# Signaling Mechanisms during the Response of Pituitary Gonadotropes to GnRH

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## I. Introduction

The pituitary gland is a versatile hormonal interface between the nervous system and the rest of the body, a warehouse of peptides waiting for nervous requisitions. In mammals, the anterior pituitary can secrete at least six major peptide hormones from at least five cell types. Each cell is under control of specific releasing hormones and neurotransmitters secreted into the pituitary portal circulation by hypothalamic neurons (Fig. 1).

Much progress has been made in the past 4 decades in defining elements of hypothalamic and pituitary signaling. Releasing hormones and pituitary hormones were isolated and sequenced by classical chemical means. Sensitive radioimmunoassays for each were developed. A periodic release of most hormones was discovered that makes pulses on a time scale of minutes to hours. The cDNAs for preproproteins of the peptide hormones and for most of their membrane receptors have been obtained by modern molecular biology. Much attention now focuses on genetic mechanisms regulating the expression of the underlying genes.

An area that has only recently become accessible to investigation concerns the intracellular signals that couple activation of releasing-hormone receptors to exocytosis of the peptide-containing secretory granules of pituitary cells. Such physiological signals, operating on a time scale from milliseconds to seconds, have been the focus of our work. They are of interest both because they share features currently under active study

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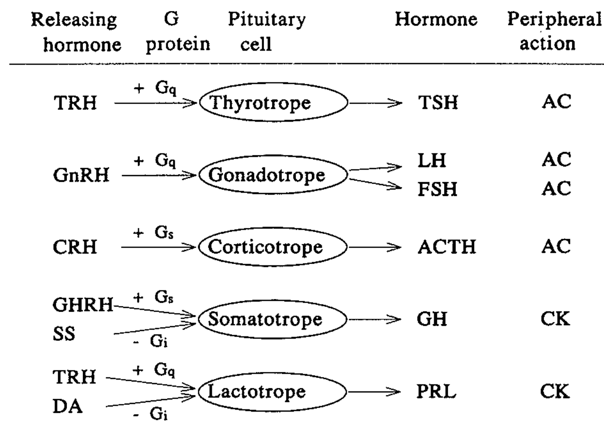


FIG. 1. Hormones and signals of the mammalian anterior pituitary. Each pituitary cell type is shown with its likely controlling inputs from the hypothalamus, the G proteins activated, the output hormone secreted, and the action of the output hormone. Based on work of many authors. TRH, Thyrotropin-releasing hormone; CRH, corticotropin-releasing hormone; GHRH, growth hormone-releasing hormone; SS, somatostatin; DA, dopamine; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; GH, growth hormone; PRL, prolactin; AC, adenylyl cyclase; CK, cytokine.

in many cell types and because they must contain explanations for the dependence of peptide secretion on the history and hormonal milieu of the animal. One might have expected that the different pituitary cells, having all differentiated from common precursor cells of Rathke's pouch, would share a common strategy for this coupling. Surprisingly, it is not so. According to the probably correct hypothesis of Douglas (1968), the final common pathway for hormone secretion is a rise in intracellular calcium ($[Ca^{2+}]_i$). However, as Fig. 1 shows, the releasing-hormone receptors of different pituitary cells couple to different GTP-binding proteins (G proteins), so the early steps of signaling are not the same and need to be investigated in each cell type. This has been greatly facilitated by the development of single-cell assays based on patch-clamp techniques (Hamill *et al.*, 1981).

Our laboratory has been studying G protein-mediated modulation of ion channels. First, we worked with muscarinic activation of a $K^+$ channel in cardiac atrial cells (Pfaffinger *et al.*, 1985), and then we turned to the well-known GnRH and muscarinic inhibition of a $K^+$ channel (M current) in frog sympathetic ganglia (Pfaffinger, 1988; Pfaffinger *et al.*, 1988; Bosma and Hille, 1989), where GnRH is a neurotransmitter. In these frog neurons we found that GnRH reduces the $K^+$ current via a pertussis toxin (PTX)-



insensitive G protein and that it also stimulates phosphoinositide (PI) turnover and initiates a transient rise of $[Ca^{2+}]_i$. This work with GnRH led us finally to the gonadotrope, where we have been able to describe a chain of the events set in motion by GnRH. Substantial recent contributions with these cells have been made by several other laboratories, including those of K. J. Catt, P. M. Conn, D. A. Leong, W. T. Mason, R. P. Millar, and Z. Naor and colleagues.

## II. Methods

We began by studying a mouse cell line, αT3-1, of the gonadotrope lineage (Windle *et al.*, 1990), but our recent studies have been on identified rat gonadotropes in primary culture. The gonadotropes, obtained from pituitaries of 5- to 6-week-old male rats by enzymatic dissociation, are positively identified as cells that secrete luteinizing hormone (LH) in response to GnRH, by use of the reverse hemolytic plaque assay (Tse and Hille, 1994). Both the pituitary cells and the surrounding red cells of the plaque assay are well stuck down to coverslips coated with concanavalin A on gelatin. This makes it possible to do the plaque assay on the day of cell dissociation and to keep the coverslips in culture conditions for up to a week without disturbing the clear plaques that mark the individual gonadotropes to be studied. For comparison to other published work, it should be noted that most other laboratories have studied cells from random cycling or ovariectomized females.

Our measurements are all single-cell assays using the identified cells. Typically we use the whole-cell configuration of the patch clamp (Hamill *et al.*, 1981), which establishes continuity between the cytoplasm and the recording pipette. This has the advantage that metabolites, inhibitors, buffers, and indicator dyes can be dialyzed from the pipette into the cytoplasm within a few minutes, and the complementary disadvantage that some essential intracellular ingredients may be lost into the pipette. With these methods, it is straightforward to characterize the ion channels of the plasma membrane by studying ionic currents under voltage clamp and to look at excitation by studying membrane potential changes. We can also get an instantaneous electrical measure of plasma membrane area changes caused by exocytosis and endocytosis (Neher and Marty, 1982; Fernandez *et al.*, 1984). This allows us to determine the time course of secretory events with subsecond time resolution, which has not been possible with radioimmune assays. There is, of course, the disadvantage that studying area changes alone does not tell us the chemical composition of the secreted substances; thus by these methods we cannot distinguish secretion of follicle-stimulating hormone (FSH) from secretion of LH.

