Life Sciences, Vol. 35, pp. 389-398
Printed in U.S.A.

Pergamon Press

GONADOTROPIN RELEASING HORMONE: REGULATION OF PHOSPHOLIPID TURNOVER AND PROSTAGLANDIN PRODUCTION IN OVARIAN GRANULOSA CELLS

Zvi Naor, Moshe Zilberstein*, Haim Zakut* and Nava Dekel

Department of Hormone Research
The Weizmann Institute of Science
Rehovot 76100, Israel
and
*Department of Obstetrics and Gynecology
Sackler Faculty of Medicine
Tel Aviv University
The Edith Wolfson Hospital
Holon, Israel

(Received in final form May 14, 1984)

Summary

The direct effect of gonadotropin releasing hormone (GnRH) upon ovarian function, is initiated by a rapid receptor-mediated increase in phosphatidylinositol (PI) turnover (~ 5 min) followed by prostaglandin E (PGE, 120 min) and progesterone (120 min) formation, oocyte maturation and induction of ovulation. In contrast, luteinizing hormone (LH) stimulation of oocyte maturation and induction of ovulation is mediated by increased adenosine 3',5'-monophosphate (cAMP, 15 min), progesterone (30 min) and PGE (180 min) production. Both LH and GnRH stimulation of oocyte maturation are inhibited by dibutyryl cAMP and 3-isobutyl-1-methylxanthine, whereas induction of ovulation by the two hormones is blocked by indomethacin. GnRH and LH differ, therefore, in the mechanism leading to PGE formation, but thereafter share a common mechanism responsible for oocyte maturation and independently for induction of ovulation.

Pituitary gonadotropin biosynthesis and release is regulated by the decapeptide gonadotropin releasing hormone (GnRH). Paradoxical antifertility effects of GnRH *in vivo* have led to the findings that the peptide and its potent analogs exert direct effects on the gonads (1).

The inhibitory effects of GnRH on FSH-induced estrogen production and LH receptor formation in the ovary, might be explained by findings that GnRH analogs reverse the inhibitory effect of FSH on phosphodiesterase activity and progressively inhibit adenylate cyclase activity (2). In addition, the inhibitory effects of GnRH on FSH-induced progesterone production can result from inhibition of the side-chain cleavage enzyme and the increase in 20-hydroxysteroid dehydrogenase activity (3). However, the mechanism by which GnRH exerts its direct 'gonadotropin-like' effects on PGE and progesterone formation, oocyte maturation and ovulation, and the relationship between GnRH and LH actions are not yet clear (4-7).

0024-3205/84 $3.00 + .00

Recently, it was reported that LH stimulates PI labeling and steroidogenesis in luteal cells, and that LH and GnRH increase PI metabolism in ovarian granulosa cells (8-10). It was also suggested that GnRH analogs increase cAMP (11), PGE, and progesterone production in granulosa cells (4-6). Therefore, it seems that a common mechanism of action might exist for LH and GnRH in stimulating ovarian functions.

We and others have recently reported that enhanced phospholipid turnover is involved in the mechanism of action of GnRH in the pituitary and the ovary (12-14). We therefore examine in this report the effect of GnRH and LH on granulosa cell phospholipid turnover in relation to PGE and progesterone formation, and compare the effects by which both GnRH and LH stimulate oocyte maturation and induce ovulation.

Methods

GnRH and its [D-Ala^6]des-Gly^{10}-N ethylamide analog (GnRHa) were purchased from Peninsula Lab. Inc. (San Carlos, Ca). Ovine LH (oLH; NIH-S19) was kindly supplied by NIAMDD, NIH. Prostaglandins were obtained from the Upjohn Co., (Kalamazoo, MI). [5,6,8,11,12,14,15-^{3}H(N)]PGE$_2$ (165 Ci/mmol) and [1,2,6,7-^{3}H]Progesterone (90 Ci/mmol) were purchased from NEN Corp. (Boston, MA). PGE and progesterone antiserum were gifts from Dr. F. Kohen.

Immature (26-day-old) Wistar-derived rats from the departmental colony were injected with 15 I.U. of pregnant mare serum gonadotropin (PMSG .V. Organon, Oss, Holland). Granulosa cells were harvested 48 h later at room temperature by puncturing the follicle with a blunt probe and applying gentle pressure to the follicles. Cells were cultured in McCoy's medium containing glutamine (2 mM), fetal calf serum (5%), penicillin (50 u/ml) and streptomycin (50 µg/ml). Cell viability was determined by trypan blue exclusion and was normally between 70 and 80%. Cells (2×10^6/dish) were then pipetted into plastic tissue culture dishes (35 mm, Falcon) in a total volume of 2 ml and cultured for 2 days in a humidified incubator.

