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EVIDENCE FOR COUPLING OF DIFFERENT RECEPTORS FOR GONADOTROPIN-RELEASING HORMONE TO PHOSPHOLIPASES C AND A_2 IN CULTURED RAT LUTEAL CELLS

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Effects of [D-Ala^6,Des-Gly^{10}]gonadotropin-releasing hormone (GnRH), ethylamide (GnRHa), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on inositol phosphate (IPs) formation and arachidonic acid (AA) release were studied in rat luteal cells of primary culture. In the cells obtained from one-day-old corpora lutea, $PGF_{2\alpha}$ (100 nM) and GnRHa (100 nM) significantly increased the IPs formation and the AA release. Antagonists of GnRH added solely or with GnRHa did not stimulate the IPs formation but did stimulate the AA release. In the cells obtained from 5-day-old corpora lutea, GnRHa failed to stimulate the IPs formation but significantly stimulated the AA release. The stimulation of both IPs formation and AA release by $PGF_{2\alpha}$ was consistently found in cells of two different luteal ages. These results suggest that GnRH receptor independently couples to both phospholipases C and A_2 through different classes of GnRH receptors.

$PGF_{2\alpha}$, GnRH and GnRH agonists are known to trigger luteolysis by the inhibition of cAMP production in rat luteal cells. It was shown in cultured luteal cells that $PGF_{2\alpha}$ and GnRH stimulated the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (1-3). These hormones are also known to stimulate the release of arachidonic acid from the cells (4). Phospholipase A_2 can be activated following ligand binding to a cell surface receptor either by a receptor coupled mechanism (5-8) or by a mechanism mediated by intra-

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ABBREVIATIONS BSA, bovine serum albumin; $[Ca^{2+}]i$, intracellular free calcium concentration; FCS, fetal calf serum; GnRH, gonadotropin releasing hormone ; hCG, human chorionic gonadotropin; IPs, inositol phosphates; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI, phosphatidylinositol; PMSG, pregnant mare's serum gonadotropin.

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cellular second messengers (9-11). Our previous results demonstrated stimulation of arachidonic acid release from cultured luteal cells by the additions of PMA and Ca ionophore A23187 (12). These results suggest the possibility that the activation of phospholipase A_2 following stimulation by $PGF_{2\alpha}$ and GnRHa is a cosequence of the hydrolysis of PIP_2 . In this paper we present the results on the mechanism of phospholipase A_2 activation mediated through the GnRH receptor in rat luteal cells. The results indicate that GnRH stimulates arachidonic acid release by a receptor-coupled mechanism.

MATERIALS AND METHODS

Cell Culture Immature (26-day-old) female Sprague-Dawley rats were injected subcutaneously with 50 IU PMSG, which was followed 64 h later by an injection of 25 IU hCG. In such rats, corpora lutea were observed on two days after hCG injection (one-day-old corpora lutea). Ovaries were removed 2 days or 6 days after the treatment with hCG, minced and digested with collagenase-dispase as described previously (12). Dispersed cells were then washed and isolated on a discontinuous density gradient (Percoll) (13). Aliquots of cell suspension in Medium 199 (5×10^5 cells/1.5 ml) were added to 35 mm culture plates (Corning, Glass Works, NY.) and cultured at 37°C under an atmosphare of 5% CO_2 in air for 4 days. Medium was changed every 2 days. At the 4th day cells became near confluent (about 7×10^5 cells/dish). The purities of the cells assessed by 3β hydroxysteroid dehydrogenase activity possessing cells was 70 to 80 % and the viability of the cells was always more than 90 %.

Analysis of Inositol Phosphates On the 3rd day medium was changed to Medium 199 supplemented with 10 % FCS and 5 μCi/ml myo-[2-^{3}H]inositol and the cells were incubated for further two days. Pre-labeled cells were washed twice with Hepes-buffered saline (145mM NaCl, 5mM KCl, 1mM Na_2HPO_4, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, 5mM glucose, 10mM Hepes-NaOH pH 7.4)(HBS) containing 0.1 % BSA. Incubation was started by adding 1 ml of the Li-HBS, in which a portion (10mM) of NaCl in HBS had been replaced with 10mM LiCl, with or without GnRHa, GnRH antagonists or $PGF_{2\alpha}$. After 15 min at 37°C, reactions were terminated by removing the media and scrapeing the cells in 1 ml ice-cold methanol. Lipids were extracted by successive additions of chloroform and water(14). The radiolabeled inositol phosphates in the aqueous phase were determined by anion exchange chromatography as described previously(14).

