml	Incubation time (hr) 9	24	48
			A. Experiment 1	
Control	—	877 ± 46.0	5784 ± 51.1	
LHRH	0.02 n*M*	933 ± 11.8	5852 ± 270.02	
TRH	11 n*M*	936 ± 19.4	5666 ± 79.3	
dbcAMP	1 m*M*	749 ± 9.0	5360 ± 109.2	
Hypothalamic extract	10 μl	918 ± 13.3	11441 ± 262.9[b]	
Hydroxyurea	25 m*M*	36 ± 3.9[b]	79 ± 19.4[b]	
			B. Experiment 2	
Control	—	1450 ± 85.9	12536 ± 703.4	27078 ± 238.5
LHRH analog	0.01 n*M*	1770 ± 33.4	13823 ± 130.4	20230 ± 46.8[c]
Somatostatin	5 n*M*	1535 ± 130.8	15688 ± 247.1	20087 ± 57.9[c]
Cerebral cortex extract	10 μl	1292 ± 72.1	32770 ± 292.3[c]	40590 ± 2813.9
Hypothalamic extract	10 μl	1448 ± 144.1	52621 ± 363.5[c]	74274 ± 187.1[c]
Estradiol	4 n*M*	1275 ± 25.0	11749 ± 575.4	19166 ± 146.9[b]
Hydroxyurea	25 m*M*	86 ± 2.3[b]	90 ± 23.4[b]	438 ± 143.7[b]
			C. Experiment 3	
Control			24575 ± 2870	
Cerebral cortex extract	5 μl		33928 ± 3166	
Cerebral cortex extract	15 μl		44835 ± 2560	
TRH	33 n*M*		23050 ± 226	
Somatostatin	15 n*M*		20394 ± 978	
Estradiol	0.4 n*M*		23222 ± 1222	
Estradiol	40 n*M*		14704 ± 643[b]	
Hypothalamic extract	10 μl		42297 ± 1272	54501 ± 3714
Hypothalamic extract (heated)	10 μl		28922 ± 424[d]	41031 ± 2142[d]
Hypothalamic extract (dialyzed)	10 μl		25238 ± 1268[d]	33481 ± 1476[d]

[a] Results expressed as mean cpm ± SEM (counts not corrected), incorporated by the cells from 4 Petri dishes.
[b] $P < 0.01$ as compared with the respective control group.
[c] $P < 0.05$ as compared with the respective control gorup.
[d] $P < 0.01$ as compared with the control containing untreated hypothalamic extract.

incorporation of labeled thymidine by pituitary cells, this nucleotide was tested at 1 m*M* concentration. After 9 hr and 24 hr incubation time (Table IA) dbcAMP had no significant effect. However, a significant inhibitory effect of dbcAMP on the incorporation of labeled thymidine was observed using rat chondrocytes in tissue culture (9).

Estradiol, 0.4 and 4.0 n*M*, had no significant effect but was inhibitory at a concentration of 40 n*M* (Table IB, C). Maurer and Gorski (16) reported that *in vivo* estradiol treatment did not increase the pituitary content of DNA, but markedly stimulated the increase in the incorporation of [^{3}H]-thymidine. Several other *in vivo* studies have suggested that estrogens stimulate DNA synthesis in the pituitary gland (17, 18). All the above *in vivo* studies indicate that the action of estrogen probably is not directly on the pituitary gland. Hydroxyurea, (25 m*M*) an inhibitor of DNA synthesis (19), completely inhibited the incorporation of tritiated thymidine into DNA after different incubation periods (Table IA, B).

The increment in the incorporation of [^{3}H]thymidine by hypothalamic or cerebral cortex extracts could be due to the presence of FGF in these extracts, since bovine brain and pituitary are rich in FGF (20). Alternatively, the stimulatory effect may be due to vasopressin as has been reported by Miller *et al.* (9).

The findings of the present report indicate that there exists some type of control by the hypothalamus over the synthesis of hypophysial DNA, which appears to be independant of the three well known hypothalamic hormones, LHRH, TRH and somatostatin. Studies are in progress to isolate and characterize the substance(s) responsible for this activity.

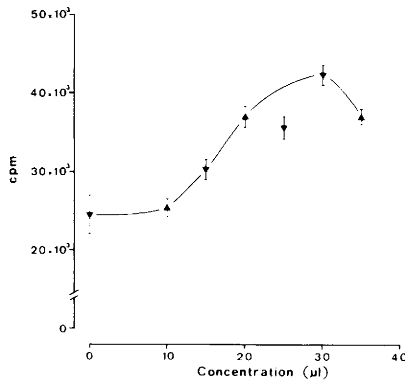

FIG. 1. Effect of different concentrations of hypothalamic extract on the incorporation of [^{3}H]-thymidine by pituitary cells. The culture was incubated for 24 hr and 30 µl of the hypothalamic extract is equivalent to one hypothalamus.

