

Effects of Cellular Interactions on Calcium Dynamics in Prolactin-Secreting Cells*

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ABSTRACT

Signals derived from other pituitary cells can have a dramatic effect on PRL gene expression and secretion by mammothropes. However, the intracellular mechanisms by which these effects are manifested on the target cell remain unexplored. Inasmuch as calcium is a key modulator of both gene expression and hormone export in mammothropes, we evaluated the effects of cell to cell contact *vs.* specific cellular interactions on calcium dynamics within these cells. This was accomplished by digital-imaging fluorescence microscopy of fura-2 in pituitary cells that were isolated in culture (singles) or adjoining one other cell (doublets). After calcium imaging, we then subjected cells to immunocytochemistry for PRL. Doublets were further categorized into mammothropes attached to another mammothrope (M-M) or to a nonmammothrope (M-nonM). We then calculated and compared Mean $[Ca^{2+}]_i$ values as well as Oscillation Indices (which

reflect the oscillatory behavior of cells) in singles and doublets and found that they were not different ($P > 0.05$). However, the phenotype of the adjoining cell had a profound influence on both of these calcium parameters, such that the presence of one mammothrope could consistently decrease ($P < 0.05$) the Mean $[Ca^{2+}]_i$ value (39.17 ± 3.83 *vs.* 56.24 ± 5.56 in M-nonM) and Oscillation Index (10.19 ± 1.76 *vs.* 21.21 ± 3.73 in M-nonM) of its neighboring counterpart. A more detailed analysis of oscillatory patterns in these cells revealed that nonoscillators were more abundant in M-M (23%) than in M-nonM (12%) doublets. Taken together, our results indicate that PRL-secreting cells convey a signal that *dampens* the oscillatory behavior of neighboring mammothropes. Thus, it appears that it is the phenotype rather than the physical presence of a neighbor that controls intercellular regulation of calcium dynamics among mammothropes. (*Endocrinology* 139: 2988–2993, 1998)

THERE IS NOW overwhelming experimental evidence to support the existence and importance of intercellular communication among cells of the anterior pituitary gland, and the PRL-secreting mammothrope has been a model of choice for studies of this phenomenon. Evidence in support of this view derives from studies demonstrating that secretion of a hormone, such as PRL, is influenced by the addition or elimination of other secretory cell types of the pituitary (see Refs. 1–3 for reviews). Moreover, cell density-dependent modulation of hormone secretion within pituitary cultures also suggests the existence of cell-cell interactions (4, 5). Indeed, mammothropes exist in a three-dimensional configuration of a tissue. Here, they are in intimate contact with other cells from which they receive a variety of signals that have the potential to regulate both gene expression and hormone release. Yet, despite the wealth of information favoring intercellular control of mammothrope function, surprisingly little is known about how these effects are transduced within the target cell. One particularly strong candidate to function as an intermediary in this process is calcium. Clearly, calcium plays a pivotal role in cell to cell adhesion of many cell types, including those of the pituitary, and cell-cell or cell-matrix adhesions have been shown to dramatically influence the expression of a number of hormonal genes (6–9). Like most pituitary cells, mammothropes exhibit spontaneous oscillations

of intracellular free calcium ($[Ca^{2+}]_i$) that are driven by electrical activity (10–12) and are positively correlated with basal hormone release (13, 14). In addition, pharmacological manipulation of $[Ca^{2+}]_i$ with agonists or antagonists evokes predictable changes of PRL release and messenger RNA accumulation (15, 16). Thus, calcium is a pivotal regulator of the PRL biosynthetic pathway from the point of gene transcription to that of hormone export, but the effects of cell to cell signaling on $[Ca^{2+}]_i$ dynamics remain unexplored.

In a recent study on intercellular communication and gene expression, we attempted to partition the effects upon a mammothrope of having a neighbor (cell to cell contact) as opposed to the nature (phenotype) of that neighbor, and found that the latter consideration was far more important for PRL gene expression than the former (17). Our strategy for that investigation was to make “real-time” measurements of PRL gene expression in living, primary pituitary cells and to follow this with immunocytochemistry (for *post facto* identification of the phenotype of a given cell or its neighbor). The paradigm for the present study was conceptually identical to this, with the only major departure being that digital-imaging fluorescence microscopy of a calcium-sensitive dye was substituted for analysis of gene expression. In this manner, we could attempt to determine whether the internal calcium dynamics of a mammothrope were also modulated by the presence and/or secretory nature of a neighbor.

