Characterization of Functional Opioid Delta Receptors in a Luteinizing Hormone-Releasing Hormone-Producing Neuronal Cell Line*

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ABSTRACT

Endogenous opioids participate in the regulation of gonadotropin secretion through an influence on the release of the hypothalamic LHRH. However, it is not clear whether opioids exert a direct effect on LHRH-producing neurons or interfere with other systems able to influence LHRH release.

A neuronal LHRH-producing cell line (GT1) developed recently provides a good model to study the mechanisms controlling LHRH release. In the present study, the presence of opioid receptors on a subclone of GT1 cells (GT1–1) has been investigated. A specific and saturable binding of the ^{3}H-labeled nonselective opioid ligand diprenorphine ([^{3}H]DIP) was detected by a receptor binding assay on both intact GT1–1 cells and crude membrane preparations obtained from these cells. Analysis of saturation curves revealed that [^{3}H]DIP apparently binds to a single class of sites with a K_d of 0.2 nM and a binding capacity of 125 fmol/mg protein, corresponding to approximately 20,000 sites/cell. Selective displacement of the binding of [^{3}H]DIP to GT1–1 cells by [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin,

[D-Pen^2,D-Pen^5]enkephalin (DPDPE), and U50488H, which are selective ligands, respectively, for μ-, δ-, and κ-receptors, was also evaluated. Only the specific δ-ligand DPDPE produced a significant inhibition of the binding of [^{3}H]DIP. [D-Ala^2,N-Me-Phe^4,Gly^5-ol]Enkephalin and U50488H were totally ineffective. The inhibitory effect of the agonist DPDPE on the binding of [^{3}H]DIP was decreased by the presence of sodium ions, a typical characteristic of the binding of agonists to opioid receptors. Finally, it has been observed that treatment with prostaglandins E_1 and E_2 produces a dramatic increase in cAMP accumulation in GT1–1 cells, and DPDPE is highly effective in suppressing this effect. On the basis of these results, it is possible to postulate the presence of functional δ-opioid receptors on GT1–1 cells. By extrapolation, one might suggest that endogenous opioids may affect LHRH neurons by two mechanisms: a direct one, acting via δ-receptors, and an indirect one, through the activation of neurons impinging on the LHRH system, which uses μ-receptors. (Endocrinology 136: 289–295, 1995)

T HE SECRETION of anterior pituitary gonadotropins is under the control of the hypothalamic hormone LHRH (1, 2). Several studies have shown that the neurons that synthesize LHRH are innervated by multiple neuronal pathways, which use different neurotransmitter systems. It is via these pathways that extrahypothalamic structures may modify LHRH release and, consequently, modulate gonadotropin secretion (3, 4). The study of the neurons that synthesize LHRH is made difficult by the peculiar anatomy of the LHRH system, which is composed of only a few hundred neurons scattered in the hypothalamic area.

A promising tool for the investigation of these interactions became available recently, when an immortalized line of LHRH-producing neurons (the GT1 cell line) was developed; this was obtained by targeted tumorigenesis in transgenic mice (5). The three subclones of the GT1 cells so far obtained (GT1–1, GT1–3, and GT1–7) (5) show the phenotypic characteristics of neuronal cells and synthesize and secrete abundant amounts of LHRH (5–7). If one assumes that these cell lines reflect the biological characteristics of physiological LHRH-synthesizing neurons, they may be used to extend our

knowledge on the physiology of LHRH-secreting cells. Recently, it has been shown that the GT1 cells possess the receptors for several families of neurotransmitters known to modify LHRH secretion, such as those for norepinephrine (8, 9), dopamine (9, 10), γ-aminobutyric acid (11), excitatory amino acids (12, 13), endothelin (14), etc.

It is known that among the transmitters involved in the control of LHRH release, the endogenous opioid peptides (Met- and Leu-enkephalin, dynorphin, and β-endorphin) play a particularly relevant role (1, 15–18). It has been repeatedly shown that opioid agonists (e.g. morphine) inhibit gonadotropin release, and that opioid antagonists (e.g. naloxone) stimulate LH output (19–21). However, little is known about the site where opioids act to modulate gonadotropin secretion. There is no clear-cut evidence on a possible direct effect of natural and synthetic opioids on LHRH-producing neurons, because of the methodological difficulty in studying LHRH neurons in vivo. The majority of the reports available support the view that the effects of opioids on the LHRH-secreting system might be indirect, via the interaction with intermediate neuronal systems that influence LHRH secretion through the release of their specific neurotransmitters (18).

