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The Relationship between Pulsatile Secretion and Calcium Dynamics in Single, Living Gonadotropin-Releasing Hormone Neurons*

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ABSTRACT

It is well established that pulsatile release of GnRH regulates the reproductive axis, but little is known about the mechanisms underlying this pulsatility. Recent findings that GT1 cells, a line derived from the mouse embryonic hypothalamus, release GnRH in a pulsatile manner indicates that this rhythmic activity is an intrinsic property of GnRH neurons. In several attempts to uncover the intracellular basis for this pulsatile phenomenon, it was revealed that intracellular calcium concentrations change in a rhythmic fashion in GnRH neurons and that cellular depolarization, which triggers a secretory event, is associated with profound calcium changes in the cells. These findings raised the intriguing possibility that periodic alterations in intracellular calcium concentrations may underlie the phenomenon of pulsatile secretion in GnRH neurons. To address this, we first adapted the use of FM1–43 fluorescence to monitor changes of secretion in individual GT1–7 cells and then combined this approach with simultaneous measurement of intracellular free calcium ($[Ca^{2+}]_i$, fura 2 method). In initial validation experiments, we found that stimulation of exocytosis with K^+ (75 mM) or *N*-methyl-D-aspartate (NMDA, 100 μM) predictably evoked dynamic increases of both FM1–43 and fura 2 fluorescence. Later measurement of calcium dynamics and exocytotic activity in unstimulated cells revealed that $[Ca^{2+}]_i$ underwent transitions from quiescence to high oscillatory behavior, and that these shifts were frequently associated with exocytotic events. Moreover, these calcium oscillatory transitions and associated changes in secretory activity occurred synchronously among most adjacent cells and at a frequency similar to that reported for pulsatile release of GnRH by entire cultures of GnRH neurons. Taken together, these results indicate that the intrinsic secretory pulsatility of GnRH neurons appears to be a consequence of coordinated, periodic changes in the pattern of calcium oscillations within individual cells. (*Endocrinology* **141:** 2012–2017, 2000)

THE PULSATILE secretion of GnRH serves as the driving force for the reproductive axis by governing the pulsatile release of gonadotropic hormones from the anterior pituitary into the circulation. The source of these GnRH pulses consists of a few thousand neurons (1) scattered in the preoptic and mediobasal hypothalamic areas (2). Even though these neurons are diffuse and do not appear to be highly organized morphologically, it is quite clear that they are functionally interconnected as evidenced by the collective pulsatile release of GnRH from the hypothalamus (3–7). Recent development by Mellon and co-workers (8) of a continuous line of GnRH neurons (GT1 cells) has provided a powerful tool to aid in the elucidation of the mechanism(s) underlying pulse generation. The GT1 cell line, derived from the mouse embryonic hypothalamus, shares most of the functional characteristics of normal GnRH neurons. These transformed cells release GnRH in a pulsatile fashion (9–12). Moreover, the frequency at which GnRH pulses are elaborated by GT1 cells is quite similar to that reported *in vivo* (5–7) and for primary cultures of monkey embryonic GnRH neurons (13). In fact, the pulsatile release of GnRH from cultures of GT1 cells, which are devoid of connection to any other cell types, suggests that secretory pulsatility is an intrinsic property of the GnRH neuron (12, 14). Despite the importance of this pulsatility in reproduction, little information is available concerning the manner in which these pulses are generated.

Recent evidence suggests that calcium is a critical component in the initiation and propagation of these pulses. First, it was reported that single GT1 cells exhibit spontaneous firing of action potentials that are associated with oscillations of intracellular free calcium ($[Ca^{2+}]_i$) (15). Interestingly, the blockade of calcium channels by pharmacological means or by removal of extracellular calcium, a maneuver that inhibited spontaneous electrical activity, abolished GnRH release in GT1 cells (10, 15, 16). Similarly, manipulation of calcium levels in primary GnRH neurons derived from embryonic rhesus monkeys also markedly dampened GnRH release (13). These observations indicate that calcium is necessary for GnRH secretion. A second line of evidence reveals a shifting pattern of intracellular free calcium that would be consistent with pulsatile activity in a cell population. Charles and Hale (17) found that, in some cases, GT1–7 cells exhibit spontaneous oscillations of intracellular calcium that appeared to be synchronized among cells, suggesting that GnRH secretory pulsatility may follow synchronization of calcium oscillations. Terasawa and co-workers (18) also demonstrated a synchronization of calcium oscillations in cultured GnRH