Materials and Methods

Animals and cell culture

Anterior pituitary glands from primiparous lactating rats (days 5–12 postpartum) were collected after decapitation and enzymatically dispersed into single cells as described previously (18). Cells were allowed

Received December 22, 1997.

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* This study was supported by funds from NIH Grant DK-38215 and a USDA-Competitive Research Grant (95-37206-2438).

to attach onto poly-lysine coated, gridded, glass coverslips at a density of 250,000 cells/90 μ l defined medium [equal volumes of phenol-free M-199 with Nutrient mix F-12 (Life Technologies, Grand Island, NY), supplemented with 0.1% BSA, insulin-transferrin-selenium Premix, and antibiotics] and then placed into 35-mm plastic Petri dishes. After a 1-h attachment period, defined medium containing 5% FBS was added, and the cells were cultured at 37 C in 95% air: 5% CO₂ for 2 days until they were used for [Ca²⁺]_i imaging or gap-junction coupling experiments.

Digital-imaging fluorescence microscopy of fura-2

Loading of cells. On day 2 of culture, cells were washed and loaded with 2 μ M of the calcium-sensitive dye fura-2/AM (Molecular Probes, Eugene, OR) in phenol-free DMEM (Life Technologies) supplemented with 5 mM D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 5 mM sodium bicarbonate, 10 mM HEPES, and 34 mM sodium chloride (pH 7.4). After 1.5 h, cells were washed 3 times with the same medium and placed on the heated stage (at 37 C) of an inverted microscope equipped with a 40 \times objective. Cells were alternately epi-illuminated with a UV-light at 340 and 380 nm excitation wavelength. Light emitted above 520 nm was recorded for 20 min and analyzed using an Attofluor Ratio Vision System (Atto Instruments, Rockville, MD). For every raw image, a total of 4 videoframes (2 frames per wavelength) were acquired. Then, a ratio of the mean fluorescence intensity at 340/380 was calculated with an overall time resolution of 4 sec. Calibration of fluorescence ratios [R] was performed according to the formula described by Grynkiewicz *et al.* (19). Fura-2 standards were used to construct a calibration curve. This curve was then used to convert ratios [R] into [Ca²⁺]_i (20, 21).

Analysis of [Ca²⁺]_i. In the current study, we calculated two functional parameters of Ca²⁺ dynamics: the Mean [Ca²⁺]_i value and the Oscillation Index. As described elsewhere, these parameters reflect distinctly different ways of evaluating the same data set (22, 23). The Mean [Ca²⁺]_i was obtained by integrating values collected at 4-sec intervals and dividing them by time of measurement. The Oscillation Index, on the other hand, denotes the relative change in [Ca²⁺]_i during the collection period. We achieved this by calculating the difference in [Ca²⁺]_i values between consecutive intervals of measurement over the duration of sampling. Thus, it is possible that a given cell could have a high Oscillation Index (because of high frequency oscillations) even though its Mean [Ca²⁺]_i value is low, or *vice-versa*.

Immunocytochemistry. After [Ca²⁺]_i imaging, cells were washed and fixed with B-5 buffered formalin for 45 min. They were then subjected to immunocytochemical detection for PRL as controlled and described previously (18). PRL-positive cells were revealed after exposure to diaminobenzidine (Sigma, St. Louis, MO) solution for 6 min, and the same field previously used for [Ca²⁺]_i imaging was reidentified by its position on the coverslip, which was photoengraved with a numbered/lettered grid pattern.

Gap-junctions

The possible existence of functional gap-junctions between anterior pituitary cells was determined by comicroinjecting Lucifer yellow (4%) and dextran-rhodamine (0.1%) into one of a pair of adjoining cells. Microinjection was achieved through finely pulled, glass capillaries in series with an Eppendorf semiautomated microinjection system (17). Ten minutes post injection, coverslips were assembled into Sykes-Moore chambers that were filled with serum-free DMEM. This assembly was then transferred to the stage of an Axiophot microscope equipped with a UV light source and appropriate excitation-emission filters for fluorescent visualization of Lucifer yellow and rhodamine. As a positive control, we also coinjected confluent cultures of GT1-1 neurons (a clonal GnRH cell line) with the same ratio of fluorescent dyes used for pituitary cells. Previous reports by other investigators confirmed the presence of functional gap-junctions in the majority of these neurons (24).

