PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 605–609 (1978)

Prolactin Secretion from Clonal Pituitary Cells following Incubation with Estradiol, Progesterone, Thyrotrophin Releasing Hormone and Dopamine (40106)

DAMON C. HERBERT, HIROSHI ISHIKAWA, MASATAKA SHIINO, AND EDWARD G. RENNELS

Department of Anatomy, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

In vitro studies utilizing anterior pituitary tissue have been employed for a number of years to study the physiologic role of specific agents on one or more of the hypophyseal hormones. One of the cells that remains viable and secretes its hormonal product in copious amounts when placed into an artificial environment is the prolactin cell (1, 2). A number of compounds have been used to determine their effect on prolactin (PRL) secretion *in vitro*. Some of those which have been shown to be stimulatory are estrogen (3–5), testosterone (5), thyrotrophin releasing hormone (TRH) (5–7), cyclic AMP (8), and cations (9, 10), while inhibition has been observed with dopamine hydrochloride (DA), (8, 11, 12), apomorphine (8), progesterone (P) (5) and the ergot alkaloids (8, 11, 13).

Several investigators have obtained enriched cell fractions of rat anterior pituitary cells from normal hypophyses (14–16). Hymer and coworkers have successfully isolated a PRL-rich fraction of cells from adult female rat pituitary glands by velocity sedimentation at unit gravity (17) and have used these cells to examine the kinetics of PRL secretion (18). Clones of pituitary cells have been established by Yasumura *et al.* (19) and Tashjian *et al.* (20) from a rat pituitary tumor. These cells were first reported to produce either adrenocorticotrophin (ACTH) or growth hormone (GH) (19). In a later study several of the GH-secreting strains were also shown to secrete PRL (21). An attempt was made by Steinberger *et al.* (22) to establish gonadotrophin secreting cell clones from normal rat pituitary tissue. They found that a majority of their clonal cells secreted neither gonadotrophic hormone and that gonadotrophs could not be well maintained in culture. Recently, Ishikawa *et al.* (23) reported the establishment from Rathke's pouch tissue derived from normal rat fetuses of 65 clonal strains of pituitary cells which produce PRL, GH and/or ACTH. The following investigation utilized one of these clones (2B8) to examine the direct effects of several compounds on PRL secretion.

Materials and methods. A clone of pituitary PRL cells, designated 2B8, was derived from the epithelium of Rathke's pouch as previously described (23). The cells were placed into Falcon culture dishes (60 × 15 mm, #3002) containing growth medium which consisted of 85% Ham's F10 medium and 15% virus screened fetal calf serum (Gibco Biological Supplies), 50 μg/ml streptomycin, 50 U/ml penicillin and 0.5 μg/ml fungizone. Each dish contained a total volume of 4 ml of growth medium and approximately 1×10^5 cells. After seeding, the dishes remained at room temperature for 2 hr and were then transferred to a humidified CO_2 incubator, and maintained at 37° with a gas mixture of 5% CO_2, 5% O_2, and 90% air for 10 days. Following this, the medium was discarded from each dish and replaced with fresh growth medium containing 10^{-8} *M* E_2 [estradiol sodium sulfate (Steraloids, Inc.)],[1] 2×10^{-9} *M* TRH (Beckman, Inc.), or a mixture of both 10^{-8} *M* E_2 and 2×10^{-9} *M* TRH. After 3 days, the medium was again discarded and replaced with fresh growth medium containing the aforementioned concentrations of E_2, TRH, or E_2 and TRH alone or in combination with 10^{-6} *M* DA (Sigma Chemical Corp.). After an additional 6 hr, the medium was collected and stored frozen for subsequent quantitation of PRL content by radioimmunoassay. The 2B8 cells were also cultured using the same protocol (10 days growth medium-3 days growth medium containing secretagogue-6 hr growth medium containing secretagogue or inhibitor) with

[1] The estradiol and progesterone were initially dissolved in absolute alcohol and then put into the growth medium. The final concentration of alcohol in the steroid-containing medium was 0.5%.