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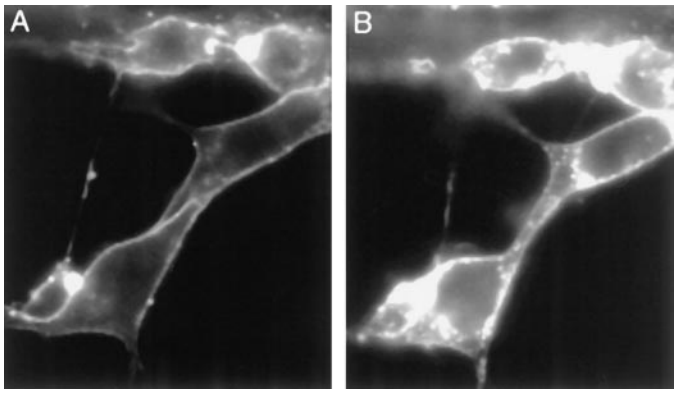

FIG. 1. Digital imaging fluorescence microscopy of FM1–43 in individual GT1–7 cells. GT1–7 cells were exposed continuously to the membrane-turnover probe FM1–43 (2 μM) and 10 min later subjected to light excitation. Light emitted above 515 nm was recorded before (A) and 10 min after (B) addition of the glutamate receptor agonist NMDA (100 μM), a well-known stimulator of GnRH release. Note the rapid increase in fluorescence intensity (indicative of secretion) after treatment.

neurons from embryonic rhesus monkeys. Thus, in addition to the role that calcium may play in the export of GnRH, it appears that the oscillatory pattern of this component is quite consistent with the unique rhythmic activity that is the hallmark of GnRH neuronal function. Attempts to further elucidate a relationship between pulses of GnRH release and intracellular calcium dynamics has been hindered by the lack of technology enabling the measurement of both calcium and secretion in the same, living neurons. In the present study, we acquired this technology by combining digital-imaging fluorescence microscopy of fura-2 (to monitor calcium changes) with continuous measurement of fluorescence emitted after excitation of FM1–43 ([*N*-(3-triethylammoniopropyl)-4-(p-dibutylaminostyryl) piridinium dibromide]), a membrane-turnover probe. FM1–43 is relatively nonfluorescent in aqueous solution, but becomes highly fluorescent in the lipid environment of cell membranes (the quantum yield of FM1–43 increases up to 350-fold; Ref. 19). Endocytocis, which invariably follows exocytotic release from cells, results in the internalization of the probe and the increase of fluorescence emission after excitation (20). These combined approaches were used in the following experiments to determine the relationship between calcium changes and the pulsatile release of GnRH in GT1–7 cells.

Materials and Methods

Cell culture

GT1–7 cells (kindly provided by Richard I. Weiner, University of California at San Francisco) were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone. The cultures were maintained in a water-saturated atmosphere of 95% air and 5% $C0_2$. For experiments, cells were plated (at 10^5 cells/ml) onto poly-L-lysine (0.01 mg/ml) coated glass coverslips and cultured for 4 to 6 days before fluorescence measurements.