Statistical analysis

Comparisons between any two treatment groups were made with a two-tailed, Student's *t* test and results were expressed as mean + SEM. Data reported in Table 1 were analyzed using a two-way ANOVA followed by a multiple comparisons test (Fisher's least significant difference). Differences were considered significant at *P* < 0.05.

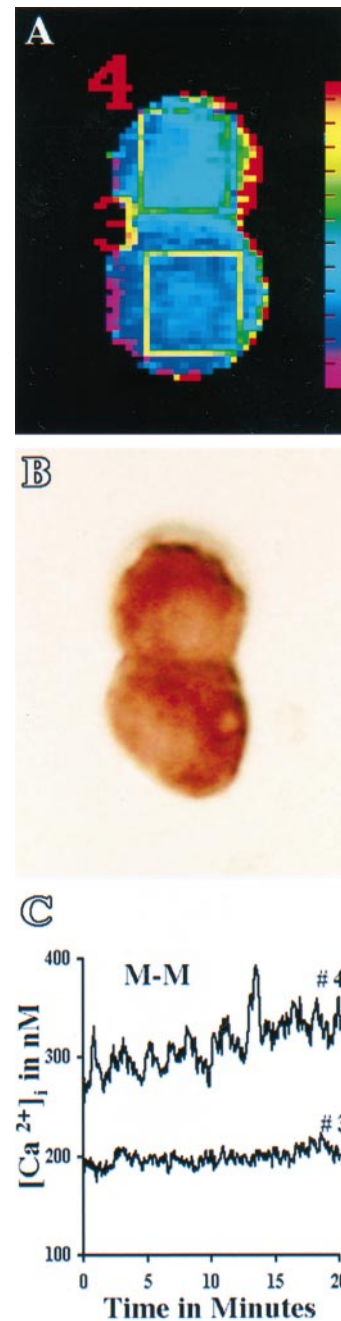


FIG. 1. Experimental protocol employed in this study. A, Anterior pituitary cells (one doublet shown) were subjected to [Ca²⁺]_i measurements by digital-imaging fluorescence microscopy of fura-2. The perimeter of each box demarcates the area in which all fluorescence measurements were recorded in that cell. Note that each box corresponds to individual cell and does not overlap with the adjacent one. Relative [Ca²⁺]_i is indicated in panel A by the warmer colors on the ascending pseudocolor scale to the right. B, After [Ca²⁺]_i measurements, the same cells were fixed and subjected to immunocytochemistry for PRL. In this particular instance, the doublet shown is comprised of two mammotropes. C, [Ca²⁺]_i transients of the same cells are illustrated. Note that the spontaneous oscillations were asynchronous.

Results

Our initial aim was to evaluate the effect of cell to cell contact on [Ca²⁺]_i oscillations in mammotropes. To this end,

we combined digital-imaging fluorescence microscopy of fura-2 with immunocytochemistry for PRL (Fig. 1, A and B). Identified mammotropes that were cultured in isolation (singles) or in physical contact with just one other cell (doublets) were then compared in terms of the aforementioned $[Ca^{2+}]_i$ parameters. As shown in Fig. 2, Oscillation Index values for single mammotropes and those in the doublet configuration were virtually identical ($P > 0.05$). Likewise, the Mean $[Ca^{2+}]_i$ values for singles and doublets were also indistinguishable ($P > 0.05$). These results demonstrate that cell to cell contact *per se* does not influence Ca^{2+} dynamics in mammotropes cultured under basal conditions.