Finally, the pipette may contain fluorescent $Ca^{2+}$ indicators (Grynkiewicz *et al.*, 1985), which permit us to measure changes of $[Ca^{2+}]_i$ optically in one cell while also measuring secretion and electrical events. All of our work is done at a room temperature of 21–25°C, so we might expect that events at body temperature occur three to four times faster than described here.

## III. Initial Steps of the GnRH Response

### A. GnRH-INDUCED MEMBRANE POTENTIAL CHANGES

Like other pituitary cells and excitable cells in general, identified gonadotropes and cells of the αT3-1 cell line express many types of ion channels. They have a variety of voltage-gated $K^+$ channels, at least two voltage-gated $Ca^{2+}$ channels, and a tetrodotoxin-sensitive $Na^+$ channel (Marchetti *et al.*, 1987, 1990; Stutzin *et al.*, 1988; Mason and Sikdar, 1988, 1989; Chen *et al.*, 1989b; Bosma and Hille, 1992; Tse and Hille, 1992, 1993). Both low voltage-activated transient and high voltage-activated, more slowly inactivating $Ca^{2+}$ currents are seen. Half or more of the high voltage-activated current is sensitive to 1,4-dihydropyridine blockers of L-type $Ca^{2+}$ channels, and none is sensitive to ω-conotoxin-GVIA, a N-type $Ca^{2+}$ channel blocker. Unlike most neurons, these cells do not express fast, ligand-gated channels of the kind found in synapses, and their resting potential is relatively low (−35 to −45 mV) so that some of the voltage-gated channels are inactivated.

Application of GnRH to a gonadotrope initiates periodic hyperpolarizations of the membrane potential (Tse and Hille, 1992, 1993; Kukuljan *et al.*, 1992; Stojilkovic *et al.*, 1992a). The potential hyperpolarizes to values of −75 to −95 mV for a few seconds and then returns to rest for several seconds, usually firing a few regenerative action potentials when it depolarizes (Fig. 2, upper trace). These action potentials are carried by an influx of $Na^+$ and $Ca^{2+}$ ions through voltage-gated $Na^+$ and $Ca^{2+}$ channels, whose inactivation was removed during the hyperpolarization. The periodic hyperpolarization is due to rhythmic opening of $Ca^{2+}$-sensitive $K^+$ channels and actually reflects underlying oscillations of $[Ca^{2+}]_i$ that are induced by GnRH (Fig. 2, lower trace). Under voltage clamp conditions one can measure the rhythmic opening and closing of these $K^+$ channels (Fig. 3, top trace) and show that they are sensitive to the bee venom toxin apamin (Tse and Hille, 1992). This classifies them as members of the small conductance (SK) $Ca^{2+}$-activated $K^+$ channel family. The rise and fall of the easily measured $K^+$ current can serve as a convenient monitor of $[Ca^{2+}]_i$ changes in experiments without intracellular $Ca^{2+}$ indicators. It is by far



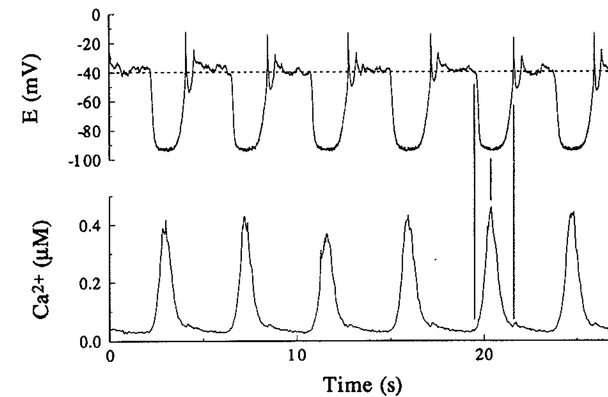


FIG. 2. GnRH-induced oscillations of membrane potential and $[Ca^{2+}]_i$. Recorded in the continued presence of 2 n*M* GnRH using a recording pipette containing 100 μ*M* indo-1. The dashed line indicates the resting potential of this cell before GnRH was applied. Three vertical lines are drawn to emphasize the synchroneity of each membrane hyperpolarization with the elevation of $[Ca^{2+}]_i$. Note also the very small $[Ca^{2+}]_i$ rises caused by the membrane action potentials.

the dominant current in GnRH-exposed cells held at a fixed potential between −140 and −20 mV. This apamin-sensitive current is far less well expressed in αT3-1 cells. It is detected in only a small fraction of the αT3-

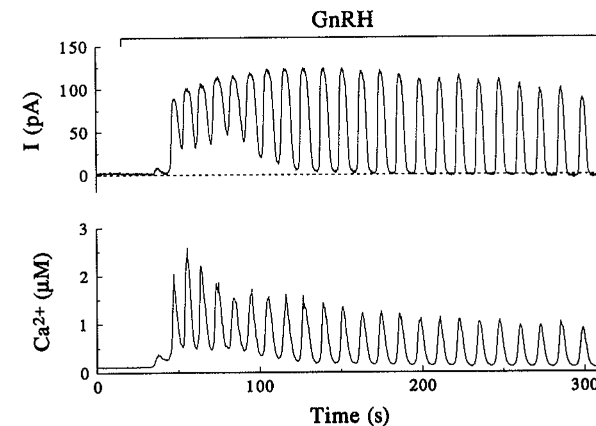


FIG. 3. GnRH-induced oscillations of outward $K^+$ current and $[Ca^{2+}]_i$. The $K^+$ current is measured under voltage clamp conditions at −50 mV, and $[Ca^{2+}]_i$ is measured simultaneously with 50 μ*M* indo-1 in the pipette. GnRH (2 n*M*) is perfused in the bath during the period marked with a bar. The opening of $K^+$ channels is strictly synchronous with $[Ca^{2+}]_i$ elevations.

1 cells and then has a much lower conductance than in gonadotropes (Bosma and Hille, 1992).

