A Potential Code of Luteinizing Hormone-releasing Hormone-induced Calcium Ion Responses in the Regulation of Luteinizing Hormone Secretion among Individual Gonadotropes*

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Luteinizing hormone-releasing hormone (LHRH) induces two Ca²⁺ responses in single gonadotropes: a Ca²⁺ spike/plateau or oscillation. Similar receptor-mediated Ca²⁺ signals have been reported in many cell types but their functional significance is obscure. Accordingly, we have determined the concentration-response properties of LHRH-induced luteinizing hormone (LH) release at the single cell level. We demonstrate a critical single cell LHRH threshold for LH release. Each gonadotrope had a particular LHRH threshold value and a range of different single cell thresholds was distributed in the gonadotrope population. The physiological significance of the threshold was demonstrated by a striking reduction (ΔED⁰₀ = 153 nM) of the LHRH threshold immediately before the preovulatory surge of LH release. The metestrous phenotype of secretion resembled a quantal process in contrast with the graded process of the proestrous phenotype. That is, the quantity of hormone secreted per metestrous gonadotrope was independent of LHRH concentration and more all-or-none than graded. The LHRH threshold and the quantal secretion process of metestrous gonadotropes was further studied by measuring cytosolic Ca²⁺ using fura-2 and digital imaging microscopy. We provide evidence suggesting that the Ca²⁺ spike/plateau and oscillation are the respective responses to subthreshold and suprathreshold concentrations of LHRH. It is proposed therefore that the Ca²⁺ oscillation and spike/plateau response form a binary intracellular signaling code that functions as an on-off switch. It is proposed that this potential code unraveled here for the regulation of hormone secretion may also regulate other gonadotrope functions. Thus, while the Ca²⁺ spike/plateau response is strongly associated with LH release, it may be associated with reduced levels of LH-β mRNA, and reduced numbers of LHRH receptors. Conversely, while the Ca²⁺ oscillation appears to be unrelated to LH release, it may be associated with increased levels of LH-β mRNA, and increased numbers of LHRH receptors. This model may explain in molecular terms the long-standing observation that an invariant, albeit pulsatile, pattern of LH release is sufficient to support the preovulatory surge of LH release.

The mechanisms of receptor-mediated Ca²⁺ responses associated with changes in phosphoinositide metabolism are being unraveled at the level of single cells. Particular attention has focused on the phenomenon of receptor-mediated Ca²⁺ oscillations that have been reported in a large number of different cell types (1-6). The mechanism of Ca²⁺ oscillations is much debated (7-11). While it is implicit that Ca²⁺ oscillations are of fundamental importance, relatively few studies have directly addressed their function. In some cell types (1, 4, 6) the frequency of the cytosolic oscillator is directly related to the agonist concentration and this observation has lead to the proposal that the Ca²⁺ signal is frequency-encoded. However, the potential role(s) of frequency encoding appear limited: the frequency of the Ca²⁺ oscillator has been reported to be sensitive to a limited range of agonist concentrations of no more than one to two orders of magnitude, where secretory responses are generally responsive over three to five orders of magnitude. Here, a potentially general Ca²⁺ hypothesis for the regulation of multiple cellular functions is proposed to account for responses to a broad range of agonist concentrations.

LHRH has been demonstrated to regulate LH release, LH-β mRNA levels, and LHRH receptor number in cell population studies. What is remarkable about the gonadotrope is that the in vitro effects of LHRH are strikingly dependent upon the LHRH concentration. Thus, relatively low concentrations of LHRH promote increased levels of LH-β mRNA (12), and increased numbers of LHRH receptors (13), with very little effect on LH release. In contrast, high concentrations of LHRH oppose increased LH-β mRNA levels (12), reduce the number of LHRH receptors (13), and markedly stimulate LH release. How LHRR brings about these concentration-dependent changes is poorly understood. We have formulated a hypothesis that accounts for the concentration-dependent duality of LHRH function.

