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Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: Intrinsic properties of the GT1-1 GnRH neuronal cell line

(chronobiology/pacemaker/single-cell oscillator/neuron)

GONZALO MARTÍNEZ DE LA ESCALERA*†, AMY L. H. CHOI*, AND RICHARD I. WEINER*

*Reproductive Endocrinology Center, University of California, San Francisco, CA 94143-0556; and †Instituto de Investigaciones Biomédicas, National University of Mexico, Mexico City, Mexico 04510

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ABSTRACT **The immortalized neuronal cell line GT1-1 was used to investigate the endogenous pattern of GnRH release. The GT1-1 cell line was derived from a GnRH-secreting tumor in a transgenic mouse induced by genetically targeted expression of the potent simian virus 40 oncogene encoding tumor antigen. Cells attached to coverslips were superfused in Sykes–Moore chambers with Locke's medium, Ca^{2+}-free Locke's medium, or Opti-MEM (another defined medium) for 2 hr, and samples were collected at 4-min intervals. Release of GnRH in 17 of 18 superfusion chambers was seen to be pulsatile when data were analyzed by cluster analysis. No significant differences were observed whether only one or both of the coverslips forming the chamber were coated with cells. Pulses exhibited a mean interpulse interval of 25.8 ± 1.5 min, a mean duration of 18.8 ± 1.4 min, and a mean amplitude of 150.5 ± 6.0% above preceding nadir. The removal of Ca^{2+} from the Locke's medium resulted in the progressive reduction of the amplitude and eventually in the absence of identifiable pulses. Pulses reappeared after the return of Ca^{2+} to the medium. It is concluded that the GT1-1 cell line secretes GnRH in a rhythmic pattern. These findings suggest that the pulsatile release of GnRH (GnRH pulse generator) may be an intrinsic characteristic of the GnRH neurons. Synchronization of pulsatile release from individual neurons could be mediated via numerous cell-to-cell contacts observed in the cultured cells on coverslips. Synchronization of GnRH release from cells on two physically separated coverslips forming a chamber would appear to be accomplished by a diffusible mediator.**

Extensive data support the idea that GnRH (gonadoliberin) is released into the portal vessels in a pulsatile ultradian pattern (1–4), initiating a cascade of hormonal events that play a central role in gonadal regulation. Numerous neuropeptides and transmitters modulate the secretion of GnRH (for a review see ref. 5). A synchronous and intermittent discharge of multiple GnRH neurons (6) is required for episodic increases in the concentration of GnRH in hypophyseal portal blood. This intermittent secretion is the result of an oscillator, the so-called GnRH pulse generator, localized in the mediobasal hypothalamic–anterior hypothalamic–preoptic area (7–9). In both the rat (7) and monkey (8) surgical deafferentation of the mediobasal hypothalamus does not interfere with GnRH pulses. Lesions of the arcuate nuclei in primates, which destroy many of the GnRH neurons, abolish the ultradian pattern of release, and neural activity within this region has been correlated with the pulses (6, 9). Furthermore, superfused hypothalamic fragments *in vitro* release GnRH in a pulsatile fashion (10). However, little is known about the precise nature of this GnRH pulse generator, and the identity and organization of the pacemaker remain to be elucidated. Answering the question of whether the pulse generator resides within GnRH neurons, in cells synapsing with them, or both has been hampered by the paucity of GnRH neurons, their diffuse localization, and the complexity of the neuroanatomy of the hypothalamus (11). In an attempt to determine whether GnRH neurons possess an intrinsic secretory rhythm we used the newly developed GT1-1 cell line, one of three subclones of GT1 cells, which were derived from a GnRH tumor induced in a transgenic mouse by genetically targeted tumorigenesis (12).