As no data are available on the presence of opioid receptors on the neurons synthesizing LHRH, it was felt to be of interest to verify whether opioid receptors might be present on GT1 cells. To this purpose, the possible binding of the nonselective opioid ligand diprenorphine (DIP) on GT1–1 cells was first analyzed, using both membrane preparations

and intact cells. As it is known that opioids exert their effects through the interaction with at least three subclasses of specific binding sites (named, respectively, μ, δ, and κ) (22, 23), it was subsequently attempted to clarify whether one or more subclasses of opioid receptors might be present on this LHRH-producing cell line.

Materials and Methods

Cell cultures

GT1-1 cells, generously provided by Dr. R. I. Weiner (San Francisco, CA) through Dr. B. Marchetti (Catania, Italy), were routinely grown in monolayer at 37 C in a humidified CO_2 incubator in Dulbecco's Minimum Essential Medium containing 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10 mg/liter phenol red (Biochrom KG, Berlin, Germany) and supplemented with 10% fetal calf serum (Gibco, Grand Island, NY). The medium was replaced at 2-day intervals. Subconfluent cells were routinely harvested by trypsinization and seeded in 57-cm^2 dishes (1 × 10^6 cells) for propagation. GT1-1 cells within six passages were used throughout the experiments. To obtain crude cell membrane preparations, GT1-1 cells at subconfluence were harvested in Dulbecco's PBS solution (DPBS) without calcium and magnesium salts and containing 0.4% EDTA; the cellular pellet, obtained by centrifugation, was subsequently washed in DPBS. The cells were then homogenized in 0.32 M sucrose solution in 5 mM Tris-HCl, pH 7.4, by seven strokes in a glass-Teflon potter kept in ice. The cell homogenate was then centrifuged (750 × g at 4 C for 10 min); the resulting supernatants were incubated for 30 min at 37 C and then further centrifuged 30 min at 40,000 × g at 4 C. The supernatant was decanted, and the pellet (crude membrane preparation) was used in the receptor binding assay.

For receptor binding assay on intact cells and cAMP experiments, GT1-1 cells were plated in 12- or 24-wells plates (0.5 × 10^6 cells/cm^2) and used after 3 days of culture. All samples were assayed for protein content using a microassay (24) with human serum albumin as a standard.

Receptor binding assay

The receptor binding assay was performed using [15,15-N-^{3}H]DIP (SA, 31 Ci/mmol; Amersham, Milan, Italy) as opioid ligand. The binding assay on crude membrane preparations was performed as previously described (25). The competition curves were performed by incubation of 0.1 nM [^{3}H]DIP in the absence and presence of the selective opioid agonists [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAGO), [D-Pen^2,D-Pen^5]enkephalin (DPDPE; Sigma Chemical Co., St. Louis, MO), and trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cycloexyl]benzenacetamide methane sulfonate (U-50488H), kindly provided by Upjohn Co. (Kalamazoo, MI).

The binding of [^{3}H]DIP to intact cells was evaluated using the method described by Cone et al. (26) with minor modifications. Briefly, GT1-1 cells growing in multiwell plates were preincubated for 10 min in 0.5 ml binding buffer [50 mM Tris-HCl (pH 7.4), 1% BSA, and 0.5 mg/ml bacitracin] containing 100 mM NaCl at room temperature. During the last 2 min of the preincubation, the plates were kept at 4 C to adapt the cells to the assay incubation temperature. The preincubation buffer was then replaced with 0.5 ml binding buffer containing the tracer [^{3}H]DIP. Nonspecific binding was assessed by the addition of 1 μM unlabeled naltrexone. For competition experiments, unlabeled opioids were added simultaneously to the tracer. After incubation at 4 C for 2 h, the cell monolayers were washed three times with DPBS, solubilized in 0.5 ml 0.2 M NaOH, neutralized with 1 M HCl, mixed with 7 ml scintillation cocktail (Instagel, Packard, Milan, Italy), and counted in a Packard 1600 CA liquid scintillation spectrometer with 60% efficiency.

cAMP assay

The intracellular cAMP accumulation was measured over a 15-min incubation period with 1 μM prostaglandin E_1 (PGE$_1$) or 10 μM prostaglandin E_2 (PGE$_2$; Sigma) as activators of adenylyl cyclase after a

10-min preincubation with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). A commercially available binding protein assay kit (Amersham) was used to evaluate cAMP levels in ethanol-extracted cells according to the manufacturer's instructions.

