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Differential Influences of Gender and Physiological Status on Calcium Dynamics and Prolactin Gene Expression in Rat Mammotropes

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The rate of prolactin (PRL) secretion is influenced by the gender and physiological state of an animal, but little is known about the mechanisms involved. In the present study, we assessed possible contributions of Ca^{2+} dynamics and PRL gene expression to these differences. This was accomplished by monitoring spontaneous $[Ca^{2+}]_i$ changes and PRL promotor-driven reporter activity in pituitary cultures derived from rats comprising a broad spectrum of PRL secretory capacities: male, cycling female, and lactating rats. We found that Ca^{2+} oscillatory activity exhibited a rank order of lactating > cycling females > males, consistent with the reported secretory capacities of mammotropes from these sources. Interestingly, we observed that the basal level of PRL promotor-driven reporter activity was the same for all three models, but that mammotropes from males were the most responsive to stimulation of PRL gene expression by elevation of $[Ca^{2+}]_i$. Collectively, our findings reveal gender- and state-specific differences in Ca^{2+} dynamics and induction of PRL gene expression. These likely contribute to reported differences in secretory capacity.

Key Words: Pituitary; calcium oscillations; transcription.

Introduction

The concentration of intracellular free calcium ($[Ca^{2+}]_i$) regulates many physiological processes including hormone secretion and gene expression *(1)*. In general, Ca^{2+} functions as a second messenger, linking events initiated at the plasma membrane to an ultimate biological response. The prolactin (PRL)-secreting mammotrope has been and continues to be a model of choice for elucidating the roles of Ca^{2+} and other second messengers in stimulus-secretion coupling. $[Ca^{2+}]_i$ in mammotropes is modulated not only by the activation of various receptors linked to discrete second-messenger pathways but also by spontaneous $[Ca^{2+}]_i$ oscillations that arise subsequent to the activation of voltage-sensitive Ca^{2+} channels. These channels also appear to be targets for a number of hypophysiotropic agents that modulate the PRL secretory pathway at various sites *(2,3)*.

In addition to the well-characterized effect of $[Ca^{2+}]_i$ on PRL release *(4,5)*, changes in $[Ca^{2+}]_i$ also play an important role in regulating PRL gene expression *(6,7)*. This is evidenced most dramatically by studies in which perturbation of Ca^{2+} channel activity (by treatment with Ca^{2+} channel blockers, Ca^{2+} agonists, or modulators of other second-messenger pathways linked to Ca^{2+} mobilization) led to predictable changes of PRL gene expression *(8–11)*.

There is abundant evidence to indicate that the gender and physiological status of a pituitary donor can have a striking influence on the amount of PRL released. Indeed, mammotropes from female rats secrete more PRL both in vivo and in vitro than do their male counterparts, and the secretory rate for lactating rats exceeds that for cycling females *(12–14)*. Given the critical role played by Ca^{2+} in both PRL release and gene expression, it seems reasonable to propose that changes in calcium dynamics are responsible, at least in part, for the aforementioned differences in mammotrope activity. Unfortunately, available data serve neither to substantiate nor to refute this possibility. In fact, progress in gaining a mechanistic understanding of this phenomenon has been hindered, because most studies on the relationship between $[Ca^{2+}]_i$ modulation and PRL secretion/gene expression have been conducted with PRL-secreting, clonal cell lines (e.g., *see* refs. *15–18*), or, more rarely, primary cultures containing mammotropes derived from pituitary donors representing a single physiological state or gender *(19,20)*. Thus, direct comparisons relevant to these relationships have not been undertaken.