After determining that the mere presence of another cell

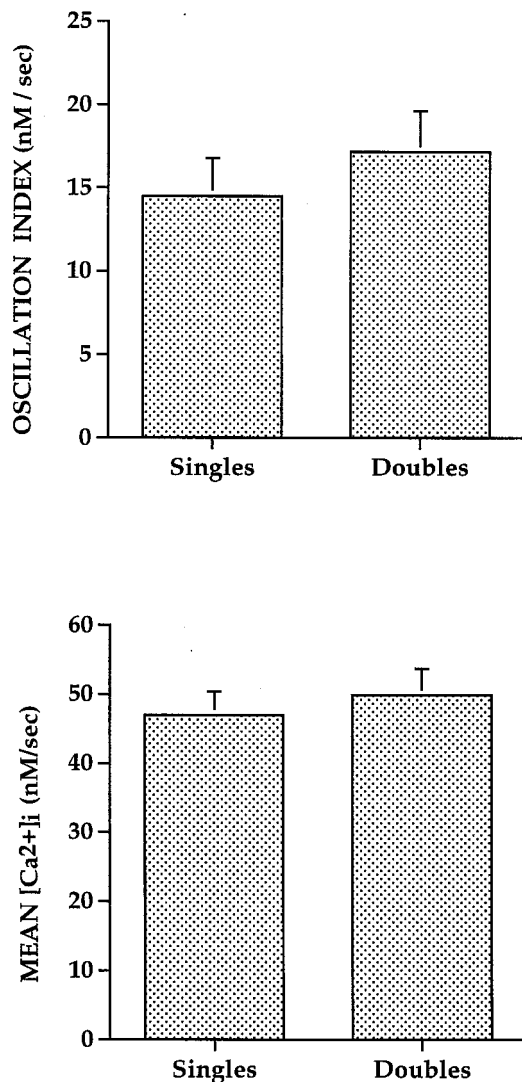


FIG. 2. Cell-to-cell contact has no effect on spontaneous $[Ca^{2+}]_i$ oscillations. Bars (mean + SEM) in panels A and B represent Oscillation Index and Mean $[Ca^{2+}]_i$ of mammotropes that were isolated from other cells (singles; $n = 160$ cells) or in contact with one other cell (doublets; $n = 141$ cells). Cells obtained from nine different dispersions were used. Note that the $[Ca^{2+}]_i$ value in the traces shown in Figs. 1C and 5 are higher than those depicted in Figs. 2 and 3 because the trace represents $[Ca^{2+}]_i$ values recorded at 4-sec intervals, whereas Mean $[Ca^{2+}]_i$ represents the sum of all recorded values divided by total time (*i.e.* nM/sec).

had no measurable effect on $[Ca^{2+}]_i$ in mammotropes, we next assessed whether the nature (phenotype) of the neighbor might have an influence. Accordingly, we performed $[Ca^{2+}]_i$ imaging on only those cells in the doublet configuration and followed this with immunocytochemical detection for PRL as before. Identified mammotropes in the doublet configuration were subsequently categorized on the basis of whether they were attached to another mammotrope (M-M) or to a nonmammotrope (M-nonM; in these cells $[Ca^{2+}]_i$ values of mammotropes and not those of nonmammotropes were analyzed). Interestingly, we found that both Oscillation Index and the Mean $[Ca^{2+}]_i$ values were significantly lower ($P < 0.05$) for the M-M doublets when compared with their M-nonM counterparts (Fig. 3). Inasmuch as "averaged" responses do not reflect the oscillatory behavior of individual mammotropes in the M-M or M-nonM categories, we plotted the Mean $[Ca^{2+}]_i$ value for each mammotrope against its corresponding Oscillation Index value. As shown in Fig. 4, doublets in the M-nonM configura-