For phosphatidylinositol turnover determination, culture medium was removed and replaced by phosphate-free Krebs-Ringer-bicarbonate. Endogenous phospholipids were labeled for 60 min with 50 µCi [^{32}P]-Pi/dish before addition of GnRHa, [D-pGlu^1, pclPhe^2, D-Trp3,6]-GnRH (GnRH antagonist), ovine FSH (NIH-FSH-S7), or ovine-LH for another 60 min.

Cells were harvested in methanol and transferred to Eppendorf conical tubes. Cell phospholipids were then extracted using chloroform-methanol (2:1), followed by sonication for 1 min. After centrifugation (20,000 x g for 10 min), the supernatant was collected, evaporated under nitrogen, and redissolved in 0.5 ml chloroform. Samples were washed with 0.5 ml chloroform-methanol-water (3:4:3), and the lower phase was collected, dried, and redissolved in 25 µl chloroform-methanol (2:1). Samples were then applied to Silica gel G-coated plates (Kieselgel 60, Merck, Darmstadt, West Germany), and phospholipids were identified by two-dimensional thin layer chromatography with chloroform-methanol-methylamine (65:35:15), followed by a second separation with chloroform-acetone-methanol-acetic acid-water (100:40:20:30:10). The plates were exposed to autoradiography (Curix RP2, AGFA-GEVAER, Belgium), the radioactive spots were identified with markers, and the corresponding areas of the various phospholipids were removed and counted by the Cerenkov technique. The inorganic phosphorus contents of total lipid extracts and individual phospholipids were determined according to the method of Bartlett (19). For statistical analyses, data were pooled from several experiments, and the means compared with control values by Student's t test.

Cyclic AMP, prostaglandin E and progesterone formation

To follow GnRHa- and LH-induced cAMP, prostaglandin and progesterone formation, cultured cells were incubated in medium 199 containing 25 mM HEPES, 0.2 mM 3-isobutyl-1-methylxanthine (MIX) and 1 mg/ml BSA. Incubations were performed in triplicate at 37°C and test substances were added in 10 µl medium. Incubations were stopped by collecting the medium from the dishes and freezing the samples until assayed. When cAMP was determined, a portion of the medium (0.3 ml) was added to 0.3 ml of acetate buffer (50 mM, pH 4) and the sample was immediately boiled for 5 min. Cyclic AMP levels were determined by a competitive protein binding assay as previously described (20), and prostaglandin E and progesterone by radioimmunoassay (20,21). Significance was evaluated by Student's t test.

Oocyte maturation

Follicles were dissected from 26-day-old female rats injected subcutaneously with 15 I.U. of PMSG and killed 48 h later. Individual follicles were incubated in 2.5 ml of Leibovitz's L-15 tissue culture medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sera-Lab, England), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco), in the presence or absence of the indicated concentrations of the following agents: GnRHa, GnRH antagonist, ovine LH, dibutyryl 3':5'-cyclic monophosphate (DBC) or methylisobutylxanthine (MIX) (Sigma Chemical Co.). Incubations were carried out in a N_2:O_2 (1:1) atmosphere in an oscillating bath at 37°C. At the indicated time the follicles were incised and the cumulus-oocyte complexes were recovered. The oocytes were examined using an inverted microscope equipped with Nomarski interference contrast optics. Resumption of meiosis was indicated by the absence of the germinal vesicle (GV) in the oocyte. For each study, the data of several individual experiments were combined and the results are reported as the fraction of oocytes with GV breakdown (GVB).

Induction of ovulation

Hypophysectomy was performed on mature Wistar proestrous female rats and followed by a subcutaneous injection of 15 I.U. of PMSG in 0.1 ml of 0.9% NaCl. An intraperitoneal injection of either human chorionic gonadotropin (hCG, Pregnyl, Organon, Holland; 4 I.U./rat) or GnRHa (500 ng/rat) was given 24 h later. A second group of the hypophysectomized rats was injected with one of these hormones in combination with either 5 µg per rat of a GnRH-antagonist or 2 mg per rat of indomethacin (1-[p-chlorobenzoyl]-5- methoxy-2-methylindol-3-acetic acid, Assia Chemical Labs., Israel). The rats were killed by cervical dislocation 20 h after injection. The oviducts were removed and examined for the presence of ovulated oocytes.