In light of these considerations, we attempted, in the present study, to evaluate the effects of gender and physiological status on Ca^{2+} dynamics and expression of the PRL gene. This was accomplished by monitoring and comparing among primary

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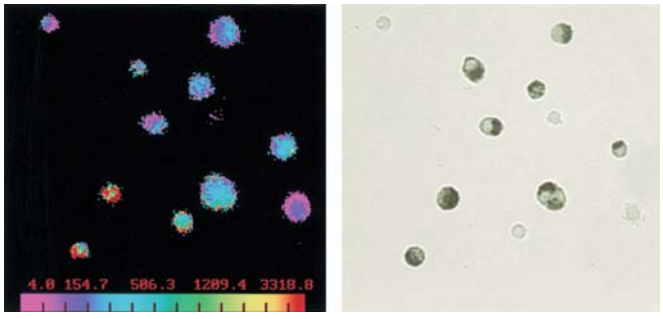

Fig. 1. Procedure for measuring calcium dynamics and identification of mammotropes. **(Left)** Ca^{2+} measurements were performed by subjecting cells on gridded glass cover slips to digital-imaging fluorescence microscopy of the calcium-sensitive dye fura-2. The ratios of fluorescence emission were calculated and converted into $[Ca^{2+}]_i$ values by comparison with fura-2 standards. Higher $[Ca^{2+}]_i$ is indicated by warmer colors on the ascending pseudocolor scale (×400). **(Right)** The same cover slips used for $[Ca^{2+}]_i$ imaging were fixed, and mammotropes were revealed by indirect immunocytochemistry for PRL. Specific cells within a field used in the calcium-imaging experiments were localized by their relative position on the numbered/lettered grid. For illustrative purposes, we have selected a field enriched in mammotropes (×400).

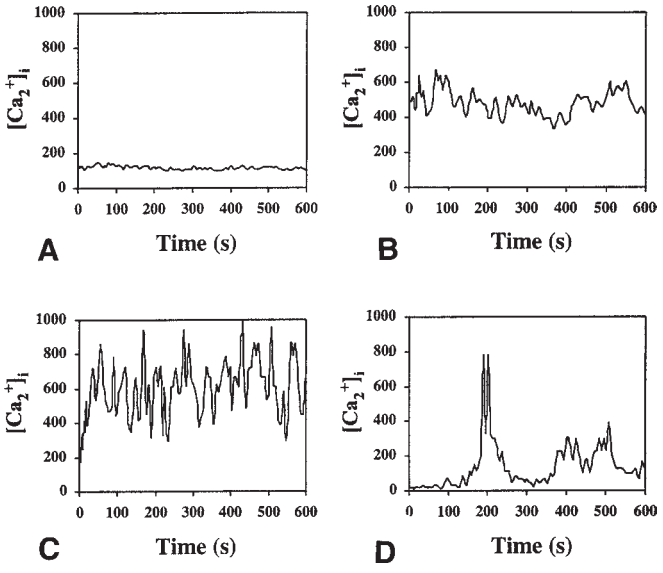

Fig. 2. Patterns of spontaneous calcium oscillations. Mammotropes from the three physiological donor types exhibited each of the four major patterns **(A–D)** of Ca^{2+} oscillations shown here and characterized previously *(20)*. See Results for descriptions of these patterns.

mammotropes derived from male, cycling female, and lactating rats (1) spontaneous Ca^{2+} oscillations in individual cells; and (2) PRL promotor-driven reporter responses to pharmacological manipulation of $[Ca^{2+}]_i$ at the population level.

Results

Our initial goal was to evaluate the influence of gender and physiological status on $[Ca^{2+}]_i$ oscillatory dynamics in

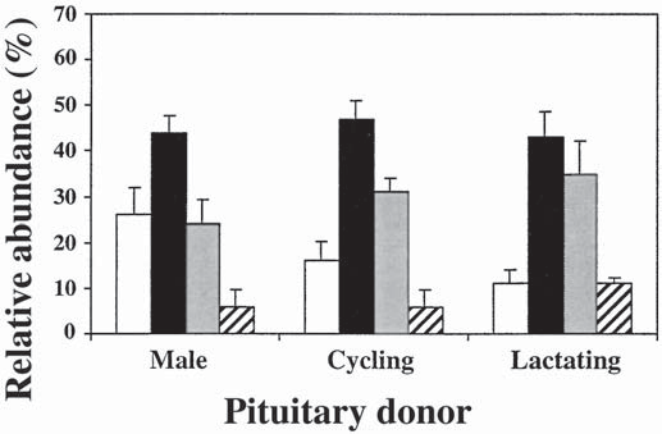

Fig. 3. Physiologic status affects distribution of oscillatory subtypes. The proportion of mammotropes exhibiting each of the four oscillatory subtypes (□, ■, ▨, ▧) varied depending on the gender and physiological status of the pituitary donor. Quantitative data presented here and in Figs. 4 and 5 and Table 1 represent mean ±SEM.