## B. CALCIUM OSCILLATIONS AND $IP_3$

The GnRH-induced oscillations of $[Ca^{2+}]_i$ are measured directly by ratio photometry with the $Ca^{2+}$ indicator indo-1 in the recording pipette (bottom traces of Figs. 2 and 3). Calibration of such cellular $Ca^{2+}$ measurements is subject to difficulties and variation between laboratories, but according to our methods (Tse and Hille, 1994), the resting $[Ca^{2+}]_i$ averages 100 n*M* and the peak values reached in each cycle range from 1 to 4 $\mu M$. The oscillations have a cycle period of 3–20 sec that tends to shorten a little as the GnRH concentration is raised from near threshold values (<5 p*M*) to supramaximal values (100 n*M*) but is not a steep function of the agonist concentration (Shangold *et al.*, 1988; Leong and Thorner, 1991; Leong, 1991; Iida *et al.*, 1991; Stojilkovic *et al.*, 1992b, 1993; Tse and Hille, 1992; Hille *et al.*, 1994). These GnRH stimuli are within the physiological range since sampling of pituitary portal blood suggests that GnRH rises at least to hundreds of picomolar for several minutes during each episode of pulsatile release (Moenter *et al.*, 1992). Surprisingly, the waveform of the $[Ca^{2+}]_i$ oscillation is similar whether gonadotropes are held at a fixed potential under voltage clamp (Fig. 3) or are free to hyperpolarize and depolarize (Fig. 2), the more physiological condition. As is seen later, each elevation of $[Ca^{2+}]_i$ is associated with a secretory burst of exocytosis from the cell.

Intracellular oscillations should not be confused with the much slower pulsatility of release of gonadotropins that is well known in the endocrine literature (Gay and Sheth, 1972; Levine and Duffy, 1988; Moenter *et al.*, 1992). Such pulses are controlled by rhythms originating in GnRH-secreting neurons of the hypothalamus. In male rats, a pulse of GnRH normally appears in the portal circulation every 20–300 min, depending on the physiological state. Presumably, this GnRH pulse initiates 10–80 cycles of internal $Ca^{2+}$ oscillation in gonadotropes during its several minute duration, and LH and/or FSH would be secreted from a cell every few seconds each time $[Ca^{2+}]_i$ is high. The internal oscillations would not be synchronized among gonadotropes in the pituitary, and no indication of them should be detectable in a hormone sampling regime that looks at the population output.

Where does this $Ca^{2+}$ come from? If extracellular $Ca^{2+}$ is removed and a $Ca^{2+}$ chelator is perfused in the bath around a stimulated cell, the hormone-induced $Ca^{2+}$ oscillation may continue for 10 min, gradually slowing, and eventually stopping (Fig. 4; Iida *et al.*, 1991; Tse and Hille, 1992). Hence, each rise of cytoplasmic $Ca^{2+}$ must represent release from



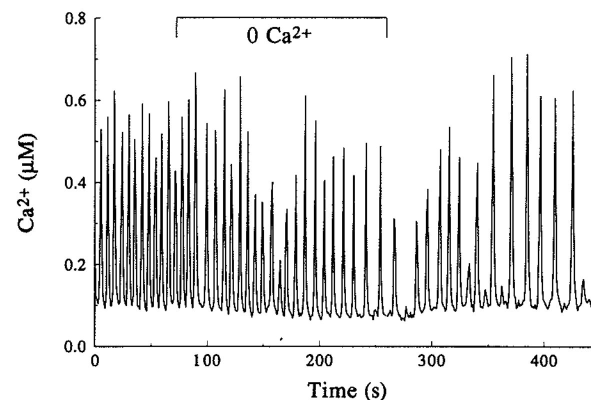


FIG. 4. Persistence of $Ca^{2+}$ oscillations in $Ca^{2+}$-free medium. This gonadotrope was bathed with 5 n*M* GnRH starting several minutes before the record and continuing throughout. At first the saline medium contains 2 m*M* $Ca^{2+}$ and 1 m*M* $Mg^{2+}$, but during the period marked with a bar, the solution was a $Ca^{2+}$-free medium containing 3 m*M* $Mg^{2+}$ and 1 m*M* EGTA. The cell was held continuously under voltage clamp at −50 mV.

intracellular stores. Furthermore, since the cell often can continue to produce >30 cycles of oscillation after being shifted to a $Ca^{2+}$-free medium, we can conclude that only a tiny fraction of the total $Ca^{2+}$ in intracellular pools is lost to the extracellular medium per cycle. Therefore, the intracellular stores may be a reservoir containing vastly more $Ca^{2+}$ than is released in an individual cycle and/or $Ca^{2+}$ extrusion across the plasma membrane is slow relative to $Ca^{2+}$ reaccumulation by internal stores. Our working hypothesis is that the $Ca^{2+}$ release in each cycle may be on the order of 10% of what is in the stores and that most of this released $Ca^{2+}$ is taken up again by the stores instead of being lost by extrusion at the plasma membrane. We have developed optical measures of the filling of intracellular stores that support this hypothesis (F. Tse *et al.*, 1994).

In gonadotropes, $\alpha$T3-1 cells, neurons, and mRNA-injected oocytes, activation of GnRH receptors stimulates PI turnover (Naor and Catt, 1981; Huckle and Conn, 1987; Morgan *et al.*, 1987; Pfaffinger *et al.*, 1988; Naor, 1990; Horn *et al.*, 1991; Tsutsumi *et al.*, 1992). Intracellular inositol trisphosphate ($IP_3$) is generated, and the activity of protein kinase C (PKC) is increased. Our own experiments have explored only the signals needed for the initial $Ca^{2+}$ oscillation and exocytosis, during at most a few minutes of GnRH application. In this time scale, we feel that $IP_3$ is the necessary and sufficient second messenger and that PKC is not as central. PKC activation may, however, be more important for subsequent stages of recovery and in responses to protracted or repeated stimulation. The importance of $IP_3$ is revealed both by mimickry of the GnRH response

when raising cytoplasmic $IP_3$ and by blockage of the GnRH response with intracellular heparin, a competitive antagonist of $IP_3$ action: When the recording pipette contains 10–100 $\mu M$ $IP_3$ or a nonhydrolyzable analogue of $IP_3$, a rhythmic, outward $K^+$ current and oscillations of $[Ca^{2+}]_i$ are induced within seconds after breakthrough to the whole-cell recording configuration (Tse and Hille, 1992; Stojilkovic *et al.*, 1993), and when the pipette contains 10 $\mu M$ caged $IP_3$, illumination with ultraviolet light to uncage the $IP_3$ induces transient rises of $[Ca^{2+}]_i$ (Fig. 5; Tse *et al.*, 1993a).