0037-9727/78/1574-0605$01.00/0

varying concentrations of E_2 or P [4-pregnen-3,20 dione (Mochida Pharmaceuticals Co. Ltd.)][1] ranging from 10^{-11} *M* to 10^{-6} *M*, 10^{-8} *M* E_2 plus 10^{-6} *M* P, or with 10^{-9} *M* to 10^{-5} *M* of DA. In all studies, 10 dishes were cultured in triplicate for each dose level of secretagogue or inhibitor employed. In addition, two types of control cultures were routinely performed using the aforementioned 10 day-3 day-6 hr protocol. In the first, a series of dishes containing only growth medium and no cells were utilized ("medium only" control), and in the second, the dishes contained growth medium and 2B8 cells only ("cells only" control). After each experiment, the cells were stained with 0.1% trypan blue and counted using a hemocytometer.

The PRL concentration in the media was measured by radioimmunoassay using a homologous rat PRL assay system employing reagents supplied in kits by NIAMDD. The rat PRL was iodinated using a slight modification of the lactoperoxidase procedure as described by Aubert *et al.* (24). The inter- and intra-assay coefficients of variation were 8.2% and 6.7%, respectively. All values are expressed in terms of NIAMDD rat PRL-RP-1. The data were statistically analyzed by analysis of variance and by the *t* test for comparison of differences between means.

Results. The amount of PRL released into the media by the 2B8 cells incubated with varying concentrations of E_2 and P is shown in Fig. 1. The concentration of PRL in the dishes containing 10^{-11} *M* E_2 was essentially the same as the "cells only" control. In contrast, there was a dose-related increase in PRL levels in the media from cells incubated with 10^{-10} *M*–10^{-8} *M* E_2, with the maximum amount of PRL secretion occurring with 10^{-8} *M* E_2. When 10^{-7} *M* and 10^{-6} *M* E_2 were utilized, PRL release declined to levels which closely approximated values recorded in the "cells only" controls. In this study as well as in the subsequent experiments, PRL was not detectable in the "medium only" control. A dose-related decrease in PRL secretion was observed following the incubation of the 2B8 cells with concentrations of P ranging from 10^{-11} *M* to 10^{-6} *M*. Moreover, an inhibition in the amount of PRL secreted by the cells was recorded when 10^{-8} *M* E_2 and 10^{-6} *M* P were simultaneously added to the culture me-

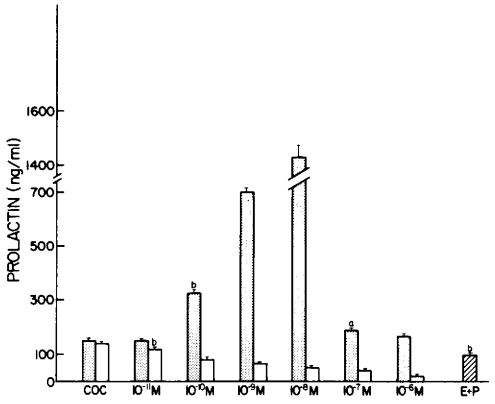

FIG. 1. Prolactin concentration (mean ± SE) in culture media from 2B8 cells incubated with E_2 (stippled bars) or P (open bars), from 10^{-11}*M* to 10^{-6} *M*, or 10^{-8} *M* E_2 and 10^{-6} *M* P (cross hatched bar). a = ns, b = $P < 0.001$ as compared to "cells only" control (COC).

dium. This, however, was not as great as the decrease noted with P alone. Besides affecting PRL production by the 2B8 cells, E_2 and P also induced changes in cell proliferation. E_2 stimulated a significant increase in cell number at all concentrations examined which was dose-related. This increase ranged from 5.4% in the group incubated with the lower concentration of E_2 to 42.3% in the group incubated with 10^{-6} *M*. The dose of E_2 which caused the maximum release of PRL only stimulated a 19.3% increase in cell number. In contrast, P inhibited cell proliferation. This effect was statistically significant only with concentrations of 10^{-8} *M*–10^{-6} *M*. No change in cell number was observed when the two steroids were incubated together.

There was nearly a twofold rise in PRL release from cells cultured with 10^{-9} *M* DA (Fig. 2). However, with higher concentrations of DA, a decline in PRL release was observed which was significantly lower than the "cells only" control at dosages of 10^{-7} *M*–10^{-5} *M*. The total cell number in dishes containing DA-supplemented media was the same as that present in the "cells only" control.