GT1 cells show a rapid large increase in GnRH secretion when depolarized with veratridine or high concentrations of K^+ (12). Treatment of the cells with tetrodotoxin, a specific blocker of fast Na^+ channels, prevents the action of veratridine but not the effect of a high concentration of K^+. Furthermore, treatment with tetrodotoxin alone significantly inhibits 60% of basal GnRH release, suggesting that GT1 cells are capable of generating spontaneous propagated action potentials that result in hormone secretion.

In view of these studies suggesting that GT1 cells release GnRH spontaneously, we have characterized the basal release of the peptide over time, using Sykes–Moore chambers in which two coverslips separated by a gasket form the superfusion chamber. In some experiments only one coverslip was coated with cells, thereby constituting a system in which cells could synchronize secretion of the peptide by cell-to-cell contacts. In other experiments both coverslips of the chamber were coated with cells, which were physically separated by the width of the gasket.

Some of these results have been reported in preliminary form.‡

MATERIALS AND METHODS

Cells. GnRH cell lines GT1-1, GT1-3, and GT1-7 were developed in transgenic mice by genetically directed expression of the oncogene simian virus 40 tumor (T) antigen linked to the promoter/enhancer domains of the GnRH gene (12). The cell lines have a neuronal phenotype, express neuronal but not glial markers, and express the GnRH gene at high levels.

Culture and Superfusion. Mouse GT1-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin on 25-mm plastic coverslips (Thermanox, Miles) coated with Matrigel (Collaborative Research) until they reached 50–70% conflu-

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Abbreviations: GnRH, gonadotropin-releasing hormone (gonadoliberin); ANOVA, analysis of variance.

‡Martínez de la Escalera, G., Choi, A. L. H. & Weiner, R. I., 20th Annual Meeting of the Society for Neuroscience, Oct. 28–Nov. 2, 1990, St. Louis, Vol. 16, abstr. 126.2.

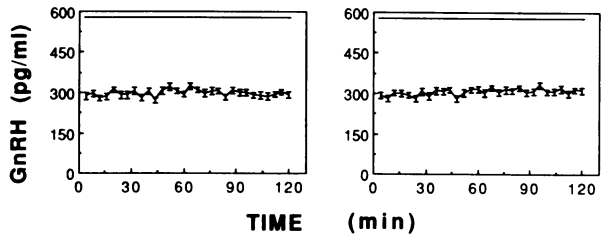

FIG. 1. Determination of GnRH by radioimmunoassay from samples obtained by continuous superfusion with exogenous GnRH of two Sykes–Moore chambers containing no cells and samples collected every 4 min. Each data point was assayed in duplicate and the SD is shown. The graphic representation of the analysis of the data with the pulse detection algorithm cluster analysis is shown at the top of each panel. No significant pulses were observed.

ency. Medium was then replaced by defined medium (Opti-MEM, GIBCO) without serum for 2 days. Cells were superfused in Sykes–Moore chambers (Bellco Glass) consisting of two coverslips separated by a rubber gasket. The coverslips were compressed against the gasket to form a chamber with a volume of 200 μl, which was superfused with medium (13). Two superfusion configurations were used. In some experiments only one cell-coated coverslip was used, while in others both coverslips contained cells. Media used for superfusion were as follows: (*i*) Locke's medium (in mM), NaCl, 154/KCl, 5.6/$CaCl_2$, 2.2/$MgCl_2$, 1/$NaHCO_3$, 6/glucose, 10/Hepes, 2; (*ii*) the same without $CaCl_2$; or (*iii*) the defined medium Opti-MEM. All media were supplemented with 20 μM bacitracin (Sigma) and superfused at a flow rate of 0.08–0.15 ml/min. After a 60-min equilibration period (except for the constant Ca^{2+}-free experiments), samples were collected every 4 min for 120 min. GnRH in the superfusate was determined by radioimmunoassay (14) using the rabbit polyclonal antibody R1245, kindly supplied by T. Nett (Colorado State University), which is specific for the decapeptide (14). The limit of detection was 5 pg/ml and the intraassay coefficient of variation was 4.3%. All samples from an experiment were analyzed in the same assay.