As in other examples of second-messenger signaling, there is much potential for ripples of additional chemical interactions to spread throughout the cell. There are many known actions of $[Ca^{2+}]_i$ and of PKC on receptors, cyclases, phosphodiesterases, and other kinases in other well-characterized systems. The literature on intracellular signaling and hormone secretion in gonadotropes is complex and contains evidence both for changes of and for actions of cyclic AMP, cyclic GMP, arachidonic acid, and the lipoxygenase pathway, as well as diacylglycerols and lipids derived through activation of phospholipase D (Naor, 1990; Dan-Cohen *et al.*, 1992). Both stimulatory and inhibitory effects of PKC activators are reported, and the literature also contains statements that $IP_3$ is not important (Hawes *et al.*, 1992). All of these interesting and sometimes contradictory findings need clarification. Nevertheless, we feel that the case for a primary role of $IP_3$ is very strong: PI turnover is stimulated by

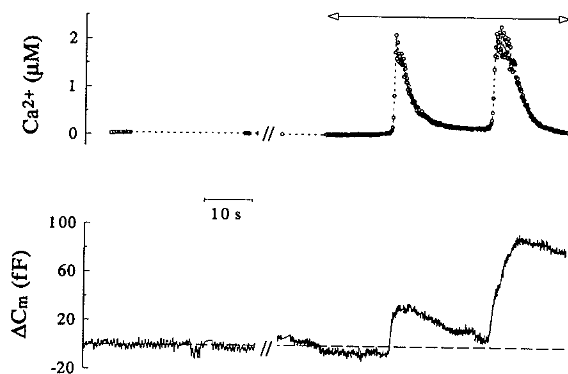


FIG. 5. Cytoplasmic $IP_3$ elevates $[Ca^{2+}]_i$ and induces exocytosis. Upper trace: $[Ca^{2+}]_i$ recorded with 100 $\mu M$ indo-1 in the pipette. Lower trace: Membrane capacitance changes, a measure of changes in the plasma membrane surface area. Increases of area signify net exocytosis, and decreases signify endocytosis. The whole-cell pipette contains 10 $\mu M$ of the inactive, caged $IP_3$. During the period marked with horizontal arrows, continuous ultraviolet light is applied to liberate active $IP_3$ in the cytoplasm. The cell is held under voltage clamp at −80 mV. (From Tse *et al.*, 1993a.)



GnRH, $IP_3$ mimics GnRH, and blockage of $IP_3$ receptors blocks the action of GnRH.

### C. A PERTUSSIS TOXIN-INSENSITIVE HETEROTRIMERIC G PROTEIN

Most or all of the actions of GnRH are mediated via a PTX-insensitive G protein (Naor, 1990). The cloned GnRH receptor has the seven hydrophobic segment character typical of hundreds of G protein-coupled receptors, but with the unusual feature that it lacks a cytoplasmic tail at the carboxy-terminal (Tsutsumi *et al.*, 1992). The GnRH-induced reduction of M current in frog neurons and the initiation of $Ca^{2+}$ oscillations in identified rat gonadotropes are blocked by GDP$\beta$S and made irreversible by GTP$\gamma$S applied intracellularly via whole-cell pipettes (Pfaffinger, 1988; Tse and Hille, 1992). Prior treatments with pertussis toxin do not diminish the reduction of M current in frog neurons or the activation of phospholipase C(PLC) in $\alpha$T3-1 cells (Pfaffinger, 1988; Anderson *et al.*, 1993). Antibodies to the $G_q$ class ($G_q$ and $G_{11}$) of PTX-insensitive G protein $\alpha$-subunits show the presence of immunoreactive subunits of the expected molecular weights in $\alpha$T3-1 cells and block the activation of phospholipase C by GnRH in membrane preparations from these cells (Hsieh and Martin, 1992; Anderson *et al.*, 1993). Thus the heterotrimeric G proteins coupling GnRH receptors to phospholipase C are very likely in the $G_q$ class. There are two reports of a decrease in GnRH effectiveness after PTX treatments (Rosenthal *et al.*, 1988; Hawes *et al.*, 1993). We are inclined to attribute this to crosstalk through reduction of a tonic PTX-sensitive input that alters the state of the $IP_3$ signaling pathway or of the exocytotic machinery during the long incubation in culture medium with PTX. However, alternative interpretations that cannot be ruled out now are that the GnRH receptor actually couples to several classes of G protein, a property that has been documented for parathyroid receptors and $\alpha_{2A}$-adrenergic receptors (Bringhurst *et al.*, 1993; Conklin and Bourne, 1993), or that PTX-sensitive G proteins participate directly in some later phase of $Ca^{2+}$ release or exocytosis.

## IV. The Calcium Economy of Gonadotropes

### A. NATURE OF THE OSCILLATION

There are numerous models for explaining how $Ca^{2+}$ oscillations arise (see Tsien and Tsien, 1990; Fewtrell, 1993). As mentioned earlier, gonadotropes have a rich repertoire of voltage-gated $K^+$, $Na^+$, and $Ca^{2+}$ channels

and fire action potentials during their cyclic membrane potential changes. In several endocrine cell types, including pancreatic $\beta$ cells and probably also somatotropes and lactotropes, rhythmic $[Ca^{2+}]_i$ elevations are a consequence of rhythmic membrane potential changes (Santos *et al.*, 1991; Ingram *et al.*, 1986; Chen *et al.*, 1989a). In these cells, a depolarization opens voltage-gated $Ca^{2+}$ channels, allowing $Ca^{2+}$ to enter from outside; opening of voltage-gated $K^+$ channels and multiple actions of elevated $[Ca^{2+}]_i$ cause the membrane to hyperpolarize until $[Ca^{2+}]_i$ becomes low again and the cycle starts over. In contrast, the $Ca^{2+}$ oscillation in gonadotropes depends little on membrane potential changes or on $Ca^{2+}$ permeability changes and $Ca^{2+}$ entry at the plasma membrane: In cells held under voltage clamp, the $[Ca^{2+}]_i$ oscillations continue, with only a small change of frequency as the membrane is held at potentials ranging from −140 to −10 mV (Tse and Hille, 1992; Hille *et al.*, 1994), and as we have said, there is no immediate need for $Ca^{2+}$ in the external solution. Entry of $Ca^{2+}$ from the medium is eventually essential to maintain the intracellular stores. The changes of membrane potential in gonadotropes are a consequence rather than a cause of the $[Ca^{2+}]_i$ oscillation. Hence, the concept of a "plasma membrane oscillator" does not capture the essence for gonadotropes. Instead, the oscillation must originate from intracellular signals and feedback.