Results

Cultured granulosa cells from preovulatory follicles were incubated with ^{32}P-Pi to label endogenous phospholipids (Fig. 1a) and later stimulated with GnRHa, FSH or LH for up to 60 min (Fig. 1b). GnRHa (10^{-9}M) increased the specific labeling of PI and phosphatidic acid (PA) by 4.5 and 3.5 fold respectively ($p < 0.02$) and the effect was abolished by the potent GnRH antagonist [D-pGlu^1, pclPhe^2, D-Trp^{3,6}]GnRH (Fig. 1b). The effect was dose-related (ED_{50}=10^{-9}M) and could be detected after 5-10 min of incubation (13). Both LH and FSH had no stimulatory effect on phospholipid labeling during 60 min of incubation.

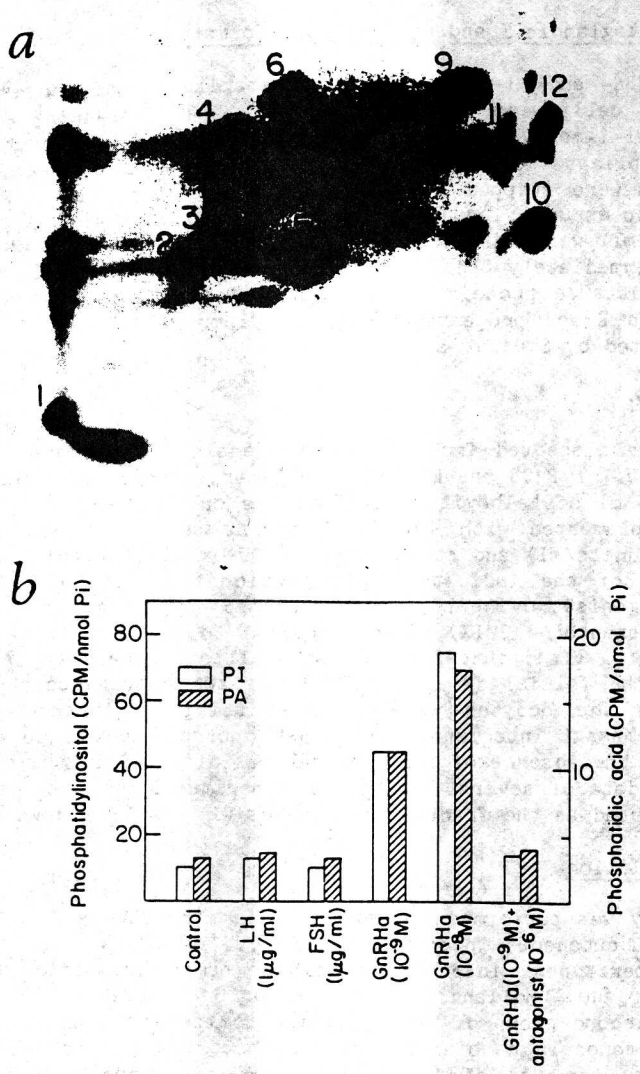

FIG. 1:

Effect of GnRH-analog (GnRHa), LH and FSH on phospholipid labeling. Granulosa cells were prepared from 26-day-old female rats pretreated with 15 I.U. of PMSG. Endogenous phospholipids were labeled for 60 min with 50 μCi [^{32}P]-Pi/dish before addition of GnRHa, [D-pGlu^{1}, pclPhe^{2}, D-Trp^{3,6}]-GnRH (GnRH antagonist), Ovine FSH, or ovine-LH for another 60 min. Cell phospholipids were extracted and separated by two dimensional thin layer chromatography as described. Plates were then exposed to autoradiography (curix RP2, AGFA) for 2 days and the spots were identified with markers, removed and counted by the Cerenkov technique. Inorganic phosphorus (Pi) was determined according to Bartlett (19) a. The numbers on the autoradiograph denote the relative migration of the phospholipids: 1) origin, 2) unidentified, possibly phosphatidylinositol plasmalogen, 3) lysophosphatidylcholine, 4) phosphatidylcholine plasmalogen, 5) phosphatidylinositol, 6) phosphatidylethanolamine plasmalogen, 7) phosphatidylserine, 8) phosphatidylcholine, 9) phosphatidylethanolamine, 10) phosphatidic acid, 11) phosphatidyl-

glycerol, 12) cardiolipin. b. Results are the mean of triplicate determinations representing three separate experiments (SE < 10%). Labeling of other phospholipids such as phosphatidylethanolamine, phosphatidylserine, choline plasmalogen, phosphatidylglycerol and cardiolipin was not affected by the GnRHa treatment, while a small increase in phosphatidylcholine and lysophosphatidylcholine was observed.