The results of the study in which E_2 (10^{-8} *M*), TRH (2×10^{-9} *M*) and DA (10^{-6} *M*) were incubated alone or in combination are shown in Fig. 3. Alone, E_2 and TRH markedly stimulated PRL release. There was no statistical difference in the amount of PRL secreted by the cells incubated in the presence

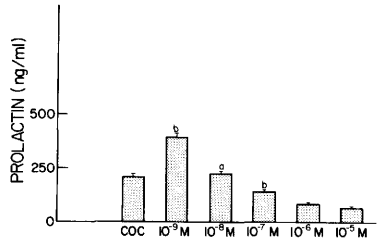

FIG. 2. Dose-related secretion pattern of PRL (mean ± SE) by 2B8 cells following incubation with 10^{-9} *M* to 10^{-5} *M* DA. a = ns, b = $P < 0.001$ as compared to "cells only" control (COC).

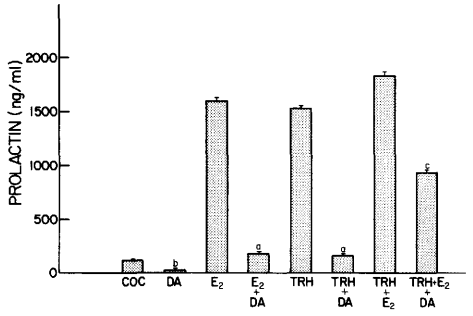

FIG. 3. Effect of 10^{-6} *M* DA, 10^{-8} *M* E_2 and 2×10^{-9} *M* TRH alone or in combination with each other on PRL secretion (mean ± SE) from 2B8 cells. a = ns, b = $P < 0.001$ as compared to "cells only" control (COC).

of E_2 as compared to the response from TRH. When the cells were cultured with a combination of E_2 and TRH, significantly more hormone was released ($P < 0.001$) than when each was incubated alone, albeit this effect was not additive. Simultaneous incubation of E_2 and DA or TRH and DA resulted in a marked decrease in PRL release to levels which were similar to that observed in the "cells only" control. The inhibitory effect of DA on the combination of TRH and E_2, however, was not as great. TRH and E_2 alone, in combination with each other or with DA significantly increased cell proliferation. DA alone had no effect.

Discussion. The present investigation demonstrates the first use of a normal diploid clone of pituitary cells for the study of the regulatory mechanisms that control PRL secretion. The 2B8 clone has previously been shown by radioimmunoassay to secrete only PRL (23). The only other viable clones of pituitary cells that have been extensively used experimentally are those developed by Tashjian and coworkers from rat pituitary tumors (19, 20). While these were initially found to produce more than one hormone (19–21), subsequent studies with subclones derived from these strains have shown that certain strains secrete only GH or PRL (21, 25).

In our experiments, the 2B8 cells have been shown to be responsive to E_2, P and TRH much like the GH tumor cells. In comparing our observations with those of Haug and Gautvik (5), it can be noted that the tumor cells initially responded to tenfold less E_2 than the 2B8 cells; however, both cell strains produced a maximum amount of PRL with 10^{-8} *M* E_2. At higher concentrations (up to 10^{-6} *M*), PRL secretion by the tumor cells plateaued, while that of the 2B8 cells fell precipitously. Observations similar to ours with varying doses of E_2 have been obtained *in vitro* using organ cultures of anterior pituitary tissue (4) and in ovariectomized rats given dosages of 0.1–500 μg estradiol benzoate (26). At all dose levels of P tested, there was significantly less PRL released from 2B8 cells than occurred in the "cells only" control cultures. In contrast, Haug and Gautvik (5) observed significant changes in the amount of PRL released by the tumor cells only with 10^{-8} *M*–10^{-6} *M* P. Although slightly less PRL was seen in the media containing 10^{-9} *M* P, they reported no significant inhibition with doses of 10^{-11} *M*–10^{-9} *M* P. When the tumor (5) or the 2B8 cells were incubated simultaneously with 10^{-8} *M* E_2 and 10^{-6} *M* P, less PRL was secreted than with E_2 alone. The suppressive effect of P on E_2 was greater on the 2B8 cells than on the GH tumor cell line. In the presence of the two steroids, the tumor cells produced 124% more PRL than their control cultures (5) while the 2B8 cells secreted 28% less than the "cells only" controls. An inhibition of the stimulatory effect of E_2 by P has also been observed *in vivo* following the simultaneous administration of 1 μg E_2 and 0.5, 1 or 4 mg P to ovariectomized rats (26). Both the 2B8 and the tumor cells were stimulated by TRH (Fig. 3; 5, 27). When the 2B8 cells were incubated with TRH and E_2, a 19% increase in PRL secretion was recorded over that obtained with TRH alone. In contrast, the GH_3 cells produced more than twice as much PRL under similar conditions (5).