Fluorescence microscopy of FM1–43

FM1–43 uptake was measured essentially as reported previously by Shorte *et al.* (21). Briefly, GT1–7 cells were washed with imaging medium

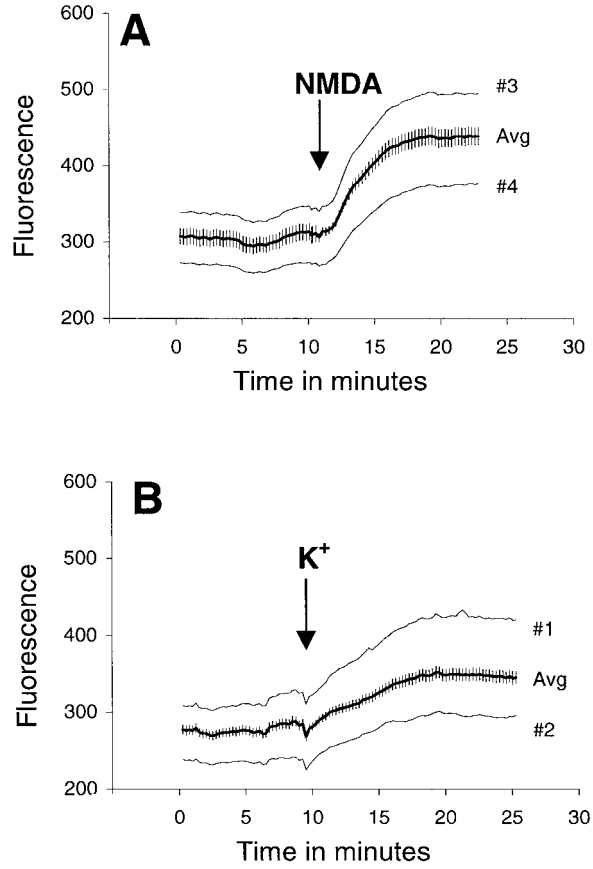

FIG. 2. Monitoring of stimulus-induced membrane retrieval with FM1–43. Cells processed as in Fig. 1 were stimulated to secrete with high-K^+ medium or NMDA. Representative examples of responses of individual cells (#1–4) recorded in the same microscopic fields are shown. Also shown are the averaged traces (Avg; mean ± SEM) for all cells present in each of the microscopic fields. Data are representative of 66 (K^+) and 50 cells (NMDA) studied in 3 independent experiments. These results demonstrate that stimulation of secretion in GT1–7 cells evokes the uptake of the membrane-turnover probe FM1–43, and that this process can be monitored continuously.

(22) composed of a mixture of Medium 199 and Nutrient mixture F-12 [1:1] (Life Technologies, Inc.) in which sodium bicarbonate had been replaced by HEPES buffer. The coverslips were mounted in a Sykes-Moore chamber (23) and placed under the microscope (Carl Zeiss Axiovert 135 TV, Jena, Germany) in a temperature-controlled chamber (37 C). Then, cells were exposed for 10 min to the membrane-turnover probe FM1–43 (2 μM, Molecular Probes, Inc., Eugene, OR). Finally, cells were epi-illuminated with 490 nm light (100 msec every 20 sec) through a Carl Zeiss Fluor 40× 1.3 n.a. objective using a fiber optic light scrambler (Technical Video, Woods Hole, MA) and a 500 nm dichroic mirror. The light emitted was passed through a barrier filter (515 nm long pass, Chroma Technologies, Brattleboro, VT) and was collected continuously at the corresponding times by a cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). The fluorescent signal of FM1-43 loaded cells was very intense, and the excitation light was reduced by use of neutral density filters (Chroma Technologies). Fluorescent images were stored and analyzed using the Metafluor software from Universal Imaging (West Chester, PA).

Combination of FM1–43 fluorescence and $[Ca^{2+}]_i$ measurements

For combination of FM1–43 fluorescence measurements and digital imaging fluorescence microscopy of fura-2, we used the protocol developed by Shorte *et al.* (21) with minor modifications. In brief, GT1–7