MATERIALS AND METHODS
Measurement of Hormone Secretion from Single Gonadotropes—The RHPA was performed essentially as described previously (14, 15). Briefly, the RHPA quantitates hormone secretion as follows: a monolayer of pituitary cells was surrounded by hundreds of specialized erythrocytes manipulated to specifically bind hormone via antibodies coated on their cell surface. Immune complexes were formed specifically on the erythrocytes surrounding a pituitary cell that secreted a particular hormone. Immune complex formation was then

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¶ The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; RHPA, reverse hemolytic plaque assay; EGTa, [ethylenebis(oxyethylene)nitro]tetracetate acid; GTPγS, guanosine 5'-O-(thiotriphosphate); Ins(1,4,5)P₃, inositol 1,4,5-triphosphate.
visualized when complement was added to trigger hemolysis of erythrocytes. A radial zone of hemolysis (a plaque) was formed around the hormone-secreting cell. Pituitary cells were obtained from female Sprague-Dawley rats (150-250 g, Charles River Co.). Estrous cycle status was determined by daily cytological examination of a vaginal smear. A rat was selected for this study only if at least four consecutive 4-day cycles were recorded. A single rat was killed at 0600 h on the morning of metestrus or at 1300 h of proestrus (a few hours before the in vivo LH surge). The anterior pituitary gland was dissociated in 0.1% trypsin to a single cell preparation. Dissociated pituitary cells were mixed with an excess of protein A-conjugated sheep erythrocytes. A monolayer of the cell mixture was established within 45 min on poly-L-lysine coated Cunningham chambers. The cells were treated with LHRH (0.0, 0.01, 0.1, 1.0, 10.0, 1000 nM) and a rabbit anti-bovine LH antiserum (final dilution 1:35, a generous gift from Dr. John G. Pierce, UCLA) and incubated at 37 °C for 2 h. The number of cells secreting LH was counted under bright-field microscopy and first expressed as a percentage of all the pituitary cells in the chamber to compensate for intercellular variability of the number of pituitary cells. The results were subsequently normalized by division against the maximum response that occurred on proestrus (see Fig. 2). A field containing at least 1000 pituitary cells was analyzed. Each determination represents the mean ± S.E. of three to four experiments using a different cell preparation. Plaque area was determined with the assistance of a semiautomated image analysis system (Bioquant MEG IV image analysis system). At least 100-200 individual plaque area determinations were made whenever possible.

A RHPA of high sensitivity was used. Few gonadotropes formed LHRH-stimulated plaques that exceeded 40,000 μm² suggesting that the upper sensitivity limit of the assay was seldom exceeded. The lower limit of sensitivity of the assay was a plaque of 250 μm². The conditions of the assay were set so that the smallest plaque observed exceeded the lower limit of the assay by 5-fold (750 μm²). The assay was sensitive enough to detect LH release in the absence of LHRH. The plaque area was heterogeneous among gonadotropes and the frequency distribution of plaque areas was always monophasic rather than biphasic (data not shown). The RHPA is specific for cells that secrete LH according to the following criteria: (i) only a few small plaques were observed spontaneously; (ii) LHRH, but not a 100 nM dose of thyrotropin-releasing hormone, human growth hormone-releasing hormone, or ovine corticotropic-releasing hormone, promoted LH plaque formation in a concentration-dependent fashion; (iii) incubation of the bovine LH antiserum overnight with 1.0 μg/ml rat LH (NIADDK-1-5), but not a 100 nM dose of thyrotropin-releasing hormone, human growth hormone-releasing hormone, or ovine corticotropin-releasing hormone, completely blocked plaque formation; (iv) LH plaque formation was completely abolished if any of the following were omitted: LH antiserum, complement, or pituitary cells.

Measurement of Cytosolic Ca²⁺ by Digital Imaging Microscopy—Cytosolic Ca²⁺ was measured using fura-2 and digital imaging microscopy as previously described (14, 15). Pituitary cells from a metestrous rat were challenged with LHRH in nominally Ca²⁺-free conditions (no added Ca²⁺ + 2.5 mM EGTA). Ratio measurements were calculated using a single wavelength excitation of 380 nm. The average fluorescence emission before stimulation (F₀) was divided by each serial value after stimulation (Fᵢ) to form F₀/Fᵢ. Experiments were performed at room temperature (22 °C). Ca²⁺ measurements were performed on a group of about 10-25 pituitary cells and the gonadotrope(s) were identified retrospectively in the mixed-cell population with the RHPA (14, 15) and a large LHRI concentration (10.0 nM).