Data Analysis. GnRH pulses were identified and their parameters were determined by the computer algorithm cluster analysis developed by Veldhuis and Johnson (15). Individual point standard deviations were calculated by 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%. In six control experiments in which synthetic GnRH (Sigma) was continuously infused through the Sykes–Moore chambers no pulses were detected (Fig. 1). These findings eliminated the possibility of pulses being aberrantly generated by the experimental conditions. The statistical significance of the pulse parameters was tested by using a one-way analysis of variance (ANOVA).

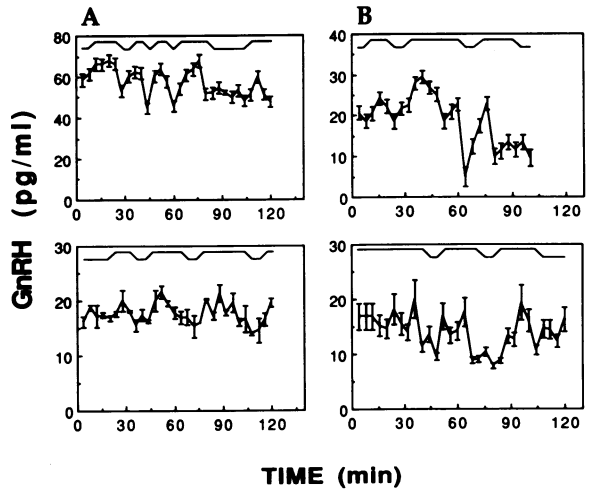

FIG. 2. Representative spontaneous pulses of GnRH release from superfused GT1-1 cells. A single coverslip containing a 50–70% confluent culture of cells was superfused with Locke's medium (*A*) or defined medium (Opti-MEM) (*B*), with samples collected every 4 min after a 1-hr equilibration period. The deflections in the graphic representation of pulses mark significant pulses. Other conditions as in Fig. 1.

RESULTS

Spontaneous Release of GnRH by GT1-1 Cells. Release of GnRH from many of the superfusion chambers was clearly pulsatile when data were analyzed by cluster analysis. In the experiments shown in Fig. 2 only one coverslip contained cells. Fig. 2*A* shows two representative experiments of GT1-1 cells superfused with Locke's medium. The upper line in each is a graphical representation of the results of the cluster analysis. In these particular examples, four significant upstrokes and three or four significant downstrokes were identified, characterizing four and three pulses, respectively. Fig. 2*B* shows two experiments also using a single cell-coated coverslip but superfused with Opti-MEM. To date we have performed 12 independent superfusions under these conditions (Table 1) in which the basal secretion level was well above the threshold of the assay, and 11 of these showed significant GnRH pulses.

Fig. 3 shows four representative experiments of chambers containing two cell-coated coverslips. When this configuration was used, significant pulses of GnRH secretion were

Table 1. Parameters of secreted GnRH pulses identified by cluster analysis with different configurations and conditions of superfusion

Group	Number of exps/peaks/valleys*	Mean interval between peaks, min	Mean peak width, min	Peak height, % increase	Mean valley width, min
One coverslip, Locke's medium	6/18/20	26.3 ± 3.19	19.10 ± 1.82	132.4 ± 12.8	7.20 ± 0.3
One coverslip, Opti-MEM	5/11/11	28.0 ± 1.13	18.50 ± 1.02	142.2 ± 11.4	8.00 ± 0.1
Two coverslips, Locke's medium	6/18/16	24.0 ± 0.86	16.44 ± 1.09	168.1 ± 9.3	7.25 ± 0.4
Two coverslips, Locke's medium after 4 hr of Ca^{2+}-free Locke's medium	6/21/19	25.2 ± 0.96	18.30 ± 1.42	162.3 ± 12.7	7.30 ± 0.2

Values are mean ± SEM. No significant differences among groups were identified by ANOVA.
*Number of independent experiments with values above the threshold of the assay is given, followed by the numbers of peaks and valleys. A peak is a section of the curve flanked by a significant increase and a significant decrease. A valley is a section of the curve flanked by a significant decrease and a significant increase and containing no significant internal variations.