On the other hand, LH, but not GnRHa, produced a time-related increase in cAMP production in cultured granulosa cells (Fig. 2). At concentrations of 10^{-12}-10^{-7}M, GnRHa had no stimulatory effect on cAMP accumulation up to 4 h of incubation (Fig. 2 and data not shown). However, both LH and GnRHa produced a dose- and time-related increase in progesterone formation which was first observed after 30 min of incubation and became significant ($p<0.05$ and $p< 0.02$) after 30 and 120 min for LH and GnRHa respectively. A 2-3 fold increase in progesterone formation was detected at the end of the incubation period (Fig. 2).

It is assumed that LH-induced cAMP formation mediates PGE_2 and progesterone formation (15-18), therefore we also measured PGE levels in the culture medium. GnRHa and LH increased the formation of PGE in a time related manner and the effect could be detected after 2 and 3 hours of incubation for GnRHa and LH respectively ($p< 0.001$ and $p< 0.05$ respectively). At the end of the incubation period there was a 3 fold increase in PGE production by GnRHa and LH.

It was recently reported that GnRH analogs can mimic the effect of LH on stimulation of both meiosis resumption of follicle enclosed rat oocyte in vitro, and induction of ovulation in hypophysectomized rats (4-7). We therefore investigated whether the formation of PGE by GnRHa mediates the stimulatory effect on oocyte maturation and ovulation. As shown in Fig. 3, administration of indomethacin blocked the stimulatory effect of both LH and GnRHa on induction of ovulation but had no effect on the peptide-induced oocyte maturation, indicating that PGE is involved in induction of ovulation but not in oocyte maturation. On the other hand, the stimulatory effect of both LH and GnRHa on resumption of meiosis in follicle-enclosed rat oocyte in vitro was blocked by DBC and MIX. These results suggest that GnRH and LH share a common mechanism leading to resumption of oocyte maturation. The mechanism by which DBC and MIX inhibit hormone induced oocyte maturation is not yet known (22).

The possibility that gonadal GnRH-like material (23,24) is involved in mediating LH actions on oocyte maturation and ovulation was excluded by demonstrating that GnRH antagonist capable of inhibiting GnRH-induced stimulatory effects failed to influence LH actions (Fig. 3).

Discussion

In this report we present evidence that following its binding to specific receptors in granulosa cells (25), GnRH, but not LH, activates the PI response (26,27). Increased PI turnover is believed to precede the opening of calcium channels, and PA was recently implicated as an endogenous calcium ionophore (28,29). The enhanced labeling of PA suggest the involvement of a PI-specific phospholipase C in GnRH action (30). The PA thus formed can activate a PA-specific phospholipase A_2 that will liberate free arachidonic acid (AA) and produce lysophosphatidic acid. The lysoderivative and the re-

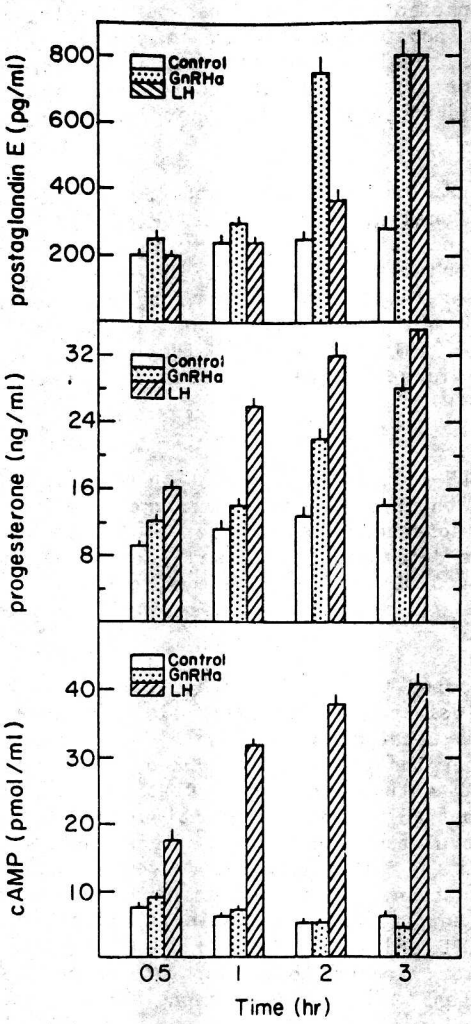

FIG. 2:

Effect of GnRH analog and LH on cAMP, progesterone and prostaglandin E formation in cultured granulosa cells. Granulosa cells were prepared from preovulatory follicles as described. After 2 days in culture the medium was changed to medium 199 containing HEPES (20 mM), MIX (0.2 mM), and BSA (1 mg/ml). GnRHa (10^{-7}M) and oLH(1 µg/ml) were added for the time indicated. Medium was collected for radioimmunoassay of progesterone and prostaglandin E, and cAMP was determined in the medium by a competitive protein binding assay. Results are the mean ± standard error of triplicate determinations representing one of 3 similar experiments.

maining PA can serve as calcium-ionophores and activate phospholipase A_2 that liberate AA from other phospholipids such as phosphatidylcholine and phosphatidylethanolamine (31). The accumulated AA is then converted to PG's and PGE mediates GnRHa induction of ovulation. On the other hand LH stimulation of PGE production is mediated by cAMP formation in the absence of elevated PI

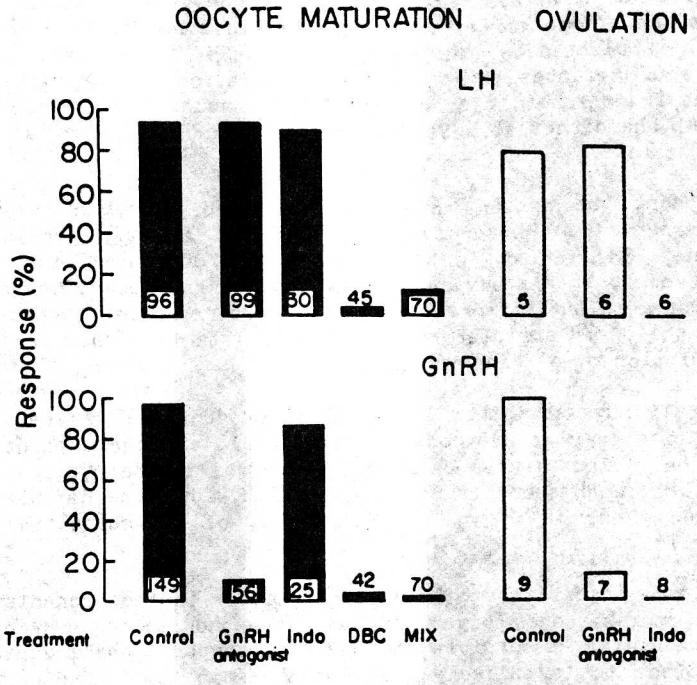

FIG. 3:

Comparative studies on GnRH and LH stimulation of ovarian functions. To study oocyte maturation, follicles were isolated from the ovaries of immature rats 48 h after PMSG (15 IU/rat) priming and incubated in the presence of either oLH (0.1 μg/ml) or GnRHa (10^{-7} M) with or without either GnRH antagonist, (10^{-5} M), dibutyryl cyclic AMP (DBC, 5 mM), MIX (0.2 mM) or indomethacin (Indo 10 μg/ml). The incidence of maturation in the oocytes recovered from the incubated follicles after 20 h was indicated by the breakdown of the germinal vesicles. The numbers at the bars represent for this study the amount of oocytes analyzed for each experimental group. Ovulation was studied in mature rats hypophysectomized on the morning of proestrus and later (2 p.m.) injected with hCG (4 IU/rat) or GnRHa (500 ng/rat) with or without a combined treatment with either GnRH antagonist (5 μg/rat) or indomethacin (2 mg/rat). The oviducts of the treated rats were examined for the presence of ovulated oocytes 20 h after the injections. The numbers at the bars represent the number of rats (SE < 10%).

labeling. Further studies are needed to support our proposal of the cascade of events involved in GnRH-induced PGE formation.