RESULTS AND DISCUSSION

Single Cell Thresholds for Hormone Secretion—Our hypothesis for the function of Ca²⁺ signals is based on studies of LHRH-induced LH release at the single cell level. We report evidence for the existence of a single cell LHRH threshold for LH release among individual gonadotropes. For a given gonadotrope, a critical LHRH threshold for LH release was observed, and our evidence for this phenomenon is described in this section. These findings provide the basis for the proposal to be developed that the single cell LHRH threshold may critically regulate LH release, LH-β gene expression, and LHRH receptor number, by generating a distinct Ca²⁺ signal at subthreshold and suprathreshold LHRH concentrations.

LH release was estimated from single pituitary cells using a highly sensitive reverse hemolytic plaque assay (RHPA) (14-16). The RHPA employs sheep erythrocytes to measure hormone secretion via an antibody-directed complement-mediated mechanism. Hormone secretion was detected by the formation of a radial zone of hemolyzed erythrocytes (a hemolytic plaque) around cells that release LH. To measure changes among single gonadotropes during the estrous cycle, pituitary cells were obtained from metestrous or proestrous rats and separate batches were then challenged with different LHRH concentrations. One advantage of the RHPA method of measuring hormone secretion is that many thousands of cells are concomitantly studied. This permits the population to be sampled at the single cell level unlike most other techniques that limit measurements to one cell/experiment.

In the analysis of the RHPA findings, two intrinsic parameters of hormone secretion were determined: the number of cells that release hormone (Fig. 1, top panels) and the mean amount of hormone released/cell (Fig. 1, bottom panels).

A small group of gonadotropes were found to release hormone under resting conditions. Graded increases of LHRH increased the percentage of gonadotropes that released hormone until an upper limit was reached (Fig. 1). The number of gonadotropes at high LHRH concentrations was similar for metestrous and proestrous gonadotropes. Our findings demonstrate a range of threshold LHRH concentrations for hormone secretion among gonadotropes of the population.
The heterogeneous distribution of single cell LHRH thresholds among gonadotropes provides a mechanism for the direct regulation of the number of gonadotropes that release hormone. That is, low LHRH concentrations stimulated secretion in relatively few gonadotropes while increasing the LHRH concentration progressively increased the number of gonadotropes that released hormone. Comparison of the metestrous and proestrous phenotypes demonstrates that a substantial reduction (ΔE<sub>0</sub> = 153 nM) of the LHRH threshold preceded the proestrous surge of secretion (Fig. 1, top panels). These findings show that the LHRH threshold is regulated during the estrous cycle and suggests a critical role in the regulation of gonadotrope function.

Our findings fail to confirm a previous report of a 2-fold increase in the number of hormone-releasing gonadotropes observed at proestrus using maximal LHRH concentrations (17). While the reason for this discrepancy is not clear, the physiological significance of measurements using LHRH concentrations in excess of 100 nM (17) is most probably irrelevant when the amount of LHRH measured in hypophysical portal blood is in the picomolar range (18). While we also employed high LHRH concentrations to define the broad properties of LHRH-induced responses, the physiological range will almost certainly correspond to the lower LHRH concentrations that were used (Fig. 1).

Comparison of the quantity of hormone released/gonadotrope among proestrus and metestrus gonadotropes revealed further differences. Among metestrus gonadotropes, the mean amount of hormone released/gonadotrope was not significantly altered by changing LHRH concentration (Fig. 1). This all-or-none response of individual gonadotropes at metestrus suggested a quantal process of secretion. This quantal process contrasted with the more graded process of secretion of the proestrus phenotype where the quantity of hormone released/gonadotrope was clearly a function of LHRH concentration. The maximum capacity of proestrous gonadotropes to release hormone was 10-fold greater than metestrus gonadotropes (Fig. 1, bottom panels). The dissociation between the number of cells releasing hormone and changes in the amount of hormone secretion strongly suggests distinct mechanisms of regulation. Whether this involves distinct second messenger mechanisms will be the subject of future investigation.

Single Cell Thresholds and Calcium Responses—The single cell LHRH threshold for secretion was investigated further by determining how an intracellular messenger such as Ca<sup>2+</sup> responds as a function of LHRH concentration. The metestrus gonadotrope was used throughout the remaining studies to investigate the intracellular signals associated with this quantal process of secretion. The LHRH receptor is coupled to the inositol phospholipid signaling pathway that elicits Ins(1,4,5)P<sub>3</sub> (19-21) and sn-1,2-diacylglycerol (22, 23). Ins(1,4,5)P<sub>3</sub> induces intracellular Ca<sup>2+</sup> release from internal stores in the gonadotrope (24) while diacylglycerol activates a family of protein kinase C isoenzymes (25).