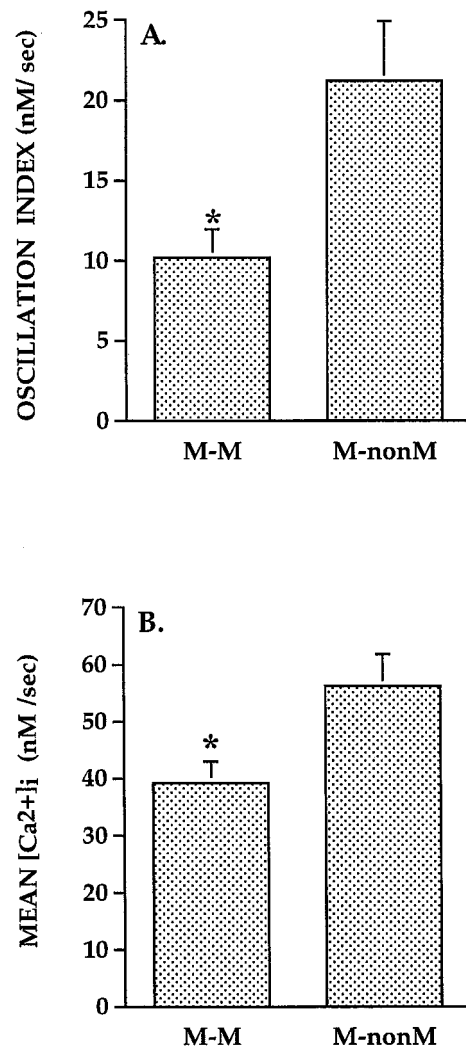


FIG. 3. Spontaneous $[Ca^{2+}]_i$ oscillations are influenced by the phenotype of a neighboring cell. Bars (mean + SEM) in panels A and B represent Oscillation Indices and Mean $[Ca^{2+}]_i$ values, respectively, for mammotropes in contact with another mammotrope (M-M; $n = 52$ cells) or a nonmammotrope (M-nonM; $n = 89$ cells). Cells obtained from nine different dispersions were used. *, $P < 0.05$ vs. M-nonM.

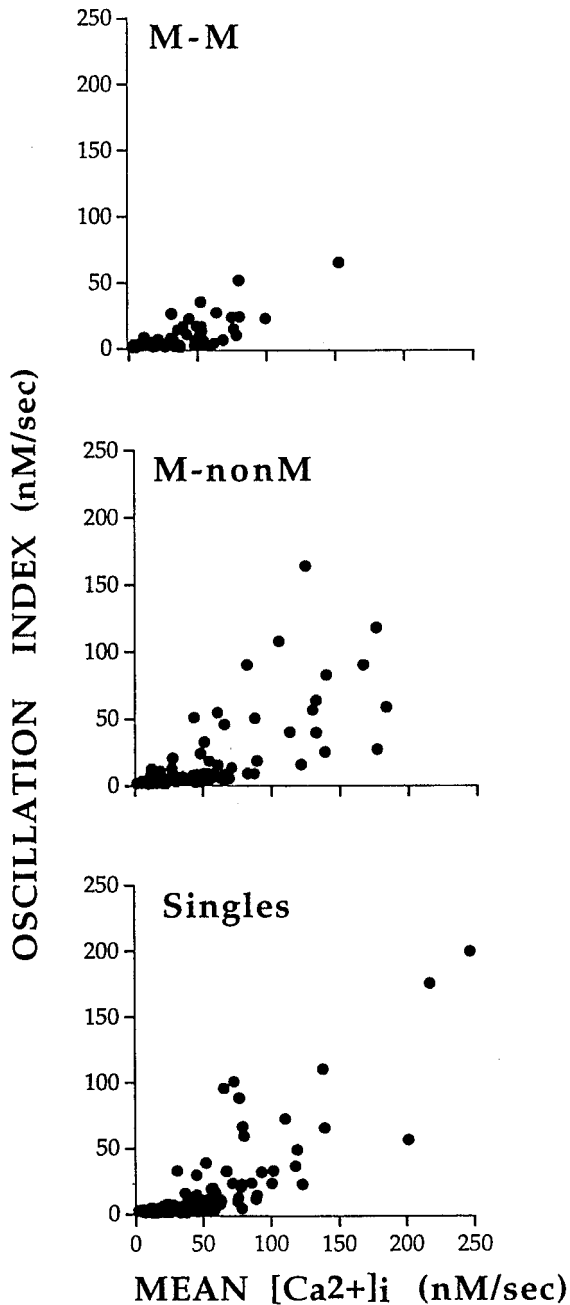


FIG. 4. Scatter plots of mammothropes maintained as singles, M-nonM, and M-M doublets are depicted here. Note that subpopulations of mammothropes that exhibit high Oscillation Indices and Mean $[Ca^{2+}]_i$ values are restricted to single mammothropes and those in the M-nonM configuration. The raw data from experiments shown in Figs. 2 and 3 were used for this plot.

tion and singles displayed a discrete subpopulation with a relatively high Oscillation Index and Mean $[Ca^{2+}]_i$ value. A corresponding group of highly active mammothropes was virtually absent for doublets in the M-M category. Thus, the inhibitory effect of one mammothrope upon another appears to be manifested preferentially upon those cells that are most active in terms of Ca^{2+} dynamics.