Figure 6 diagrams some of the steps we have discussed. What are the feedback signals? One could, for example, envision action back on the GnRH receptor, to the coupling G proteins, or to PLC. Although they may normally occur, such changes seem secondary and nonessential since

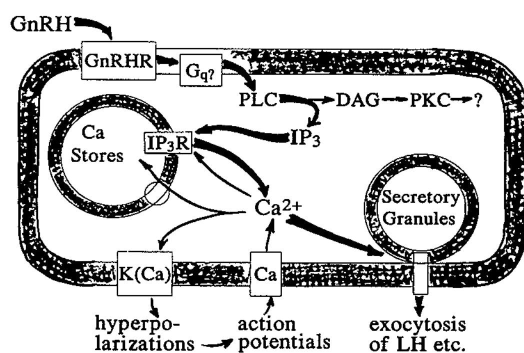


FIG. 6. Activation of a gonadotrope by GnRH. Diagram of intracellular signaling events leading to exocytosis. $G_q$, A pertussis toxin-insensitive G protein; PLC, phospholipase C; PKC, protein kinase C; $IP_3R$, receptor for $IP_3$; K(Ca), $Ca^{2+}$-activated $K^+$ channel; Ca, $Ca^{2+}$ channel; LH, luteinizing hormone.



oscillations can be initiated without GnRH by including $IP_3$ in the pipette, and oscillations are maintained for 20 min after a short GnRH pulse if the pipette contains the poorly hydrolyzable GTP analogue GTP$\gamma$S (Tse and Hille, 1992). A small action of PKC on the oscillation is evidenced when phorbol esters or diacylglycerol analogues are applied to the gonadotrope. In our experience with cells under voltage clamp, such PKC stimulators do not change the resting level of $[Ca^{2+}]_i$. However, if a $[Ca^{2+}]_i$ oscillation is already occurring because of GnRH in the bath or GTP$\gamma$S in the cell, application of PKC stimulators leads to a small slowing of the oscillation frequency (Tse *et al.*, 1993b). This slowing is partially reversed by the PKC inhibitor staurosporine. Interestingly, PKC stimulators do not change the oscillation frequency if the oscillation was initiated by $IP_3$ in the recording pipette. This somewhat complicated result suggests a modulatory, but not essential, role of PKC at an early stage in the signaling pathway between the activated G protein and the action of $IP_3$. Perhaps PLC is one of the modulated targets.

As yet, we lack a robust mechanistic explanation for reports in the literature that activators of PKC can stimulate hormone secretion from previously resting cells (Naor, 1990). The only relevant observation we have made is that treatments with PKC activators or with GnRH increase the amplitude of voltage-gated $Ca^{2+}$ currents by a small amount in $\alpha$T3-1 cells (Horn *et al.*, 1991; Bosma and Hille, 1992). On the other hand, Merelli *et al.* (1992) conclude that PKC activators reduce $Ca^{2+}$ influx in L-type $Ca^{2+}$ channels on the basis of measurements with $Ca^{2+}$ indicators in $K^+$-depolarized $\alpha$T3-1 cells. Perhaps PKC also alters the resting potential or stimulates exocytosis directly in gonadotropes.

At present, we favor a model for oscillations like that of DeYoung and Keizer (1992), which makes use of the bell-shaped $Ca^{2+}$ sensitivity of the $IP_3$ receptor channel opening probability described by Bezprozvanny *et al.* (1991) and Finch *et al.* (1991). The idea is that a small $Ca^{2+}$ release induced by $IP_3$ helps to open more $IP_3$-sensitive channels, but as $[Ca^{2+}]_i$ continues to rise to the micromolar level, the $Ca^{2+}$ release channels are shut down again. The model requires the $Ca^{2+}$-induced shut-down to last for several seconds. This time delay allows the $Ca^{2+}$-ATPase on membranes of the intracellular store to pump the $Ca^{2+}$ out of the cytoplasm and back into the store, and the cycle can start again. Several observations agree with assumptions of the model. We can sometimes trigger regenerative, spike-like $Ca^{2+}$ rises by modest elevations of $[Ca^{2+}]_i$ brought about by slow hydrolysis of caged $IP_3$, by a depolarizing voltage clamp pulse, or by inhibitors of intracellular $Ca^{2+}$ pumps. Like pituitary melanotropes (Thomas *et al.*, 1990), gonadotropes do not release $Ca^{2+}$ from intracellular stores when challenged with caffeine (10 m$M$). Their oscillations are also

insensitive to ryanodine (10 $\mu M$) and to cyclic ADP ribose (200 n$M$; A. Tse, unpublished), so the apparent $Ca^{2+}$-stimulated $Ca^{2+}$ release probably involves the $IP_3$-activated release channel rather than ryanodine receptors that mediate $Ca^{2+}$-induced $Ca^{2+}$ release in other cells. In addition, as would be predicted, oscillations in gonadotropes are blocked by treating cells with high concentrations of inhibitors of endoplasmic reticulum $Ca^{2+}$ pumps, such as thapsigargin, cyclopiazonic acid, or 2,5-di-*tert*-butyl-1,4-benzohydroquinone (Iida *et al.*, 1991; A. Tse *et al.*, 1994). We actually believe that some of the released $Ca^{2+}$ passes through other intracellular compartments (mitochondria?) before returning to the $IP_3$-sensitive store (Tse *et al.*, 1994a,b). One aspect of the model still needs more attention. That is the postulation of a several second shut-down of release channels following the $Ca^{2+}$ elevation. Some kind of memory, such as a covalent modification, may be needed to achieve this delay.