mammotropes, a process best explored at the single-cell level. Accordingly, we subjected anterior pituitary cells in primary culture to measurements by digital-imaging fluorescence microscopy of fura-2 (Fig. 1, left), and mammotropes were identified *post facto* by indirect immunocytochemistry for PRL (Fig. 1, right). Various parameters of calcium dynamics were then measured and compared for mammotropes derived from rats in different physiological states.

As a first approximation, we decided to evaluate qualitative and quantitative aspects of oscillatory patterns and found that mammotropes from all three physiological states exhibited the four major oscillatory subtypes (Fig. 2) reported previously *(20)*. These included cells that were nonoscillatory (type A), as well as those exhibiting high-frequency, low-amplitude oscillations (type B) with increments above baseline of about 100 n*M*. Type C cells were characterized by high-frequency, high-amplitude oscillations of which at least two exhibited an increment of 500 n*M* during the sampling period. Finally, type D cells presented with at least one slow wave of $[Ca^{2+}]_i$, and assignment was made with little consideration of frequency/amplitude parameters. Each cell in the present study was assigned to one of these phenotypes on the basis of its oscillatory behavior, and the relative abundance of each subtype was calculated. As shown in Fig. 3, we found that the proportion of mammotropes displaying each of the oscillatory subtypes appeared to shift according to the gender or physiological status of the pituitary donor. More specifically, mammotropes from lactating rats tended to exhibit the greatest proportion of type C and D oscillators, and the fewest type A (nonoscillatory) cells. Males, on the other hand, tended to exhibit the highest fraction of type A (nonoscillating) mammotropes. The proportional abundance of oscillatory subtypes for cycling females was

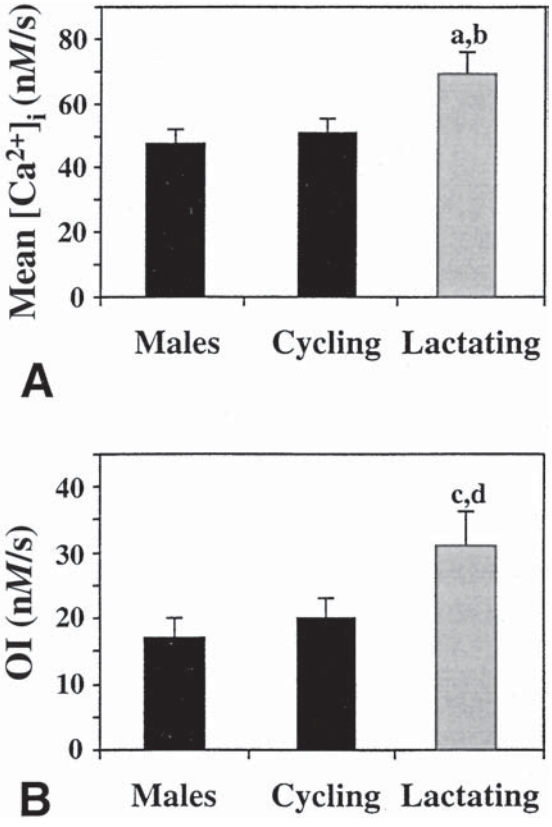

Fig. 4. Mean $[Ca^{2+}]_i$ and OI were significantly greater in mammotropes derived from lactating donor rats than from males or cycling females. The two parameters of Ca^{2+} dynamics presented here, mean $[Ca^{2+}]_i$ and OI, were calculated from $[Ca^{2+}]_i$ measurements acquired at 4-s intervals. **(A)** Mammotropes from lactating rats had the highest values of mean $[Ca^{2+}]_i$ vs male or cycling female rats (a: $p < 0.01$; b: $p < 0.02$ respectively). **(B)** Lactating rats had the highest values of OI vs male and cycling female rats (c: $p < 0.04$, d: $p < 0.03$ respectively). Data represent the average values for 61 cells for male, 73 cells for cycling female, and 54 cells for lactating rats studied in three independent experiments.

intermediate to that of male and lactating females except in the case of subtype B cells, which did not appear to change as a function of donor status. We hasten to point out that differences between specific groups may not have achieved statistical significance. However, the trends observed here were reinforced when more quantitative aspects of calcium oscillations were considered.