Having established that the average oscillatory activity of M-M cells is lower than for M-nonM cells, we then set out to

determine the basis for these differences. This was accomplished by analyzing the pattern of spontaneous $[Ca^{2+}]_i$ oscillations in M-M and M-nonM doublets. Previous studies conducted in our laboratory successfully characterized spontaneous $[Ca^{2+}]_i$ oscillations of mammothropes into the following major patterns: (A) nonoscillating or "silent"; (B) high frequency, low amplitude oscillations; and (C) high frequency, high amplitude oscillations (20). In the present study, we found that although M-M and M-nonM cells displayed similar types of oscillatory profiles (Fig. 5), the relative proportions exhibiting patterns A and C as well as the corresponding Oscillation Indices and Mean $[Ca^{2+}]_i$ values of the latter profile were strikingly different (Table 1). Specifically, nonoscillators were 2-fold more abundant for M-M (23%) than for M-nonM (12%) cells. More importantly, the Oscillation Index and Mean $[Ca^{2+}]_i$ of "C" type oscillators were 2-fold higher in M-nonM than in M-M cells. Thus, the higher frequency and amplitude of spontaneous $[Ca^{2+}]_i$ oscillations seen in those mammothropes associated with nonmammothropes appear to contribute, at least in part, to the increased oscillatory activity observed in this subset of PRL secretors. Conversely, decreased oscillatory activity in neighboring mammothropes may in part be due to an increase in the proportion of nonoscillators in this configuration.

Although our results showed clearly that Ca^{2+} dynamics of mammothropes were influenced by the neighboring cell, they did not provide any insights as to whether the communication was paracrine or juxtacrine. In an attempt to distinguish between these possibilities, we coinjected Lucifer yellow and dextran-rhodamine into cells maintained as doublets. The former fluorescent dye passes readily through gap-junctions, whereas the cell membrane is impermeable to the latter. Thus, the rhodamine complex served both as a means for identifying the injected cell and as a control for membrane integrity. Of 137 doublets studied in this manner, only three exhibited functional gap-junctions, whereas the remainder showed no dye-transfer between adjoining cells. Inasmuch as 60–65% of the cells in our pituitary cultures were PRL-secretors, these data demonstrate clearly that juxtacrine signaling mediated by gap-junction coupling cannot account for the interactions we observed between mammothropes and their neighbors. In contrast, gap-junction coupling was observed in the majority of GT1-1 neurons coinjected with the same mix of dyes. A final point worthy of mention is that we found no evidence to suggest that adjacent cells were electrically coupled. As illustrated by the representative example in Fig. 1C, cells in the M-M configuration (or M-nonM, for that matter) were just as likely to exhibit dissimilar as similar patterns of $[Ca^{2+}]_i$ oscillations.

Discussion

Our results provide compelling evidence that cell to cell communication has a dramatic influence on $[Ca^{2+}]_i$ dynamics within living mammothropes. To be more specific, we found that the physical presence of a neighboring cell *per se* had no influence on the Ca^{2+} activity of a given mammothrope but that the secretory nature of the neighbor was of paramount importance. Indeed, when one mammothrope was in close physical apposition with another, there was a striking