Like the literature for other anterior pituitary cells, that on gonadotropes contains frequent references to two phases in the $[Ca^{2+}]_i$ rise and secretory responses following agonist. The first phase, said to last several minutes, is attributed to $Ca^{2+}$ mobilization from internal stores, and the second is attributed to entry of extracellular $Ca^{2+}$ via dihydropyridine-sensitive $Ca^{2+}$ channels (Smith *et al.*, 1987; Shangold *et al.*, 1988; Stojilkovic *et al.*, 1992a). A sharp distinction of two phases is less clear in our work. Regular oscillations of $[Ca^{2+}]_i$ can continue relatively robustly for up to 20 min during protracted exposures to GnRH—or even after a brief exposure if the cell has been loaded with GTPγS (Fig. 4). We take this as a sign that $IP_3$ is continuously synthesized and that a cyclic release of $Ca^{2+}$ from intracellular stores continues to produce each rise during a long response. At the same time, we feel that a small quantity of $Ca^{2+}$ enters the cell through $Ca^{2+}$ channels during each cycle and that this compensates for a small loss that occurs through extrusion during each $[Ca^{2+}]_i$ elevation. Thus during excitation in our system, there seem to be repeated cycles of $Ca^{2+}$ release from stores and continual, but very slow, $Ca^{2+}$ entry from outside, and if one blocks plasma membrane $Ca^{2+}$ channels or removes extracellular $Ca^{2+}$, the intracellular stores eventually would be depleted. In this way the $[Ca^{2+}]_i$ elevations could be regarded as initially not requiring $Ca^{2+}$ entry and subsequently failing without it, although the mechanism of oscillation actually does not change. In our work we have so far failed to find evidence for a "capacitative" $Ca^{2+}$-loading signal, a signal that initiates extra $Ca^{2+}$ entry in response to depletion of stores (Putney, 1990; Penner *et al.*, 1993). If there is such a mechanism in these cells, it would not account for the reported dihydropyridine sensitivity of the secondary phase.



## B. CYTOPLASMIC CALCIUM BINDING

The cytoplasmic compartment of any cell contains many molecules that bind $Ca^{2+}$ quickly and lower its free concentration by acting as endogenous $Ca^{2+}$ buffers. Some of these are high-affinity $Ca^{2+}$-switch proteins such as calmodulin and troponin; others are $Ca^{2+}$-binding proteins with no known signaling function; and still others are low-affinity small molecules of intermediary metabolism. In addition, $Ca^{2+}$ transporters (pumps and exchangers) act as stoichiometric $Ca^{2+}$-binding sites. In a quantitative model of $Ca^{2+}$ dynamics in the cell, the properties of these rapid $Ca^{2+}$ buffers become important. They determine how many $Ca^{2+}$ ions have to be moved into the cytoplasm to achieve a given increment in $[Ca^{2+}]_i$ and they influence the length of time it takes for cellular $Ca^{2+}$ to return to baseline after an elevation.

This problem has been studied extensively in the literature on contraction of skeletal muscle (Baylor *et al.*, 1983; Brum *et al.*, 1988), where the sarcoplasm contains approximately 1 m$M$ of high-affinity $Ca^{2+}$-binding sites on known proteins. For all other cells, much less is known. Thomas and colleagues (1990) applied depolarizing voltage clamp steps to melanotropes and compared the $[Ca^{2+}]_i$ increment reported by Fura-2 with the $Ca^{2+}$ entry calculated by integrating the recorded voltage-gated $Ca^{2+}$ current. They found roughly that for every 100 $Ca^{2+}$ ions entering the cell, only 1 remained free and the rest were effectively instantly (<100 ms) bound to the cytoplasm or to the dye. Similar results were reported by Lledo *et al.* (1992) in $GH_3$ cells. Neher and Augustine (1992), using similar arguments with adrenal chromaffin cells, but extrapolating to a situation with no intracellular dye, found a bound/free ratio of 75–90. This kind of experiment has been repeated with gonadotropes, giving a ratio near 100 (A. Tse *et al.*, 1994). In order for $[Ca^{2+}]_i$ to rise by 3 $\mu M$ during a $Ca^{2+}$ oscillation, the intracellular stores must deliver 300 μmol/liter $Ca^{2+}$. At the same time, if the intracellular stores have a volume that is, let us say, only 10% that of the rest of the cytoplasm, the total $Ca^{2+}$ within the stores must drop by more than 3 mmol/liter during release. [Like the $Ca^{2+}$ stores of other cells, those of gonadotropes probably also contain their own $Ca^{2+}$-binding proteins of the calsequestrin/calreticulin family (Milner *et al.*, 1992)]. Then after the release, $Ca^{2+}$ is pumped back into the stores with a time constant of about 2 sec (A. Tse *et al.*, 1994), and the $Ca^{2+}$-ATPase consumes about 150 $\mu M$ ATP (two $Ca^{2+}$ ions are transported per ATP consumed). If we consider the 2-sec decay time of $[Ca^{2+}]_i$ transients, the apparent first-order rate constant for pumping would be 1/(2 sec) = 0.5 $sec^{-1}$. However, we must also take into account that, for each free

$Ca^{2+}$ ion removed from the cytoplasm, pumps would have to remove 100 more bound to intracellular buffers. This makes the corrected pumping rate constant 100 times larger, or 50 $sec^{-1}$. For comparison, in fast twitch skeletal muscle, which can relax in tens of milliseconds from a twitch, the corresponding corrected rate constant is eightfold higher, about 400 $sec^{-1}$ (Melzer *et al.*, 1986), reflecting presumably a considerably higher density of $Ca^{2+}$ pumps per unit volume and an endogenous buffer binding that is only 30–50% of that in gonadotropes. For chromaffin cells, which do not show $[Ca^{2+}]_i$ oscillations, the corrected rate constant for $Ca^{2+}$ removal is about fivefold lower than for gonadotropes (Neher and Augustine, 1992).

## V. Calcium and Exocytosis

The important endpoint of GnRH action is hormone secretion. We have used the membrane electrical capacitance as a rapid, electrical monitor of membrane area, and hence of exocytosis in gonadotropes. We find that when GnRH induces a $[Ca^{2+}]_i$ rise, there is exocytosis (Fig. 7). We always observe significant exocytosis when $[Ca^{2+}]_i$ rises about 300 n*M* (Tse *et al.*, 1993a), and if there is a delay in the initiation of exocytosis, it must be less than the 100-msec time resolution of our $[Ca^{2+}]_i$ measurements.