cells were washed with imaging medium and loaded with fura-2 by incubating them for about 60 min at room temperature with fura-2/AM (5 μM, Molecular Probes, Inc., Eugene, OR). Cells were then mounted in a chamber as described above and incubated under the microscope for 10 min with FM1–43 (2 μM). Cells kept in FM1–43 containing medium were then epi-illuminated alternately with excitation light at 340 nm and 380 nm for 100 msec every 5 sec and at 490 nm for 300 ms every 50 sec. This was achieved by passing the excitation light through an alternating wheel containing 10 nm (340, 380 for fura-2) or 5 nm (490 for FM1–43) band-pass interference filters. In this protocol, we used a 400 nm dichroic mirror. Emitted light was passed through a barrier filter (515 nm long pass) and collected continuously at the corresponding times as described above. The images were stored and analyzed using Metafluor software. Pixel by pixel ratios of consecutive frames obtained at 340 and 380 nm excitation were produced and $[Ca^{2+}]_i$ values were estimated by comparison with fura-2 standards (24). In experiments using FM1–43 alone or in combination with fura 2, we did not observe any deleterious effect during prolonged exposure of loaded cells to excitation light. For long-term experiments, the chamber was closed to keep conditions stable and avoid changes in medium concentration due to evaporation. In contrast, the chamber was opened for short-term experiments to facilitate addition of treatments. K^+, NMDA and nimodipine were added at final concentrations of 75 mM, 100 μm, and 100 mM, respectively. Each agent was tested by addition of 40 μl of a more concentrated solution (also containing 2 μM FM1–43) into the open chamber containing 800 μl of imaging medium. The chambers that included stimulating agents were imaged immediately. Those containing nimodipine were incubated for 10 min before imaging. Each agent remained in contact with the cells for the entire course of the experiment.

Results and Discussion

As a first step, we had to develop a method for continuously monitoring secretory activity within individual GnRH neurons. In pursuit of this, we assessed whether the membrane-turnover probe FM1–43 could be used for this purpose. This dye is nonfluorescent in aqueous solution, but becomes fluorescent upon incorporation into the lipid environment of the plasma membrane (19). During a secretory event, granule vesicles fuse with the plasma membrane exposing more membrane surface area to the dye. Molecules of FM1–43 that have inserted between phospholipids in the outer leaflet of the plasma membrane then enter the cell through endocytosis as the vesicle membranes are recycled. Collectively, the extensive membrane recycling that occurs following a secretory event increases the level of fluorescence in the cell. Figure 1 provides a photomicrograph of GT1–7 cells imaged in the presence of FM1–43 (2 μM) and subsequently exposed to excitation light. Fluorescence images of the cells were recorded before (Fig. 1A) and after (Fig. 1B) exposure to the glutamate receptor agonist *N*-Methyl D-aspartate (NMDA), a well-established stimulator of GnRH secretion. As shown, the dye appeared to be distributed over both the soma and neurites of the GT1–7 cells under basal conditions. In most of the cells, an increase in membrane incorporation of the probe followed stimulation, as evidenced by the local increase of fluorescence intensity after treatment. The responsiveness of fluorescent signal to stimulation is illustrated more quantitatively in Fig. 2 for NMDA (100 μM, panel A) as well as for high K^+ (75 mM, panel B) medium. The latter treatment is used commonly to depolarize cells and thereby causes the exocytosis of stored secretory product. Examples of responses from individual cells (#1–4) or the averaged traces (Avg.; mean ± SEM) are shown. Clearly, each of these treatments initiated a rise in fluorescence within minutes of administration, consistent with the time-frame reported for the endocytosis that occurs subsequent to exocytosis in neurons (19). Addition of NMDA stimulated membrane retrieval in 92% of the cells (n = 50, 3 independent experiments). Treatment with high-K^+ medium resulted in an increase of FM1–43 fluorescence in 92% of the cells studied (n = 66 cells, 3 independent experiments). Our demonstration that unequivocal increases of FM1-43 fluorescence occurred subsequent to stimulations with agents known to elicit strong secretory responses serves to confirm the validity of this probe for making dynamic measurements of secretion in GT1–7 cells.

