

Expression of Prostacyclin Receptors in Luteinizing Hormone-Releasing Hormone Immortalized Neurons: Role in the Control of Hormone Secretion*

F. PIMPINELLI, G. E. ROVATI, V. CAPRA, F. PIVA, L. MARTINI, AND R. MAGGI

Department of Endocrinology, Institute of Pharmacological Sciences (G.E.R., V.C.), University of Milan, Milan, Italy

ABSTRACT

PGs of the E series are involved in the control of LHRH secretion. The present experiments were conducted to clarify whether PGI₂ (prostacyclin) might be also involved in such a control, using multiple methodological approaches on immortalized LHRH-secreting neurons. A RT-PCR procedure to detect mouse PGI₂ receptor (IP) messenger RNA was first applied, and the results obtained showed the presence of a specific transcript in two cell lines of immortalized LHRH neurons (GT1-1 and GN11 cell lines). Receptor binding assays on membrane preparations from GT1-1 cells showed the presence of a single specific and saturable class of binding sites ($K_d = 4.6$ nM; 10,000 sites/cell) for [³H]iloprost, a stable analog of PGI₂. Competition experiments showed that the binding sites labeled by [³H]iloprost

possess the pharmacological characteristics of IP receptors. In functional studies, PGI₂ and its analogs, iloprost and cicaprost, were able to stimulate LHRH release from the GT1-1 cells with elevated potencies ($EC_{50} = 0.6$ – 4.3 nM); PGE₁ was only slightly less active ($EC_{50} = 28.5$ nM), whereas PGE₂, considered the major PG involved in LHRH secretion, was poorly effective ($EC_{50} = 921$ nM). The relative potencies (EC_{50}) of these compounds in stimulating the intracellular accumulation of cAMP were in line with their LHRH-releasing activities.

In conclusion, these results indicate that immortalized LHRH-secreting neurons express IP receptors through which PGI₂ may exert relevant effects on LHRH release. (*Endocrinology* **140**: 171–177, 1999)

THE PEPTIDE LHRH, synthesized by specific hypothalamic neurons, is the major regulator of the secretion of pituitary gonadotropins. The secretion of LHRH is under the control of multiple nervous pathways, which involve different neurotransmitters, several neuropeptides, steroid hormones, and other neuroactive factors (*e.g.* PGs, cytokines *etc.*) (1–3).

The LHRH system is composed of only a few hundred neurons, which are not confined to a discrete nucleus but are distributed as clusters extending from the preoptic to the anterior hypothalamic regions (4). Such an organization makes it difficult to determine, by *in vivo* or *ex vivo* experiments, whether the different regulatory factors exert their effects directly on the LHRH-synthesizing neurons or whether they act via the activation and/or inhibition of other neuronal systems impinging on the LHRH-secreting neurons (2).

A promising tool for the investigation of the possible excitatory or inhibitory inputs reaching the LHRH-secreting neurons is represented by the utilization of immortalized hypothalamic cell lines, such as GT1 (5) and GN (6) cells. Among these, GT1 cells have been extensively studied, and it has been found that they retain many characteristics of hypothalamic LHRH-secreting neurons, such as a neuronal morphology, the expression of neuronal markers, an elevated synthesis of the LHRH gene, and the typical pulsatile

secretion of LHRH (7–9). In the last few years, the receptors for several families of neurotransmitters known to modify LHRH secretion have been identified on GT1 cells, and the stimulatory or inhibitory activity of the corresponding ligands on LHRH secretion has been documented (8). In particular, the presence of functional opioid receptors of the δ -type has been ascertained in GT1-1 cells, a subclone of the GT1 cell line (10). It has also been reported that the activation of these receptors leads to an inhibition of the release of LHRH when this is stimulated by either forskolin or PGs (11).

Prostanoids (PGs, thromboxanes, and leukotrienes), which derive from arachidonic acid metabolism, have been shown to affect reproductive functions (12–15). Among the various PGs, PGE₂ appears to be the one mainly involved in the control of LHRH secretion *in vivo* (16, 17). PGE₂ is also able to induce a significant release of LHRH from GT1 cell line (11, 18, 19), an effect accompanied by an increase in intracellular cAMP levels and probably mediated by the interaction with PG receptors of the EP series (see below) (20). However, the involvement of PGE₁ in the control of the secretion of LHRH *in vivo* has also been reported (13, 21), and previous results obtained in the authors' laboratory have shown that PGE₁ is more potent than PGE₂ in stimulating both LHRH release and cAMP accumulation in GT1-1 cells (10, 11). According to the most recent classification of the PG receptors (22), PGE₁ appears to interact with the putative PGI₂ receptor (IP receptor) with an affinity and a specificity higher than those found for any other member of the PG receptor family (*e.g.* EP).

The strong effect exerted by PGE₁ on the release of LHRH from GT1-1 cells supports the hypothesis that IP receptors might be present on these cells, and that their activation

Received April 15, 1998.

Address all correspondence and requests for reprints to: Roberto Maggi, Ph.D., Department of Endocrinology, University of Milan, Via G. Balzaretti 9, 20133 Milan, Italy. E-mail: roberto.maggi@unimi.it.

* This work was supported by funds from Telethon (Grant E.523), by CNR through the Project Aging (95.01020PF40), and by MURST.

might influence the secretion of LHRH. This hypothesis has been verified by evaluating the expression of IP receptors in two immortalized LHRH neuronal cell lines (GT1-1 and GN11) using RT-PCR. Moreover, GT1-1 cells were used to perform specific binding assays for IP receptors and to measure the intracellular accumulation of cAMP and the release of LHRH after treatment with PGI₂, its synthetic analogs iloprost and cicaprost, PGE₁, and PGE₂.