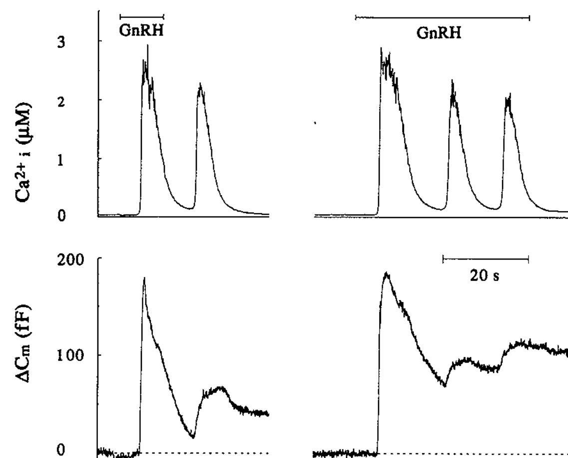


FIG. 7. Exocytosis during GnRH-induced $Ca^{2+}$ oscillations. This gonadotrope was exposed briefly to 40 n*M* GnRH applied by a puffer pipette (bars). Two applications are shown in separate panels. Upper traces $[Ca^{2+}]_i$ recorded with the dye indo-1. Lower traces: Membrane capacitance changes. The cell is held under voltage clamp at −80 mV.



Exocytosis is seen whether the $[Ca^{2+}]_i$ rise is induced by GnRH or simply by slow photohydrolysis of caged $IP_3$ (Fig. 5), a maneuver that presumably releases $Ca^{2+}$ without the potent stimulation of PLC and PKC that occurs with the natural stimulus. The induction of exocytosis by GnRH can be prevented by loading the cell with $Ca^{2+}$ buffers that blunt the rise of $[Ca^{2+}]_i$. Thus $Ca^{2+}$ satisfies many criteria of a necessary and sufficent signal for exocytosis: It rises with GnRH addition; exocytosis shows a close temporal coupling with the $[Ca^{2+}]_i$ rise; and blocking the rise blocks exocytosis.

When the $[Ca^{2+}]_i$ rise is oscillatory, exocytosis occurs in each elevation (Fig. 7). Interestingly, however, close examination of each cycle shows that the rate of exocytosis reaches its maximal value early in the cycle as the $[Ca^{2+}]_i$ is still rising (Tse *et al.*, 1993a). The net membrane area increase becomes small or even negative while the $[Ca^{2+}]_i$ is still at maximal values—above 1.5 $\mu M$. It is as if the available pool of readily releasable secretory granules has been depleted. Nevertheless, 5–20 sec later, when the $[Ca^{2+}]_i$ rises again, a new round of exocytosis occurs, as if new granules had been mobilized and docked for release in the intervening time. By the third or fourth cycle, however, the number of additional granules released diminishes. Thus, in a several minute pulse of GnRH, much of the secretion of the LH is likely to take place in the first minute. This may help explain why physiological GnRH pulses are only a few minutes long.

We estimate from published micrographs that a gonadotrope may contain 10,000 secretory granules, the great majority of which are not apposed to the plasma membrane. From the diameter of a granule, we estimate that each may add about 1.3 fF of electrical capacitance when it fuses with the plasma membrane. On this basis about 150 granules would be released in the typical 200-fF capacitance rise that occurs in one of the initial cycles of $Ca^{2+}$ oscillation (Fig. 7). Usually in our experiments, much of the membrane area added rapidly to the plasma membrane during a $[Ca^{2+}]_i$ elevation is recovered (by endocytosis) within a few minutes. In the cell of Fig. 7, endocytosis, seen as a capacitance decrease, followed the exocytosis within a second. The time course is quite variable.

These interpretations fit well with measurements on melanotropes and adrenal chromaffin cells. With these cells, the time course of exocytosis has been measured after an abrupt and persistent elevation of $[Ca^{2+}]_i$ to near 100 $\mu M$ by a rapid photolysis of caged $Ca^{2+}$ (Thomas *et al.*, 1993a,b; Neher and Zucker, 1993). In both cell types, there is immediate and rapid exocytosis that slows within a fraction of a second, as if readily available secretory granules were quickly depleted and then slower mobilization processes become rate limiting. In those experiments, the peak rate of

exocytosis can be several orders of magnitude higher than in GnRH-stimulated gonadotropes, probably because the flash-induced $[Ca^{2+}]_i$ rise is vastly faster and larger; however, the total capacitance increase in the first 500 msec is several hundred femtofarads and is not much larger than in the gonadotrope. The initial rate of exocytosis in melanotropes is a graded function of the $[Ca^{2+}]_i$ level reached by rapid photorelease of caged $Ca^{2+}$ (Thomas *et al.*, 1993b). The concentration dependence is cooperative with a midpoint near 30 $\mu M$. These results suggest that the exocytotic machinery of gonadotropes is not maximally activated by hormone-induced $[Ca^{2+}]_i$ rises and is operating in a range of steep sensitivity to the $[Ca^{2+}]_i$ level. Endocrine exocytosis, which operates at moderate rates, seems to contrast with the extremely rapid and brief (<1 msec) exocytosis at fast chemical synapses. In chemical synapses, the exocytotic machinery and synaptic vesicles may be docked on the plasma membrane in a complex with voltage-gated $Ca^{2+}$ channels of the active zone. The $[Ca^{2+}]$ might therefore rise locally and nearly instantly to several hundred micromolar to achieve exocytosis within a fraction of a millisecond (Roberts *et al.*, 1990). In contrast, in those endocrine cells dependent on $Ca^{2+}$ release from intracellular stores, the exocytotic machinery cannot be as close to the $Ca^{2+}$ release channels, and their more moderate secretory rate reflects the lower $[Ca^{2+}]_i$ levels (1–50 $\mu M$) reached. Although many proteins associated with synaptic vesicles and secretory granules have been identified (Bennett and Scheller, 1993), we do not yet know what the $Ca^{2+}$ sensor for exocytosis is.