To explore the relationship between intracellular calcium dynamics and secretory activity in the same GT1–7 cells, we adopted a novel strategy developed by Shorte *et al.* (21) for use on anterior pituitary cells. It consists of loading cells with both the calcium sensitive probe fura-2/AM and the membrane retrieval probe FM1–43. The cells are then subjected to ratiometric determination of $[Ca^{2+}]$i (after dual excitation at 340 and 380 nm) followed by monitoring of FM1-43 incorporation after excitation at 490 nm. Figure 3 shows an example of the images of FM1–43 fluorescence (panel A) and intracellular calcium (340/380 ratios coded in pseudocolor, panel B) recorded almost simultaneously. As illustrated, this approach allows analysis of both secretory activity and

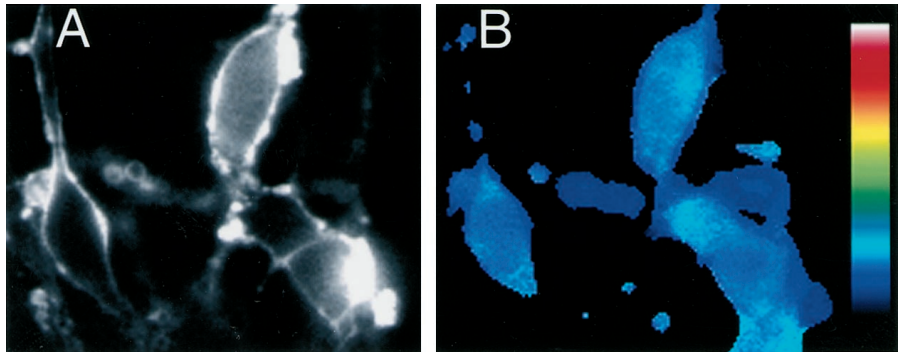

FIG. 3. Combination of FM1–43 and Fura-2 fluorescence measurements. GT1–7 cells were loaded with fura-2/AM for 1 h and then exposed continuously to FM1–43. Ten minutes later, cells were sequentially epi-illuminated with 340, 380, and 490 nm excitation light and fluorescence emissions (above 515 nm) resulting from each excitation were recorded over time. Light emitted after excitation at 490 nm reflects FM1–43 uptake (A). Ratios of emissions resulting from excitations at 340 and 380 nm were used to estimate $[Ca^{2+}]_i$. Here (B) they are represented by pseudocolor images. Relative $[Ca^{2+}]$i is indicated on the ascending pseudocolor scale to the right in which warmer colors reflect higher ratios.

