Dynamic Changes in Spontaneous Intracellular Free Calcium Oscillations and Their Relationship to Prolactin Gene Expression in Single, Primary Mammotropes

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Cytosolic calcium plays a critical role in the control of a number of genes, including that of the pituitary hormone PRL. Cells that secrete this hormone, termed mammotropes, display spontaneous oscillations of intracellular free calcium ($[Ca^{2+}]_i$) that are positively correlated to PRL release. However, the precise contribution of calcium signaling to the expression of any gene including PRL has remained obscure owing to the requirement for and lack of a strategy for monitoring both of these dynamic variables (gene expression and $[Ca^{2+}]_i$ oscillations) in the same living cell. In the present study, we overcame this technical limitation by making real-time measurements of PRL gene expression in transfected, primary rat mammotropes previously subjected to $[Ca^{2+}]_i$ determinations by digital imaging fluorescence microscopy of fura-2. Our results showed that the majority of mammotropes (75%) exhibited distinct oscillatory behaviors that could be subgrouped on the basis of frequency/amplitude of $[Ca^{2+}]_i$ changes, whereas the remainder (25%) were quiescent (nonoscillatory). Interestingly, most mammotropes displayed spontaneous transitions between oscillatory and quiescent states over the course of several hours. As a consequence of this oscillatory plasticity, there was not a positive correlation between $[Ca^{2+}]_i$ dynamics and gene expression at any point in time, as would be predicted by studies with entire populations of cells. Instead, the relationship was distinctly inverse, suggesting that dynamic changes in PRL gene expression may be regulated by temporally dissociated transitions between quiescent and oscillatory states. (Molecular Endocrinology 12: 87–95, 1998)

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INTRODUCTION

Dynamic changes in the concentration of intracellular free calcium ($[Ca^{2+}]_i$) play a pivotal role in the control of many physiological functions (1). One of these is stimulus-secretion coupling, and the PRL-secreting mammotrope has been studied extensively in this regard (2–4). Indeed, mammotropes, like other pituitary cells, display spontaneous oscillations of $[Ca^{2+}]_i$ that are driven by electrical activity and modulated by a variety of hypophysiotropic agents (5). Such oscillations are positively correlated with hormone release, in that exocytotic events are invariably associated with increased oscillatory activity (6). There is also ample evidence to indicate that cytosolic calcium imposes a major regulatory influence on the initiation of the PRL biosynthetic processes as well as the final step. The case for calcium regulation of transcription in mammotropes is compelling and derives from observations that physiological agonists (*e.g.* TRH) that stimulate PRL gene expression also evoke increases in $[Ca^{2+}]_i$ in pituitary cell lines and normal mammotropes (7), and conversely, that abolition of spontaneous or induced $[Ca^{2+}]_i$ oscillations (by removal of extracellular calcium or exposure to calcium antagonists) decreases PRL gene expression (8). Moreover, the PRL gene promoter possesses several regulatory sequences that are reported to confer transcriptional responsiveness to calcium (9, 10). Thus, calcium functions as a critical second messenger that controls key regulatory checkpoints that span the entire length of the PRL secretory pathway.

Our depth of knowledge about the regulatory role of calcium in stimulus-secretion coupling is attributable largely to the fact that many investigations have been carried out at the single cell level to obviate the confounding effects of heterogeneous responses. Thus, the capacity to make combined measurements of exocytosis (by changes in membrane capacitance or reverse hemolytic plaque development) and $[Ca^{2+}]_i$ os-

cillations (by microfluorimetry or electrophysiological measurements) on the same cell has contributed greatly to our knowledge about the temporal aspects of calcium dynamics and hormone export (11–13). Unfortunately, our level of understanding about the relationship between $[Ca^{2+}]_i$ changes and PRL gene expression is less well developed due to the fact that the consensus experimental paradigm in this area has been to pharmacologically manipulate $[Ca^{2+}]_i$ within entire cultures of pituitary cells and then measure steady state levels of PRL messenger RNA (mRNA) by Northern analysis (14, 15). Although much has been learned with this approach, further progress would be facilitated greatly by access to a single cell strategy that would enable the monitoring of these two extremely dynamic variables ($[Ca^{2+}]_i$ and gene expression) in the same cell. In the present study we circumvented this technical constraint by subjecting the same individual, living mammotropes to digital imaging fluorescence microscopy of fura-2 (for monitoring $[Ca^{2+}]_i$ dynamics) followed by real-time measurement of PRL gene expression (16). The latter technique, which is based on quantification of photonic emissions emanating from single mammotropes transfected with a PRL promoter-driven luciferase construct, provides a reliable estimate of the relative level of gene activity within a living cell (17). Results obtained with this combined approach show, interestingly, that calcium oscillatory behavior within a mammotrope can vary greatly over time. As a consequence, there is not a positive correlation between oscillatory activity and PRL gene expression in single mammotropes at a given point in time, as would be predicted by studies with entire populations of cells.