Parameters of $[Ca^{2+}]_i$ dynamics (mean $[Ca^{2+}]_i$ and oscillation index [OI]; *[20]*) were calculated for identified mammotropes from each of the pituitary donor types (Fig. 4). We found that the mean $[Ca^{2+}]_i$ (the average of the $[Ca^{2+}]_i$ values during the measurement period) was significantly greater for mammotropes derived from lactating as opposed to male or cycling female rats ($p < 0.01$ and $p < 0.02$, respectively). The OI (representing the rate of change of $[Ca^{2+}]_i$ during the sampling period) was also highest for lactating rats when compared with males or cycling females ($p < 0.04$ and $p < 0.03$, respectively). Table 1 presents more detailed comparison of calcium dynamics as a function of oscillatory subtype and physiological status. Of particular note is the finding that physiological state influenced not only the proportional abundance of multiple oscillatory phenotypes but also the frequency and/or amplitude of calcium oscillations. This is evidenced by the highest OI for type C cells (high-frequency, high-amplitude) derived from lactating as opposed to male rats ($p < 0.05$).

Having established that physiological status of the pituitary donor had a tangible influence on Ca^{2+} dynamics within mammotropes, we next asked, Would pharmacological modulators of $[Ca^{2+}]_i$ differentially influence PRL gene expression within cells from the same sources? Accordingly, we transiently transfected (with the PRL-luciferase reporter plasmid) entire cultures of pituitary cells in Petri dishes and incubated them for 48 h in either control medium or in medium containing a Ca^{2+} agonist (BayK 8644) or a Ca^{2+} channel blocker (nimodipine). Luciferase activity reflective of PRL gene promoter activation was assayed in cell extracts; and Fig. 5 presents the results. Interestingly, we found that the basal levels of reporter activity were quite similar for the three donor groups ($p > 0.05$), once the values were normalized for mammotrope numbers (which are greater in females than males and increased during lactation). As one might anticipate, increasing $[Ca^{2+}]_i$ by treatment with BayK 8644 increased PRL promoter activity in mammotropes from all three donor types, whereas exposure to nimodipine had the opposite effect (*see* Fig. 5; a: $p < 0.05$ vs control; b: $p < 0.05$ vs control). However, donor status did influence the magnitude of the response. For example, mammotropes from males showed the greatest response to the Ca^{2+} agonist BayK 8644 (a 2.3-fold increase for male vs a 1.5-fold increase for lactating rats, $p < 0.05$). Thus, the source from which mammotropes were derived influenced not only spontaneous Ca^{2+} oscillatory dynamics but also the capacity to modulate PRL gene expression in response to changes in $[Ca^{2+}]_i$.

Discussion

Mammotrope secretory status is dramatically influenced by gender and physiological status *(13)*, with female rats exhibiting greater PRL secretion than males, and lactating rats higher levels than females in other physiological conditions. PRL secretion is regulated distally by transcription of the PRL gene and more proximally by the rate of PRL biosynthesis and export, and multiple points along this pathway are controlled by $[Ca^{2+}]_i$ (5,15,21). In the present study, we attempted to elucidate the roles that PRL gene expression and Ca^{2+} dynamics play in modulating PRL secretory capacity as a function of physiological setting. To this end, we measured and compared several parameters of Ca^{2+} oscillatory dynamics along with the levels of PRL promoter-driven reporter activity among

Table 1
Effect of Physiologic Status on Multiple Parameters of Ca^{2+} Dynamics in Mammotropes[a]