Since it is thought that cAMP mediates LH effects on both PGE and progesterone production (15-18), it is obvious that LH and GnRH differ in the mechanism of action leading to PGE and progesterone formation. It is possible that GnRH-induced PI turnover is also involved in progesterone formation via the recently discovered calcium-activated, phospholipid-dependent protein kinase C (32). Nevertheless, the two pathways merge apparently at the PGE production step.

Although little is known about the process of oocyte maturation, both LH and GnRH seem to share some mechanistic pathways since the stimulatory effect of both hormones is blocked by DBC and MIX but not by indomethacin. It has been shown that PGE mediates LH-induction of ovulation (for review see 17) the finding that indomethacin also blocked GnRH-induced ovulation indicates that PGE mediates the direct stimulatory effect of GnRH on induction of ovulation.

Our data support the notion that the AA needed for GnRH-induced PGE synthesis was derived from PI turnover, as suggested for angiotensin II action in the kidney (33). Also, in terms of the time course of GnRHa action, the effect observed in this study represents, to our knowledge, the earliest biochemical response to the hormone, and hence might represent initial events leading to both the early stimulatory effects and later to inhibition of gonadotropin stimulation of ovarian function.

We have recently suggested that lipoxygenase products of AA are involved in GnRH-induced pituitary gonadotropin release (12), and the present report indicates that the cyclooxygenase pathway is involved in the ovarian actions of GnRH. Therefore, GnRH seems to be an example of a hormone capable of selectively activating one of the two major pathways of AA metabolism in different target cells.

Our data indicate that although LH and GnRH differ in the mechanism leading to PGE and progesterone formation (Fig. 4), thereafter both peptides share a common mechanism responsible for oocyte maturation and independently to ovulation. Since it is unlikely that hypothalamic GnRH reaches the gonads, it is possible that the data presented provided insight into the mechanism of action of putative GnRH-like substances in the gonads of some species (23,24). Finally, the pathway suggested for GnRH action in the pituitary (12,14) and the ovary (present results), provides a novel alternative mechanism of action for peptide hormones which do not act via cAMP formation.

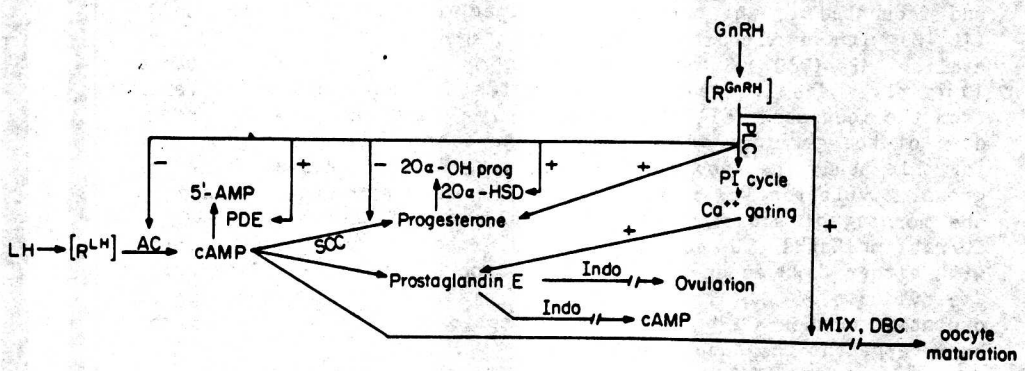

FIG. 4:

Scheme of proposed mechanism of LH and GnRH action. R, represent the respective specific receptor for LH and GnRH; PI, phosphatidylinositol; indo, indomethacin; MIX, 3-isobutyl-1-methylxanthine; DBC, dibutyryl cAMP; PLC, phospholipase C; AC, adenylate cyclase; PDE, phosphodiesterase; SCC, side chain cleavage enzyme; 20α-HSD, 20α-hydroxysteroid dehydrogenase.

Acknowledgements

We thank Mrs. Yona Eli for excellent technical assistance, Dr. D. Coy for the GnRH antagonist, and Drs. A. Tsafriri and U. Zor for the interest taken in this study. Z.N. and N.D. are the Incumbents of the Charles H. Revson Career Development Chairs. M.Z. is a recipient of Grant-in-Aid from the Chief Scientist's Bureau of the Ministry of Health, Israel. We also thank Mrs. M. Kopelowitz for typing the manuscript.