Materials and Methods

Chemicals and reagents

PGE₁, PGE₂, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO); prostacyclin (PGI₂) was purchased from Cayman Chemical (Ann Arbor, MI). Iloprost was a gift from Schering SpA (Segrate, Milan, Italy) and Schering AG (Berlin, Germany), and cicaprost was provided by U. Habenicht (Schering AG). The stock solutions of these compounds were made in ethanol and stored at -20 C. The cell culture media and additives were obtained from Biochrom KG (Berlin, Germany); FBS was obtained from Life Technologies (Grand Island, NY). [³H]Iloprost (12.7 Ci/mmol) and [γ -³²P]deoxy-ATP were purchased from Amersham (Aylesbury, UK). All other analytical grade reagents were obtained from Merck (Darmstadt, Germany) and Sigma Chemical Co.

Cell cultures

GT1-1 and GN11 cells were provided by R. I. Weiner (San Francisco, CA) and S. Radovick (Children's Hospital, Boston, MA), respectively. The cells were routinely grown in monolayer at 37 C in a humidified CO₂ incubator in DMEM containing 1 mM sodium pyruvate, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 10 mg/liter phenol red and supplemented with 10% FBS. The medium was replaced at 2-day intervals. Subconfluent cells were routinely harvested by trypsinization and seeded in 57-cm² dishes (1 \times 10⁶ cells). GT1-1 and GN11 cells within six passages were used throughout the experiments.

RT-PCR analysis

Expression of the specific messenger RNA (mRNA) coding for mouse IP receptors was tested after nucleic acid amplification, using a PCR procedure (RT-PCR), of reverse transcribed total RNA extracted from GT1-1 and GN11 cells. Cultured cells were solubilized in Ultra-Spect reagent (Biotex Laboratories, Inc., Houston, TX) and processed for total RNA extraction according to the protocol included in the reagent kit. RT and PCR were performed using a GeneAmp kit (Perkin Elmer, Branchburg, NJ) on 2 μ g total RNA from each sample. Samples of total RNA of adult mouse spleen, known to contain high levels of IP receptor mRNA (23), were obtained as described for the cells and used as positive controls. All of the synthetic oligonucleotides, obtained from Pharmacia Biotech (Uppsala, Sweden), were deduced from the complementary DNA (cDNA) sequence of the mouse IP receptor (GenBank accession no. D26157) and verified with the program Amplify (Bill Engels, University of Wisconsin, Madison, WI). The synthetic oligonucleotides used as primers were designed to amplify sequence 500-1128 of the IP cDNA, and their sequences were as follows: upstream primer, 5'-CAC.CCA.TCG.GCC.TTT.GCG.GT-3'; and downstream primer, 3'-TA.GTA.CCG.GCA.CAC.GAG.GGA-5'. To exclude the presence of contamination with genomic DNA, a parallel set of the RNA samples was assayed for RT-PCR, but reverse transcriptase was omitted from the incubation mixture.

The RT conditions were 42 C for 45 min, followed by 5 min at 95 C, using a final concentration of 1 mM of each deoxy-NTP, 1 U ribonuclease inhibitor, 2.5 U murine leukemia virus reverse transcriptase, and the downstream primer in a final volume of 20 μ l. The same buffer (50 mM KCl, 10 mM Tris-HCl, and 2 mM MgCl₂) was used for both transcription and amplification. After this step, 2.5 U Ampli-Taq polymerase and the upstream primer were added in a final volume of 100 μ l. Samples were amplified by repeated cycles (35 cycles) at 95 C for 1 min, followed by 1 min at 42 C and 1 min at 72 C.

Amplification products were separated by electrophoresis in 2% aga-

rose gel and detected by ethidium bromide staining. The amplified cDNAs were transferred to blotting membrane (Hybond Nfp, Amersham) by capillary elution in 10 \times SSC (standard saline citrate) and fixed at 80 C for 2 h. A synthetic antisense oligonucleotide (3'-GC.AAG.GCG.GGT.CGG.ACC.GCC-5' complementary to the sequence 893-912 of IP cDNA) was used as radiolabeled probes in Southern analysis. The labeling reactions were performed at the free 5'-end of the oligoprimers using T4 DNA polynucleotide kinase, and [γ -³²P]deoxy-ATP. The cDNAs fixed on the membrane were incubated at 45 C for 4 h with a prehybridizing solution; after then, the ³²P 5'-end-labeled oligonucleotide probes (1 \times 10⁶ cpm/ml) were added, and the hybridization reaction was left at 45 C for 16 h. After high stringency washes, the membranes were exposed to x-ray films (Hyperfilm, Amersham).

RT-PCR blanks were performed using distilled water instead of mRNA and simultaneously subjected to RT-PCR-Southern blotting with the same reagents and conditions described above; in any of the experiments performed, no specific signal could be obtained, indicating that no contamination by any reagent occurred in these studies.