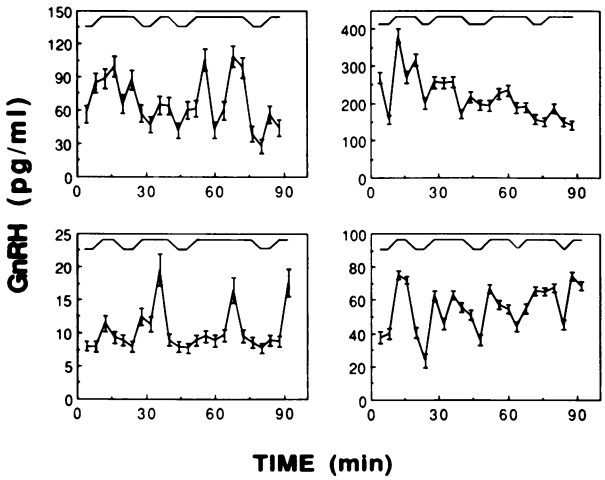

FIG. 3. Representative spontaneous pulses of GnRH release from superfused GT1-1 cells. Chambers with two cell-coated coverslips were superfused with Locke's medium and samples were collected every 4 min after a 1-hr equilibration period. Other conditions as in Fig. 1.

again identified by cluster analysis. Table 1 summarizes the parameters of the significant peaks and valleys identified by cluster analysis in six independent experiments. Comparison of pulse parameters using one or two cell-coated coverslips and Locke's or defined medium revealed no significant differences between groups as analyzed by ANOVA. Overall the results showed 47 spontaneous GnRH pulses secreted by GT1-1 cells in 17 of 18 independent superfused chambers with a mean interpeak interval of 25.8 ± 1.58 min, a mean pulse width of 18.83 ± 1.46 min, a mean peak height of 150.5 ± 6.01% above the preceding nadir, and a mean valley length of 7.25 ± 0.27 min between pulses.

Ca^{2+} Dependency of GnRH Pulses. No significant GnRH pulses were identified by cluster analysis when GT1-1 cells, cultured in Ca^{2+}-free Locke's medium for 4 hr, were superfused with Locke's medium without Ca^{2+} for an additional 2 hr. Fig. 4*A* shows two representative experiments in which chambers containing two cell-coated coverslips were superfused with Ca^{2+}-free Locke's medium without a preceding equilibration period.

The acute withdrawl of Ca^{2+} for 72 min from the superfusion medium (Fig. 4*B*) did not abolish the identification of GnRH pulses by cluster analysis. However, the pulse amplitude tended to decrease with time.

Return of Ca^{2+} to the superfusion medium, after a period of 4 hr in Ca^{2+}-free Locke's medium, resulted in significant spontaneous pulses of GnRH (Fig. 4*C*). As shown in Table 1, no significant differences in the parameters of the pulses were observed between this group and the respective control (two coverslips coated with cells superfused with complete Locke's medium).

DISCUSSION

In this paper we show that the GT1-1 cell line (12) secretes GnRH in discrete pulses that show a rhythmic pattern. Since GT1-1 cells are a clonal cell line, this result shows that the ability to generate pulses is an inherent property of the cells. In addition to implying the existence of a pacemaker or single-cell oscillator (16), the observations of discrete pulses imply the existence of a mechanism for synchronization of the release of GnRH. Coverslips used in these experiments contain sufficient cells to cover 50% of their surface. Numerous cell-to-cell contacts as well as synapse-like connections were observed at the ultrastructural level (12), similar to those found between GnRH-containing neurons *in vivo* in the rat (17) and monkey (18). Therefore, a model of cell-to-cell communication via intercellular contacts could explain synchronization of pulses in the experiments performed with a single cell-coated coverslip per superfusion chamber. Synchronization could be mediated by synaptic mechanisms or possibly electrotonic coupling via gap junctions.

