Receptors and neurosecretory actions of endothelin in hypothalamic neurons

(gonadotropin-releasing hormone neuron receptors/inositol phospholipid hydrolysis/gonadotropin-releasing hormone secretion)


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ABSTRACT Primary cultures of rat hypothalamic neurons were found to secrete the potent calcium-mobilizing and mitogenic peptide endothelin (ET) and to contain specific ET binding sites with higher affinity for ET-1 and ET-2 than ET-3. ET receptors of similar specificity were also identified in two gonadotropin-releasing hormone (GnRH) neuronal cell lines (GT1-1 and GT1-7). In both primary cultures and GnRH neurons, receptor binding of ETs led to marked and dose-dependent increases of inositol phosphates; inositol bis-, tris-, and tetrakisphosphates increased promptly, reached a peak within 2 min, and returned toward the steady-state levels during the next 10 min. ET-1 was more potent than ET-3 in mobilizing inositol phosphates, consistent with its greater affinity for the ET receptors in these cells. ET also stimulated GnRH secretion from perfused hypothalamic cultures and GnRH cell lines, with a sharp increase followed by a prompt decline to the basal level. These data show that ET is produced in the hypothalamus and acts through calcium-mobilizing ET receptors in normal and transformed secretory neurons to stimulate GnRH release. These actions of locally produced ETs upon GnRH-secreting neurons indicate that the vasoconstrictor peptides have the capacity to regulate neurosecretion and could participate in the hypothalamic control of anterior pituitary function and gonadotropin secretion.

Endothelin (ET) is a 21-amino acid peptide, originally identified as a potent vasoconstrictor produced by endothelial cells and presumably acting as a local hormone (1, 2). The ET family includes several peptides (ET-1, ET-2, and ET-3) that differ from each other by a few amino acids (3, 4). Such structural differences and the individual binding characteristics and physiological actions of the ETs have suggested the existence of several different ET receptor subtypes (4–7). Two distinct ET receptor subtypes, A and B (ET₄ and ET₆), have been characterized by molecular cloning as members of the guanine nucleotide-binding regulatory protein-coupled family of rhodopsin-type receptors (8, 9). ET₄ receptors have higher affinity for ET-1 and ET-2 than for ET-3, whereas ET₆ receptors do not discriminate between the three ETs.

ETs have a wide spectrum of pharmacological effects at locations other than blood vessels (5–7), and receptors specific for ET-1 and ET-2 or for ET-3 are present in several tissues including the intestine, heart, lungs, kidney, adrenal gland, pituitary, and brain (10–15). The mRNA for ET₄ receptors is widely distributed in the central nervous system (16, 17). It has been proposed that ET-1 and ET-3 have different targets in the brain and may have separate functions (18). It has also been suggested that ET is produced by glial cells and acts upon both glia and neurons in an autocrine-paracrine fashion (14). Two reports have indicated the potential involvement of ET in neuroendocrine regulation; ET-like immunoreactivity was demonstrated in paraventricular and supraoptic nuclear neurons with terminals in the posterior pituitary gland (19), and ET-1 was found to stimulate pituitary hormone secretion in vitro (15).

The present studies have demonstrated specific receptors for ¹²⁵I-labeled ET-1 in primary cultures of fetal hypothalamic cells and in gonadotropin-releasing hormone (GnRH)-secreting neuronal cell lines (20). Activation of the neural ET receptors is coupled to inositol phospholipid hydrolysis, inositol phosphate production, and release of GnRH. These findings suggest a role for the ET system at the hypothalamic–pituitary level, where ET peptides may participate in the physiological control of neurosecretion and anterior pituitary function.

EXPERIMENTAL PROCEDURES

Cell Cultures. Hypothalamic tissue was removed from fetuses of 17-day pregnant female rats. The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margin of the mammillary bodies, and laterally by the hypothalamic sulci. After dissection, hypothalami were placed into ice-cold Heps dissociation buffer (HDB) (21). Enzyme-dispersed hypothalamic cells were prepared by minor modifications of the method of Peterfreund and Vale (21); each hypothalamic yield about 1.5 × 10⁶ cells. GT1-1 and GT1-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 medium, supplemented with 10% (vol/vol) fetal calf serum and gentamicin (100 μg/ml), as described (20).