RESULTS

Characterization of Spontaneous $[Ca^{2+}]_i$ Oscillations in Normal Mammotropes

To establish oscillatory subtypes, we subjected anterior pituitary (AP) cells in primary culture to $[Ca^{2+}]_i$ measurements by digital imaging fluorescence microscopy for about 30 min. Mammotropes were identified *post-facto* by their ability to express the luciferase structural gene driven by the PRL promoter. We found that all identified mammotropes exhibited one of the four patterns illustrated in Fig. 1. To be specific, about a quarter of the cells showed no spontaneous $[Ca^{2+}]_i$ oscillations during the entire $[Ca^{2+}]_i$ measurement period (quiescent cells; Fig. 1A). The remaining cells exhibited discrete patterns of spontaneous $[Ca^{2+}]_i$ oscillations, the most common of which was characterized by high frequency, low amplitude $[Ca^{2+}]_i$ transients (Fig. 1B). A less common pattern was characterized by high frequency, high amplitude $[Ca^{2+}]_i$ oscillations (Fig. 1C). Finally, a minority subset of mammotropes displayed slow waves of $[Ca^{2+}]_i$ (Fig. 1D). For quantification of $[Ca^{2+}]_i$ oscillations in normal

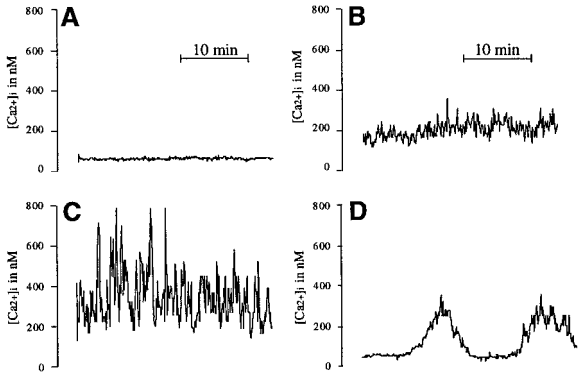

Fig. 1. Patterns of Spontaneous $[Ca^{2+}]_i$ Oscillations in Normal Mammotropes

AP cells in primary culture were subjected to $[Ca^{2+}]_i$ measurements for 30 min. Mammotropes were identified thereafter by their ability to express the luciferase structural gene under the control of the PRL gene promoter, as stated in *Materials and Methods*. Cells were categorized into subgroups on the basis of whether they were quiescent (nonoscillating; A), or exhibited one of three oscillatory phenotypes (B–D). Cells displaying $[Ca^{2+}]_i$ changes higher than 50 nM but lower than 500 nM were assigned to the low amplitude, high frequency group (B). For the high amplitude, high frequency category, we selected those cells exhibiting $[Ca^{2+}]_i$ changes higher than 500 nM (C). Finally, those cells exhibiting slow waves of low amplitude $[Ca^{2+}]_i$ oscillations comprised their own category (D). The examples shown are representative of those obtained when 112 mammotropes were studied in 10 independent experiments.

mammotropes, we employed two parameters previously developed to analyze $[Ca^{2+}]_i$ levels and oscillations in pituitary cells: the mean $[Ca^{2+}]_i$ value and the oscillation index (18, 19). The former represents an average of the $[Ca^{2+}]_i$ values during the measurement period. The oscillation index reflects an average of the changes in $[Ca^{2+}]_i$ and is largely independent of the actual level of $[Ca^{2+}]_i$. Table 1 shows the averaged values of mean $[Ca^{2+}]_i$ and oscillation index for the cells pooled according to the various $[Ca^{2+}]_i$ oscillatory patterns shown in Fig. 1.

Relationship between $[Ca^{2+}]_i$ Oscillations and PRL Gene Expression in Individual Mammotropes

Having established the heterogeneous nature of $[Ca^{2+}]_i$ oscillations in normal mammotropes, we next attempted to elucidate the relationship between $[Ca^{2+}]_i$ oscillations and transcriptional activity of the PRL gene in the same living mammotropes. To this end, AP cells previously microinjected with the rat PRL-LUC plasmid and used for $[Ca^{2+}]_i$ measurements were subjected immediately thereafter to photon counting for quantification of PRL gene expression. The results of this combined protocol are illustrated in Fig. 2. Specifically, a fluorescence image was captured after $[Ca^{2+}]_i$ measurements to identify the cells studied (Fig. 2A). Then, specific photonic emissions

Table 1. Parameters of Spontaneous $[Ca^{2+}]_i$ Oscillations and PRL Gene Expression in Normal Mammotropes