The Ca<sup>2+</sup>-sensitive fluorescent indicator fura-2 (26) and digital imaging microscopy were employed to measure serial cytosolic Ca<sup>2+</sup> levels among single living cells. Gonadotropes were stimulated with LHRH in nominally Ca<sup>2+</sup>-free medium (medium was exchanged 30 s before the stimulus to allow equilibration) to measure cytosolic Ca<sup>2+</sup> changes following release from the intracellular Ca<sup>2+</sup> store. In our first study (Fig. 2), LHRH-induced Ca<sup>2+</sup> responses were measured for 3 s, a time course sufficient to determine the presence or absence of intracellular Ca<sup>2+</sup> release from the internal store(s). While this time course was insufficient to resolve the decay phase of the Ca<sup>2+</sup> transient it was entirely clear that the gonadotropes mobilized intracellular Ca<sup>2+</sup> release after stimulation with 1.0 μM and 0.01 nM LHRH in a similar fashion. LHRH-induced intracellular Ca<sup>2+</sup> release was unchanged even at 1.0 fm LHRH although in a few gonadotropes the rate and amplitude of the rise was variable. The interval between the presentation of stimulus and the onset of the Ca<sup>2+</sup> response was a direct function of LHRH concentration. This latency period may be a function of the time required to accumulate a sufficient threshold amount of Ins(1,4,5)P<sub>3</sub> to trigger intracellular Ca<sup>2+</sup> release (7, 10), a view supported by our finding that stimulus-induced Ca<sup>2+</sup> release from the intracellular store appeared to be an “all-or-none” phenomenon. Our findings also demonstrated that LHRH-induced intracellular Ca<sup>2+</sup> release was not a function of LHRH concentration either in terms of the rate of rise or the maximal amplitude of the rise (Fig. 2). It is of considerable interest that our findings show that each individual gonadotrope was able to sense LHRH concentrations with a Ca<sup>2+</sup> response (Fig. 2) that in separate experiments failed to initiate hormone secretion (Fig. 1).

The amplitude and rate of rise of the initial Ca<sup>2+</sup> response are independent of LHRH concentration and therefore cannot explain the single cell LHRH threshold for hormone secretion. When the time course of Ca<sup>2+</sup> measurements was extended to 60 s two distinct modes of LHRH-induced Ca<sup>2+</sup> response were observed among gonadotropes, either the Ca<sup>2+</sup> oscillation, or the spike/plateau (Fig. 3). The period of Ca<sup>2+</sup> oscillations was always about 3-5 s, and there was no apparent change of
frequency of the oscillation with increasing LHRH concentration. Of considerable interest, the percentage of gonadotropes responding with the Ca\(^{2+}\) spike/plateau increased with increasing LHRH concentration (Fig. 4, top right), whereas the Ca\(^{2+}\) oscillation decreased (bottom right), according to LHRH concentration. The LHRH-induced Ca\(^{2+}\) spike/plateau is strongly correlated with the number of gonadotropes that release hormone (reproduced from Fig. 1, top left). In contrast, the LHRH-induced Ca\(^{2+}\) oscillation is strongly correlated with the number of gonadotropes that did not release hormone (numbers obtained by subtraction from the total, Fig. 1, bottom left). Experiments were performed as described in Fig. 2.

A precise quantitative correlation between the separate calcium and the hormone secretion studies would not be anticipated since, for technical reasons, different time course and temperature conditions were employed. Even with this constraint, the time course of measured hormone secretion (2 h) for those gonadotropes that did not release hormone can be directly compared with the calcium findings (1.0 min) since time resolution is irrelevant for gonadotropes not releasing hormone. Thus, it is very difficult to associate either the Ca\(^{2+}\) oscillation with cells that do release hormone, or the Ca\(^{2+}\) spike/plateau with cells that do not release hormone, based on our analysis of the cell population at the single cell level (Fig. 4). It is therefore proposed that the stimulus-induced Ca\(^{2+}\) spike/plateau or oscillation operates as an intracellular signaling code to switch hormone secretion either on or off. Our findings may be relevant for other cell types where it is at least empirically recognized that low concentrations of stimulus favor the appearance of the Ca\(^{2+}\) oscillation and high concentrations favor the spike/plateau (3, 4, 11).