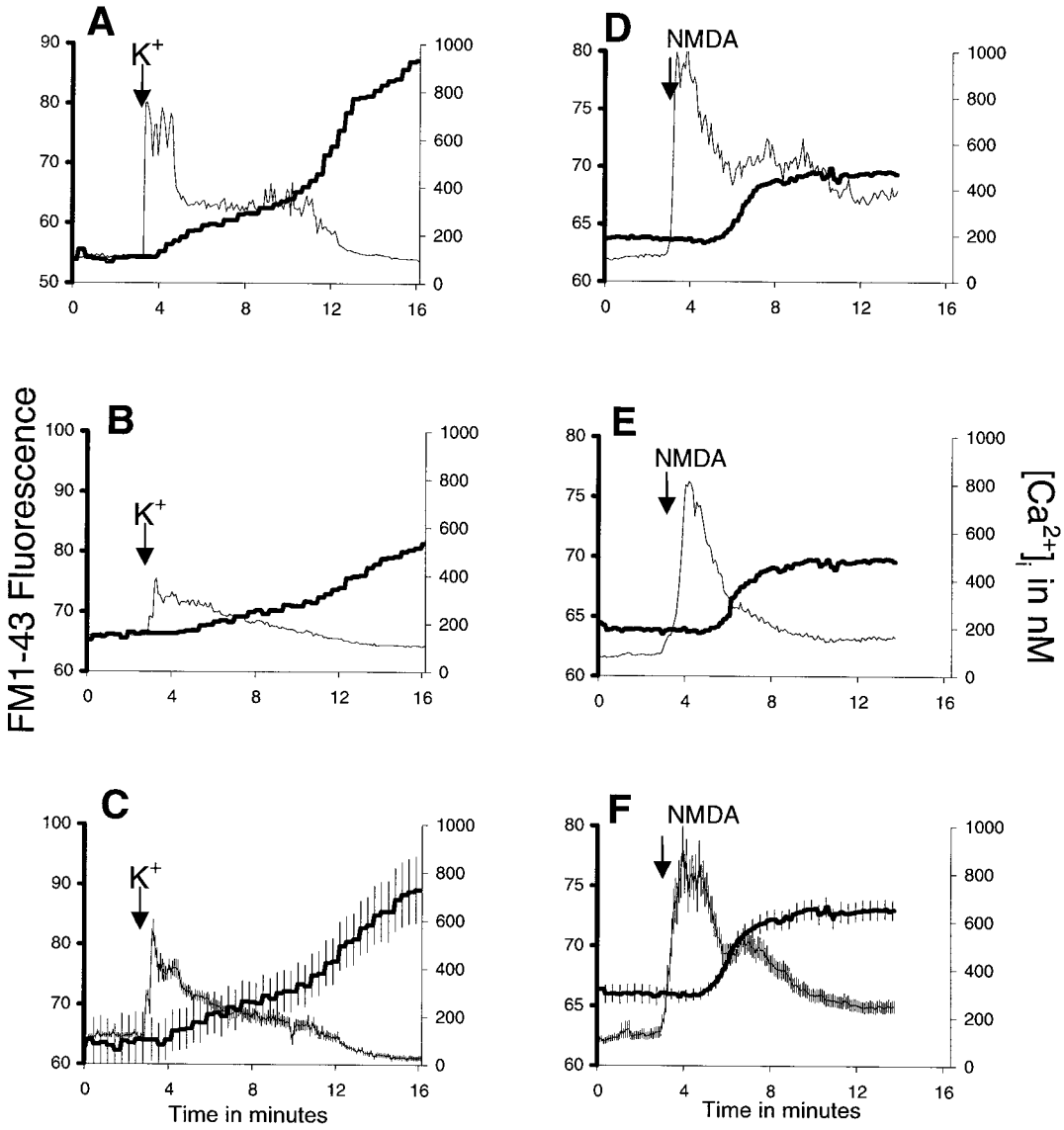

FIG. 4. Simultaneous measurements of secretagogue-induced changes of intracellular calcium and membrane retrieval in individual GT1–7 cells. Cells processed as in Fig. 4 were stimulated with high-K^+ medium (*left panel*) or the glutamate receptor agonist NMDA (*right panels*). Stimulation induced an increase of $[Ca^{2+}]_i$ (*lighter lines*), which was followed shortly thereafter by exocytosis, as revealed by the increase in FM1–43 fluorescence (*bold lines*). Representative examples of responses obtained from individual cells (A, B, D, E) recorded in the same microscopic fields are shown. Data are representative of 62 and 60 cells, respectively, studied in three independent experiments for each agonist.

$[Ca^{2+}]_i$ in the same neurons. With the goal of validating this combinatorial approach, we stimulated GT1–7 cells with either high-K^+ medium or NMDA as before and recorded both of the above mentioned variables. As illustrated in Fig. 4, A–C, stimulation with high K^+ evoked an increase of $[Ca^{2+}]_i$ (*lighter lines*), which was followed shortly thereafter by exocytosis, as revealed by the increase of FM1–43 fluorescence (*bold lines*). We found that 89% of cells stimulated with this high-K^+ medium (n = 62, 3 experiments) responded with a $[Ca^{2+}]_i$ increase and 85% of these responders exhibited a rise in FM1–43 fluorescence. As further illustrated in Fig. 4, D–F, stimulation with NMDA also increased $[Ca^{2+}]_i$ and induced membrane retrieval in the cells imaged. This occurred in 93% of the cells studied (n = 60, 3 experiments). In those few instances in which there were no $[Ca^{2+}]$i responses following stimulation, there was also a lack of increase in FM1–43 fluorescence (data not shown). Taken together, these data demonstrate the feasibility of making essentially simultaneous measurements of calcium dynamics and secretory events in the same living GT1–7 neuron.