Pattern	%	Mean $[Ca^{2+}]_i$	Oscillation Index	Photonic Emissions
A	25	16.0 ± 1.3	1.8 ± 0.1	2960 ± 541
B	42	30.2 ± 2.4[a]	5.3 ± 0.4[a]	1531 ± 286[a]
C	25	71.1 ± 6.7[a,b]	36.1 ± 5.0[a,b]	1187 ± 227[a]
D	8	32.0 ± 5.6[a]	6.4 ± 1.8[a]	1666 ± 717

Transfected AP cells were subjected to measurements of $[Ca^{2+}]_i$ and photonic emissions (reflective of PRL gene expression). The proportions of cells exhibiting each of the four $[Ca^{2+}]_i$ oscillatory phenotypes (A–D) described in Fig. 1. are shown with the values for mean $[Ca^{2+}]_i$, oscillation index, and photonic emissions. These data were obtained for 112 mammotropes from 10 independent experiments.

[a] $P < 0.05$ *vs*. A.

[b] $P < 0.05$ *vs*. B.

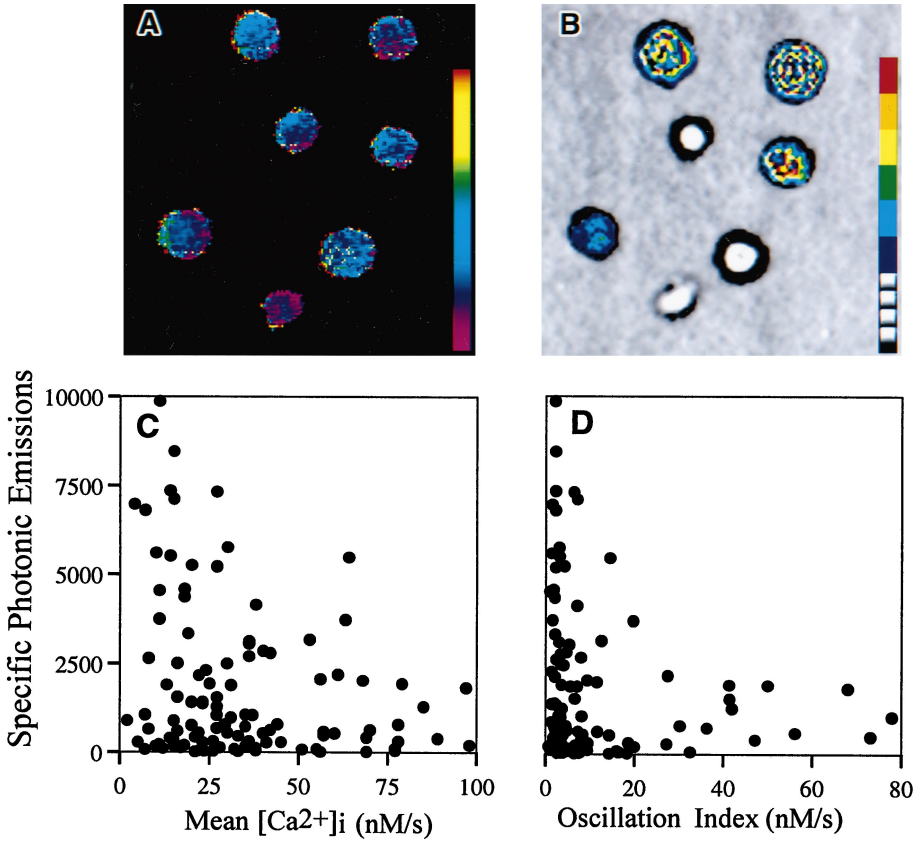

Fig. 2. Concurrent Measurements of $[Ca^{2+}]_i$ and PRL Gene Expression in the Same Cells

AP cells within a grid were microinjected with a plasmid containing the luciferase structural gene under the control of the PRL gene promoter. After 2 days, microinjected cells were relocated and subjected to $[Ca^{2+}]_i$ measurements by digital imaging fluorescent microscopy for 30 min. Then, a fluorescence image was captured for reference purposes (A). Immediately after $[Ca^{2+}]_i$ measurements, the same cells were subjected to quantification of luciferase/luciferin-generated photonic emissions reflective of PRL gene expression (B). These photonic emissions were plotted against the mean $[Ca^{2+}]_i$ level (C) or oscillation index (D) computed from the $[Ca^{2+}]_i$ traces obtained from the same cells. The data shown are from 10 independent experiments (n = 112 cells).