Many cellular processes involve membrane traffic, with vesicles docking and fusing with target membranes. Examples include vesicle movements between endoplasmic reticulum and parts of the Golgi apparatus or between endosomes and lysosomes. Such membrane traffic seems to require GTP and small G proteins related to ras proteins (Goud and McCaffrey, 1991). These monomeric G proteins, distinct from the heterotrimeric signaling G proteins, are believed to contribute to specificity of vesicle docking to target membranes. Regulated exocytosis also generally has a requirement for GTP in addition to $Ca^{2+}$ (Gomperts, 1990) and in some cases can be stimulated by intracellular GTP$\gamma$S without elevation of $[Ca^{2+}]_i$ (Fernandez *et al.*, 1984). In endocrine cells as in others, the G proteins important for exocytosis seem to be from the rab3 family. Thus, "effector peptides" modeled after rab3A sequences increase insulin secretion from permeabilized HIT-T15 cells (Li *et al.*, 1993) and decrease LH secretion and growth hormone secretion from permeabilized pituitary cells (Davidson *et al.*, 1993). The anterior pituitary actually expresses rab3B preferentially, and this G protein is likely to be essential in exocytosis of hormones, as injections of antisense oligonucleotides for rab3B (but not rab3A) block the ability of raised $[Ca^{2+}]_i$ to induce exocytosis in unidentified anterior pituitary cells (Lledo *et al.*, 1993). In addition, when $[Ca^{2+}]_i$ is strongly buffered at or below the resting level, delivery of GTP$\gamma$S to the cytoplasm via the recording pipette or by photolysis of caged GTP$\gamma$S initiates a gradual exocytosis (net increase in plasma membrane area) in melanotropes and gonadotropes (Okano *et al.*, 1993; A. Tse, F. Tse, and B. Hille unpublished). These various approaches show that stimulation and disruption of small G proteins have significant effects on exocytosis. Additional experiments are needed to clarify if the major effect is on the final, rapid steps of regulated exocytosis or on earlier steps of formation and intracellular traffic of the secretory vesicle population.



## VI. Role of Oscillations

Many cell types show $Ca^{2+}$ oscillations in response to some stimulus conditions (Tsien and Tsien, 1990; Fewtrell, 1993). In some cases, including the gonadotrope, it is clear that a $Ca^{2+}$ rise is important to a physiological response such as secretion. However, it is usually less obvious why a constant rise would not be as effective as an oscillatory one. A full answer to this question probably awaits new insights. Schulman and colleagues (1992) have suggested how $Ca^{2+}$/calmodulin-dependent protein kinase can decode pulsatile $Ca^{2+}$ signals into a graded activation of the kinase. Kasai and Augustine (1990) have suggested a "push–pull" model for agonist-stimulated salt and fluid secretion from exocrine pancreas. It relies on waves of $Ca^{2+}$ release to open $Cl^{+}$ and $K^{+}$ channels alternately at the apical pole and at the basal pole of an acinar cell. Gonadotropes are relatively round and are not part of a polarized epithelium. Nevertheless, one can imagine another type of push–pull model in which the rapid rise to high $[Ca^{2+}]_i$ levels promotes exocytosis and the subsequent drop to more modest levels somehow contributes to mobilization of a new cohort of secretory granules (cf. Heinemann *et al.*, 1993). There are insufficient experimental data at present to test such a model. In addition, there are qualitative and common suggestions that (1) processes are easier to control by pulse-like signals varying in frequency than by precisely set steady levels, and that (2) all other things being equal, it is less "damaging" or "ATP costly" to cells to alternate periods of high and low $[Ca^{2+}]_i$ than to maintain a steady modest elevation in response to a long physiological stimulus.

In the gonadotrope, the oscillation may have the advantage of assisting in the replenishment of intracellular $Ca^{2+}$ stores. While $[Ca^{2+}]_i$ is high, the membrane is hyperpolarized and then, as the $[Ca^{2+}]_i$ drops, the membrane depolarizes and fires several action potentials (Fig. 2). The action poten-

tials open voltage-gated $Ca^{2+}$ channels and should let in a small amount of $Ca^{2+}$, calculated to be approximately 600 nmol/liter per cycle before any is bound (Tse and Hille, 1993), corresponding to an increment of only 6 n*M* in the free $[Ca^{2+}]_i$ (see Fig. 2). This is 50-fold less than the amount of $Ca^{2+}$ released by the stores in each cycle so it has a negligible effect on exocytosis, but it may be enough to balance the presumed small loss that occurs in each cycle through $Ca^{2+}$ pumping at the plasma membrane, thus keeping the cell $Ca^{2+}$ content in a steady state during prolonged stimulation.

## VII. Conclusions

The primary secretory response to GnRH is unambiguously attributable to a steady rise of $IP_3$ that leads to an oscillatory release of $Ca^{2+}$ from intracellular stores. Each elevation brings $[Ca^{2+}]_i$ well above the 300 n*M* level that suffices to initiate exocytosis of numerous secretory granules. Exocytosis is tightly coupled to the rise of $[Ca^{2+}]_i$. GnRH treatments also lead to rises in other second messenger molecules. Although several of them affect hormone release when given exogenously, their essential involvement and roles in the normal secretory response are not well established. It is quite possible that they are not necessary in the initiation of secretion but may coordinate important slower processes in the life of the gonadotrope.

The extracellular medium is necessarily the ultimate source of $Ca^{2+}$ ions released from intracellular stores. However, unlike for some endocrine cells, the secretory stimulus for gonadotropes does not initiate a major entry of extracellular $Ca^{2+}$ as a primary signal for exocytosis. The electrophysiological responses of stimulated gonadotropes include a hyperpolarization during periods of $[Ca^{2+}]_i$ rise instead of a depolarization. As $[Ca^{2+}]_i$ falls again, the cell depolarizes and fires a few action potentials, letting in an aliquot of $Ca^{2+}$ ions through voltage-gated $Ca^{2+}$ channels that is much smaller than the amount released by $IP_3$ from intracellular stores. Additional work is needed to look for more subtle possible effects of GnRH and other hormones on membrane potential and on $Ca^{2+}$ permeability that could have effects on the secretory response.

Several additional signaling questions are ready for further study. A general cell biological question that probably has universal answers is to elucidate the molecular events of hormone granule exocytosis and its control by $Ca^{2+}$ and by other synergistic signals. A question that may combine universals with cell-specific details is to clarify the feedback and modulatory signals on $Ca^{2+}$ oscillations and to explain why an oscillation is preferable to a steady signal. Several questions are specific to gonadotropes: What determines the proportion of follicle-stimulating hormone versus LH that is stored and secreted from gonadotropes? Is there a significant difference between gonadotropes of males and females? How much does their cellular physiology—the availability and state of molecules in the excitation–response pathway—change through the estrous cycle? Are the events during the prolonged GnRH stimulation of the preovulatory surge significantly different from those of the much briefer pulsatile episodes of other times? To what degree are the GnRH responses normally subject to modulation by steroids, neurotransmitters, and other peptide hormones of the portal circulation and pituitary? Many of these questions can be addressed by the newer single-cell methods.



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