Receptor binding assay

[³H]Iloprost (12.7 Ci/mmol), purchased as a solution in organic solvent, was evaporated under nitrogen stream, and the residue was taken up in 10 mM Tris-HCl, pH 8. PGs stock solutions were diluted in 10 mM Tris-HCl buffer immediately before use. The program Design (24) was used to optimize the binding protocols, by selecting the lowest number of most appropriate concentrations. Homologous iloprost curves were always performed according to a mixed type protocol, combining both saturation (the first three concentration points of the curves, 1, 3, and 10 nM) and displacement (the last four concentration points, from 30 nM to 10 μ M) curves (25). By effectively combining both saturation and competition protocols in a single curve, one can reach high concentrations of the ligand without consuming an excessive amount of labeled ligand (competition part of the curve), yet have adequate radioactivity in the lower concentration range (saturation part of the curve). Equilibrium binding studies were performed as previously described (26) with minor variations. Briefly, GT1-1 membranes (0.1-0.15 mg/sample) were incubated for 5 min at 30 C in 10 mM Tris-HCl, pH 8 (final volume, 100 μ l), with 1, 3, and 10 nM [³H]iloprost and unlabeled homologous and heterologous ligands at the indicated concentrations. Unbound ligand was separated from bound ligand by rapid vacuum filtration onto glass-fiber filters (GF/C, Whatman, Clifton, NJ), and the filters were washed twice with 2 ml ice-cold 10 mM Tris-HCl. Radioactivity was then measured by liquid scintillation counting (Filter Count, Packard Instruments Co., Meriden, CT). Nonspecific binding was calculated by Ligand (see below) as one of the unknown parameters of the model and it ranged between 25-30% of the total binding of 10 nM [³H]iloprost.

Static culture studies

GT1-1 cells were plated in 24-well plates (0.5 \times 10⁶ cells/cm²) and used after 5 days of culture. All of the samples were assayed for protein content using a microassay (27) with human serum albumin as a standard. No variations in total protein per well were detected in any of the experimental groups (data not shown).

On the day of the experiment, GT1-1 cells were washed with 1 ml DMEM (prewarmed at 37 C) before addition of the compounds to be tested, made from 1000-fold concentrated ethanolic solution.

cAMP accumulation. GT1-1 cells were preincubated for 15 min at 37 C in DMEM containing the phosphodiesterase inhibitor IBMX (0.5 mM). Intracellular cAMP accumulation was then measured over a 15-min incubation period at 37 C in DMEM with the various compounds in the presence of IBMX using a commercial assay kit (Amersham) after ethanolic extraction.

LHRH secretion. GT1-1 cells were incubated for 30 min at 37 C in DMEM with the test compounds. At the end of the incubation period the incubation medium was collected and centrifuged for 5 min at 12,000 rpm, and the supernatant was stored at -70 C until RIA. The content of LHRH in the culture medium was determined by RIA using a commercial antibody (L-8391, Sigma Chemical Co.) and iodinated LHRH (Amersham). Synthetic LHRH (NovaBiochem, Laufelfingen, Switzerland) was used as standard. All samples from the same experiment were run in

duplicate in the same assay. Inter- and intraassay variations were 9.4% and 6.6%, respectively. The sensitivity of the assay was 3.9 pg/ml.

Data analysis and statistical evaluation

Analysis of equilibrium ligand binding data were performed by means of the computer program Ligand (28). Statistical analysis of the concentration-response curves was performed using the program Allfit (29), which calculates the lower and upper plateaus, the slope, and the EC_{50} and allows the comparison of two or more curves. Selection of the best fitting model and evaluation of the statistical significance of the parameters were based on the F test for the extra sum of square principle. A statistical level of significance with $P < 0.05$ was accepted. All curves shown were computer generated.

Results

RT-PCR analysis of the IP receptor transcript in immortalized LHRH-secreting neurons

The presence of a specific transcript for the IP receptor was initially investigated in GT1-1 and GN11 immortalized LHRH-secreting neurons. Figure 1 shows the autoradiographic data obtained after Southern analysis performed on the amplification products of the RT-PCR. Lane 1 corresponds to an internal control, in which no RNA was added to the PCR incubation mixture. The presence of a specific transcript of the expected size (628 bp) was detectable in RNA samples obtained from mouse spleen (lane 2), which was used as the positive control (23). The results also show that a specific amplification of a transcript for the IP receptor was detected in both GT1-1 (lane 3) and GN11 (lane 4) cells; no amplification fragments were obtained from RNA samples when the incubation with RT was omitted (lanes 5 and 6), a fact that excludes contamination of the samples with genomic DNA.

Further assessment of the biological role(s) of the presence of IP receptors on immortalized LHRH-secreting neurons was performed using GT1-1 cells, because these cells represent a more mature form of LHRH neurons than GN11 cells, which show low levels of expression and release of LHRH. In addition, many aspect of the physiology of the GT1 cells have been widely characterized (8).

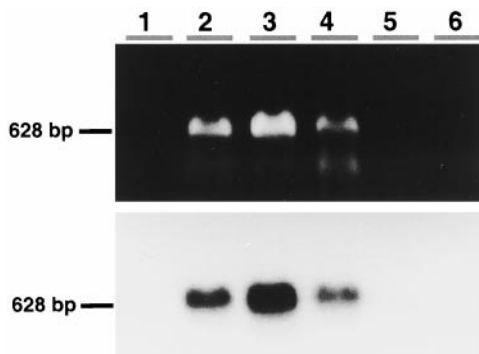


FIG. 1. IP receptor transcripts, evidenced by ethidium bromide staining (upper panel) and Southern blot (lower panel), detected by RT-PCR in RNA samples extracted from mouse spleen (lane 2) and immortalized hypothalamic neuronal cell lines GT1-1 (lane 3) and GN11 (lane 4). Lanes 5 and 6 represent the reaction products of RNA samples from GT1-1 and GN11 cells, respectively, not incubated with reverse transcriptase. The negative control was performed using distilled water instead of RNA (lane 1; see details in *Materials and Methods*).