from the same cells were quantified, and the resulting data were stored as computer files for latter analysis (Fig. 2B). To explore a possible relationship between $[Ca^{2+}]_i$ or $[Ca^{2+}]_i$ oscillations and PRL gene expression, specific photonic emissions for each cell were plotted against its corresponding mean $[Ca^{2+}]_i$ value (Fig. 2C) or oscillation index (Fig. 2D). Consistent with our previous observation (17), we found that basal expression of the PRL promoter-driven reporter construct varied considerably from cell to cell, with values ranging from 50–10,000 specific photonic emissions/10 min·cell. Interestingly, cellular values for mean $[Ca^{2+}]_i$ and oscillation index were likewise extremely heterogeneous, in that they ranged from 2 to

about 100 nM/s for both parameters. Surprisingly, there was not a positive correlation between the degree of PRL gene expression (photonic emissions) and either of the two calcium parameters quantified. However, it is noteworthy that the highest levels of PRL gene expression were generally associated with cells exhibiting the lowest values of mean $[Ca^{2+}]_i$ or oscillation index. A possible relationship between oscillatory phenotype and gene expression was next explored by calculating the average level of photonic emissions for each of the oscillatory subtypes described in Fig. 1. As shown in Table 1 (*last column*), the relative degree of gene expression was very similar for the three categories of mammotropes that exhibited spontaneous $[Ca^{2+}]_i$ oscillations (groups B-D). However, when these values were considered collectively and compared with those of their nonoscillatory counterparts, the latter was found to be 2-fold higher than that of the former (Fig. 3). In contrast, mean $[Ca^{2+}]_i$ values were about 3-fold greater for oscillatory than for nonoscillatory cells (Fig. 3). Thus, at any point in time, there was a distinct inverse relationship between $[Ca^{2+}]_i$ and PRL gene expression within individual cells.

One might argue that the observed relationship between PRL gene expression and calcium dynamics is attributable to the protracted half-life of luciferase in our system and/or the time required for synthesis of new luciferase reporter enzyme. The former possibility can be discounted by the data presented in Fig. 4, which shows that the half-life of luciferase in primary mammotropes is relatively short (~60 min). The issue of the time course for luciferase synthesis is extremely difficult to address directly because we currently lack

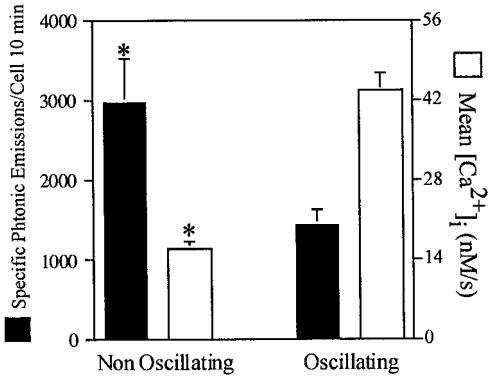

Fig. 3. The Level of PRL Gene Expression Is Higher in Quiescent Mammotropes Than in Cells Exhibiting Spontaneous $[Ca^{2+}]_i$ Oscillations

All of the mammotropes studied were pooled into two groups: quiescent (n = 28) and oscillating cells (n = 84). Specific photonic emissions reflective of PRL gene expression (*solid bars*) and mean $[Ca^{2+}]_i$ values (*open bars*) were then averaged for both groups. Quiescent cells exhibited a significantly higher level of PRL gene expression than those cells displaying oscillations of $[Ca^{2+}]_i$ (*, $P < 0.05$ *vs.* oscillating cells).

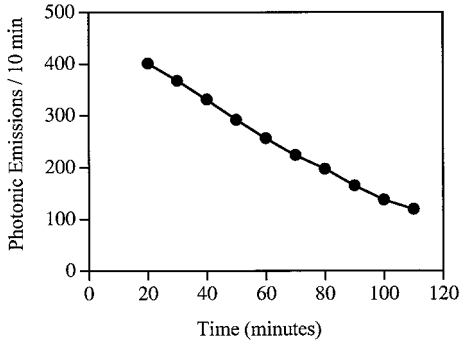

Fig. 4. Functional Half-Life of Luciferase

AP cells in primary culture were microinjected with purified firefly luciferase protein (Sigma) at a dose (1 mg/ml), which was designed to achieve a level of photonic activity comparable to that detected in cells transfected with our reporter plasmid. Immediately after microinjection, coverslips were immersed in luciferin-containing medium and placed in our photon-counting system to monitor luciferase activity over time. The mean activity of 30 single cells is shown. The results obtained in this experiment are representative of those from two others.

of a method capable of discriminating recently synthesized luciferase from that already present in the same living cell. Nevertheless, one way to approach the problem is to measure the time required to first detect light after transfection of cells with the PRL promoter-driven luciferase plasmid, and our results indicate that this can frequently be achieved within the first hour after transfection (data not shown). Thus, the time required for synthesis and degradation of reporter enzyme might contribute to the lack of correlation between gene expression and calcium dynamics, but probably not to a major extent.