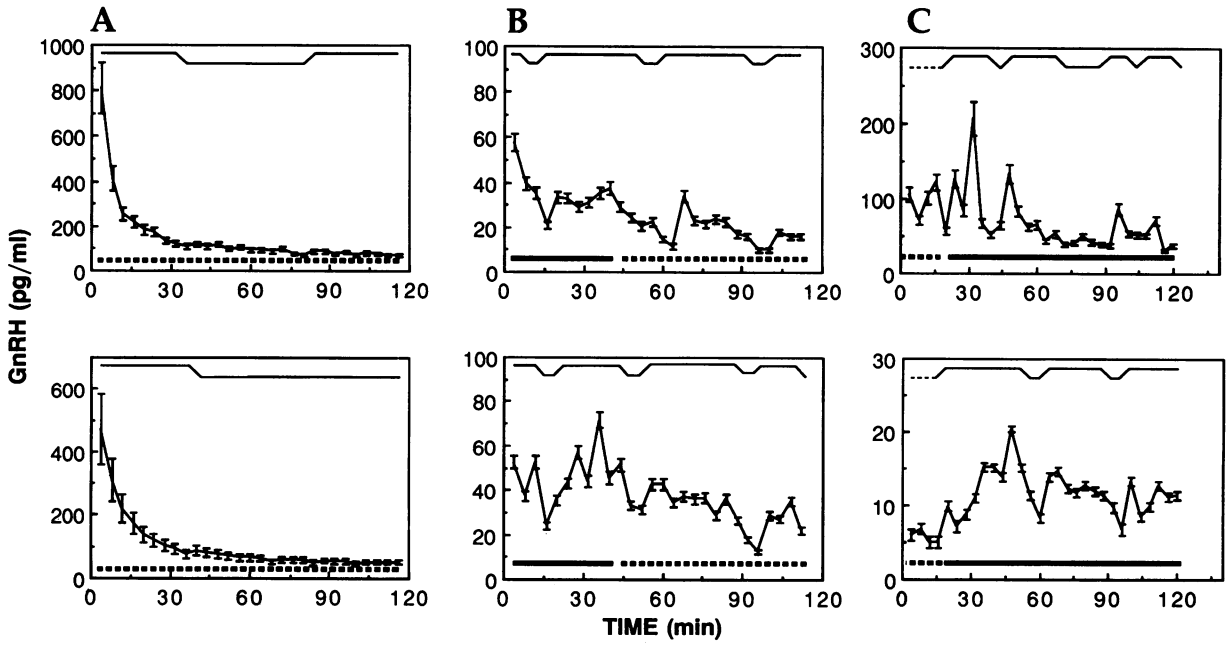

FIG. 4. Representative spontaneous GnRH release profiles from superfused GT1-1 cells. Cell were challenged with the chronic (*A*) or acute (*B*) withdrawal of Ca^{2+} or the acute return of Ca^{2+} after a chronic incubation in Ca^{2+}-free medium (*C*). Chambers with two cell-coated coverslips were superfused as follows: Ca^{2+}-free Locke's medium for 2 hr, after a 4-hr culture in the same medium [without superfusion equilibration period (*A*)]; complete Locke's medium for 40 min, followed by Ca^{2+}-free Locke's medium for the last 72 min (*B*); or complete Locke's medium for 100 min after a 240-min incubation in Ca^{2+}-free Locke's medium (*C*). Solid bars, complete Locke's medium (2.2 mM $CaCl_2$); broken bars, Ca^{2+}-free Locke's medium. Other conditions as in Fig. 1.