¹²⁵I-Labeled ET-1 Binding Experiments. Binding sites for ET were analyzed in cultured hypothalamic cells in situ. ¹²⁵I-labeled ET-1 (Amersham) was added to monolayers of hypothalamic cells or GT1 cells cultured in 12-well Falcon plates at 22°C in DMEM. Nonradioactive peptides (Peninsula Laboratories) were added in 100 μl to evaluate their abilities to compete with the radioligand. After incubation to equilibrium for 90 min at room temperature, the cells were washed rapidly three times with ice-cold phosphate-buffered saline/0.1% bovine serum albumin (BSA). The cells were then solubilized in 1 M NaOH containing 0.1% SDS and analyzed for bound radioactivity in a γ-spectrometer. Calculations of

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Abbreviations: ET, endothelin; GnRH, gonadotropin-releasing hormone; InsP₂, Ins(1,3,4)P₃, Ins(1,4,5)P₃, and InsP₄, inositol bisphosphate, inositol 1,3,4-trisphosphate, inositol 1,4,5-trisphosphate, and inositol tetrakisphosphate, respectively; BSA, bovine serum albumin.

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Production of Inositol Phosphates. Four weeks after preparation of primary cultures (or 6 days after subculturing GT-1 cells), the culture medium in the four-well plates was changed to 0.5 ml of inositol-free medium 199 with Hanks' solution, containing 25 mM HCO₃, 0.1% BSA, and 10 µCi myo-[³H]inositol (DuPont/New England Nuclear; 1 Ci = 37 GBq). After a 48-hr incubation, the cells were washed three times with inositol-free medium 199 containing 25 mM Hepes and 0.1% BSA. After a 5-min preincubation in the same medium, ET-1 (100 nM) and ET-3 (100 nM) or solvent (controls) were added and the cells were incubated at 37°C for the indicated times. The radioactivity incorporated into the individual or total inositol phosphates was determined as described (23).

Measurement of Secretory Responses. The release of GnRH in primary cultures of hypothalamic neurons and cell lines was examined under two experimental conditions: in perfused neurons (Krebs/Ringer buffer; flow rate, 10 ml/hr) cultured on beads (20 × 10⁶ cells per column) and in static cultures (2 × 10⁶ cells per well, 12-well plates). Attachment of the cells to Cytoxid beads (Pharmacia) was performed in 50-ml tubes containing 1.5 × 10⁶ cells and 0.3 ml of preswollen Cytoxid-2, incubated for 24 hr in 5% CO₂/95% air. Next day, cells were transferred into 30-mm dishes and cultured in DMEM/Ham's F-12 medium, with L-glutamine (365 mg/liter), high glucose (4500 mg/liter), and gentamicin (100 µg/ml) supplemented with 10% fetal calf serum. The culture medium was changed every 3 days. Before each perfusion the cell/bead mixture was collected by centrifugation, resuspended in Krebs/Ringer buffer containing BSA (1 mg/ml), glucose (1 mg/ml), and 20 µM bacitracin (pH 7.4), gassed with 5% CO₂/95% O₂ for 1 hr, and loaded into a temperature-controlled 0.5-ml chamber (Endotronics, Minneapolis). Cells were perfused for at least 1 hr before testing at a flow rate of 10 ml/hr to establish a stable baseline. Fractions were collected every 5 min and stored at −20°C prior to RIA.

GnRH assay was performed as described (24), using ¹²⁵I-labeled GnRH from Amersham, unlabeled GnRH from Peninsula Laboratories, and primary antibody donated by V. D. Ramirez (Urbana, IL). The intra- and interassay coefficients of variation at 80% binding in standard samples (15 pg/ml) were 12% and 14%, respectively. Measurement of ET in samples from perfused primary cultures was performed by RIA using antibodies specific for ET-1/ET-2 (Amersham) or ET-3 (Peninsula Laboratories). The IC₅₀ of the ET-1/ET-2 assay was 28 pg per tube, and of the ET-3 assay was 30 pg per tube. All samples were analyzed in the same assay with an intraassay coefficient of variation of 4.1%.

RESULTS

ET Secretion from Primary Hypothalamic Cultures. Immunoreactive ETs (ET-1 and ET-3) are present in the human hypothalamus and the pituitary gland (25). In primary cultures of rat hypothalamic cells, we observed release of readily measurable amounts of ET-1/ET-2 (214 ± 3 pg/ml) and ET-3 (33 ± 3 pg/ml) into the culture medium after 3 hr of incubation.

