Muscarinic Regulation of Intracellular Signaling and Neurosecretion in Gonadotropin-Releasing Hormone Neurons

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ABSTRACT

Agonist activation of cholinergic receptors expressed in perfused hypothalamic and immortalized GnRH-producing (GT1–7) cells induced prominent peaks in GnRH release, each followed by a rapid decrease, a transient plateau, and a decline to below basal levels. The complex profile of GnRH release suggested that acetylcholine (ACh) acts through different cholinergic receptor subtypes to exert stimulatory and inhibitory effects on GnRH release. Whereas activation of nicotinic receptors caused a transient increase in GnRH release, activation of muscarinic receptors inhibited basal GnRH release. Nanomolar concentrations of ACh caused dose-dependent inhibition of cAMP production that was prevented by pertussis toxin (PTX), consistent with the activation of a plasma-membrane G protein. Micromolar concentrations of ACh also caused an increase in phosphoinositide hydrolysis that was inhibited by the M1 receptor antagonist, pirenzepine. In ACh-treated cells, immunoblot analysis revealed that membrane-associated G\textsubscript{af/i3} immunoreactivity was decreased after 5 min but was restored at later times. In contrast, immunoreactive G\textsubscript{a1d} was decreased for up to 120 min after ACh treatment. The agonist-induced changes in G protein \( \alpha \)-subunits liberated during activation of muscarinic receptors were correlated with regulation of their respective transduction pathways. These results indicate that ACh modulates GnRH release from hypothalamic neurons through both M1 and M2 muscarinic receptors. These receptor subtypes are coupled to \( G_\alpha \) and \( G_\beta \) proteins that respectively influence the activities of PLC and adenylyl cyclase/\( \alpha \)ion channels, with consequent effects on neurosecretion. (Endocrinology 139: 4037–4043, 1998)

THE DIVERSE actions of acetylcholine (ACh) are mediated by activation of nicotinic (ionotropic) and muscarinic (metabotropic) classes of receptors. Nicotinic receptors (nAChRs) are ligand gated receptor channels that are cation-specific but do not distinguish readily among cations (1). Of the five subtypes (M1–M5) of muscarinic receptors (mAChRs), M1, M3, and M4 are coupled to \( G_\text{i3/11} \) and mediate activation of phospholipase C (PLC) and InsP\textsubscript{3}/Ca\textsuperscript{2+} signaling. The M2 and M4 subtypes are coupled to \( G_\text{i3/11} \) and mediate inhibition of cAMP production (2). Both classes of cholinergic receptors are expressed throughout the brain and are abundant in the hypothalamus, where monoaminergic neurons that terminate on GnRH and other peptidergic neurons are located (3). The GnRH neurons of the hypothalamus are innervated by noradrenergic neurons located in the hindbrain, and catecholamines have been implicated in the regulation of GnRH release. In contrast, there is less evidence for a role of cholinergic innervation in the control of GnRH secretion. In early studies, a rapid atropine-sensitive cholinergic component was found to be involved in the release of ovulating hormone in rats and rabbits (4, 5). Subsequently, intraventricular administration of atropine was shown to block the release of LH, FSH, and PRL from the pituitary gland, implicating cholinergic pathways in the control of gonadotropin secretion (6). Direct evidence for actions of ACh at the hypothalamic level was provided by studies on the release of LH and FSH from hypothalamic and pituitary tissue (7–9). More recently, muscarinic and nicotinic receptors were found to be involved in the stimulatory and inhibitory actions of ACh on GnRH and LH release (10–15). ACh has also been shown to have a significant role in the regulation of lordosis by acting on muscarinic receptors in the ventromedial nucleus (16).

In the present studies, the direct actions of ACh on GnRH release were analyzed in cultured hypothalamic neurons and GnRH-producing (GT1–7) cells.