Statistical analysis

Receptor binding experiments were optimized with the program Design (27), and the results were further analyzed by the program Ligand (28), adapted to a Macintosh computer by Dr. G. E. Rovati (Institute of Pharmacological Sciences, Milan, Italy).

The dose-response curves were analyzed by means of a Macintosh version of the program Allfit (29), kindly provided by Dr. V. Guardabasso (Cyanamid, Catania, Italy).

Results

Studies on GT1-1 cell membrane preparations

Receptor binding experiments have shown the presence of specific binding of the opioid ligand [^{3}H]DIP to crude membrane preparations obtained from GT1-1 cell homogenates. Such binding attains equilibrium in 30 min at 25 C and is totally inhibited by an excess (1 μM) of unlabeled nonselective opioid ligands such as bremazocine and naltrexone, which leave a low (5%) residual nonspecific binding (data not shown).

As illustrated in Fig. 1, the binding of [^{3}H]DIP to membrane preparations of GT1-1 cells is saturable and characterized by a high affinity (K_d, 0.2 nM), which is of the same order of magnitude as that in membranes obtained from the whole mouse brain (K_d, 0.1 nM). The maximal binding capacity (B_{max}) of [^{3}H]DIP, normalized per mg protein incubated, seems to be lower in GT1-1 cell membranes than in mouse brain (125 fmol/mg protein for GT1-1 cell membranes vs. 156 fmol/mg protein for mouse brain). However, the two results are not directly comparable due to the dif-
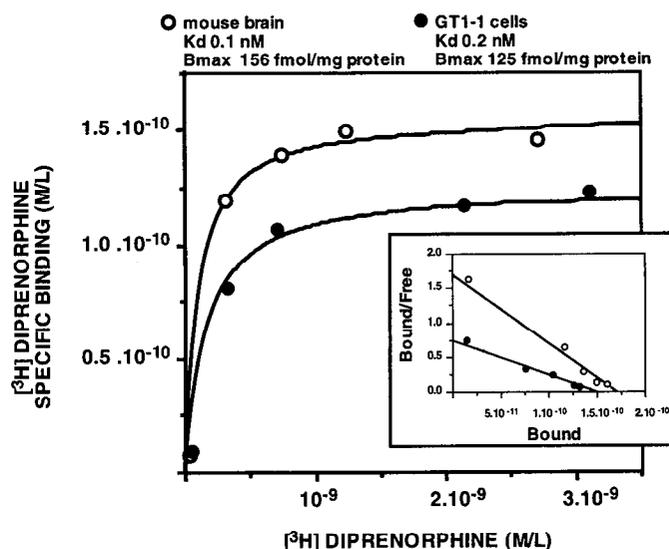

FIG. 1. Specific binding of increasing concentrations of [^{3}H]DIP to mouse brain and GT1-1 cell membrane preparations. Nonspecific binding was determined in the presence of 1 μM naltrexone. The saturation isotherms were analyzed by the Ligand program (28), and the binding parameters B_{max} and K_d were determined. The data are also represented as a Scatchard plot (inset) and are representative of two independent experiments performed in duplicate.

ferent sources of the starting materials (*i.e.* a homogeneous cell line *vs.* an extract of heterogeneous brain tissues containing different types of cells).

As [³H]DIP is known to label with approximately equal affinity brain μ, δ, and κ sites, the possible presence in GT1–1 cells of the different subclasses of opioid receptors and their relative proportions were further investigated by selective inhibition of the specific binding of [³H]DIP. The selective opioid ligands DAGO, DPDPE, and U50488H were used to inhibit the binding of [³H]DIP, respectively, to μ-, δ-, and κ-receptors. Two concentrations of each selective ligand were used to obtain a submaximal and a maximal mask of their relative binding sites. The results show that at 1-nM concentrations, the three specific ligands had no significant inhibitory effect on [³H]DIP binding (Fig. 2). At higher concentrations (100 nM), only the specific δ-ligand DPDPE inhibited (up to 60%) the specific binding of [³H]DIP to GT1–1 membranes (Fig. 2). The pure μ- and κ-ligands DAGO and U50488H were practically ineffective in inhibiting [³H]DIP binding even at the higher concentration (Fig. 2). These results may be indicative of the presence of only δ-opioid-binding sites on the membranes of GT1–1 cells.