ET Receptors in Hypothalamic and Neuronal Cultures. In addition to being produced by cultured hypothalamic cells, ET also bound to hypothalamic cells and GnRH neuronal cell lines (GT1-1 and GT1-7) in a dose-, time-, and temperature-dependent manner. At room temperature, binding reached equilibrium within 60 min of incubation. Nonspecific binding (estimated in the presence of 1 µM unlabeled ET-1) to the culture wells (without cells) was 3−5% of the total added radioactivity. In 4-week-old cultures, specific binding (Bₒ) was 50% of the total added tracer. As shown in Fig. 1A, binding of ¹²⁵I-labeled ET-1 to hypothalamic cells in 4-week-old primary cultures was more potently inhibited by ET-1 (IC₅₀ = 1.5 nM) than by ET-3 (IC₅₀ = 27 nM). ¹²⁵I-labeled ET-1 also bound specifically to the GT1-1 and GT1-7 neuronal cell lines. Fig. 1B shows the displacement of the labeled peptide by ET-2, ET-1, and ET-3 in GT1-7 cells, with IC₅₀ values of 0.27, 0.34, and 165 nM, respectively. Similar results were obtained in GT1-1 cells, with IC₅₀ values of 0.30, 0.32, and 145 nM for ET-2, ET-1, and ET-3, respectively (data not shown). The specificity of binding was indicated by the inability of several unrelated peptides, including thyrotropin-releasing hormone, GnRH, angiotensin II, vasopressin, and oxytocin (all 100 nM) to inhibit binding of ¹²⁵I-labeled ET-1.

Inositol Phosphate Production in ET-Stimulated Cultures. ET-1 (100 nM) and ET-3 (100 nM) caused marked increases in the levels of four inositol phosphates [inositol bisphosphate (InsP₂), inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), and inositol 1,3,4,5-tetakisphosphate] in [³H]inositol-labeled hypothalamic cell cultures. In 4-week-old primary cultures, the several inositol phosphates increased rapidly to a peak at around 2 min and fell gradually thereafter (Fig. 2A). The peak inositol phosphate response to ET-1 was dose-dependent from 10 pM to 300 nM, with an EC₅₀ value of 7–9 nM (Fig. 2B). ET-1 was more effective than ET-3 in stimulating production of InsP₂.
Threshold concentrations (InsP4) (Fig. 2) were expressed as percent of the maximum responses. The results are the mean ± SEM of triplicate determinations.

Ins(1,3,4)P3, Ins(1,4,5)P3, and inositol tetrakisphosphate (InsP4) (Fig. 3A); it was also more potent, as shown by the threshold concentrations of 0.1 nM for ET-1 vs. 1.0 nM for ET-3 (Fig. 3B).

ET also increased inositol phosphate production in hypothalamic-labeled GT1-7 cells, consistent with its stimulatory effect on inositol phospholipid hydrolysis. As shown in Fig. 3C, statistically significant increases in InsP2, Ins(1,3,4)P3, and Ins(1,4,5)P3 were observed 2 min after stimulation with 100 nM ET. Consistent with its receptor binding activity, the amplitude of the inositol phosphate response to ET-2 was the highest, followed by ET-1 and ET-3 (Fig. 3D).

Stimulation of GnRH Release by ET. GnRH released from perfused cultured hypothalamic cells in a pulsatile manner, with small intermittent spikes and a mean level of ~20 pg/ml. Such pulsatility was abolished when the extracellular Ca²⁺ concentration was reduced from 1.25 mM to 0.1 mM (data not shown). In 2-week-old cultures perfused at a flow rate of 10 ml/hr, a 15-min pulse of 100 nM ET-3 stimulated a sharp increase in GnRH secretion, followed by a prompt decline to the basal level (Fig. 4A). Similar GnRH responses were elicited by ET-1 in perfused hypothalamic cells (data not shown). In static cultures, both ET-1 (100 nM) and ET-3 (100 nM) increased GnRH release during a 24-hr incubation. The amplitude of the response to ET-1 was higher than that to ET-3 (Fig. 4A Right; ET-1 = 41 ± 9 pg/ml; ET-3 = 29 ± 1 pg/ml; controls = 13 ± 4 pg/ml; P < 0.01).

GnRH secretion in perfused GT1-7 cells was also pulsatile, with a mean basal concentration of 30 ± 4.2 pg/ml. Stimulation with both ET-1 (data not shown) and ET-3 (Fig. 4B Left) significantly enhanced GnRH secretion. The stimulatory effects of ET-1 and ET-3 on GnRH release were also seen in static 24-hr cultures of GT1-7 neurons (Fig. 4B Right; ET-1 = 40.2 ± 5.2 pg/ml; ET-3 = 31 ± 2 pg/ml; controls = 14.8 ± 2.6 pg/ml; P < 0.01) and in GT1-1 cells (data not shown).

ET Receptors and Duration of Primary Culture. Competitive inhibition of 125I-labeled ET-1 binding by unlabeled ET-1 (from 10 pM to 300 nM) was analyzed at three ages of culture.