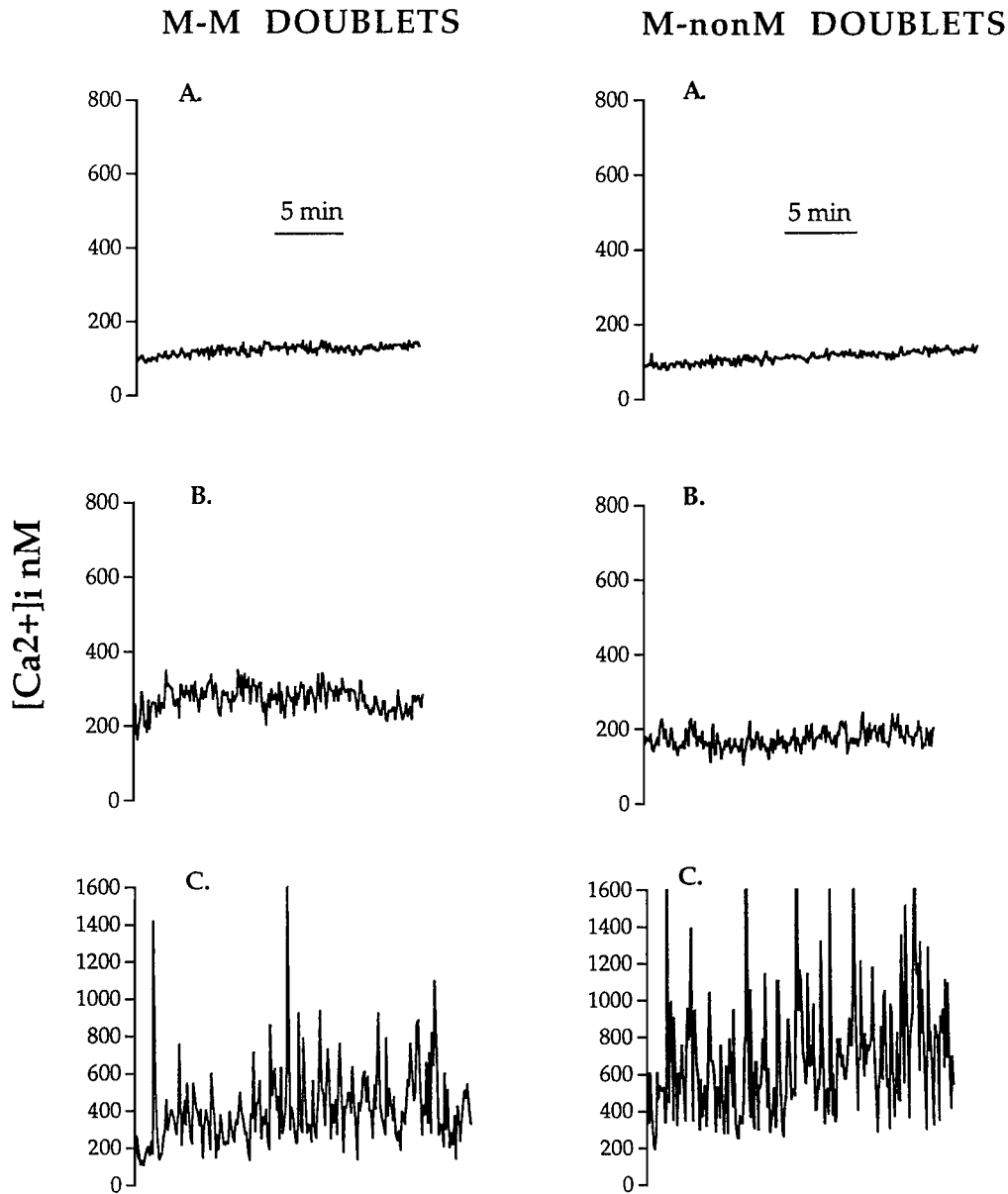


FIG. 5. Patterns of spontaneous $[Ca^{2+}]_i$ oscillations in M-M and M-nonM doublets. All identified mammothropes were subgrouped based on their oscillatory patterns into (A) nonoscillating, (B) high frequency, low amplitude oscillations, and (C) high frequency, high amplitude oscillations. Representative traces of each profile are depicted for M-M and M-nonM cells. Note that there were no differences between A and B type oscillators, but that the amplitude of "C" type oscillators tended to be higher in M-nonM than in M-M cells.