The characteristics of the inhibitory effect of DPDPE on [³H]DIP binding were further investigated by heterologous displacement curves. The analysis of these curves shows that DPDPE inhibits the specific binding of 1 nM [³H]DIP on GT1–1 cell membranes in a dose-related fashion, with a K_i of 5.5 nM and maximal inhibition (>90%) at the 1-μM concentration (Fig. 3).

One of the characteristics of opioid receptors resides in the decrease in the binding of opioid agonists (but not of opioid antagonists) induced by the presence in the medium of monovalent ions, in particular sodium ions (30, 31). The potency of the agonist DPDPE in displacing [³H]DIP binding on GT1–1 membrane preparations was then assessed in the presence of sodium ions. As expected, the binding of the
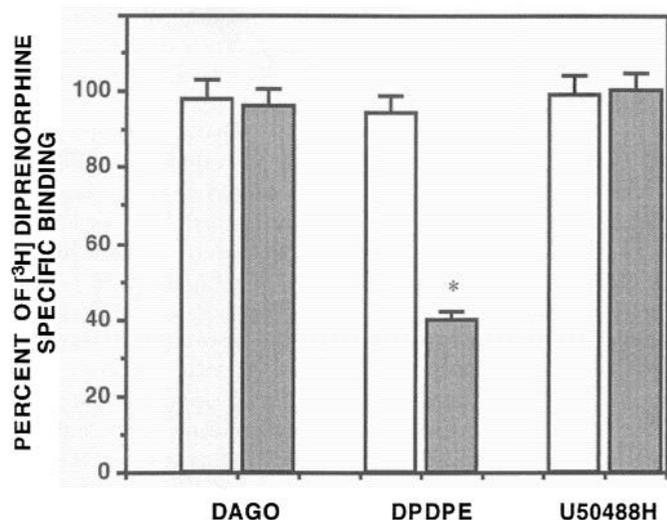

FIG. 3. Inhibition of the binding of [³H]DIP to GT1–1 cell membrane preparations by the selective δ-agonist DPDPE. [³H]DIP (0.5 nM) was incubated with various concentrations of DPDPE. The values represent one of two independent experiments performed in triplicate. The K_i of DPDPE was calculated using the Ligand program (28).

antagonist [³H]DIP was not changed in the presence of 100 mM NaCl (data not shown), whereas a significant increase in the IC_{50} of DPDPE was observed (Table 1).

Studies on intact GT1–1 cells

To rule out the possibility that the results obtained might be due to artefacts linked to the mechanical disruption of membrane integrity and cytoskeletal ultrastructures during homogenization, the binding of [³H]DIP was also assessed in intact GT1–1 cells. The analysis of the saturation curve of [³H]DIP to intact GT1–1 cells shows that in these conditions, specific binding of the labeled ligand occurs; this is characterized by the same K_d value (0.23 nM) previously observed in membrane preparations. The density of receptors labeled by [³H]DIP in intact cells appears to be 38 fmol/mg protein, corresponding to approximately 20,000 sites/cell, with a nonspecific binding of 5% (Fig. 4). The binding of [³H]DIP to intact GT1–1 cells is presumably due to an interaction with receptors located on the cellular membrane (26) and not to internalization of the tracer. In fact, an acid wash (PBS, pH 3, for 6 min at 4 C) performed on intact GT1–1 cells previously incubated under standard conditions with [³H]DIP (see *Materials and Methods*) removes more than 85% of the [³H]DIP bound to these cells. Selective inhibition of the specific binding of [³H]DIP to intact GT1–1 cells also shows that only those ligands that bind preferentially the δ-opioid receptors (*i.e.* DPDPE) are able to inhibit the binding of the tracer (Table 2). DPDPE inhibits the binding of [³H]DIP to intact cells with a K_i of 14.5 nM, and its inhibitory potency is significantly