Analysis of Arachidonic Acid Release On the fourth day of culture, cells were incubated for 4 h with Medium 199 containing 1 μCi/ml of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid. Prelabeled cells were washed three times with HBS containing 0.1 % fatty acid free BSA and then preincubated with the same buffer for 15 min at 37°C. Incubations were started by the addition of 1 ml of the buffer with or without hormone. After 15 min at 37°C, media were collected and extracted with 1.5 ml of chloroform-methanol (1:2, v/v). The chloroform-methanol extract was partitioned into two phases by adding 1 ml each of chloroform and water. The radioactivity of the lower phase was determined by liquid scintillation counting. More than 92 % of the radioactivity in the lower phase was located at the position of free fatty acid by TLC. Where indicated, the cells were preincubated with mepacrine (200 μM) for 15 min ,which was followed by an incubation for 15 min with or without hormone in the presence of mepacrine.

Analysis of lysophospholipids On the 3rd day of the culture, medium was changed to a culture medium containing 0.1 μCi/ml of [2-^{14}C]glycerol and the cells were labeled for two days. Prelabeled cells were washed twice with HBS containing 0.1 % BSA and incubated for 15 min at 37°C in 1 ml of the buffer with or without hormone. Media were removed and cells were scraped into 1 ml of cold methanol. Lipids were extracted by the method of Folch et al.(15). Carrier phospholipid mixture was added to the sample and lysophospholipids were separated by two dimensional TLC as described previously (16). Area of the individual phospholipid were detected by I_2 vapors, scraped into scintillation vials and counted.

Determination of $[Ca^{2+}]i$ Cells were harvested by scraping with a rubber policeman on the 4th day after plating. The cells were loaded with fura 2 by incubating in Medium 199 containing 1.5 μM fura 2/acetoxymethyl ester at 37°C for 30 min. After being loaded, cells were washed three times with HBS and suspended in the same saline. Changes in fura 2 fluorescence were recorded at 37°C as described (17).

Chemicals myo-[2-^{3}H]inositol (15.8 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (83.6 Ci/mmol) and [2-^{14}C]glycerol (15.0 mCi/mmol) were obtained from New England Nuclear Corp. (Boston,M.A.). $PGF_{2\alpha}$ was a gift from Ono Pharmaceuticals (Osaka). PMSG and hCG were supplied by Teikoku Hormone MGF (Tokyo). Commercial sources of other chemicals were as follows: Medium 199 , Nissui,Tokyo; Fetal calf serum, Gibco, Grand Island, NY.; Type I dispase, Godo Shusei, Tokyo; Type I collagenase, fatty acid free BSA, quinacrine dihydrochloride (mepacrine), Sigma, St Louis, MO.; [D-Ala^{6}, Des-Gly^{10}]GnRH ethylamide (GnRHa), [D-Phe^{2},Pro^{3},D-Phe^{6}]GnRH (GnRH antagonist I), [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]GnRH (GnRH antagonist II), Peninsula Laboratories, Belmont,CA.; TLC plates, Merck, Darmstadt; fura 2 /penta acetoxymethyl ester, Dojin Chemicals, Kumamoto, Japan.