Materials and Methods

Tissue and cell culture

Hypothalamic tissue was removed from fetuses of 17-day pregnant Sprague-Dawley rats. The borders of the excised hypothalami were delineated by the anterior margin of the optic chiasm, the posterior margin of the mamillary bodies, and laterally by the hypothalamic sulci. After dissection, hypothalami were placed in ice-cold dissociation buffer containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na\textsubscript{2}HPO\textsubscript{4}, 26 mM HEPES, and 100 mg/liter gentamicin, pH 7.4. The tissues were washed and then incubated in a sterile flask with dissociation buffer supplemented with 0.2% collagenase, 0.4% BSA, 0.2% glucose, and 0.05% DNase I. After 60 min incubation in a 37°C water bath with shaking at 60 cycles/min, the tissue was gently triturated by repeated aspiration into a smooth-tipped Pasteur pipette. Incubation was continued for another 30 min, after which the tissue was again dispersed. The cell suspension was passed through sterile mesh (200 μm) into a 50-ml tube, sedimented by centrifugation for 10 min at 200 × g, and washed once in dissociation buffer and once in culture medium consisting of 500 ml DMEM containing 0.584 g/liter l-glutamate and 4.5 g/liter glucose, mixed with 500 ml F-12 medium containing 0.146 g/liter l-glutamine, 1.8 g/liter glucose, 100 μg/ml gentamicin, 2.5 g/liter sodium bicarbonate, and 10% heat-inac-
tivated FCS. Each dispersed hypothalamus yielded about 1.5 × 10⁶ cells. Immortalized GnRH neurons (GT1–7 cells) obtained from Dr. R. I. Weiner (University of California at San Francisco) were cultured under the same conditions as primary hypothalamic cells.

Perifusion procedure

Cells were incubated in 50-ml tubes containing 1.5 × 10⁷ cells, 0.3 ml preswollen Cytodex-2 beads, and 30 ml of culture medium for 24h in 5% CO₂/air. The suspension was then transferred into 60-mm dishes and culture was continued for 14–60 days, with replenishment of culture medium every second day. Before perifusion, the cell-bead mixture was collected by sedimentation and resuspended in Krebs-Ringer buffer containing 1 mg/ml BSA, 1 mg/ml glucose, 20 µM bacitracin, pH 7.4. After gassing for 1 h with 95% O₂/5% CO₂, the beads were loaded into a temperature-controlled 0.5 ml chamber. Perifusion was performed at a flow rate of 10 ml/h at 37 C for at least 1 h to establish a stable baseline before addition of agents made up in the same medium. Fractions were collected at either 1- or 5-min intervals and stored at −20 C before RIA using ¹²⁵I-GnRH, GnRH standards, and primary antibody donated by Dr. V. D. Ramirez (University of Illinois, Urbana, IL). The intra and interassay coefficients of variation at 80% binding in standard samples (15 pg/ml) were 12–14%, respectively. GnRH pulses were identified and their parameters were determined by algorithm cluster analysis. Individual point sums were calculated using a power function variance model from the experimental duplicates. A 2 × 2 cluster configuration and a t-statistic of 2 for the upstroke and downstroke were used to maintain false-positive and false-negative error rates below 10% (17). The statistical significance of the pulse parameters was tested by one-way ANOVA.

cAMP production and phosphoinositide hydrolysis

For studies on cAMP release, GnRH-producing cells were stimulated in serum-free medium (1:1 DMEM/F-12) containing 0.1% BSA, 30 mg/liter bacitracin, and 1 mM IBMX. RIA of cAMP was performed as previously described (18), using a specific cAMP antisera at a titer of 1:5000. This assay showed no cross-reaction with cGMP, 2-3' cAMP, ADP, GDP, CTP, or IBMX.

Inositol phosphate production

Cells were labeled for 24 h in inositol-free DMEM medium containing 20 µCi/ml [³H]inositol as described previously (19) and then washed with inositol-free M199 medium and stimulated at 37 C in the presence of 10 mM LiCl. Incubations were terminated by the addition of ice-cold perchloric acid (5% vol/vol) final concentration. The inositol phosphates were extracted and analyzed by anion exchange chromatography as previously described. The combined InsP₃ + InsP₄ fractions were eluted from the columns by washing twice with 1 mM ammonium formate in 0.1 M formic acid (3 ml/wash), and their radioactivities were measured by liquid β-spectrometry.

Immunoblot analysis of membrane-associated G proteins

After stimulation, the cells were washed twice with TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.4), scraped from the plates, and lysed by freeze-thawing. After centrifugation at 12,000 × g, 15 min at 4 C, the pellet was resuspended in TE buffer and stored at −70 C until assayed. Protein contents were measured by the Pierce BCA protein assay kit (Pierce, Rockford, IL). SDS-gel electrophoresis was performed on 12.5% acrylamide gels (20), followed by blotting with PVDF membrane of 0.45 µm pore size. The blots were incubated with first antibody, followed by peroxidase-coupled goat-antirabbit IgG (H + L), and visualized by chemiluminescence. The immunoreactive bands were analyzed as three-dimensional digitized images using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). The optical density (D.O.) of images is expressed as volume (O.D. × area) adjusted for the background, which gives arbitrary units of adjusted volume (Adj. Volume).