Spontaneous $[Ca^{2+}]_i$ Oscillations Can Fluctuate over Time

One possible explanation for the lack of positive correlation between $[Ca^{2+}]_i$ oscillatory behavior and PRL gene expression is that the former may change over time. In this scenario, the level of gene expression at any particular point in time might actually reflect changes in $[Ca^{2+}]_i$ oscillatory behavior that occurred up to several hours earlier. To test this line of reasoning, we subjected the same identified mammotropes to $[Ca^{2+}]_i$ measurements on three separate occasions over a 24-h period. Representative examples of the results obtained are presented in Fig. 5. We found that most cells maintained the same $[Ca^{2+}]_i$ oscillatory pattern over a 3-h period (*e.g.* Fig. 5, cells A, C, and D), although some mammotropes changed their $[Ca^{2+}]_i$ oscillatory pattern during this time frame (*e.g.* cell B in Fig. 5). In contrast, cells exhibited dramatic changes in oscillatory patterns over longer (24-h) periods of time (see cells A, B, and C as opposed to D in Fig. 5). To be more specific, the fraction of cells that changed from one of the four oscillatory patterns to another in-

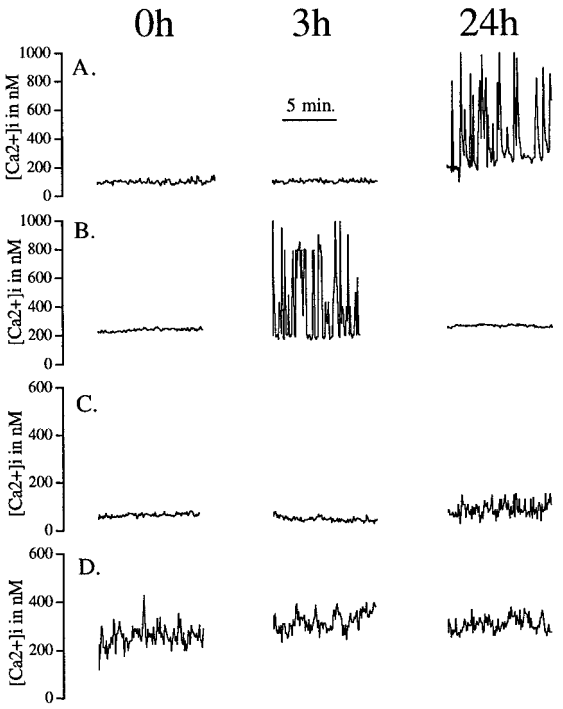

Fig. 5. Individual Mammotropes Exhibit Changes in the Pattern of $[Ca^{2+}]_i$ Oscillations over Time

Identified mammotropes were subjected to 10-min $[Ca^{2+}]_i$ measurements at the three times indicated. The pattern of $[Ca^{2+}]_i$ oscillations changed over time in most mammotropes. These four traces (A–D) are representative examples of 56 individual mammotropes that were studied in three independent experiments.

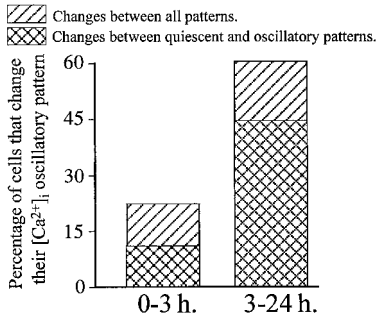

Fig. 6. Percentage of Mammotropes That Exhibit Changes in the Patterns of $[Ca^{2+}]_i$ Oscillations over Time

Mammotropes that were subjected to $[Ca^{2+}]_i$ measurements at three separate times (0, 3, or 24 h) were analyzed to determine whether they changed their $[Ca^{2+}]_i$ oscillatory pattern from one of the four identified previously (Fig. 1) to another. After 3 h, only 22% of the mammotropes changed their pattern of $[Ca^{2+}]_i$ oscillations. This percentage increased to 60% after 24 h. The percentage of cells exhibiting transitions between quiescent and oscillatory patterns represented 50% and 75% of all of the changes at 3 and 24 h, respectively. Therefore, transitions between quiescent and oscillatory patterns account for the majority of changes observed.

creased from 22% between 0–3 h to 60% between 0–24 h (Fig. 6). Further analysis of the same dataset revealed that the majority of these changes (50% and 75% at 3 and 24 h, respectively) was attributable to transitions between quiescent and oscillatory states or *vice versa*, rather than to changes among the four basic oscillatory phenotypes. Despite the oscillatory plasticity exhibited by individual mammotropes, the relative proportion of all cells that displayed the four $[Ca^{2+}]_i$ oscillatory patterns (illustrated in Fig. 1) remained remarkably constant during the three $[Ca^{2+}]_i$ measurement periods (data not shown). These results indicate clearly that spontaneous $[Ca^{2+}]_i$ oscillations remain quite constant over a few hours, but tend to change over longer periods. Moreover, most transitions result from excursions between quiescent and oscillatory states.