Specific binding of [3 H]iloprost to GT1-1 cell membrane preparations

Binding experiments were then performed on GT1-1 cell membrane preparations using the stable PGI_2 analog [3 H]iloprost as the ligand for the IP receptors. The results of homologous binding curves show that [3 H]iloprost bound to GT1-1 membrane preparations in a dose-dependent manner (0.1 nM to 10 μ M; Fig. 2a). Computer analysis of the results revealed that [3 H]iloprost interacts with a single class of binding sites with an equilibrium dissociation constant (K_d) of 4.6 nM and a maximal binding capacity of 85 fmol/mg protein, which corresponds to about 10,000 sites/cell.

Specificity of [3 H]iloprost binding to GT1-1 cell membranes

To verify the specificity of the binding sites labeled by [3 H]iloprost, the competing activity of different unlabeled PGs was investigated. The results of homologous and heterologous competition curves are shown in Fig. 2b. It is evident that the specific binding of [3 H]iloprost on GT1-1 cell membrane is inhibited by unlabeled PGs in the following order of potency: iloprost > PGI_2 = PGE_1 \gg PGE_2 . In particular, as summarized in Table 1, unlabeled iloprost competes for the binding of [3 H]iloprost with an equilibrium inhibition constant (K_i) of 4.6 nM; PGI_2 and PGE_1 are 14- to 20-fold less potent than iloprost (K_i = 83.8 and 65.8 nM, respectively). On the other hand, PGE_2 exerts a very weak competition on [3 H]iloprost binding, with a potency significantly smaller (K_i = 2783.5 nM) than that of either PGI_2 or PGE_1 . These results indicate the presence of biologically active IP receptors specific for PGI_2 and its analogs on GT1-1 cell membrane preparations.

Effects of different PGs on intracellular accumulation of cAMP in GT1-1 cells

It has been extensively reported that IP receptors are positively coupled to adenylate cyclase, and that PGI_2 strongly stimulates cAMP formation in neural cells (30). A series of experiments were then performed to verify the effects of different PGs on the accumulation of intracellular cAMP in GT1-1 cells. In the experimental conditions adopted in the

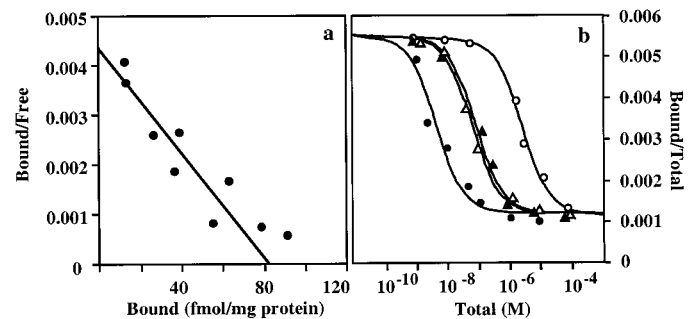


FIG. 2. Pharmacological characterization of IP receptors present on GT1-1 cells. a, Scatchard plot representation of the specific binding of [3 H]ilprost to GT1-1 cell membrane preparations. b, Selective inhibition of [3 H]ilprost-specific binding by unlabeled iloprost (\bullet), PGI_2 (\blacktriangle), PGE_1 (\triangle), and PGE_2 (\circ). The binding isotherms were analyzed by the Ligand program, and the binding parameters B_{max} , K_d , and K_i were determined. The data are representative of two independent experiments performed in duplicate.

TABLE 1. Competing activities of different PGs on the binding of [³H]iloprost on GT1-1 cell membrane preparations

Competitor	K _i (nM)
Iloprost	4.6 ± 0.8
PGE ₁	65.8 ± 5.9
PGI ₂	83.8 ± 7.6
PGE ₂	2783.5 ± 759.9

Values are expressed as calculated K_i values ± SD.

present study, the basal level of cAMP was 110 pg/mg total protein.

The dose-response curves obtained in these experiments are reported in Fig. 3; because of the differences observed in maximal responses (see below), the curves are represented as percent values. It has been found that PGI₂ as well as its synthetic agonists iloprost and cicaprost are similarly potent in stimulating cAMP accumulation in GT1-1 cells and more potent than PGE₁ and PGE₂. The potencies of the different substances are reported in Table 2; it is evident that iloprost induces a sustained accumulation of cAMP with an EC₅₀ of 0.48 nM; PGI₂ and its other stable analog cicaprost are slightly less potent and stimulate cAMP formation with similar potencies (EC₅₀ 1.08 and 1.28 nM, respectively). PGE₁ and PGE₂ are far less potent than PGI₂, with EC₅₀ of 46.8 and 1216.8 nM.

Table 2 also shows that the compounds under study exert different maximal responses on cAMP formation. This parameter corresponds to the response at the "infinite" dose of each compound, as calculated by the Allfit program using absolute values. Iloprost appears to be the most efficient agent, inducing a 49-fold accumulation of cAMP over basal levels. PGI₂ and cicaprost show similar elevated maximal activities (33- and 38-fold, respectively). PGE₁ and PGE₂ possess an efficiency significantly lower than those of PGI₂ and its analogs, showing a maximal cAMP increase corresponding, respectively, to 21 and 8 times the basal levels.