FIG. 2. Selective inhibition of the specific binding of [³H]DIP (0.5 nM) by μ (DAGO)-, δ (DPDPE)-, and κ (U50488H)-selective ligands. [³H]DIP was incubated with 1 nM (□) or 100 nM (■) of the specific opioid agonists. Values are the mean ± SEM of triplicate determinations obtained from two independent experiments. *, $P < 0.05$ *vs.* [³H]DIP-specific binding in the absence of opioid agonists.
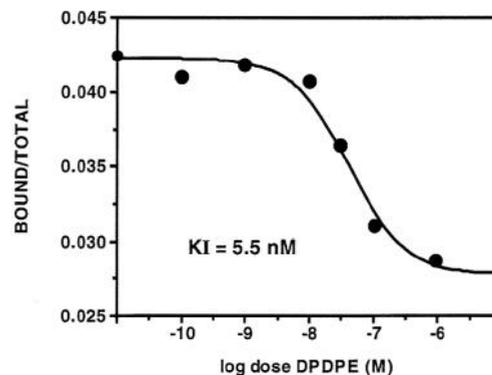
TABLE 1. Effect of sodium ions on the potency of DPDPE to displace the binding of [³H]DIP to GT1–1 cell membrane preparations

Treatment	IC_{50} (nM)
Control	15.1 ± 1.6
NaCl (100 mM)	63.7 ± 7.7^{a}

Values ± SD were obtained by Allfit (29) analysis of competition curves, performed in triplicate.
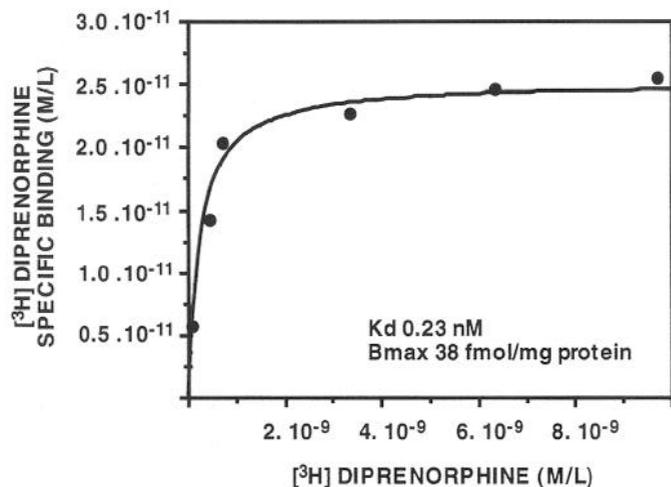[a] $P < 0.05$ *vs.* control.

FIG. 4. Specific binding of increasing concentrations of [³H]DIP to intact GT1–1 cells. Nonspecific binding was determined in the presence of 1 μM naltrexone. The saturation isotherms were analyzed by the Ligand program (28), and the binding parameters B_{max} and K_d were determined. Data are representative of two independent experiments performed in duplicate.

TABLE 2. Effects of several opioid agonists on the binding of [³H]DIP to intact GT1–1 cells

Agonists (100 nM)	% of [³H]DIP specific binding
DAGO	102.0 ± 3.9
DPDPE	81.9 ± 0.6[a]
U50488H	103.7 ± 2.4

Data are expressed as the mean ± SEM of two experiments performed in triplicates.
[a] $P < 0.05$ vs. percent specific binding of [³H]DIP in the absence of the agonists (100%).

reduced, as in the studies on membrane preparations, by the presence of 100 mM NaCl in the incubation medium (Table 3).

Opioid inhibition of cAMP accumulation in GT1–1 cells

The effect of DPDPE on the activation of adenyl cyclase induced by different stimuli was studied in GT1–1 cells by measuring the intracellular accumulation of cAMP. As illustrated in Fig. 5, the basal levels of cAMP found in control cells were not modified by a 10-μM concentration of the δ-opioid agonist DPDPE. The addition to GT1–1 cells of 1 μM PGE₁ dramatically enhanced cAMP accumulation (8.6-fold over prestimulated levels at the end of the 15 min of treatment). PGE₂ also showed a strong stimulatory effect on cAMP accumulation. However, the stimulatory effects on the intracellular cAMP concentration induced by both PGE₁ and

TABLE 3. Effect of sodium ions on the potency of DPDPE to displace the binding of [³H]DIP to intact GT1–1 cells

Treatment	IC₅₀ (nM)
Control	92.1 ± 12.0
NaCl (100 mM)	1138.2 ± 106.9[a]