The concept that hormone secretion (and other functions) may be actively switched on and off by LHRH has important implications. The release state of a single cell is apparently influenced by the LHRH-induced Ca\(^{2+}\) spike/plateau (switch on) or Ca\(^{2+}\) oscillation (switch off). Accordingly, a digital mode of Ca\(^{2+}\)-hormone secretion coupling operates at the single cell level since the quantity of hormone released/cell appeared to be independent of LHRH concentration in the metestrus phenotype (Fig. 1, bottom left). In contrast, the whole gland or entire cell population responds to graded changes of LHRH with commensurate graded increases of hormone secretion. Thus, the macroscopic whole gland (or
the population) is graded and shows no thresholds, and therefore, a conversion from digital to analogue responses is required to account for the transition from the single cell (quantal) to the macroscopic (graded) population response. The heterogeneous distribution of single cell LHRH thresholds in the gonadotrope population satisfies the requirement for digital-to-analogue conversion. The heterogeneity of the single cell LHRH threshold among gonadotropes results in a graded population response to different LHRH concentrations based on the quantal responses of individual cells. According to our findings, such a digital-to-analogue conversion scheme operates for both metestrus and proestrus gonadotropes (Fig. 1). In addition, a further analogue mode of release is required to account for the properties of the proestrus phenotype where single proestrus gonadotropes clearly are able to respond with graded hormone secretion when stimulated with graded increments of LHRH in the suprathreshold range (analogous to the behavior of single presynaptic cell of squid giant axon) (27). These findings suggest stimulus-secretion coupling involves at least two different modes of release among single cells that can be operationally distinguished as digital and analogue mechanisms of release. These separate mechanisms of digital versus analogue release may be critical to the interpretation of receptor-mediated hormone secretion at the single cell level.

Neher and colleagues (28–30) have matched simultaneous measurements of Ca\(^{2+}\) and hormone secretion in nonexcitable and excitable cells and shown that intracellular GTP\(_{\gamma}\)S or Ins(1,4,5)P\(_3\), stimulated either in the Ca\(^{2+}\) oscillation or spike/plateau responses. The Ca\(^{2+}\) oscillation invariably failed to initiate hormone secretion although many cells released hormone in association with the Ca\(^{2+}\) spike/plateau response. Hormone secretion coincided precisely with the plateau phase and was never associated with the preceding spike phase. The authors concluded in a provocative vein that “calcium is neither necessary nor sufficient for hormone secretion” based on the failure of Ca\(^{2+}\) spikes to elicit a corresponding transient pattern of hormone secretion. In other words, the direct relationship between Ca\(^{2+}\) concentration and “analogue” release classically recognized in single cells of the squid giant axon (27) was not obtained in hormone secreting cells. One alternate possibility, raised by our findings, is that the plateau phase of Ca\(^{2+}\) is involved in the digital mechanism of release, i.e. in switching release on or off. Thus, the receptor-mediated Ca\(^{2+}\) spike/plateau response may be a necessary condition for hormone secretion. Neher and colleagues noted that a few cells exhibited the Ca\(^{2+}\) spike/plateau response that still did not release suggesting that the Ca\(^{2+}\) spike/plateau alone is not a sufficient condition for hormone secretion. A potential role of Ca\(^{2+}\) in the analogue release mode will require further studies to determine if the amplitude of the plateau Ca\(^{2+}\) phase is directly regulated by suprathreshold concentrations of stimulus.

In a previous study from this laboratory (31), it was shown that the amount of hormone secretion was directly correlated with both the amplitude and frequency of Ca\(^{2+}\) oscillations in single pituitary somatotropes (31). The pituitary somatotrope and gonadotrope are contrasting cell types. In the somatotrope, the plasma membrane Ca\(^{2+}\) channels spontaneously provide low amplitude “membrane” oscillations of Ca\(^{2+}\) entry by a mechanism independent of the intracellular Ca\(^{2+}\) stores. The “cytosolic” oscillator of LHRH-stimulated gonadotropes employs Ca\(^{2+}\) from the internal stores and generates high amplitude Ca\(^{2+}\) oscillations (Fig. 3). It is apparent that intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) entry have entirely different functional consequences.