Effects of different PGs on secretion of LHRH from GT1-1 cells

To verify whether the activation of cAMP-mediated intracellular pathways elicited by agents stimulating the IP receptor plays a physiological role in GT1-1 cells, the effects of various PGs on the release of LHRH were subsequently investigated. The results are summarized in Fig. 4 and Table 3. The evaluation of LHRH accumulation in the culture medium under the effects of different PGs shows that cicaprost exerts the highest potency as an LHRH secretagogue (EC₅₀ = 0.6 nM), followed by iloprost and PGI₂, which are able to stimulate LHRH release from GT1-1 cells with similar potencies (EC₅₀ = 4.3–4.7 nM). PGE₁ is less potent than PGI₂ and its stable analogs (EC₅₀ = 28.5); PGE₂ shows the lowest potency (215 times less than that of PGI₂; EC₅₀ = 921 nM). The maximal stimulation of LHRH release evoked by the various PGs is comparable and equivalent to an increase of 1- to 1.5-fold the basal levels measured in the medium of unstimulated cells (Table 3).

Discussion

The present study provides the first evidence of the presence of specific prostacyclin receptors (IP receptors) on im-

TABLE 2. Effects of different PGs on the intracellular accumulation of cAMP in GT1-1 cells

Compounds	EC ₅₀ (nM)	Maximal efficacy	
		cAMP (pmol/ml) ^a	Fold induction (vs. control)
Control		0.72 ± 0.1	1.0
Iloprost	0.48 ± 0.05	35.6 ± 6.0	49.1
PGI ₂	1.08 ± 0.31	28.0 ± 11.6	38.9
Cicaprost	1.28 ± 0.24	24.2 ± 7.2	33.6
PGE ₁	46.80 ± 18.06 ^b	15.6 ± 4.3 ^b	21.7
PGE ₂	1216.80 ± 187.91 ^b	6.12 ± 4.3 ^b	8.5

Values are expressed as the mean ± SD.

^a cAMP levels obtained at the "infinite" dose of each compound and corresponding to the upper plateau of the dose-response curve obtained using absolute values.

^b P < 0.05, significant vs. PGI₂ by Allfit analysis.

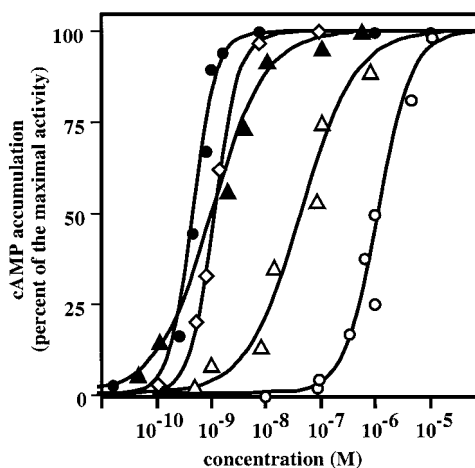


FIG. 3. Dose-related effect of PGI₂ (▲), its synthetic analogs iloprost (●) and cicaprost (◇), PGE₁ (△), and PGE₂ (○) on the intracellular accumulation of cAMP in GT1-1 cells. Cells were incubated for 30 min with IBMX (0.5 mM) in serum-free medium, PGs were then added for the last 15 min of culture. Results are expressed as a percentage of the maximal effect obtained from each compound determined from two independent experiments using quadruplicate wells for each determination.

mortalized hypothalamic LHRH-producing neurons. The results reported here also show the presence of a specific transcript related to a sequence of the cloned mouse IP receptor (23) in two different cell lines of LHRH neurons, GT1-1 and GN11 cells. The presence of an IP transcript in both cell lines suggests that the expression of IP receptors is conserved among LHRH-secreting neurons and strongly supports the possibility that hypothalamic LHRH neurons *in situ* might also express IP receptors.

The experiments devoted to the clarification of the binding characteristics of the IP receptors and their possible physiological roles were performed in GT1 cells, as many mechanisms controlling LHRH release have been well studied in this cell line (8, 31, 32). First, high affinity (K_d = 4.6 nM) binding sites for [³H]iloprost (23, 33) were found in crude GT1-1 cell membrane preparations. The binding characteristics of [³H]iloprost to GT1-1 cells and its specificity are in agreement with those reported for the same tracer when tested on other cells and tissues (33–36) as well as on Chinese hamster ovary cells transfected with the mouse IP receptor

TABLE 3. Effects of different PGs on the secretion of LHRH from GT1-1 cells

Compounds	EC ₅₀ (nM)	Maximal efficacy	
		LHRH (pg/ml) ^a	Fold induction (vs. control)
Control		94.4 ± 10.4	1.0
Cicaprost	0.6 ± 0.4 ^{b,c}	185.6 ± 19.8	1.9
PGI ₂	4.3 ± 1.7	225.2 ± 28.4	2.4
Iloprost	4.7 ± 1.8	190.4 ± 21.5	2.0
PGE ₁	28.5 ± 4.7 ^b	230.9 ± 18.5	2.4
PGE ₂	921.8 ± 178.8 ^b	209.2 ± 16.9	2.2

Values are expressed as the mean ± SD.

^a LHRH levels obtained at the "infinite" dose of each compound and corresponding to the upper plateau of the dose-response curve obtained using absolute values.

^b $P < 0.05$, significant vs. PGI₂, by Allfit analysis.