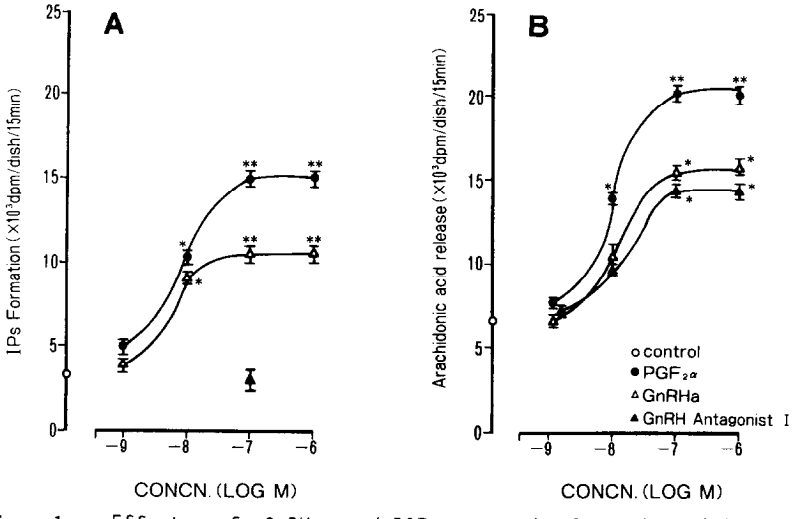

Fig. 1. Effects of GnRHa and $PGF_{2\alpha}$ on the formation of inositol phosphates (A) and the release of arachidonic acid (B) in day 5 luteal cells. Prelabeled luteal cells were incubated for 15 min in the presence of various concentrations of each hormone. Each point represents the mean ± SEM for triplicate experiments.
* P <0.01, ** P <0.001 as compared with identical incubations without hormones.

RESULTS AND DISCUSSION

[^{3}H]Inositol phosphate formation and [^{3}H]arachidonic acid release in cultured luteal cells obtained from one-day-old corpora lutea. Rat luteal cells obtained from one-day-old corpora lutea were cultured for 4 days before experiment (day 5 luteal cells). Day 5 luteal cells were incubated for 15 min with various concentrations of $PGF_{2\alpha}$ and GnRHa. As shown in Fig.1.A, addition of more than 10^{-8}M of each hormone stimulated the accumulation of inositol phosphates in the cells. Increases in the intracellular free calcium ion concentration ($[Ca^{2+}]i$) in response to $PGF_{2\alpha}$ (100 nM) and GnRHa (100 nM) are shown in Fig. 2. A and C. These results show that $PGF_{2\alpha}$ and GnRHa activate PIP_2 phsopholipase C. Similar observations have been reported by other workers (1-3). Antagonists of GnRH (I and II) added alone or with GnRH did not stimulate phospholipase C (Table I). The addition of $PGF_{2\alpha}$ or GnRHa increased the release of arachidonic acid from the luteal cells into the medium (Fig. 1.B, Table I). The effects of these hormones on release of arachidonic acid was abolished by the addition of mepacrine, an inhibitor of phospholipase A_2 (Fig. 3).

We studied the sources of free arachidonic acid to know whether they originated from any special phospholipid. As shown in Fig. 4, the contents of lysophosphatidylinositol and lysophosphatidylcholine significantly increased and those of phosphatidylinositol and phosphatidylcholine significantly decreased after the addition of $PGF_{2\alpha}$ or GnRHa. These results show that the stimulation of arachidonic acid release was a result of phospholipase A_2 activation. Antagonists of

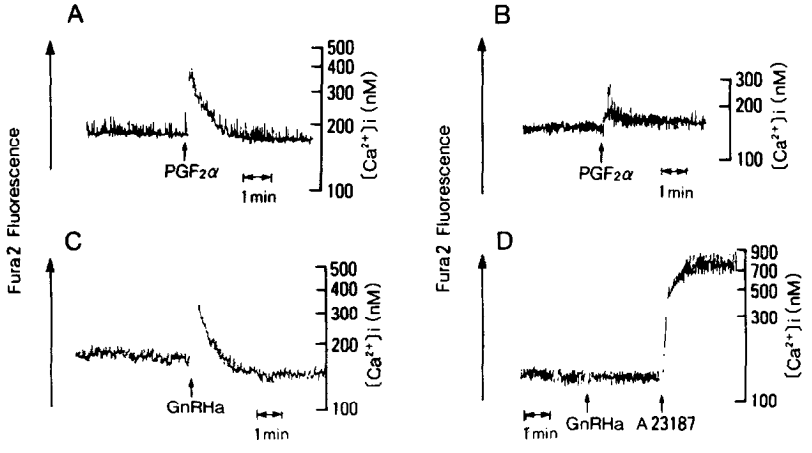

Fig. 2. Effects of $PGF_{2\alpha}$ (A, B) and GnRHa (C, D) on the increase in $[Ca^{2+}]i$. Day 5 luteal cells (A, C) or day 9 luteal cells (B, D) were suspended in 1 ml HBS at 37°C. To the suspension ($1x10^6$ cells/ml) 100 nM of each hormone was added at indicated time.