The discrepancy in the two studies may be due in part to the difference in the amount of TRH employed, which in our experiment was 100 times less than that used by Haug and Gautvik (5); both investigations utilized the same concentration of E_2. The stimulation of PRL secretion by either TRH or E_2 was suppressed to control levels by DA, while simultaneous incubation of the 2B8 cells with TRH, E_2 and DA reduced PRL levels by about 50%. These observations are consistent with those of Hill-Samli and MacLeod (7).

Proliferation of the 2B8 cells was stimulated by TRH and E_2, inhibited by P and not affected by DA. There was no correlation between cell proliferation and PRL release or inhibition. Cell number was at its maximum with the dose of E_2 that failed to stimulate PRL release. Incubation of the cells with TRH or E_2 lead to a 15-fold increase in PRL release but only a 19–20% increase in cell number. DA inhibited hormone release but had no effect on cell proliferation. The degree of inhibition of PRL release by P was much greater than the extent to which cell number was reduced. In their studies with the GH_3 tumor cells, Haug and Gautvik (5) found no change in cell growth (as determined through quantitation of cell protein) with either E_2 or P. They speculated that either the cells had lost the ability to proliferate under the influence of E_2 or that the factor(s) responsible for cell growth were of hypothalamic origin. In light of our findings, the latter hypothesis seems unlikely.

Heretofore, the only reported investigation in which stimulation of PRL release was observed with DA was that of Malarkey *et al.* (28). They found an increase in PRL release by GH tumor cells using 4.5×10^{-5} *M* DA. A similar phenomenon was consistently observed from the 2B8 cells with 10^{-9} *M* DA. Since concentrations of DA in the hypophyseal portal blood have been reported to be in excess of 10^{-9} *M* (29), the significance of our observations remain unclear. The secretory pattern of the 2B8 cells was unaffected by 10^{-8} *M* DA. Likewise, no change in PRL release was observed following incubation of hemipituitary glands with approximately 1.2×10^{-8} *M* DA (30). Other investigators (7, 8, 12, 17) including ourselves who have employed DA at concentrations which more closely approximate those values found in the hypophyseal portal blood (29) have obtained a marked inhibition of PRL secretion. From these reports one would assume that DA acts to inhibit the secretion of PRL from the anterior pituitary gland. However, this premise has recently been challenged by Neill *et al.* (31) who failed to obtain a significant correlation between peripheral PRL levels and DA concentration in the portal blood during the estrous cycle. This then raises the question of what the physiological role of DA is in the regulation of PRL secretion.

Summary. Prolactin (PRL) release was assessed using clonal cells from the 2B8 strain grown in the presence of estradiol (sodium sulfate) (E_2), progesterone (P), thyrotrophin-releasing hormone (TRH) and/or dopamine hydrochloride (DA). Using concentrations of E_2 ranging from 10^{-11} *M* to 10^{-6} *M*, it was found that a significant amount of PRL was released with doses of 10^{-10} *M* to 10^{-8} *M*. No effect was observed with either higher or lower concentrations of the steroid. Approximately the same amount of PRL was released from pituitary cells incubated with 2×10^{-9} *M* TRH as was noted with 10^{-8} *M* E_2. When these two secretagogues were combined, significantly more PRL was present in the medium than when either was employed alone. Prolactin release was significantly inhibited from cells cultured with 10^{-7} *M*–10^{-5} *M* DA; lower concentrations were either ineffective or were found to stimulate PRL secretion. In combination with 10^{-8} *M* E_2 or 2×10^{-9} *M* TRH, 10^{-6} *M* DA significantly reduced the stimulatory effect of either of these two compounds. However, when E_2 and TRH were incubtaed together in the presence of DA, less of an inhibition of PRL was noted than when DA was combined with either E_2 or TRH. Inhibition of PRL release also occurred when the cells were incubated with 10^{-11} *M*–10^{-6} *M* P. In addition, the stimulatory effect of 10^{-8} *M* E_2 was found to be suppressed by 10^{-6} *M* P. The results indicate that the 2B8 clonal strain is sensitive to agents known to effect PRL secretion *in vivo*.