Materials

¹²⁵I-GnRH, [³H]inositol, ECL, and Western blotting reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL); collagenase (149 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ); DNase I, trypsin, bacitracin, 3-isobutyl-1-methylxanthine (IBMX), CTP, GDP, GMP, 2',3'-cAMP, and BSA were from Sigma Chemical Co. (St. Louis, MO); cytodex-beads were from Pharmacia Biotech (Piscataway, NJ); the perifusion system was from Acusyst-S Celllex Biosciences (Minneapolis, MN); standard GnRH was from Peninsula Laboratories, Inc. (Belmont, CA); ACh, carbachol, nicotine, muscarine, methoctramine, atropine, pirenzepin were from Research Biochemicals International (Natick, MA); ²⁻³⁻⁹⁻cAMP was from Covance Laboratories, Inc. (Vienna, VA); protein assay was from Pierce. Membrane Immobilon-P was from Millipore Corp. (Bedford, MA). Peroxidase-coupled goat-antirabbit IgG (L + H), FBS, and DMEM/F12 1:1 powder were from Gibco BRL-Life Technologies (Gaithersburg, MD). Antibodies to Gα₁/Gα₁, Gα₅, Gα₁₅, Gα₁₃ and Gα₁₃ were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), as well as the corresponding standard peptides; anti-Gα₁₅ was a gift from MBL International Corporation (Watertown, MA). Other reagents, if not specified, were obtained from Sigma Chemical Co.

Results

GnRH release from perifused hypothalamic cells and GT1–7 neurons

Treatment of both hypothalamic neurons and GT1–7 cells with ACh elicited prominent changes in GnRH release. An initial rapid increase occurred during the first 15 min, followed by a decline to a transient plateau phase and a subsequent fall to below the initial basal level. Recovery from the late inhibitory phase was slow, and GnRH release was suppressed for up to 60 min after the cessation of ACh treatment (Fig. 1, A and B). Such biphasic responses to ACh suggested that GnRH release was differentially regulated by the activation of individual cholinergic receptor subtypes. Treatment of perifused hypothalamic cells and GT1–7 cells with carbachol also produced an initial stimulatory response, followed by sustained inhibition of GnRH release (not shown).

Selective activation of nicotinic receptors stimulated GnRH release in both hypothalamic and GT1–7 cells and was followed by a return to the basal pattern of pulsatile release (Fig. 1, C and D). The secretory action of nicotine was similar to that of K⁺-induced depolarization, consistent with the operation of nicotinic receptor channels that promote Na⁺ and Ca²⁺ entry, with consequent effects on GnRH release. In contrast, selective activation of muscarinic receptors inhibited GnRH release in both hypothalamic and GT1–7 cells, followed by a return to the basal pulsatile pattern (Fig. 1, E and F). These results suggest that muscarinic receptor subtypes are responsible for the ACh-induced inhibition of GnRH secretion. Basal GnRH release was potentiated by the nonselective cholinergic receptor antagonist, atropine, as well as by the selective M₂ antagonist, methoctramine (Fig. 2).

Stimulation of phosphoinositide hydrolysis by cholinergic agonists in hypothalamic cells and GT1–7 neurons

Treatment of both hypothalamic cells and GT1–7 cells with ACh caused time- and dose-dependent stimulation of phosphoinositide hydrolysis. The maximal stimulatory effect of 1 mM ACh was reached after 5 min, and the production of inositol phosphate declined to the basal level after 2 h (Fig. 3). ACh caused dose-dependent increases of inositol phosphate production in both hypothalamic cells and GT1–7 neu-
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FIG. 1. Biphasic actions of acetylcholine on GnRH release from perfused hypothalamic cells and GT1–7 neurons. In both hypothalamic cells (A) and GT1–7 neurons (B), treatment with acetylcholine (ACh, closed circles) caused a transient increase in GnRH release followed by a decline to below the basal level. Treatment of hypothalamic cells (C) and GT1–7 neurons (D) with nicotine (closed circles) caused prominent increases in GnRH release, followed by a return to the basal level (open circles). Treatment of hypothalamic cells (E) and GT1–7 neurons (F) with muscarine (closed circles) caused inhibition of GnRH release, followed by a return to basal pulsatile release (open circles).