	Male $n = 55$			Cycling $n = 62$			Lactating $n = 65$		
Pattern	Mean $[Ca^{2+}]_i$	OI	%	Mean $[Ca^{2+}]_i$	OI	%	Mean $[Ca^{2+}]_i$	OI	%
A: Nonoscillating	19.5 ± 1.8	2.6 ± 0.2	26	23.0 ± 3.7	1.8 ± 0.1	16	22.3 ± 6.6	1.9 ± 0.3	11
B: High frequency/ low amplitude	44.3 ± 5.5	7.8 ± 0.7	44	30.9 ± 2.6	5.9 ± 0.7	47	42.5 ± 3.5	7.0 ± 0.7	43
C: High frequency/ high amplitude	90.0 ± 8.6	41.1 ± 5.0	24	96.7 ± 8.9	39.6 ± 6.1	31	121.5 ± 7.7	60.2 ± 0.9	35
D: Wave	16.1 ± 8.3	5.3 ± 1.5	6	63.0 ± 6.3	12.9 ± 1.0	6	34.7 ± 6.2	10.2 ± 3.5	11
Overall mean	47.3 ± 4.6	17.1 ± 2.8		51.9 ± 4.9	19.9 ± 3.1		69.1 ± 6.2	31.0 ± 5.3	

[a]Note that the gender and physiological status of the pituitary donor influenced not only the proportional abundance (%) of oscillatory phenotypes but also the frequency and/or amplitude of calcium oscillations (OI).

pituitary cultures derived from donors that secrete PRL at quite different rates: male, randomly cycling female, and lactating rats.

There are several possible mechanisms that might contribute to gender- and physiological state–specific differences in mammotrope secretory capacity. Prominent among these are state-specific differences in (1) basal PRL gene expression; (2) responsiveness of PRL gene expression to modulators of $[Ca^{2+}]_i$; and (3) quantitative and/or qualitative aspects of Ca^{2+} dynamics and oscillatory profiles. Each one of these is considered in detail next.

With respect to the first of these possibilities, we found that differences in basal PRL promoter-driven gene expression cannot account for the disparate PRL secretory capacities of the three donor types studied. Indeed, we observed that pituitary cultures from male, cycling female, and lactating rats exhibited similar levels of basal reporter activity, once these values were normalized for the relative abundance of mammotropes present. Inasmuch as we measured only a single index of PRL gene expression (one most reflective of transcriptional activity), our results should not be interpreted to mean that there are no differences in gene expression as a whole. In fact, posttranscriptional mechanisms of gene expression, which are operative in mammotropes and were not examined here, could very well contribute in part to the differences in secretory capacities reported *(11,22)*. These considerations notwithstanding, our observations are counterintuitive to the view that secretory capacity, at least in mammotropes, is dictated largely by the rate of transcription.

Is the PRL gene in mammotropes from male, cycling female, and lactating rats differentially responsive to stimulation by changes in $[Ca^{2+}]_i$? The results of our pharmacological studies aimed at perturbing $[Ca^{2+}]_i$ strongly indicate that this is the case. With each of our three models, we found that potentiation of voltage-gated calcium channels and, presumably, calcium oscillations predictably led to an augmentation of PRL promoter-driven gene expression. By contrast, inhibition of the same channels resulted in a significant decrease of reporter activity. Taken together, these results indicate that spontaneous Ca^{2+} oscillations regulate resting PRL gene activity in all three models studied. A surprising and novel aspect of our findings was that the degree to which modulators of $[Ca^{2+}]_i$ augmented PRL promoter activation was inversely proportional to the reported secretory capacity of the mammotrope source. Thus, mammotropes from male exhibited a larger augmentation of PRL promoter-driven reporter activity after $[Ca^{2+}]_i$ elevation than did those from lactating rats. Therefore, our observations do not support the possibility, elaborated previously, that the higher secretory capacity of female mammotropes, when compared to males, is a consequence of the greater Ca^{2+} responsiveness of the PRL gene in females.