^c $P < 0.05$, significant vs. iloprost, by Allfit analysis.

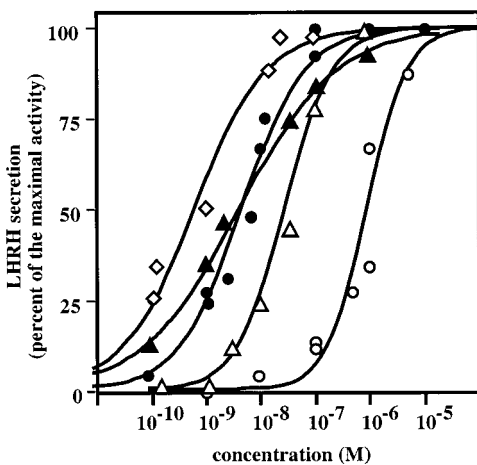


FIG. 4. Dose-related effect of PGI₂ (▲), its synthetic analogs iloprost (●) and cicaprost (◇), PGE₁ (△), and PGE₂ (○) on the release of LHRH from GT1-1 cells. Cells were incubated for 30 min with PGs, and the content of LHRH released in the culture medium was measured by RIA. Results are expressed as a percentage of the maximal effect obtained from each compound determined from three independent experiments using quadruplicate wells for each determination.

gene (23). Selective competition experiments performed with several PGs further confirm that the binding sites labeled by [³H]iloprost on GT1-1 cells belong to the IP class of PG receptors (22, 23).

It is well known that IP receptors are positively coupled to adenylyl cyclase and that their activation leads to an accumulation of cAMP in several cells and tissues (37). The results presented here clearly show that PGI₂ and its analogs, iloprost and cicaprost, are strong stimulators of cAMP accumulation in GT1-1 cells, whereas PGE₂ possesses the lower potency. These data are consistent with previous findings showing that prostacyclin stimulates the intracellular accumulation of cAMP in nonneural and neural cells (23, 30, 38) and indicate, in addition, that this effect is induced via the activation of IP receptors.

The low efficacy of PGE₂ on cAMP accumulation was surprising in view of the preliminary identifications in GT1-1 cells of transcripts for the EP1, EP2, and EP3 PG receptors (20) to which PGE₂ preferentially binds (22). However, the presence of biologically active receptors of the EP series in GT1-1

cells has yet to be demonstrated by functional or binding experiments.

The functional role of IP receptors detected on GT1-1 cells has been further confirmed in the present study by the finding that prostacyclin and its analogs stimulate the release of LHRH in a dose-dependent manner. In general, this effect reflects the pharmacological profile of the activation of the IP receptors as measured in the other experiments here described (*e.g.* binding studies and cAMP accumulation). However, even though there is a good correlation between the potencies (EC₅₀) and the efficacies of the various PGs in stimulating cAMP production, this is less evident when cAMP production and LHRH release are analyzed. First, PGI₂, iloprost, and cicaprost stimulated cAMP accumulation with similar potencies; however, the latter compound proved to be much more potent than PGI₂ and iloprost in stimulating LHRH release. In this context, it is important to remember that the release of LHRH from GT1 cells is under the control of various signaling pathways and that each of these may operate via multiple steps (PKA, PKC, cGMP, ion channels, *etc.*) (8, 31, 32). It has also been reported that PGE₂-stimulated LHRH release involves intracellular calcium (39). Because of these considerations, the apparent divergence in the potencies of cicaprost on cAMP accumulation and LHRH release may be indicative of the activation of signaling systems, other than cAMP (40), induced by cicaprost in GT1-1 cells.

Another point that deserves some comment is the fact that the PGs considered in the present study stimulate cAMP accumulation with different efficacies even if all of the compounds tested show similar maximal LHRH-releasing activity. One possible explanation of this quantitative discrepancy may be that the increase in intracellular levels of cAMP induced by the less effective agent (*i.e.* PGE₂) is already sufficient to totally discharge the pool of releasable LHRH present in GT1-1 cells. On the other hand, the elevated formation of cAMP induced by PGI₂ and its analogs might subserve additional cellular functions [for instance, the morphological differentiation of the cells we observed in preliminary experiments (Maggi, R., *et al.*, in preparation)].

If one accepts that immortalized hypothalamic neurons represent a good model for study of the physiological functions of LHRH-producing neurons (8, 9), the obvious conclusion derived from these results is that the selective activation of IP receptors (by PGI₂ or by PGE₁) may play an important role in the control of LHRH secretion.

The physiological relevance of the presence of IP receptors in and around the LHRH neuronal system is supported by the identification of similar amounts of PGE₂ and 6-cheto-PGF_{1 α} , the main metabolite of PGI₂, in extracts of rat median eminence (41). In addition, it has been reported that estrogens may affect the production of PGI₂ in different *in vivo* and *in vitro* systems (42).

The data reported here do not permit identification of the possible source of the PGI₂ interacting with the IP receptors present on hypothalamic LHRH neurons; nevertheless, some hypotheses may be proposed. The first takes into consideration a possible autocrine function of PGI₂. It has been found that LHRH-liberating agents (*e.g.* endothelin) facilitate the release of LHRH from GT1 cells via stimulation of the pro-

duction of arachidonic acid derivatives (18). It is then possible that PGI₂ might be produced by the LHRH neurons themselves in response to stimulatory signals. A second hypothesis is derived from the recently discovered involvement of glial cells in the control of LHRH release (43, 44). In particular, it has been observed that transforming growth factor- α may stimulate the production and release of PGE₂ from astrocytes impinging on the LHRH neurons; in turn, this and other PGs would induce LHRH release (43). Finally, it may be recalled that at the level of the median eminence, LHRH axons are in close association with the endothelium of the pituitary portal vessels, and that some vasoactive factors released by the blood vessels (e.g. endothelin and nitric oxide) have been found to affect the release of LHRH (18, 45). It is then attractive to hypothesize that PGI₂, which is also produced by the vascular endothelium, might create an additional functional link between the portal vessels and the hypophysiotropic neurons, leading to a synergizing effect.