DISCUSSION

The overall goal of this study was to elucidate the relationship between calcium dynamics and PRL gene expression in single mammotropes. *A priori*, we envisioned at least three aspects of calcium signaling that might be relevant to regulation of the PRL gene: the overall pattern of $[Ca^{2+}]_i$ oscillations, the mean $[Ca^{2+}]_i$ value, and the oscillation index. Inasmuch as all three variables can be quantified from the same dataset, we chose as a point of departure an experiment aimed at characterizing in a narrow frame time (30 min) the overall patterns of $[Ca^{2+}]_i$ oscillations in single living mammotropes. We found that the majority (75%) of mammotropes from lactating rats exhibited distinct oscillatory behaviors that were amenable to subgrouping on the basis of differences in the frequency/amplitude of $[Ca^{2+}]_i$ changes. In contrast, a minority subset accounting for only 25% of all the mammotropes studied were relatively quiescent (*i.e.* nonoscillatory). Our results differ from those of Hinkle and co-workers (20), who found that spontaneous oscillations were common among clonal, PRL-secreting GH_3 cells (63%), but were rarely observed in primary cultures derived from retired breeder, female rats (21). Lewis *et al.* (22), on the other hand, found that 22% of cycling female mammotropes displayed oscillations, and Ho *et al.* (23) reported a value of 43% for mammotropes derived from the same physiological model. Given that oscillatory activity is positively correlated to secretion (24), and that the predicted rank order for the rate of PRL export from mammotropes is lactators $>$ cycling females $>$ retired breeders, it seems reasonable to propose that the preponderance of spontaneous oscillators observed in the present study is a simple reflection of the highly active secretory status of mammotropes from lactators.

The time-resolved analysis of calcium dynamics and gene expression in the same cell was made possible by the combined application of digital imaging fluorescence microscopy of fura-2 with real-time mea-

surement of PRL gene expression. That the latter strategy provides a reliable estimate of gene activity in living cells is evidenced by our previous observations that expression of the transfected reporter construct is highly cell specific (restricted to mammotropes in this instance) and that treatments with agents known to either increase or decrease PRL gene expression by entire populations of cells have identical, predictable effects on the rates of photonic emissions from single, transfected mammotropes (17). Moreover, our observation that the functional half-life of luciferase in this system is fairly short (60 min) lends confidence that rapid changes in the rate of gene expression (reflected by newly synthesized luciferase) would not be obscured by a high background of preexisting reporter activity. Armed in the present study with this powerful and responsive tool, we measured PRL gene expression in living cells previously subjected to measurement of calcium dynamics and found, quite surprisingly, that these two variables were not positively correlated. In fact, the relationship was distinctly inverse regardless of the measure of internal calcium activity used for comparison with the relative index of gene expression (photonic emissions). These results cannot be interpreted to mean that calcium is unimportant for the regulation of PRL gene expression, for if that were the case, one would expect to find no correlation between these two variables as opposed to a negative one. The distinct inverse correlation between these variables measured in the same living cell might indicate that a given mammotrope temporally dissociates hormonal secretion (which is associated with an increased frequency of calcium oscillations) from biosynthesis. In this scenario, a particular series of $[Ca^{2+}]_i$ oscillations would act over the short term to evoke hormone release and over the long term to induce gene expression required for replenishment of hormonal stores. Our recent observation on the dissociation between PRL gene transcription, mRNA storage, and hormone release within the same mammotropes is entirely consistent with this possibility (25).

Our present findings on single, primary mammotropes are difficult to reconcile with those from population studies (largely with tumor cell lines) that suggest that calcium is a pivotal modulator of PRL gene activity. As indicated above, one possible explanation for these seemingly disparate observations is that a given mammotrope might change its $[Ca^{2+}]_i$ oscillatory pattern from time to time, and that changes in PRL gene expression induced by such signals would naturally lag behind, owing to the fact that oscillatory phenotypes could conceivably change in seconds, whereas modulation of gene expression would require many minutes or even hours for full manifestation. When we tested this idea experimentally, we found that individual mammotropes could indeed change their oscillatory phenotype over the course of just a few hours, although they were more likely to do so from one day to the next. Most interestingly, the vast majority of such transitions occurred between quiescent and oscillatory phenotypes as opposed to between one oscillatory subtype and another. To our knowledge, this is the first report that normal mammotropes in primary culture have the capacity to switch spontaneously between quiescent and oscillatory states. Whether this also occurs *in vivo* remains to be established. Parenthetically, it is noteworthy that individual mammotropes from lactating rats undergo striking day to day fluctuations in both the relative amount of PRL secreted (26) and the basal level of PRL gene expression (17). On the basis of these collective observations, it is tempting to speculate that spontaneous transitions in the calcium oscillatory behavior of mammotropes may mechanistically underlie day to day variations that occur in both of these calcium-dependent phenomena.