reduction of the Oscillation Index (51.9%), and, to a lesser extent, an attenuation of the Mean $[Ca^{2+}]_i$ value (30.3%). Inasmuch as the former parameter is a more direct and reliable indicator of frequency/amplitude changes than the latter, it seems reasonable to propose that most of this inhibitory effect was attributable to a diminution of oscillatory activity as opposed to modulation of the average amount of Ca^{2+} present. Moreover, analysis of spontaneous $[Ca^{2+}]_i$ oscillations indicate that inhibition of such activity in M-M cells is not attributable to the presence or absence of discrete oscillatory phenotypes exhibited by M-nonM doublets. Instead, it is due to a different distribution of mammothropes exhibiting each pattern. To be more specific, there was a much higher proportion of nonoscillatory cells in the M-M

group than in the M-nonM category, and this increment was achieved largely at the expense of the high frequency, high amplitude oscillators. It appears, then, that PRL-secreting cells convey a signal that *dampens* the oscillatory behavior of neighboring mammothropes. Of course, an alternative interpretation that deserves consideration is that nonmammothropes emit a signal that *stimulates* $[Ca^{2+}]_i$ oscillations in adjoining mammothropes. If this were the case, one would expect to see a preponderance of cells with both high Oscillation Indices and $[Ca^{2+}]_i$ values when mammothropes were attached to nonmammothropes. In reality, this was not the case at all: the scatter plots for M-nonM doublets and singles (Fig. 4) were extremely similar. Thus, although we cannot completely exclude the possibility of a stimulatory

TABLE 1. Parameters of calcium dynamics in M-M and M-nonM doublets

| | A | B | C |
|---------------------------------------|--------------|--------------|----------------------------|
| M-M | | | |
| % | 23 | 50 | 19.2 |
| Oscillation Index | 1.97 ± 0.22 | 6.52 ± 0.79 | 29.93 ± 5.26 ^a |
| Mean [Ca ²⁺] _i | 21.75 ± 4.27 | 34.19 ± 4.04 | 67.63 ± 11.2 ^c |
| M-nonM | | | |
| % | 12.3 | 52 | 25.8 |
| Oscillation Index | 2.14 ± 0.09 | 5.62 ± 0.34 | 65.18 ± 9.76 ^b |
| Mean [Ca ²⁺] _i | 13.6 ± 2.31 | 34.81 ± 3.23 | 119.29 ± 12.5 ^d |

The proportions and values for cells exhibiting each of three major oscillatory patterns are presented: A, nonoscillatory; B, high frequency, low-amplitude oscillations; C, high frequency, high amplitude oscillations. A minority of mammothropes (8–10%) changed from one pattern to another during the collection period and therefore were not classified into any specific pattern. They were, however, included in the total cell number for the purpose of calculating percentages. Values with *different letters* are significantly ($P < 0.05$) different from each other.

signaling mechanism to explain this phenomenon, our data are more consonant with an inhibitory one.

Results conceptually similar to these were obtained in our previous study in which we found that M-M doublets displayed a greatly reduced level of PRL gene expression compared with their M-nonM counterparts (17). The striking similarities of these responses raise the possibility that inhibitory interactions between mammothropes on PRL gene expression are mediated by changes of [Ca²⁺]_i oscillations. In support of this possibility are observations that pharmacologic diminution of [Ca²⁺]_i leads to a decrease of PRL gene expression, whereas an increase of [Ca²⁺]_i has an opposite effect (25–27).

How might such signals be transmitted from one mammothrope to another? One can envision at least three possible mechanisms. The first of these, gap-junctions, is rendered implausible by our direct observation that this form of communication was exceedingly rare under the present experimental conditions. A second possibility is electrical coupling of adjacent cells. This scenario also seems highly unlikely because electrically coupled cells typically display synchronized oscillations, and we did not find any evidence for this in the present study, even in M-M doublets. A final possibility entirely consistent with our results is paracrine communication. Indeed, there is a wealth of information supportive of paracrine actions on mammothropes (1–3, 28), and our efforts are currently focused on isolating and identifying this putative signal.

Although the physiologic relevance of inhibitory interactions among mammothropes remain to be unequivocally established, they may serve to hold PRL gene expression and secretion in check until the mammothrope is presented with a challenge from other regulatory (hypophysiotropic) signals. This may be particularly relevant in areas where there is a concentration of mammothropes such as near the poles of the anterior pituitary (29).

Acknowledgments

The authors thank Dr. R. I. Weiner for providing the GT1–1 cells and Dr. L. Nuñez for sharing her cultures with us. We would also like to thank W. J. Faught for assistance with microinjection studies and J. Nicholson for access to the Attofluor Vision system. We also thank Drs. S. T. Willard, M. D. Amstutz, and L. Nuñez and Ms. A. Gore for expert critique of the manuscript.

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