In conclusion, the present results indicate that not only PGE₂, but also PGs acting on the IP receptors, may participate in the activation of LHRH release. As it is known that PGs represent the final mediator in many biological systems, the present results prompt additional studies on the reciprocal interactions between the most classical neurotransmitter systems known to induce the release of LHRH (norepinephrine, epinephrine, dopamine, nitric oxide, histamine, excitatory amino acids, etc.) and the ubiquitous system of arachidonic acid derivatives.

Acknowledgments

We thank Prof. Nicoletta Nicosia for the useful suggestions and for having kindly provided access to her laboratory facilities.

References

- Weiner RI, Findell PR, Kordon C 1987 Role of classic and peptide neuromediators in the neuroendocrine regulation of LH and prolactin. In: Knobil E, Neill JD (eds) *The Physiology of Reproduction*. Raven Press, New York, pp 1235–1281
- Kalra SP 1993 Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. *Endocr Rev* 14:507–538
- Kalra S, Horvath T, Naftolin F, Xu B, Pu S, Kalra P 1997 The interactive language of the hypothalamus for the gonadotropin releasing hormone (GnRH) system. *J Neuroendocrinol* 9:569–576
- Merchenthaler I, Gorcs T, Setalo G, Petrusz P, Flerko B 1984 Gonadotropin-releasing hormone (GnRH) neurons and pathways in the rat brain. *Cell Tissue Res* 237:15–29
- Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 5:1–10
- Radovick S, Wray S, Lee E, Nicols D, Nakayama Y, Weintraub B, Westphal H, Cutler Jr G, Wondisford F 1991 Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. *Proc Natl Acad Sci USA* 88:3402–3406
- Liposits Z, Merchenthaler I, Wetsel WC, Reid JAJ, Mellon PL, Weiner RI, Negro-Vilar A 1991 Morphological characterization of immortalized hypothalamic neurons synthesizing luteinizing hormone-releasing hormone. *Endocrinology* 129:1575–1583
- Wetsel W 1995 Immortalized hypothalamic luteinizing hormone-releasing hormone (LHRH) neurons: a new tool for dissecting the molecular and cellular basis of LHRH physiology. *Cell Mol Neurobiol* 15:43–78
- Gore A, Roberts J 1997 Regulation of gonadotropin-releasing hormone gene expression *in vivo* and *in vitro*. *Front Neuroendocrinol* 18:209–245
- Maggi R, Pimpinelli F, Martini L, Piva F 1995 Characterization of functional opioid δ receptors in a luteinizing hormone-releasing hormone producing neuronal cell line. *Endocrinology* 136:289–295
- Maggi R, Pimpinelli F, Martini L, Piva F 1995 Inhibition of luteinizing hormone-releasing hormone secretion by δ -opioid agonists in GT1-1 neuronal cells. *Endocrinology* 136:5177–5181
- Kuehl Jr FA, Humes JL, Tarnoff J, Cirillo VJ, Ham AE 1970 Prostaglandin receptor site: evidence for an essential role in the action of luteinizing hormone. *Science* 168:883–887
- Spies H, Norman R 1973 Luteinizing hormone release and ovulation induced by the intraventricular infusion of prostaglandin E1 into pentobarbital-blocked rats. *Prostaglandins* 4:131–141
- Batta S, Piva F, Martini L 1974 Prostaglandins and gonadotropin secretion. *Neuroendocrinology* 14:224–232
- Batta S, Zanisi M, Martini L 1975 Role of prostaglandins in the control of gonadotropin secretion. *Psychoneuroendocrinology* 1:115–121
- Ojeda SR, Wheaton G, McCann SM 1975 Prostaglandins E₂ induced release of luteinizing hormone releasing factor (LRF). *Neuroendocrinology* 17:283–287
- Negro-Vilar A, Valenca M, Culler M 1987 Transmembrane signals and intracellular messengers mediating LHRH and LH secretion. In: Mahesh V, Dhindsa D, Anderson E, Kalra S (eds) *Regulation of Ovarian and Testicular Function*. Plenum Press, New York, vol 219:85–108
- Moretto M, Lopez FJ, Negro-Vilar A 1993 Endothelin-3 stimulates luteinizing hormone-releasing hormone (LHRH) secretion from LHRH neurons by a prostaglandin-dependent mechanism. *Endocrinology* 132:789–794
- Negro-Vilar A, Wetsel W, Mellon PL, Valenca M, Secretagogue-stimulated secretion of LHRH, and GAP elicited from a perfused immortalized LHRH neuronal cell line. 73th Annual Meeting of The Endocrine Society, Washington DC, 1991, p 458 (Abstract)
- Rage F, Ma Y, Ojeda SR, Different prostaglandin E2 (PGE2) receptor subtypes are expressed in immortalized luteinizing hormone releasing hormone (LHRH) neurons. 24th Annual Meeting of the Society for Neuroscience, Miami Beach, FL, 1994, p 637 (Abstract)
- Ojeda SR, Jameson HM, McCann SE 1977 Hypothalamic area involved in prostaglandin (PG)-induced gonadotropin release. I. Effect of PGE₂ and PGF_{2 α} implants on luteinizing-hormone release. *Endocrinology* 100:1585–1594
- Coleman R, Smith W, Narumiya S 1994 Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* 46:205–229
- Namba T, Oida H, Sugimoto Y, Kakizuka A, Negishi M, Ichikawa A, Narumiya S 1994 cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla. *J Biol Chem* 269:9986–9992
- Rovati GE, Rodbard D, Munson PJ 1988 DESIGN: Computerized optimization of experimental design for estimating Kd and Bmax in ligand binding experiments. I. Homologous and heterologous binding to one classes of sites. *Anal Biochem* 174:636–649
- Rovati G, Rodbard D, Munson P 1989 Optimization of experimental design for ligand binding studies: improved estimation of affinity and binding capacity. *Pharmacol Res* 21:71–72
- Lombroso M, Nicosia S, Paoletti R, Whittle B, Moncada S, Vane J 1984 The use of stable prostaglandins to investigate prostacyclin (PGI₂)-binding sites and PGI₂-sensitive adenylate cyclase in human platelet membranes. *Prostaglandins* 27:321–333
- Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Munson PJ, Rodbard D 1980 LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* 107:220–239
- De Lean A, Munson PJ, Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* 235:E97–E102
- Hingtgen C, Waite K, Vasko M 1995 Prostaglandins facilitate peptide release from rat sensory neurons by activating the adenosin 3'5'-cyclic monophosphate transduction cascade. *J Neurosci* 15:5411–5419
- Stojilkovic SS, Krmanovic LZ, Spergel DJ, Catt KJ 1994 Gonadotropin-releasing hormone neurons. Intrinsic pulsatility and receptor-mediated regulation. *Trends Endocrinol Metab* 5:201–209
- Martinez de la Escalera G, Choi ALH, Weiner RI 1995 Signaling Pathways involved in GnRH secretion in GT1 cells. *Neuroendocrinology* 61:310–317
- Town M, Schillinger E, Speckenbach A, Prior G 1982 Identification and characterization of a prostacyclin-like receptor in bovine coronary arteries using a specific and stable prostacyclin analogue, ciloprost, as radioactive ligand. *Prostaglandins* 24:61–72
- MacDermot J, Blair I 1981 Prostacyclin receptors of a neuronal hybrid cell line. Divalent cations and ligand-receptor coupling. *Biochem Pharmacol* 30:2041–2044
- Hashimoto H, Negishi M, Ichikawa A 1990 Identification of a prostacyclin coupled to the adenylate cyclase via a stimulatory GTP-binding protein in mouse mastocytoma P-815 cells. *Prostaglandins* 40:491–505
- Tsai A, Hsu M, Vijjeswarapu H, Wu K 1989 Solubilization of prostacyclin membrane receptors from human platelets. *J Biol Chem* 264:61–67
- Moncada S 1982 Biological importance of prostacyclin. *Br J Pharmacol* 76:3–31
- Halushka P, Mais D, Mayeux P, Morinelli T 1989 Thromboxane, prostaglandin and leukotriene receptors. *Annu Rev Pharmacol Toxicol* 29:213–239
- Ojeda SR, Negro-Vilar A 1985 Prostaglandin E₂-induced luteinizing hormone-releasing hormone release involves mobilization of intracellular Ca²⁺. *Endocrinology* 116:1763–1770