Effects of cholinergic agonists on cAMP production in GT1–7 neurons

Micromolar concentrations of ACh also induced prominent decreases in cAMP production in GT1–7 cells. The inhibitory effect of ACh (−44%) was evident after 5 min and persisted for up to 30 min but was no longer present after 2 h (Fig. 5A). Inhibition of cAMP production was also observed after treatment of GT1–7 cells with micromolar concentrations of muscarine and was maintained for about 2 h. The inhibitory action of ACh on cAMP production was dose dependent, with an IC50 in the nanomolar range (Fig. 5B). The inhibitory effect of carbachol on cAMP production was prevented by prior treatment of cells by PTX, suggesting that it results from the coupling of muscarinic receptors to adenylyl cyclase inhibitory G proteins (Fig. 5C). After pretreatment of GT1–7 cells with the M2 muscarinic antagonist, methoctramine (100 nM), carbachol no longer inhibited cAMP production and at higher concentrations caused a slight increase above the basal level (Fig. 5D). In contrast, treatment of GT1–7 cells with nicotine had no significant effect on cAMP production.

Identification of G proteins coupled to cholinergic receptors in GT1–7 neurons

Western blot analysis of membrane preparations from GT1–7 cells with specific antibodies to Gaq/11, Gai3, Gao, and Gas revealed significant changes in G protein content. Activation of muscarinic receptors by ACh caused a dose-dependent decrease in Gao/11 immunoreactivity that was evident after 5 min (Fig. 6A). In contrast, there was only a minor change in Gas immunoreactivity during the first 5 min of ACh stimulation (Fig. 6B). After 30 min of exposure to 10 mM ACh, Gao/11 immunoreactivity returned to the control level (Fig. 6C). In contrast, immunoreactive Gas fell to a minimal level at this time (Fig. 6D), consistent with the decrease in basal cAMP production and inhibition of GnRH release. After prolonged (2 h) exposure to ACh, Gao/11 immunoreactivity remained at the control level (Fig. 6E), whereas Gas immunoreactivity was still significantly reduced (Fig. 6E).

Discussion

In the present study, concomitant analyses were performed on cultured hypothalamic neurons and immortalized
GnRH neurons (GT1 cells) to use the complementary features of both systems (heterogeneous normal cells vs. homogeneous transformed cells). Cultured hypothalamic cells and immortalized GT1 neurons exhibit pulsatile GnRH release at a frequency similar to that of GnRH secretion in vitro (21–24). This property, with their spontaneous electrical activity (25), expression of ion channels (26–28), and coexpression of GnRH and its receptors (29), suggest that GnRH neurons can operate as an elemental endogenous GnRH oscillator. The modulation of GnRH pulse amplitude and frequency by GnRH agonist and antagonist analogs in cultured hypothalamic neurons and GT1–7 cells indicates the potential role of autocrine control of GnRH release. The *in vivo* operation of the GnRH oscillator is influenced by neuropeptides (30, 31), neurotransmitters (32–36), peripheral hormones (37, 38), and feedback actions of gonadal steroids (39–42).

It is clear that GnRH release is influenced by nicotinic and muscarinic receptors, which respectively mediate transient stimulatory and sustained inhibitory actions on neurosecretion. Thus, nonselective cholinergic agonists such as ACh and carbachol exerted sequential stimulatory and inhibitory actions on GnRH release from both normal and immortalized GnRH neurons. These actions were separable into individual stimulatory and inhibitory responses in cells treated with nicotinic and muscarinic agonists, respectively. The stimulatory actions of these agonists are in part attributable to the activation of nicotinic receptor channels, and in part to activation of muscarinic receptors coupled to Gq with consequent stimulation of phosphoinositide hydrolysis. The stimulatory action of cholinergic agents on inositol phosphate production was elicited by micromolar agonist concentrations, with EC50 of 12 μM for ACh and 26 μM for carbachol. The ability of pirenzepine to prevent this response identified the M1 receptor as the mediator of this cholinergic stimulatory action on phosphoinositide hydrolysis. There was no significant change in inositol phosphate production in nicotinic-stimulated cells, in which the transient secretory response is presumably related to calcium influx through nicotinic receptor-channels.