If mammotropes from males secrete less PRL than their female counterparts but have comparable basal levels of PRL promoter-driven gene expression, and male mammotropes are the most responsive to Ca^{2+} stimulation of PRL gene expression, then it follows that PRL secretors from males may exhibit differences in resting oscillatory dynamics. We tested this line of reasoning by evaluating three parameters of Ca^{2+} oscillatory activity in single living mammotropes. The results of all analyses support the same conclusion: Ca^{2+} oscillatory dynamics in male mammotropes are suppressed when compared with those of their female counterparts. Specifically, we observed that the rank order for two of the parameters measured, mean $[Ca^{2+}]_i$ and OI, was lactating rats > cycling females > males, indicating that both the average amount of intracellular free calcium and the frequency/amplitude of spontaneous Ca^{2+} oscillations were relatively suppressed in male mammotropes examined under resting conditions. Consistent with these findings was our observation that the fractional abundance of oscillatory mammotropes (types B–D) was highest for lactating rats whereas the proportion of quiescent cells (type A) was highest for males. Once again,

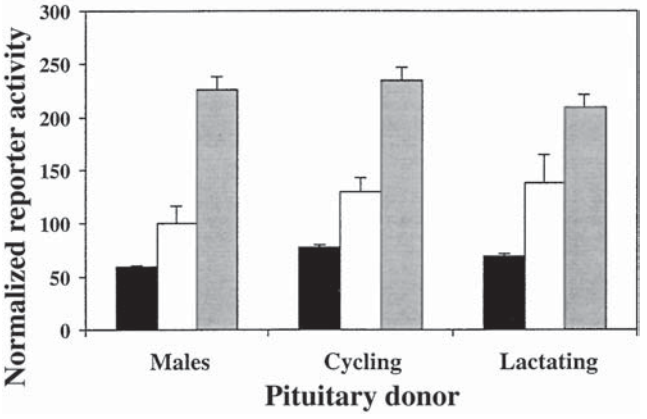

Fig. 5. Effect of nimodipine and BayK 8644 on PRL gene expression in anterior pituitary cells. Culture dishes were transiently transfected with the PRL-luciferase reporter plasmid and incubated for 48 h in vehicle (□) or vehicle containing 100 n*M* BayK 8644 (▨) or nimodipine (■). Luciferase activity was assayed in cell extracts. The percentage of mammotropes was determined by immunocytochemistry in companion cultures derived from male (37.2%), cycling female (52.8%), and lactating rats (57.2%). Data shown were adjusted for the relative abundance of mammotropes. Basal levels of reporter activity were similar for the three donor groups, and each group exhibited Ca^{2+}-dependent PRL reporter activity (a: $p < 0.05$ vs control; b: $p < 0.05$ vs control). Fold increase induced by BayK 8644 was larger for males than for lactating rats ($p < 0.05$).

proportional values for cycling female rats were intermediate to those of lactating and male rats. Taken together, our data reveal that quantitative as well as qualitative aspects of Ca^{2+} dynamics are subdued in mammotropes derived from males when compared to those of females. These findings provide at least a partial explanation for reported differences in basal secretory activity between males and females or among physiological states. We hasten to point out, however, that in these studies we did not measure PRL secretion in parallel with PRL gene expression and Ca^{2+} oscillatory dynamics. Therefore, unequivocal conclusions about the relationships among these three parameters cannot be drawn.

In summary, we have shown that Ca^{2+} oscillatory activity within mammotropes from male rats is significantly muted when compared with that of lactating rats and, to a lesser extent, cycling female rats. These data indicate that the basal state of $[Ca^{2+}]_i$ activity contributes to gender- and physiological state–specific differences in PRL secretion and gene expression in mammotropes. Clearly, the functional relationships between Ca^{2+} oscillatory dynamics and PRL gene expression, as well as between gene expression and secretion, are far more complex than envisioned previously. Unequivocal clarification of these relationships will require more sophisticated experimental paradigms that provide time-resolved information about Ca^{2+} oscillations, PRL gene expression, and secretion, preferably at the single-cell level.