For ET-1, the IC₅₀ was 0.3 nM in 2-week-, 0.5 nM in 3-week-, and 1.5 nM in 4-week-old cultures. Scatchard analysis of the displacement data showed an increase in binding capacity and decrease in binding affinity with increasing age of culture: 2-week culture, 5590 sites per cell and a K_d of 0.13 nM; 4-week culture, 175,000 sites per cell and a K_d of 1.57 nM. Cultures maintained for >2 weeks showed an increase in protein content per well (2-week culture, 435 ± 51 pg per well; 4-week culture, 1016 ± 186 pg per well) but not in cell number (2 x 10⁶ ± 2.8 x 10⁵ vs. 2.2 x 10⁶ ± 3.5 x 10⁵ cells).

The effects of culture age on ET receptor properties did not affect the ability of high ET concentrations to activate inositol phosphate hydrolysis and secretory responses. Two-month-old cultures responded to ET-1 (100 nM) with similar maximum amplitudes of inositol phosphate responses and slightly higher steady-state levels. Basal and stimulated GnRH secretion in 2-, 3-, and 4-week-old cultures was comparable [basal, 26 ± 1, 21 ± 1, and 29 ± 2 pg/ml, respectively; ET-3 (100 nM)-stimulated GnRH release, 43 ± 1, 47 ± 2, and 62 ± 2 pg/ml, respectively].

**DISCUSSION**

These studies have demonstrated that cultured hypothalamic cells produce and respond to ETs and that GnRH neurons possess ET receptors and exhibit secretory responses to the vasoconstrictor peptides. The differences in potencies of
release of vasopressin and substance P from hypothalamic slices (27, 28) and of luteinizing hormone, follicle-stimulating hormone, and substance P from pituitary cells (15, 28). The higher potency of ET-1 vs. ET-3 in displacement of $^{125}$I-labeled ET-1 and intracellular responses has also been observed in pituitary cells (29), suggesting a similarity between the pituitary and neuronal receptors. In both pituitary cells and GnRH neurons, activation of ET-1 receptors is coupled to phospholipase C-mediated hydrolysis of inositol phospholipids. In contrast to the pituitary gonadotrophs, where ET-1 induces a sharp increase in Ins(1,4,5)P$_3$ within 10 sec, followed by an exponential decrease to the low steady-state level (29), the Ins(1,4,5)P$_3$ response in hypothalamic cells is more prolonged, reaching a maximum within 2 min followed by a gradual decrease toward the basal level. The time course of the cellular response also varies among different cell types in which ET-1 receptors are activated. Whereas ET exerts long-lasting vasopressor effects, GnRH neurons and cell lines respond transiently to ET stimulation and are not refractory to repetitive stimulation with ET-1 or ET-3. On the other hand, rapid desensitization (within 10 min) occurs in pituitary gonadotrophs exposed to ET (29, 30).

The availability of the GnRH neuronal cell lines (20) has enabled us to localize the effects of ET to specific neurosecretory cells within the hypothalamus. The stimulatory action of ET on GnRH secretion in the primary hypothalamic cultures could be exerted on the GnRH cells or mediated by another cell type. However, the presence of ET receptors and secretory responses in the neuronal cell lines indicates that the effects of ET are exerted directly on the GnRH cells. The relationship between the ET-producing and effector cells within the hypothalamus is not yet clear but probably includes paracrine effects of the peptide.

It is not yet known whether the facilitatory effects of ET on GnRH release are present at all ages or are predominant at specific periods of development. Our experiments were performed with primary cultures of fetal hypothalamic cells, which showed changes in their binding characteristics for ET with increasing duration of culture; these included a decrease in binding affinity and an increase in binding capacity. Such effects could reflect developmental changes that occur in vivo or may result from prolonged culture per se. Marked changes in the appearance of the cells and in the relationships between nonneural and neural cells have been observed with increasing duration of culture (31).

In conclusion, concurrent studies in primary hypothalamic cultures and GnRH neuronal cell lines have been valuable in circumventing the limitations of each system: the variety of different cell types present in the cultures, and the possible deviation from primary functions of the cell lines. The combination of these culture systems provides a valid in vitro model for studies on the cell and molecular biology of GnRH neurons. This approach has unequivocally demonstrated the presence of ET receptors in GnRH neurons and their coupling to inositol phospholipid breakdown and GnRH secretion. It is reasonable to conclude that the ability of ETs to stimulate inositol phosphate production and GnRH release in cultured neurons reflects their role in the neurosecretory process and its regulation in vivo.

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