The inhibitory action of muscarinic agonists on cAMP release was evident at much lower concentrations than those required to stimulate inositol phosphate production. Also, it was prevented by treatment with pertussis toxin, consistent with its mediation by M2 or M4 receptors coupled to Gi regulatory proteins. The ability of methoctramine to prevent this response identified the M2 receptor as the predominant.
mediator of this cholinergic inhibitory action, which is evident at nanomolar concentrations of ACh. The increase in basal GnRH release during application of the nonselective cholinergic antagonist atropine, as well as the selective M₂ muscarinic receptor antagonist methoctramine, indicate that M₂ muscarinic receptors exert a tonic inhibitory action on GnRH release. This may occur as a consequence of its spontaneously active conformation (43), or of the local production of ACh as observed in both hypothalamic cells and GT1–7 neurons (unpublished data). The rapid inhibitory action of ACh on pulsatile GnRH release from hypothalamic cells and GT1–7 neurons could also be related to the release of βγ subunits from Gᵢ (or Gᵣ), with consequent actions on plasma membrane ion channels (44) that lead to suppression of neurosecretion. Such effects could include both inhibition of voltage-dependent calcium channels (45, 46) and activation of inwardly rectifying potassium channels (47).

An analysis of the changes in membrane-associated Gᵢ₃/₁₁ subunit levels during activation of the M₁ receptor by ACh revealed a dose-dependent decrease at 5 min, with a return to control levels at 30 and 120 min. In contrast, Gᵢ₃/₁₁ subunits showed a more extensive and sustained reduction that was evident for up to 120 min at high ACh concentrations. These changes in G proteins are consistent with the initial activation of phosphoinositide hydrolysis via M₁ receptors and the subsequent M₂ receptor-mediated inhibitory action of ACh on GnRH release from both cultured hypothalamic neurons and GT1 cells. The down-regulation of Gᵢ₃/₁₁ by ACh in GT1–7 cells is qualitatively similar to results obtained for Chinese hamster ovarian cells (CHO cells) expressing M₁ muscarinic receptors (48) and human M₁ muscarinic receptors (49). Activation of Gᵢ₃ in mouse lymphoma cells (549 cyc− cells) by cholera toxin, or reduction of α₃ GTPase activity by mutations, accelerated the rate of degradation of α₃ by 3- to 4-fold and induced a shift of the α₃ from a membrane-bound to a soluble compartment. In the same cells, the β-adrenoceptor agonist, isoproterenol, caused a rapid (<2 min) 20% shift of α₃ from the membrane-bound to the soluble compartment (50).

Our data indicate that ACh modulates GnRH release from normal and immortalized hypothalamic neurons by acting on both nicotinic and muscarinic receptor subtypes. The transient increase in GnRH release elicited by application of nicotine is consistent with the operation of nicotinic receptors as ion channels that mediate Na⁺ and Ca²⁺ entry. The similarity of the secretory responses during nicotinic receptor activation to that elicited by depolarization with potassium suggests that Ca²⁺ entry is responsible for the transient increase in GnRH release. Activation of M₁ muscarinic receptors caused a transient decrease in Gᵢ₃/₁₁ immunoreactivity and stimulation of phosphoinositide hydrolysis, followed by increased GnRH secretion. In contrast, activation of M₂ muscarinic receptors caused a sustained reduction in Gᵢ₃/₁₁ immunoreactivity and a PTX-sensitive reduction in cAMP production, with concomitant inhibition of GnRH release. The agonist-induced down-regulation of α subunits liberated after dissociation of the heterotrimeric G proteins during receptor activation may reflect their release from the plasma membrane and/or degradation by specific proteases. Such down-regulation also provides an indication of the mode(s) of G protein coupling used by individual seven transmembrane domain receptors that respectively influence the activities of phospholipase C and adenylyl cyclase. Thus, stimulation of nicotinic and muscarinic receptors in normal and immortalized hypothalamic neurons activates ligand-gated...
receptor channels and/or G protein-dependent second messenger systems and signal transduction pathways, with consequent stimulatory (nicotinic, M₁) or inhibitory (M₂) effects on neurosecretion.

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