Table I. Effects of $PGF_{2\alpha}$, GnRHa and GnRH antagonists on IPs formation and arachidoinc acid release in luteal cells obtained from one-day-old corpora lutea (day 5 luteal cells) or 5-day-old corpora lutea (day 9 luteal cells)

	Treatment	Total[^{3}H]IPs[a]	AA release[b]
		% of control	% of control
Day 5 luteal cells	$PGF_{2\alpha}$ (100 nM)	342.1 ± 2.3**	249.2 ± 3.2**
	GnRHa (100 nM)	162.9 ± 2.1**	201.5 ± 3.7**
	GnRH antagonist I (100 nM)	79.1 ± 1.5*	171.8 ± 1.1**
	GnRH antagonist II (100 nM)	80.6 ± 1.0*	161.9 ± 4.1*
	GnRHa (100 nM) + GnRH antagonist I (100 nM)	79.3 ± 1.5*	213.5 ± 5.3*
Day 9 luteal cells	$PGF_{2\alpha}$ (100 nM)	238.5 ± 1.2**	203.5 ± 8.4*
	GnRHa (100 nM)	82.9 ± 6.8	232.6 ± 1.3**

[a]Results are calculated from the sum of [^{3}H]IPs ($IP+IP_2+IP_3$) and expressed as [b]percentages of the control(n=3).
[b]Results are expressed as percentages of the control(n=3).
Control values for IPs are 5069 ± 10 d.p.m./dish in day 5 luteal cells, and 9847 ± 118 d.p.m./dish in day 9 luteal cells. Control values for arachidonic acid release are 8402 ± 123 d.p.m./dish in day 5 luteal cells, and 6041 ± 20 d.p.m./dish in day 9 luteal cells. *P<0.01, **P<0.001 as compared with control.

GnRH (I and II) unexpectedly stimulated the release of arachidonic acid from the luteal cells (Table I).

[^{3}H]Inositol phosphate formation and [^{3}H]arachidonic acid release in cultured luteal cells obtained from 5-day-old corpora lutea. The effects of luteolitic hormones were studied in cultured luteal cells obtained from 5-day-old corpora lutea (day 9 luteal cells). As shown

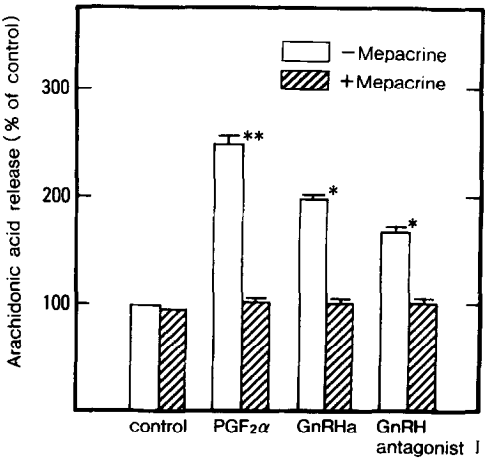

Fig. 3. Effect of mepacrine (200 μM) on the release of arachidonic acid. Values are means ± SEM (n=3) expressed in percent of the control values (8402 ± 123 d.p.m./dish), which were obtained in the incubation without mepacrine and hormones. * P < 0.01, ** P < 0.001 as compared with identical incubation without hormones.

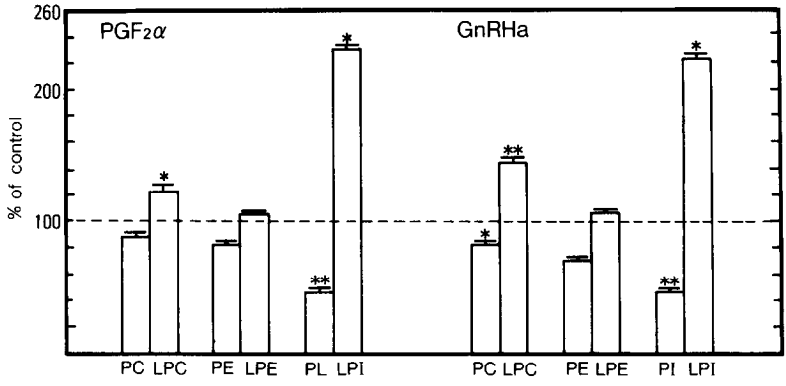

Fig. 4. The relative radioactivity found in phospholipids at 15 min after addition of $PGF_{2\alpha}$ (100 nM) or GnRHa (100 nM). The radioactivity in each phospholipid obtained from cells incubated in the absence of hormone is taken as 100 %. * $P < 0.01$, ** $P < 0.001$ as compared with control.