40. Vassaux G, Gaillard D, Ailhaud G, Négrel R 1992 Prostacyclin is a specific effector of adipose cell differentiation: its dual role as a cAMP- and Ca⁺⁺-elevating agent. *J Biol Chem* 267:11092–11097
41. Franchi A, Gimeno M, Szwarcfarb B, Carbone S, Rondina D, Moguevsky J 1995 Changes in the hypothalamic interaction between norepinephrine and prostaglandin E2 during sexual maturation in female rats. *Neuroendocrinology* 62:226–230
42. Nordo Berge L, Hansen J-B, Svensson B, Lyngmo V, Nordoy A 1990 Female sex hormones and platelet/endothelial cell interaction. *Haemostasis* 20:313–320
43. Ma Y, Berg-von der Emde K, Rage F, Wetsel W, Ojeda S 1997 Hypothalamic astrocytes respond to transforming growth factor- α with the secretion of neuroactive substances that stimulate the release of luteinizing hormone-releasing hormone. *Endocrinology* 138:19–25
44. Melcangi R, Galbiati M, Messi E, Piva F, Martini L, Motta M 1995 Type 1 astrocytes influence luteinizing hormone-releasing hormone release from the hypothalamic cell line GT1-1: is transforming growth factor- β the principle involved? *Endocrinology* 136:679–686
45. Olcese J, Middendorff R, Munker M, Schmidt C, McArdle CA 1994 Natriuretic peptides stimulate cyclic GMP production in an immortalized LHRH neuronal cell line. *J Neuroendocrinol* 6:127–130

**The Fifth International Congress of Endocrine Disorders
Sponsored by Endocrine Research Center
SBUMS & Iran Endocrine Society
Teheran, Iran, September 5–9, 1999**

For further information, please contact: Tel: 98-21-2409301-8; Fax: 98-21-2402463; E-mail: erc5c@geocities.com.