In most cell types, including the gonadotrope, the spike phase of a Ca\(^{2+}\) transient is due to intracellular release while the plateau phase is primarily accounted for by Ca\(^{2+}\) entry (32–35, Fig. 3). The calcium switch may therefore be unrelated to the profiles of the Ca\(^{2+}\) oscillation and spike/plateau per se but rather reflects distinct functional consequences of Ca\(^{2+}\) entry versus mobilization from internal stores. In direct support of this view, it has recently been reported that catecholamine release is associated with Ca\(^{2+}\) entry rather than mobilization from internal stores in single adrenal chromaffin cells (36, 37). A potentially important consideration is that the plateau phase of the biphasic spike/plateau response also includes an unexpectedly large component of residual Ca\(^{2+}\) mobilization for periods up to 1–2 min after stimulation with LHRH in single gonadotropes. Further studies are required but accumulating evidence suggests that the regulation of Ca\(^{2+}\) entry is intimately related to an empty state of the intracellular Ca\(^{2+}\) store (38, 39). The sequence of events leading to Ca\(^{2+}\) entry may be one of the critical events required for hormone secretion. Indeed, Smith and Augustine (40) have previously discussed the importance of the high local concentration of Ca\(^{2+}\) near plasma membrane Ca\(^{2+}\) channels located in neuronal active zones of release. Secretory granules localized near Ca\(^{2+}\) channels in the plasma membrane are likely to be exposed to a very high local Ca\(^{2+}\) concentration (100 μM) following influx. The conditions required to initiate hormone secretion may involve very high Ca\(^{2+}\) increases near the fusion site of secretory granule and plasma membrane.

Data from this and other laboratories (28–30, 36, 37), argue strongly against a role of “cytosolic” Ca\(^{2+}\) oscillations in hormone secretion. What, then, is the function of the cytosolic Ca\(^{2+}\) oscillator? Andrews et al. (12) reported that the concentration-response curve was bell shaped for pituitary LH-β mRNA content, such that low LHRH concentrations (10\(^{-11}\)–10\(^{-10}\) M) progressively stimulated, and paradoxically, high LHRH concentrations (10\(^{-8}\)–10\(^{-7}\) M) progressively returned, LH-β mRNA to resting levels. In agreement, other laboratories have shown that high LHRH concentrations do not alter LH-β mRNA levels (41–43). The duality of LHRH function in regulation of LH-β mRNA levels demonstrated by Andrews et al. (12) was in the context of our findings strikingly concentration-dependent. Our findings suggest that comparably low LHRH concentrations are associated with the Ca\(^{2+}\) oscillation response. And, conversely, high LHRH concentrations favor the spike/plateau Ca\(^{2+}\) response among gonadotropes. It is therefore reasonable to propose that the Ca\(^{2+}\) oscillation effectively switches on, and the Ca\(^{2+}\) spike/plateau effectively switches off, LH-β mRNA levels.

It is of further interest that the number, rather than the affinity, of LHRH receptors is regulated during the estrous cycle (44–46). Treatment with high LHRH concentrations rapidly leads to the phenomenon of homologous down-regulation of pituitary LHRH receptor numbers (47, 48). However, Loumaye and Catt (13) demonstrated that subnanomolar LHRH concentrations increased, while suprananomolar concentrations decreased, the number of LHRH receptors. This duality of LHRH function for the regulation of LHRH receptor number was thus once again strikingly concentration-dependent. In view of our findings, it is reasonable to propose that the Ca\(^{2+}\) oscillation effectively increases, and the Ca\(^{2+}\) spike/plateau effectively decreases, LHRH receptor number.

A Molecular Model for Intracellular Mechanisms of Gonadotrope Function and Implications for the Regulation of the Estrous Cycle—The proposed LHRH threshold for LH release, LH-β mRNA, and LHRH receptor number provides