However, such contacts do not adequately explain how cells on opposing coverslips become synchronized. Our finding thus suggests that a diffusible mediator may be involved. One candidate for the mediator is the decapeptide GnRH itself. Previous work suggested that GnRH was involved in an ultrashort-loop feedback (19). Other likely candidates as a mediator are the peptides processed from the pro-GnRH molecule, including the GnRH-associated peptide (GAP). However, the possibility of a factor not related to GnRH cannot be excluded. A small percentage of rat GnRH neurons have been reported to contain galanin (20) and δ sleep-inducing peptide (21). Whether these or other putative neuromediators are expressed in GT1 cells is unknown. Indeed, whether these paracrine-like interactions observed with GT1 cells actually occur *in vivo* is unclear. Paracrine effects in our experiment could be an artifact of the proximity of large numbers of GnRH cells in the chambers. A putative mediator of synchronization could reach concentrations normally seen only in a synaptic cleft. However these observations suggest that a diffusible substance released from the GT1-1 cells is involved in synchronization of the cells.

Evidence that both the pulse generator and the mechanism for synchronization reside within the medial basal hypothalamus comes from the observations that deafferentation of the medial basal hypothalamus does not inhibit pulsatile release of luteinizing hormone (7, 8) and that release of GnRH from the hypothalamus *in vitro* is pulsatile (10). However, these experiments did not exclude the role of local nonGnRH neuronal circuits in generation of the pulses. Our results suggest that the GnRH pulse generator is intrinsic to GnRH neurons. Whereas the neurotransmitter systems—e.g., γ-aminobutyric acid, acetylcholine, or glutamate—that have been implicated in the control of gonadotropin surges may do so by modulating the frequency and amplitude of an inherent pulsatile rhythm.

The pattern of GnRH pulses observed varies among species, physiological state, and method of determination (2, 22). The duration of the pulses observed with superfused GT1-1 cells is considerably longer and the relative amplitude smaller than those characteristics observed for GnRH pulses in the portal blood of the ovariectomized sheep (22). No data are available on the pattern of GnRH pulses in the mouse. However, the pattern of GnRH pulses from superfused GT1-1 cells resembles very closely that measured by push-pull perfusion of ovariectomized rats (2). It should be remembered that the shape of the pulses in the present experiments is dependent on the superfusion rate of the chambers, a parameter set by the practical considerations of assay sensitivity and sample frequency. At the flow rate at which sampling was done, it takes 80–160 sec to completely exchange the medium in the chambers. Therefore, even if GnRH was released over a short period of time, the pulses would appear more prolonged. The mean pulse frequency observed with the GT1-1 cells, one pulse every 25.8 ± 1.5 min, is in close agreement with the interpulse interval observed in castrated rats (23) and mice (R. Steiner, personal communication).

We previously showed that GnRH secretion is tightly coupled to depolarization via tetrodotoxin-sensitive Na^+ channels and that treatment with tetrodotoxin inhibited basal secretion of GnRH (12). These findings were consistent with GT1 cells having spontaneous propagated action potentials that involved fast Na^+ channels. Clearly the pulsatile release of GnRH from GT1-1 cells in culture is Ca^{2+} dependent. Removal of extracellular Ca^{2+} for a prolonged period (4 hr) resulted in the loss of spontaneous GnRH pulses from the cells. Taken together, these observations suggest that activation of voltage-dependent high-threshold Ca^{2+} conductances is triggered by membrane depolarization, resulting in an increased rate of Ca^{2+} influx and quantal release of GnRH. However, the observation of progressively smaller-amplitude GnRH pulses after the short-term (0–72 min) removal of Ca^{2+} suggests that intracellular Ca^{2+} stores (or residual extracellular Ca^{2+}) can transiently maintain episodic GnRH release. This is in agreement with the observation that K^+-induced GnRH release was initially delayed and finally blocked by the removal of extracellular Ca^{2+} in superfused rat hypothalami (24). Further studies will be necessary to determine the role of intracellular and extracellular Ca^{2+} in the pulsatile release of GnRH. The cellular mechanism or mechanisms responsible for the timing and generation of pulses remain as intriguing and important questions that should be addressable in GT1 cells.

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