How might varying the mode of calcium presentation be advantageous to the physiological regulation of PRL gene expression in living mammotropes? This question is impossible to answer directly because so little is known about the relationship between calcium dynamics and PRL gene expression in single cells, particularly those from normal pituitary glands. Nevertheless, a series of cleverly designed population studies conducted by Haisenleder and co-workers (27) has provided important insights about how these processes might interact in the same untransformed cell. Briefly, these investigators perifused pituitary cells (from adult female rats) with various agents known to modulate the pattern of calcium influx (KCl, the calcium channel activator BayK 8644, or the calcium ionophore A23187). These treatments were administered for 24 h in either a pulsatile or a continuous manner, after which the perifused cells were recovered, extracted, and subjected to PRL mRNA determinations. Interestingly, these investigators found that the agents could either increase or decrease steady state levels of PRL mRNA depending on the mode of presentation (pulsatile or continuous). To be more specific, they observed that intermittent elevation of $[Ca^{2+}]_i$ was much more effective at augmenting PRL gene expression than persistent induction of calcium influx across the mammotrope membrane, which had a profound inhibitory effect (27). These data clearly reinforce the idea that a pulsatile calcium signal is required to stimulate PRL gene expression in mammotropes. By extension, it seems reasonable to propose that spontaneous transitions between oscillatory and nonoscillatory states comprise the mechanism by which a mammotrope can optimize calcium stimulation of PRL gene expression while avoiding the deleterious effects of continuous calcium exposure.

The existence of $[Ca^{2+}]_i$ oscillations that vary in frequency, amplitude, and/or shape has led to the proposal that calcium-dependent processes are regulated by qualitative and/or quantitative modulation of oscillatory $[Ca^{2+}]_i$ signal characteristics (28–30). In this scenario, extracellular signals would be transduced to a specific calcium code that subsequently would be

deciphered by a sensor associated with the effector system (31). Our present observations on the phenotypic heterogeneity of calcium oscillations in PRL cells, the temporal dissociation between calcium oscillatory activity and PRL gene activity, and the dramatic effect that frequency modulation of $[Ca^{2+}]_i$ has on PRL gene expression (28) all provide compelling (albeit indirect) evidence that such a coding system is operative in normal mammotropes. Unfortunately, the precise nature of this putative code has defied resolution because the relevant variables (patterns of gene expression and calcium oscillations) are essentially "moving targets." It is becoming abundantly obvious that success in cracking the calcium code will require the monitoring of both dynamic variables multiple times (if not continuously) in the same living cell. Although technically onerous, this is currently our approach for ongoing investigations in this research area.

MATERIALS AND METHODS

Cell Dispersion and Microinjection

Monodispersed AP cells from primiparous, lactating (days 6–10 postpartum) rats (Sprague-Dawley Harlan, Madison, WI) were prepared as described previously (17). Cells were plated onto poly-L-lysine-coated, gridded coverslips at a density of 75,000 cells/75 μl of a defined medium [phenol red-free medium 199/nutrient mixture F-12 (1:1; Life Technologies, Grand Island, NY) in which L-valine was replaced by D-valine] supplemented with 0.1% BSA, insulin-transferrin-selenium premix, and antibiotics. Cells were incubated for 1 h to facilitate attachment, covered with 2 ml defined medium supplemented with 10% FBS, and cultured in a humidified atmosphere of 5% CO_2-95% air for 2 days. The cells within a particular grid were then microinjected with a reporter plasmid (0.2 μg/μl in 10 mM PBS) in which 2.5 kilobase pairs of the 5′-flanking region of the rat PRL gene were placed upstream of the coding sequence for firefly luciferase. After microinjection, cells were washed twice and cultured for 2 more days in phenol red-free DMEM (Life Technologies) supplemented with 10 mM HEPES, 10% FBS, 0.1% BSA, and antibiotics.

$[Ca^{2+}]_i$ Measurements in Single, Identified Mammotropes

$[Ca^{2+}]_i$ measurements were performed on primary cultures of AP cells loaded with the calcium-sensitive probe fura-2 (32). This was accomplished by digital imaging fluorescence microscopy essentially as reported previously (33). Briefly, cells were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) for 1 h at 37 C in culture medium (Sigma Chemical Co., St. Louis, MO) of the same composition as before except that it was devoid of FBS, BSA, and bicarbonate. Coverslips were then washed three times in the same medium lacking fura-2/AM and mounted over the heated stage (37 C) of an Axiovert 35 inverted microscope (Zeiss, Jena, Germany). The microscopic field containing microinjected cells was then reidentified (from its position on the numbered/lettered, gridded coverslip), and the cells were epiilluminated alternately at 340 and 380 nm. Emission of light above 520 nm was recorded and analyzed with an Attofluor Ratio Vision System (Atto Instruments, Rockville, MD). Two video frames of each wavelength were averaged with an overall resolution time of 4 sec for each pair of images at alternate wavelengths. The ratio of consecutive frames obtained at 340 and 380 nm excitation light was calculated, and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards (32).