The authors wish to express their gratitude to Pauline Cisneros-Polette, Rose Marie Gomez, and James Chambers for their technical assistance and to the NIAMDD for the rat PRL radioimmunoassay kits. This study was supported by USPHS Grant No. AM 12583.

1. Pasteels, J. L., Arch. Biol. **74,** 439 (1963).
2. Baker, B. L., Reel, J. R., Van Dewark, S. D., and Yu, Y. Y., Anat. Rec. **179,** 93 (1974).
3. Nicoll, C. S., and Meites, J., Endocrinology **70,** 272 (1962).
4. Nicoll, C. S., and Meites, J., Proc. Soc. Exp. Biol. Med. **117,** 579 (1964).
5. Haug, E., and Gautvik, K. M., Endocrinology **99,** 1482 (1976).
6. Vale, W., Blackwell, R., Grant, G., and Guillemin, R., Endocrinology **93,** 26 (1973).
7. Hill-Salmi, M., and MacLeod, R. M., Endocrinology **95,** 1189 (1974).
8. Hill, M. K., MacLeod, R. M., and Orcutt, P., Endocrinology **99,** 1612 (1976).
9. Gautvik, K. M., and Tashjian, A. H., Jr., Endocrinology **92,** 573 (1973).
10. Gautvik, K. M., and Tashjian, A. H., Jr., Endocrinology **93,** 793 (1973).
11. MacLeod, R. M., and Lehmeyer, J. E., *in* "Ciba Foundation Symposium on Lactogenic Hormones" (G. E. W. Wolstenholme and J. Knight, eds.), p. 53. Churchill Livingstone, London (1971).
12. Rennels, E. G., Blask, D. E., and Warchol, J. B., Gunma Symp. Endocrinol. **13,** 179 (1976).
13. Pasteels, J. L., Danguy, A., Frerotte, M., and Ectors, F., Ann. Endocrinol. (Paris) **32,** 188 (1971).
14. Ishikawa, H., Endocrinol. Japon. **16,** 517 (1969).
15. Hymer, W. C., Kraiser, J., Bencosme, S. A., and Haskill, J. S., Proc. Soc. Exp. Biol. Med. **141,** 966 (1972).
16. Hymer, W. C., Evans, W. H., Kraiser, J., Mastro, A., Davis, J., and Griswold, E., Endocrinology **92,** 275 (1973).
17. Hymer, W. C., Synder, J., Wilfinger, W., Swanson, N., and Davis, J., Endocrinology **95,** 107 (1974).
18. Synder, J., Wilfinger, W., and Hymer, W. C., Endocrinology **98,** 25 (1976).
19. Yasumura, Y., Tashjian, A. H., Jr., and Sato, G., Science **154,** 1186 (1966).
20. Tashjian, A. H., Jr., Yasumura, Y., Levin, L., Sato, G. H., and Parker, M. L., Endocrinology **82,** 342 (1968).
21. Tashjian, A. H., Jr., Bancroft, F. C., and Levine, L., J. Cell Biol. **47,** 61 (1970).
22. Steinberger, A., Chowdhury, A. M., and Steinberger, E., Endocrinology **92,** 18 (1973).
23. Ishikawa, H., Shiino, M., Arimura, A., and Rennels, E. G., Endocrinology **100,** 1227 (1977).
24. Aubert, M. L., Grumbach, M. M., and Kaplan, S. L., Acta Endocrinol. (Copenhagen) **77,** 460 (1974).
25. Gautvik, K. M., and Kriz, M., Endocrinology **98,** 344 (1976).
26. Chen, C. L., and Meites, J., Endocrinology **86,** 503 (1970).
27. Tashjian, A. H., Jr., Barowsky, N. J., and Jensen, D. K., Biochem. Biophys. Res. Commun. **43,** 516 (1971).
28. Malarkey, W. B., Groshong, J. C., and Milo, G. E., Nature (London) **266,** 640 (1977).
29. Ben-Jonathan, N., Oliver, C., Weiner, H. J., Mical, R. S., and Porter, J. C., Endocrinology **100,** 452 (1977).
30. Koch, Y., Lu, K. H., and Meites, J., Endocrinology **87,** 673 (1970).
31. Neill, J. D., Plotsky, P. M., Gibbs, D. M., and Tsou, R. C., *in* "Abstracts, 59th Annual Meeting, Chicago, Illinois," Abstract 74, p. 73. The Endocrine Society (1977).

Received October 24, 1977. P.S.E.B.M. 1978, Vol. 157.