\[ \text{D. A. Leong and M. O. Thorner, unpublished results.} \]
some provocative insights into the control of mammalian reproduction. It is apparent that a high LHRH threshold is maintained during most of the estrous cycle relative to the amplitude of physiological LHRH pulses. Hence, most gonadotropes respond to a physiological pulse of LHRH with a brief episode of intracellular Ca\(^{2+}\) oscillations once every 55–90 min (the duration of each LHRH pulse reaching the pituitary is uncertain, but is probably in the range of 1–5 min). The Ca\(^{2+}\) oscillation, in turn, switches off LH release (conserving LH for the proestrous surge) and switches on increased LH-\(\beta\) mRNA. The Ca\(^{2+}\) oscillation also increases the number of LHRH receptors that, in turn, effectively reduce the LHRH threshold successively with each LHRH pulse of the estrous cycle. Exactly how the LHRH threshold of single cells is constituted will require further investigation, but it is reasonable to assume that the LHRH receptor number is an important influence. Thus, as a consequence of successive episodes of Ca\(^{2+}\) oscillation the action of LHRH may indeed by self-promoting as originally proposed by Pickering and Fink (49). It is possible that the LHRH threshold eventually becomes sufficiently reduced to allow even tonic levels between LHRH pulses to evoke the LHRH-induced Ca\(^{2+}\) spike/plateau response that in turn would help elicit the LH surge. Certainly, we show that a substantial reduction in the LHRH threshold occurs by the time of the proestrous LH surge (Fig. 1). This critical transition reverses the pattern of changes such that the LHRH-induced Ca\(^{2+}\) spike/plateau switches on hormone secretion, reduces LH-\(\beta\) mRNA production, and reduces the number of LHRH receptors with the net effect of raising the LHRH threshold. Accordingly, a high LHRH threshold becomes reset among gonadotropes and the pattern of change is reversed. The cycle begins over with a new phase of LHRH-induced Ca\(^{2+}\) oscillations.

The proposed model for the cyclical mechanism of reproduction is attractive for its ability to simply reconcile the coordinate regulation of LH release on the one hand, and the converse changes of LH-\(\beta\) mRNA and LHRH receptor number on the other. It accounts for previous observations of a decrease in pituitary LH-\(\beta\) mRNA levels (50) and LHRH receptor number (45–48) coincident with the increase of LH release during the proestrous surge. The opposing changes for LH release versus LH-\(\beta\) mRNA and LHRH receptor numbers follow from individual functions being cross-coupled with the calcium on-off switch. The model also potentially reconciles the otherwise paradoxical increase in the number of pituitary LHRH receptors (45–48) and LH-\(\beta\) mRNA levels (50) that occurs about 36–48 h prior to the LH surge without a commensurate alteration in the responsivity of the whole gland to LHRH (49). It is apparent that one of the impetuses of the estrous cycle is to reduce the LHRH threshold among gonadotropes to values that match the amplitude of the physiological LHRH signal at the time of the LH surge. Accordingly, significant changes in LHRH receptor number can paradoxically occur without concomitant changes in LH release (49), provided that the value of the LHRH threshold exceeded the amplitude of the endogenous LHRH signal.

The proposed signaling mechanism by which the LHRH threshold operates is based on correlative evidence. Further studies are required to determine whether the binary Ca\(^{2+}\) response per se constitutes the code, or whether other intracellular messengers that at a prior step regulate the Ca\(^{2+}\) oscillation and the spike/plateau responses (e.g. Ins(1,4,5)P\(_3\) and diacylglycerol) constitute the code. A number of models have been proposed as the basis for Ca\(^{2+}\) oscillations (7–11). However, our findings suggest that it is equally important to understand the mechanism for the transition from the Ca\(^{2+}\) oscillation to spike/plateau. Whatever the intracellular signaling mechanism, the underlying logic of the model is that subthreshold and suprathreshold stimulus concentrations generate distinct intracellular messenger signals that oppose each others actions. Finally, the coupling pattern that links the binary signaling code to cell function provides a mechanism for the coordinate control of different cellular functions (e.g. hormone secretion, gene expression, receptor number).

The concept of a single cell threshold among individual cells that integrates multiple functions represents a novel principle for cell regulation that may be of widespread significance. It is premature to speculate on the influence of other regulators such as estradiol and progesterone (hormones that also have concentration-dependent positive and negative effects on LH release) that, at the very least, frame critical periods for LHRF action. Our unifying model for the cyclical mechanism of reproduction accounts for how the LH surge may occur in response to a relatively unchanging, albeit pulsatile, pattern of LH release (51–52). This model reconciles reports that an increased amplitude or frequency of pulsatile LH release is not observed during the periovulatory period in many individual animals (53–56). However, in the few animals, or on those occasions where the amplitude or frequency of pulsatile LHRH release during the LH surge do increase (55, 56), our findings suggest that such increases may not be redundant, but instead function to ensure that the LHRH threshold is reset to a very high value following the preovulatory LH surge. Reminiscent of the action of light to entrain circadian oscillators, a preovulatory surge of LHRH release that exceeds the regular pulsatile mode may serve to promote the fidelity of successive reproductive cycles.


REFERENCES

Function of Receptor-mediated Ca\(^{2+}\) Signals