Materials and Methods

Animals and Cell Culture

All experimental procedures were reviewed and approved by the Medical University of South Carolina Animal Care and Use Committee in accordance with established national guidelines. Anterior pituitary glands from primiparous lactating (d 6–9 postpartum), male and random cycling female (90–100 d of age) rats (Holtzman, Madison, WI) were collected after decapitation and enzymatically dispersed into single cells as described previously *(23)*. Three separate dispersions of two glands each for male and lactating female rats and five glands each for randomly cycling female rats were performed. Cells were plated onto poly-L-lysine-coated, 6-well plates at a density of 200,000 cells/35-mm well, or onto poly-L-lysine-coated glass cover slips (photoengraved with a numbered/lettered grid pattern) at a density of 25,000 cells/cover slip. Cells were cultured in phenol red–free Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% bovine serum albumin, penicillin G (100 U/mL)/streptomycin sulfate (100 μg/mL) and 10% fetal bovine serum at 37°C in 5% CO_2 until they were transfected or used directly for $[Ca^{2+}]_i$ imaging. All experimental measurements were made 2 to 3 d after dispersion, a time frame over which mammotropes are reported to maintain their secretory characteristics *(13)*.

Digital-Imaging Fluorescence Microscopy of Fura-2

On d 2 (48 h) following dispersion, cells on gridded glass cover slips were washed and incubated with a 2 μ*M* solution of the calcium-sensitive probe fura-2/AM (Molecular Probes, Eugene, OR) for 1 h at 37°C in phenol red–free DMEM. Cover slips were then washed three times in medium without fura-2/AM and mounted on the heated stage (37°C) of an Axiovert 35 inverted microscope (Zeiss, Jena, Germany). A microscopic field was then identified with the ×40 objective, and the alphanumeric position on the cover slip was recorded for identification after immunocytochemistry. Next, cells were alternately epi-illuminated with ultraviolet light 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 s for each pair of images at alternate wavelengths. The ratio of emission from 340/380 nm excitation light was calculated, and $[Ca^{2+}]_i$ was estimated by comparison with fura-2 standards *(24)*.

Analysis of $[Ca^{2+}]_i$

In this study, we utilized two parameters of Ca^{2+} dynamics: mean $[Ca^{2+}]_i$ and Ca^{2+} OI *(20)*. $[Ca^{2+}]_i$ measurements acquired at 4-s intervals were normalized for the length of the sampling period and used to calculate mean $[Ca^{2+}]_i$. The OI, representing the rate of change of $[Ca^{2+}]_i$ during the sampling period, was calculated by

averaging absolute changes of $[Ca^{2+}]_i$ (from one measurement to the next) per unit of sampling time (4 s). As previously reported, the OI is reflective of Ca^{2+} oscillation frequency and/or amplitude and is independent of the actual $[Ca^{2+}]_i$ *(20)*.

Immunocytochemistry

After $[Ca^{2+}]_i$ 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 *(25)*. Mammotropes were identified by positive staining for PRL after exposure to diaminobenzidine (Sigma, St. Louis, MO) solution for 3–6 min. The field used in the $[Ca^{2+}]_i$-imaging was reidentified by its numbered/lettered position on the cover slip.

Measurement of PRL Gene Expression

Companion cultures of anterior pituitary cells were transiently transfected after overnight culture with the –2500 rPRL-luc reporter construct as described elsewhere *(25,26)*. Following transfection, cells were incubated for 48 h at 37°C either in control medium (0.01% EtOH in supplemented DMEM) or in treatment medium containing 10^{-7} *M* BayK 8644 or nimodipine (Sigma). These test compounds were dissolved initially in EtOH to 10^{-4} *M* and diluted to final concentration in supplemented DMEM. Luciferase activity was assayed in cell extracts by use of a Promega (Madison, WI) luciferase determination kit. The assay was run according to the guidelines suggested by the manufacturer. Luciferase activity measurements were performed for 30 s/sample in an AutoLumat LB 953 luminometer (EG & C Berthold, Bad Wildbad, Germany).

Statistical Analysis

Means generated in this study were derived from three completely separate experiments for each physiological donor type. A total of 188 cells (54 lactating, 73 cycling female, and 61 male) were examined after digital-imaging fluorescence microscopy and immunocytochemistry. Microsoft Excel software was used to determine standard error. Two-way analysis of variance was utilized to determine statistical significance of differences among means at the level of $p < 0.05$.

Acknowledgments

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