Values ± SD were obtained by Allfit (29) analysis of competition curves, performed in triplicate.
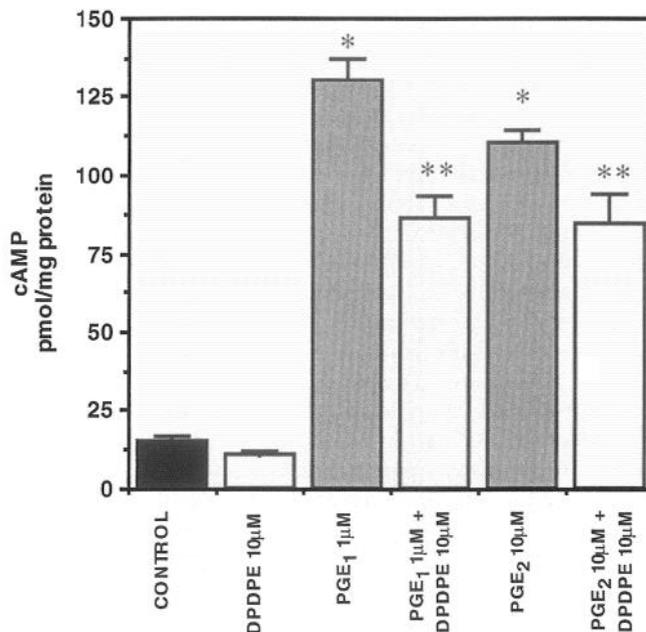[a] $P < 0.05$ vs. control.

FIG. 5. Inhibition of PGE₁- and PGE₂-stimulated cAMP accumulation in GT1–1 cells by the opioid delta agonist DPDPE. Cells were incubated for 30 min with 3-isobutyl-1-methylxanthine (0.5 mM) in serum-free medium; PGE₁ (1 μM) or PGE₂ (10 μM), alone or with DPDPE (10 μM), was then added for the last 15 min of culture. Values are the mean ± SEM of quadruplicate determinations obtained from two independent experiments. *, $P < 0.05$ vs. control; **, $P < 0.05$ vs. PG-treated cells.

PGE₂ were significantly reduced by coincubation with 10 μM DPDPE by 44% and 33% respectively (Fig. 5).

Discussion

The results described in the present study provide the first evidence showing that LHRH-producing GT1–1 cells express opioid receptors. The nonselective opioid antagonist [³H]DIP binds with high affinity (0.2 nM) to both crude cell membrane preparations as well as intact GT1–1 cells. The binding affinity of [³H]DIP to GT1–1 cells is similar to that reported for this opiate when tested on membrane preparations obtained from either rat brain or neuroblastoma cells (26, 32–35).

The binding of [³H]DIP to GT1–1 cells is characterized by low nonspecific binding and is similar to that observed in crude membrane preparations of whole mouse brain. [³H]DIP binds with approximately equal affinity to μ-, δ-, and κ-opioid receptors (22, 32, 36); consequently, it is not possible to discriminate, using only this approach, the type(s) of opioid receptor(s) present on GT1–1 cells. Selective displacement experiments performed with specific opioid ligands revealed the presence only of binding sites of the δ type. Indeed, in the membrane preparation studies, the selective ligand for δ-opioid receptors, DPDPE, strongly inhibits (>90% at 1 μM) the binding of [³H]DIP, which, on the contrary, is not affected by selective ligands for the μ- and κ-opioid-binding sites. The K_i for DPDPE (5.5 nM) calculated on the present results is very similar to the K_i or K_d found for labeled DPDPE on δ sites present in membrane preparations obtained from rodent brain or neuroblastoma cells (35, 37–

39). The presence of only binding sites of the δ type was also confirmed in intact GT1–1 cells (see below).

A further characterization of these receptors was performed by analyzing their sensitivity to sodium ions. It is well known that the binding of μ- and δ-agonists is decreased in the presence of sodium ions, a phenomenon that does not occur when opioid antagonists are used. From the data obtained, it is evident that the presence of sodium in the incubation buffer induces a significant decrease in the potency of DPDPE to inhibit the binding of [^{3}H]DIP to either membrane preparations or intact GT1–1 cells. This results in increases in the K_i and IC_{50}.

As previously mentioned, the presence of δ-opioid-binding sites was confirmed when the binding experiments were repeated on intact GT1–1 cells. However, the selective inhibitory effect of the δ-ligand DPDPE on the binding of [^{3}H]DIP was slightly lower in intact GT1–1 cells than that found in crude membrane preparations. It is well known that the binding of μ- and δ-agonists is decreased not only in the presence of sodium ions, but also by GTP (34). As the binding experiments on intact GT1–1 cells were performed in a sodium-free buffer, it is possible that the decreased displacing activity of DPDPE might be due to the presence of GTP or to a combined effect of GTP and residual intracellular sodium (31). Recently, the cloning of δ-opioid receptors has been reported (40–42); an assessment of the expression of messenger RNA for the δ-opioid receptors in GT1–1 cells is presently under way and will provide a definitive evidence of their presence in these LHRH-secreting cells.