in Fig. 2.B and D, and Table I (lower part), we found in the mature corpora lutea that the signal transduction through GnRH receptor-phospholipase C route diminished while the signal transduction through GnRH receptor-phospholiase A_2 route was still active. Changes in the generation of intracellular messengers during maturation of rat corpora lutea has been reported by Lahav et al.(3). These authors showed that cells at 7 days of luteal age were no longer stimulated by GnRH and $PGF_{2\alpha}$ to form inositol phosphates. In our luteal cells, however, $PGF_{2\alpha}$ could still activate both phospholipases C and A_2(Table I). The findings that GnRH antagonists failed to antagonize the GnRHa on the activation of phospholipase A_2 might suggest that different classes of GnRH receptors are involved in the activation of phospholipases A_2 and C. This possibility is further supported by the fact that the day 9 luteal cells which lacked the response to GnRHa by phospholipase C activation still have the response to GnRHa by phospholipase A_2 activation. The results indicate that GnRH activates phospholipase A_2 and C through two independent pathways.

Experiments using luteal cells permeabilized with saponin showed that $GTP\gamma S$ (100 μM) enhanced the effects of GnRHa and $PGF_{2\alpha}$ on phospholipases A_2 and C (Data not shown). This result implies that in rat luteal cells receptors for GnRH and $PGF_{2\alpha}$ are coupled to phospholipases A_2 and C through GTP-binding proteins. Pretreatment of luteal cells with Bordetella pertussis toxin (100 ng/ml) for 24 h had no effect on the activation of phospholipases A_2 and C by GnRHa and $PGF_{2\alpha}$. ADP-Ribosylation of 41 Kd protein was evident in the pertussis toxin treated luteal cells (Data not shown).

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REFERENCES

1. Leung,P.C., Minegishi,T., Ma,F., Zhou,F., and Ho-Yuen,B. (1986) Endocrinology, 119, 12-18.
2. Davis,J.S., Weakland,L.L., Weiland,D.A., Farse,R.V., and West,L.A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3728-3732.
3. Lahav,M., West,L.A., and Davis,J.S. (1988) Endocinology 123, 1044-1052.
4. Minegishi,T.,and Leung,P.C.K.(1985) Endocrinology 117, 2001-2007
5. Burch,R.M., Luini,A., and Axelrod,J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7201-7205.
6. Slivka,S.R., and Insel,P.A. (1987) J. Biol. Chem. 262, 4200-4207.
7. Kaya,H., Patton,G.H., and Hong,S.L. (1989) J. Biol. Chem. 264, 4972-4977.
8. Cockcroft,S.,and Stutchfield,J. (1989) Biochem. J. 263, 715-723.
9. Bell,R.L., Kennerly,D.A., Stanford,N., and Majerus,P.W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3238-3241.
10. Ho,A.K., and Klein,D.C. (1987) J. Biol. Chem. 262, 11764-11770.
11. Martin,T.W., and Wysolmerski,R.B. (1987) 262, 13086-13092.
12. Yamamoto,H., Tanaka,S., and Hayashi,H.(1987) The Sapporo Med. J. 56, 755-770.
13. Gore,S.D., and Behrman,H.R. (1984) Endocrinology 114, 2020-2031.
14. Hasegawa-Sasaki,H. (1985) Biochem. J.232, 99-109.
15. Folch,J., Lees,M., and Sloane-Stanley,G.H. (1957) J. Biol. Chem. 227, 497-509.
16. Nagao,M. (1986) The Sapporo Med. J. 55, 421-431.
17. Hasegawa-Sasaki,H., Lutz,F., and Sasaki,T. (1988) J. Biol. Chem. 263, 12970-12976.