REFERENCES

1. A.J.W. HSUEH and P.B.C. JONES, Endocrine Reviews 2, 437-461 (1981).
2. M. KNECHT and K.J. CATT, Science 214, 1346-1348 (1981).
3. P.B.C. JONES and A.J.W. HSUEH, Endocrinology 111, 713-721 (1982).
4. T. HILLENSJO and W.J. LEMAIRE, Nature 287, 145-146 (1980).
5. C. EKHOLM, T. HILLENSJO and O. ISAKSSON, Endocrinology 103, 2022-2024 (1981).
6. C. EKHOLM, M.R. CLARK, C. MAGNUSSON, O. ISAKSSON and W.J. LEMAIRE, Endocrinology 110, 288-290 (1981).
7. N. DEKEL, I. SHERIZLY, A. TSAFRIRI and Z. NAOR, Biol. Reprod. 23, 161-166 (1983).
8. J.S. DAVIS, R.V. FARESE and J.M. MARSH, Endocrinology 109, 469-475 (1981).
9. J.S. DAVIS, R.V. FARESE and M.R. CLARK, 15th Annual Meeting of the Society for the Study of Reproduction, Madison, Wisconsin, Abs. 9. (1982).
10. J.S. DAVIS, R.V. FARESE and M.R. CLARK Proc. Natl. Acad. Sci. USA 80, 2049-2053 (1983).
11. T. HILLENSJO, W.J. LEMAIRE, M.R. CLARK and K. AHREN Acta. Endocrinol. 101, 603-610 (1982).
12. Z. NAOR and K.J. CATT, J. Biol. Chem. 256, 2226-2229 (1981).
13. Z. NAOR and E. YAVIN, Endocrinology 111, 1615-1619 (1982).
14. G.D. SNYDER and J.E. BLEASDALE, Mol. Cell. Endocrinol. 28, 55-63 (1982).
15. A. TSAFRIRI, H.R. LINDNER, U. ZOR and S.A. LAMPRECHT J. Reprod. Fertil. 31, 39-50 (1972).
16. U. ZOR, B. STRULOVICI, A. NIMROD and H.R. LINDNER, Prostaglandins 14, 947-959 (1977).
17. U. ZOR and S.A. LAMPRECHT, Biochemical Actions of Hormones, Ed. G. Litwack, (Academic Press) p. 85 (1977).
18. M.R. CLARK, J.M. MARSH and W.J. LEMAIRE, Endocrinology 102, 39-44 (1978).
19. G.R. BARTLETT, J. Biol. Chem. 234, 466-468 (1959).
20. Z. NAOR, Y. KOCH, S. BAUMINGER and U. ZOR, Prostaglandins 9, 211-215 (1975).
21. F. KOHEN, S. BAUMINGER and H.R. LINDNER, Steroid Immunoassays Eds. E.H.D. Cameron, S.G. Hillier, and K. Griffiths, Alfa-Omego, Cardiff, Wales, U.K. pp. 11-32 (1975).
22. N. DEKEL, T.S. LAWRENCE, N.B. GILULA and W.H. BEERS, Devel. Biol. 86, 356-362 (1981).
23. S. YING, N. LING, P. BOHLEN and R. GUILLEMIN, Endocrinology 103, 1206-1215 (1981).
24. R.M. SHARP, H.M. FRASER, I. COOPER and F.F.G. ROMMERTS, Nature 290, 785-787 (1931).
25. T. RANTA, M. KNECHT, M. KODY and K.J. CATT, Mol. Cell. Endocrinol. 27, 233-240 (1982).
26. M.R. HOKIN and L.E. HOKIN, J. Biol. Chem. 203, 967-977 (1953).
27. R.H. MICHELL, Biochim. Biophys. Acta. 415, 81-157 (1975).
28. D.M. SALMON and T.W. HONEYMAN, Nature 284, 344-345 (1980).

29. J.W. PUTNEY, S.J. WEISS, C.M. VAN DE WALLE and R.A. HADDAS, Nature 284, 345-347 (1980).
30. S. RITTENHOUSE-SIMMONS, J. Clin. Invest. 63, 580-587 (1979).
31. E.G. LAPETINA, M.M. BILLAH and P. CUATRECASAS, Nature 292, 367-369 (1981).
32. M. CASTAGNA, Y. TAKAI, K. KAIBUCHI, K. SANO, U. KIKKAWA and Y. NISHIZUKA, J. Biol. Chem. 257, 7847-7851 (1982).
33. J.E. BENABE, L.A. SPRY and A.R. MORRISON, J. Biol. Chem. 257, 7430-7434 (1982).