It is well known that opioids may exert inhibitory effects on the stimulated accumulation of cAMP in several cells and tissues (23, 43, 44). To investigate whether the δ-opioid receptors identified on GT1–1 cells are functional, the ability of the δ-agonist DPDPE to inhibit the stimulation of cAMP accumulation induced in GT1–1 cells by treatment with two known activators of adenylyl cyclase, PGE_1 and PGE_2, was analyzed (45). The results obtained clearly showed that both PGs are strong stimulators of cAMP accumulation in GT1–1 cells, and that the activation of δ-opioid receptors by DPDPE leads to a significant decrease in the stimulated cAMP levels. The data presented here are consistent with the numerous observations of an inhibitory role exerted by the opioids on intracellular cAMP accumulation (43–47) and indicate that the δ-receptors reported on GT1–1 cells are functional. In addition, the ability to inhibit cAMP accumulation as well as the sensitivity to sodium ions suggest a functional coupling of the δ-receptor subtype present in GT1–1 cells to intracellular effector molecules such as the GTP-binding proteins (22, 23). In experiments presently in progress, it has been shown that the δ-opioid agonist DPDPE inhibits LHRH secretion in GT1–1 cells elicited by forskolin (Maggi, R., F. Pimpinelli, L. Martini, and F. Piva, in preparation).

The presence of opioid-binding sites on the subclone of GT1 cells used in the present experiments is relevant, especially in light of the possible role of these receptors in the control of LHRH release and expression. If one assumes that GT1 cells represent a good model for the study of LHRH-secreting neurons, the obvious conclusion from these data is that endogenous opioid peptides may influence LHRH se-

cretion by acting directly on LHRH-secreting neurons. A second conclusion would be that this effect of the opioids is mediated by the δ-subclass of receptors. Obviously, it must be pointed out that the present data do not exclude the possibility that opioids act on the release of LHRH via other mechanisms (see below). As mentioned in the introduction, a large body of evidence implicates opioid peptides in the central mechanisms controlling gonadotropins and LHRH secretion (1, 15, 17–20). The prevailing theory proposes that opioid neurons have synaptic contacts with intermediate neurons, which, in turn, influence LHRH neurons via the local release of their respective neurotransmitters (18, 48). However, a few reports have demonstrated that, at least in rats (49–51), monkeys (52), and cows (53), opioid neurons (particularly POMC or β-endorphin reactive) may entertain direct synaptic contacts with hypothalamic LHRH-immunoreactive neurons, suggesting the possibility of a direct effect of physiological opioids. In contradistinction with the present data, which shows the presence of δ-receptors on LHRH-producing neurons, a number of data obtained *in vivo*, especially in the rat, support the view that opioids affect LHRH release mainly through the interaction with opioid receptors of the μ type (54–57). However, a few studies have also implicated δ (58–60)- and κ (58, 59)-receptors in such a mechanism. For instance, Leadem *et al.* (58, 59) have shown that Δ-agonists, including DPDPE, induce a biphasic effect on LH secretion in ovariectomized rats after intraventricular injection. In this connection, one may also point out that β-endorphin binds with similar affinities to μ and δ sites (22, 36). It must be recalled that the relative proportions of opioid receptor subtypes are different in the brains of various animal species (61). For instance, in the forebrain of the mouse (the species from which GT1 cells are derived), there is 25% more δ-receptor and 40% less μ-receptor than in the forebrain of the rat, suggesting a predominantly δ-receptor composition in the mouse brain (61). Moreover, in the mouse, both the hypothalamus and preoptic area contain the same amounts of μ- and δ-opioid receptors (62), whereas in the rat, the concentration of Δ sites is extremely low in the hypothalamus (39, 61, 63), a structure in which a relatively high concentration of μ-receptors is present in this species (61, 63).

In conclusion, from the data here presented, when analyzed in conjunction with the evidence just described, we hypothesize that the effects of physiological opioid peptides on LHRH secretion consist of two distinct components: the first, acting directly on the δ-receptors present on the LHRH-synthesizing neurons, and the second acting via intermediate neurons, where opioids probably interact mainly with the μ-receptor subfamily.

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