Measurements of PRL Gene Expression in Single, Living Mammotropes

Within 10 min of completing $[Ca^{2+}]_i$ measurements, the coverslip containing AP cells was assembled into a Sykes-Moore chamber and transferred to the heated stage (37 C) of a photon capture system (17) for quantification of luciferase-luciferin-generated photonic emissions. This system was comprised of a Zeiss Axioskop in series with a Hamamatsu VIM photonic camera/Argus 50 image processor. To be more specific, the microscopic field in which $[Ca^{2+}]_i$ measurements were just performed was reidentified, and a brightfield image was captured for reference purposes. Next, 3 ml of the same medium used for $[Ca^{2+}]_i$ measurements were supplemented with 3 mM luciferin along with 1% dimethylsulfoxide and infused into the chamber (total volume, 0.75 ml). Seventeen minutes later, photonic signals emitted by individual cells were accumulated over a 10-min period, and the images obtained were stored as computer files. For quantification of photonic events, the image of accumulated photonic emissions was superimposed over the brightfield image of individual cells, and the number of photonic events within a window of fixed area was calculated. Photonic measurements made in at least 20 adjacent areas devoid of cells were used to compute a background value. This was subsequently subtracted from the total accumulation to calculate specific photonic emissions from each cell.

It is noteworthy that expression of the transfected reporter plasmid used in this study is specific to mammotropes. Indeed, we had shown previously (17) that all transfected pituitary cells that emitted photons after exposure to luciferin contained PRL (immunocytochemistry), released the hormone (reverse hemolytic plaque assay), or contained the corresponding mRNA (*in situ* hybridization cytochemistry). It should also be mentioned that the presence of fura-2 did not interfere with our bioluminescence measurements, as the latter were performed in complete darkness (no UV excitation), and the background values of photonic emissions did not differ for fura-2-loaded and nonloaded cells (data not shown).

Multiple Measurements of $[Ca^{2+}]_i$ from the Same Cell

In those experiments in which $[Ca^{2+}]_i$ dynamics were monitored on three separate occasions from the same transfected cells, coverslips were washed three times in culture medium after the first $[Ca^{2+}]_i$ measurement and placed in the incubator for 2 h while immersed in the same medium supplemented with 0.1% BSA and 10% FBS. Next, cells were loaded again with fura-2/AM as described above and subjected to a second period of $[Ca^{2+}]_i$ measurements. The coverslips were then subjected to photonic imaging to identify which of the cells under study were mammotropes (as determined by their ability to emit light after exposure to luciferin). Finally, the cells were washed, incubated for 23 h in the same culture medium, and subjected again to a period of loading with fura-2/AM, followed by $[Ca^{2+}]_i$ measurements. In all instances, $[Ca^{2+}]_i$ monitoring was restricted to a 10- to 15-min period to optimize the viability of cells. Relocation of the same cells was achieved by the combined use of the photoengraved coverslips and computer retrieval of information about relative positions. Cells that were not positively identified as mammotropes or that had changed their location were excluded from the analysis.

Quantification of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ Oscillations

Two relevant parameters were calculated for quantification of $[Ca^{2+}]_i$ levels and $[Ca^{2+}]_i$ oscillations in identified mammotropes as reported previously (18, 19): the mean $[Ca^{2+}]_i$ value and the oscillation index. To calculate the mean $[Ca^{2+}]_i$ value, measurements of $[Ca^{2+}]_i$ obtained at 4-sec intervals were accumulated and normalized for the length of the entire sampling period. Thus, this parameter provides a mean value of $[Ca^{2+}]_i$ over time. The oscillation index represents the rate of changes in $[Ca^{2+}]_i$ during the period of measurement. To determine this, absolute differences in $[Ca^{2+}]_i$ levels between successive measurement intervals were averaged for the time period during which data were collected. This parameter reflects the frequency and/or amplitude of $[Ca^{2+}]_i$ oscillations and is largely independent of the actual $[Ca^{2+}]_i$ value (18, 19).

Statistics

A two-way ANOVA was employed to analyze the data. Differences in mean levels of PRL gene expression, mean $[Ca^{2+}]_i$ values, and oscillation indexes for different groups of cells were compared by use of Bonferroni's multiple comparisons test. Differences were considered significant at $P < 0.05$.

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