Summary. Rat hypothalamic extracts stimulated the incorporation of [^{3}H]-thymidine into DNA derived from cultured rat pituitary cells, after 24 hr and 48 hr of incubation. A three- to fivefold increase in the incorporation of [^{3}H]-thymidine was observed in the presence of hypothalamic extracts. This stimulatory activity was significantly decreased by heating or dialysis. Rat cerebral cortical extracts also stimulated this incorporation but to a lesser extent than hypothalamic extracts. Hydroxyurea (25 m*M*) completely inhibited the incorporation of [^{3}H]-thymidine by pituitary cells. The synthetic hypothalamic hormones, LHRH, TRH, and somatostatin, had no significant effects, whereas estradiol (40 n*M*) had a significant inhibitory effect.

The authors appreciate the skillful technical assistance of Mrs. T. Taverny-Bennardo. We are indebted to Dr. Jürgen Sandow, Hoechst AG, Frankfurt, for the gift of the LHRH analog and somatostatin and to Drs. R. O. Studer and D. Gillessen, Hoffmann La Roche, Basel, for LHRH and TRH. We thank Mrs. C. Guidet for the statistical analysis. The suggestions regarding the manuscript made by Dr. A. Corbin, Wyeth Laboratories, Philadelphia, are highly appreciated. This work was supported by a grant from DGRST No 7571657 and 7670715. One of us (A.K.) is the post-doctoral fellow of Ministère des Affaires Etrangères, Paris.

1. Holley, R. W., and Kiernan, J. A., Proc. Nat. Acad. Sci. U.S.A., **60**, 300 (1968).
2. Armelin, H. A., Proc. Nat. Acad. Sci. U.S.A. **70**, 2702 (1973).
3. Clark, J. L., Biochemistry **13**, 4668 (1974).
4. Gospodarowicz, D., Jones, K. L. and Sato, G. Proc. Nat. Acad. Sci. U.S.A. **71**, 2295 (1974).
5. Gospodarowicz, D., J. Biol. Chem. **250**, 2515 (1975).
6. Gospodarowicz, D., Veseman, J., Moran, J. S., and Lindstrom, J., J. Cell Biol. **70**, 395 (1976).
7. Stratman, I. E., Ezrin, C., Kovacs, K., and Sellers, E., Z. Zellforsch **145**, 23 (1973).
8. Pawlikowski, M., Kunert-Radek, J., and Stepien, H., Symp. Int. Soc. Psychoneuroendocrinol. Visegrád. 367 pp. (1975).
9. Miller, R. P., Husain, F., Svensson, M., and Lohin, S., Endocrinology **100**, 1365 (1977).
10. Richman, R. A., Claus, T. H., Pilkis, S. J. and Friedman, D. L., Proc. Nat. Acad. Sci. U.S.A. **73**, 3589 (1976).
11. Hollenberg, M. D., and Cuatrecasas, P., J. Biol. Chem. **250**, 3845 (1975).
12. Debeljuk, L., Khar, A., and Jutisz, M., Mol. Cell. Endocrinol. **10**, 159 (1978).
13. Morley, C. G. D., and Kingdon, H. S., Anal. Biochem. **45**, 298 (1972).
14. Steel, R. G. D., and Torrie, J. H., *in* "Principles and Procedures of Statistics", Mcgraw-Hill, New York, (1960).
15. Vale, W., Rivier, C., Brown, M., Chan, L., Ling, N., and Rivier J., *in* "Hypothalamus and Endocrine Functions" (F. Labrie, J. Meites and G. Pelletier, eds.) Vol. 3, p. 397, Plenum Press, (1976).
16. Maurer, R. A., and Gorski, J., Endocrinology **101**, 76 (1977).
17. Mastro, A. and Hymer, W. C., J. Endocrinol. **59**, 107 (1973).
18. Davies, C., Jacobi, J., Lloyd, H. M., and Meares, J. D., J. Endocrinol. **61**, 411 (1974).
19. Young, C. W., and Hodas, S., Science **146**, 1172 (1964).
20. Gospodarowicz, D., Nature (London) **249**, 123 (1974).

Received January 5, 1978. P.S.E.B.M. 1978, Vol. 158.