Having established the validity and utility of using FM1–43 to measure secretion alone or in combination with fura-2 imaging of calcium, we then set out to establish the relationship between these variables under basal conditions. In preliminary experiments, we measured FM1–43 fluorescence alone and found that roughly half of the cells (52%, n = 56 cells) exhibited spontaneous changes in secretion over time and that these had an average frequency of 1.1 ± 0.1 pulses/h consistent with that reported for GnRH release from perfused GT1 cultures (10, 11). This behavior of FM1–43

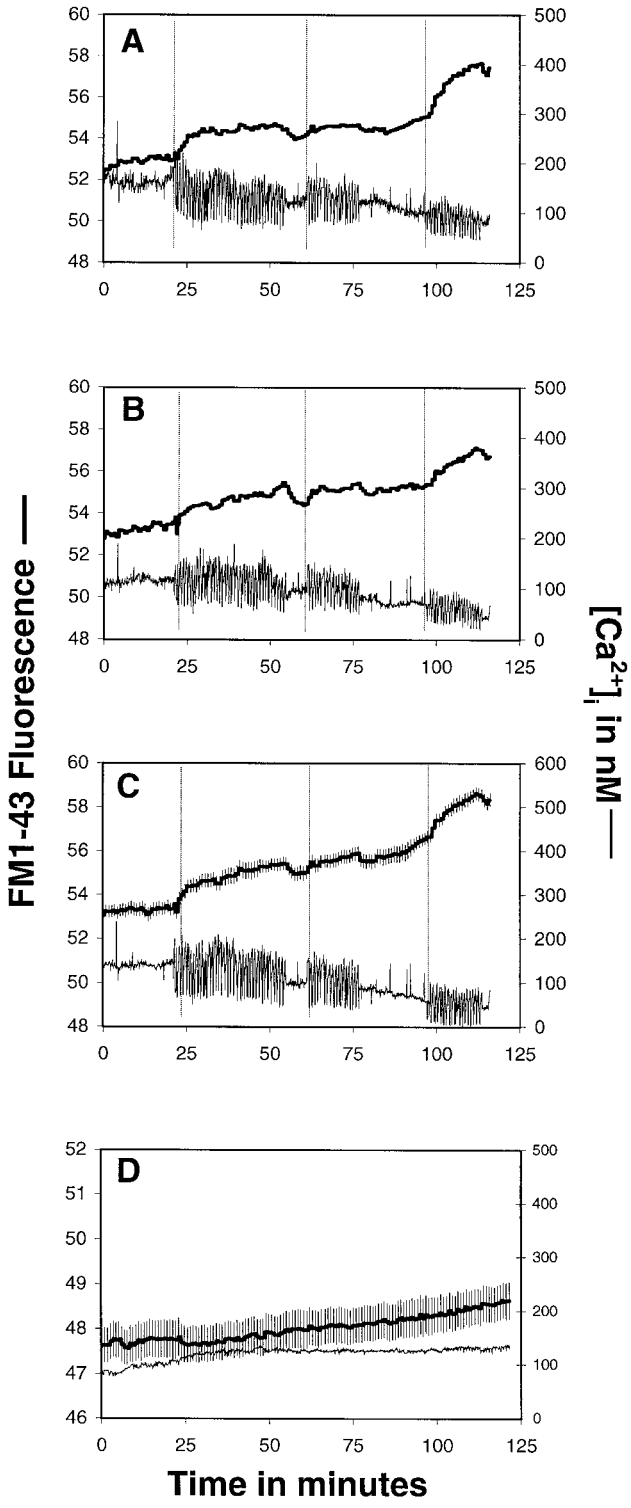

FIG. 5. Relationship between calcium and secretory dynamics in GT1–7 cells. Cells were subjected to simultaneous monitoring of both $[Ca^{2+}]_i$ (*lower lines*) and secretory activity (FM1–43 fluorescence, *upper lines*) for 2 h. The behavior of representative adjacent cells (A and B) and the average responses of all cells in the same microscopic field (C) are shown here. *Vertical lines* represent synchonic secretory episodes. This experiment is representative of three out of five in which we found synchronic secretory activity (n = 59 cells, three experiments). Shown in Panel D is the effect of nimodipine treatment on calcium oscillations and secretory activity (n = 43 cells, three experiments).

fluorescence is illustrated in Fig. 5 by representative example in adjacent cells (panels A and B) and the averaged traces of all cells in the same microscopic field (panel C). When calcium imaging was combined with analysis of secretion, we found that a change in FM1–43 fluorescence (indicating a secretory event) was always accompanied by the resumption of high level calcium oscillations after a period of relative quiescence. In these experiments, changes of both secretory activity and mode of $[Ca^{2+}]_i$ oscillations occurred at an average rate of 0.96 ± 0.15 (mean ± SEM, n = 59) pulses per hour, similar to that found in our preliminary experiments on secretion alone. Other investigators have reported that GT1 cells or cells in primary hypothalamic cultures exhibit spontaneous changes of calcium oscillatory profiles. Both Charles and Hales (17) and Spergel *et al.* (15) demonstrated the presence of spontaneous calcium oscillations in GT1–7 cells, which were associated with firing of spontaneous action potentials. Recently, the presence of periodic calcium oscillations was also demonstrated in individual primary neurons from monkeys (18). In each of these studies, it was proposed that changes in calcium oscillatory activity were associated with the rhythmic release of GnRH, but a direct demonstration of the relationship between secretion and calcium oscillations was not attempted. Our findings demonstrate that shifts occur in the overall oscillatory states of calcium and that these shifts are indeed linked to secretory events at a frequency consistent with that of GnRH pulsatility. Although all secretory events in our studies were accompanied by calcium shifts to a higher oscillatory state, we found that not all shifts in the functional state of calcium were linked to secretory episodes (62%, n = 69). Thus, calcium oscillatory transitions appear to be necessary, but not sufficient, for induction of GnRH release. There are several possible explanations for this. One possibility is that a secretory event does occur in concert with every shift to a higher oscillatory state, but the secretory bursts are not of sufficient magnitude to be detected with our system. Another explanation may be related to the differences in readiness for secretion from cell to cell. Some cells may contain abundant secretory granules and are able to respond to an appropriate stimulus for secretion such as a calcium oscillatory transition whereas others are not, even though such intracellular calcium signaling occurs. Whether or not they secrete, most of these cells do exhibit shifts in $[Ca^{2+}]_i$ oscillatory behavior (76%, n = 91) revealing an internal rhythm in the majority of cells in culture.

Our results obtained from analysis of multiple cells in the same microscopic fields further indicate that intercellular as well as intracellular signaling occurs with respect to calcium oscillatory and GnRH secretory activities. We found that adjacent cells usually (76%, n = 69) exhibited coordinated shifts in calcium oscillatory behavior. Moreover, the majority of secretory events (80%, n = 51) occurred in a synchronized manner among adjacent cells. In fact, we observed that 100% of the synchronic activity in secretion was associated with synchronic shifts in calcium oscillatory states, suggesting a causal relationship between these parameters. This possibility was confirmed when treatment with nimodipine (100 nM), a calcium channel blocker, abolished both periodic increases in FM1–43 fluorescence and spontaneous $[Ca^{2+}]_i$ os-

cillations (Fig. 5D). The mechanism by which this coordination is effected remains to be established. The possibility that GnRH neurons may communicate through synaptic transmission (25), gap junctions (26), or by release of diffusable substances (27, 28) has been under extensive investigation.

When taken together, our results invite the development of a working hypothesis to explain the generation of intrinsic secretory pulses in GT1–7 cells. It is clear from our findings that changes in oscillatory patterns and exocytotic events are closely linked in a temporal fashion. For the elaboration of a secretory pulse, it appears that relatively quiescent cells begin to display a characteristic pattern of high-frequency, high amplitude calcium oscillations. These oscillations are communicated and synchronized among adjacent cells and result in synchronized exocytotic events. Therefore, it is tempting to speculate that these tightly coupled events largely comprise the basis for the elusive "GnRH pulse generator."

Acknowledgments

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