Dynamics of Stimulus-Expression Coupling as Revealed by Monitoring of Prolactin Promoter-Driven Reporter Activity in Individual, Living Mammotropes

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Single-cell paradigms have greatly expanded our knowledge about stimulus-secretion coupling, but the understanding of stimulus-gene expression coupling has lagged behind for lack of a dynamic model sufficiently sensitive to provide single-cell resolution. In the present study, we made continuous indirect measurements within individual, living cells of expression dynamics both before and after treatment with a gene-activating secretagogue. This was accomplished by transfecting (via microinjection) individual, primary mammotropes with a PRL promoter-driven luciferase reporter plasmid, and then quantifying the rate of photonic emissions (reflective of endogenous gene activity). We found that individual cells exhibit spontaneous, random, short-term fluctuations of basal reporter activity and are extremely heterogeneous in terms of responses to a stimulatory agent (TRH). In addition, we found that responses are affected by several factors including the secretory status of the pituitary donor, the manner in which the stimulus is presented, and by the initial level of reporter activity. Moreover, the responsiveness of an individual cell can fluctuate dramatically over time. These results invite speculation that a given cell can "sense" its gene activation state and regulate its response accordingly to satisfy requirements for the corresponding secretory product. (Molecular Endocrinology 13: 1718–1727, 1999)

INTRODUCTION

Cellular responses induced by physiological and/or pharmacological agonists are generally manifested as both short- and long-term changes that range in duration from milliseconds to hours or days. Whereas the former mode of response is mediated by modulation of ligand- or second messenger-activated effector proteins, the latter requires the expression of new genes or changes in the transcriptional rate of previously activated genes. The secretion of hormones and neuropeptides is not an exception in this regard, and releasing factors and neurotransmitters that activate exocytosis or synaptic transmission usually evoke changes in the expression of genes corresponding to the secretory products (1). The use of dynamic approaches, especially those at the single-cell level, has greatly improved our knowledge about stimulus-secretion coupling (2, 3). However, our understanding of the coupling between stimulus and gene expression is much less developed, especially in those cases (such as the pituitary gland or the central nervous system) where functional complexity and heterogeneity of the tissue warrant a single-cell approach.

The PRL-secreting mammotrope, one of five major hormone-producing cell types within the adenohypophysis, is rapidly becoming a model of choice for dynamic analysis of gene expression at the single-cell level (4). This view is supported by three considerations. First, the PRL gene is extremely well characterized in terms of its 5′-regulatory sequences and how second messenger systems indirectly impinge upon them to effect modulation of gene expression (5–7). Second, flow through the PRL biosynthetic pathway has been the subject of intense investigation for almost four decades, and the mechanistic relationship between hormonal gene expression and secretion is becoming increasingly clear (8, 9). Third, and perhaps most important, the mammotrope's high level of basal PRL transcription provides the potential for monitoring hormonal gene expression at the single-cell level (9). Indeed, we have exploited this potential by developing a paradigm for making multiple measurements of PRL gene expression from the same, living mammotrope. This is accomplished by exposing mammotropes to luciferin after transfecting them with

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a PRL promoter-driven luciferase construct, and then quantifying photonic emissions [reflective of PRL promoter-driven gene expression (4, 10)] with an extremely sensitive photon capture system. Because our method of transfection (microinjection) allows delivery of a predetermined amount of reporter plasmid to every cell, this strategy also enables dynamic analysis of gene expression in normal (primary) as opposed to transformed mammotropes.

Dynamic analysis of PRL gene expression has provided a number of insights about gene activity in single cells that were unattainable previously because of technical constraints. These include, but are not restricted to, the finding that mammotropes injected with the same amount of plasmid can differ from one another by more than 100-fold in the basal level of gene expression (4). Moreover, individual mammotropes photonically sampled in narrow (10 min) windows at 24-h intervals were found to exhibit striking, random, day-to-day changes in their level of gene expression (4). In addition, not all transfected mammotropes exhibited predictable changes of photonic emissions when treated for 24 h with either dopamine or epidermal growth factor (agents reported by others to inhibit or stimulate, respectively, PRL gene expression within entire cultures of pituitary cells) (4). Although valuable and provocative, these previous findings suffered in terms of temporal resolution and interpretability owing to the fact that multiple measurements of gene expression on the same cells were made at 24-h intervals rather than continuously. Accordingly, we decided to adopt a strategy that involves continuous monitoring of gene expression (11) both before and after exposure to a secretagogue. We chose TRH as our prototypic secretagogue for the present study because it is not only the consensus PRL-releasing factor in mammals but is also a well documented, physiological stimulator of PRL gene transcription (12, 13). Armed with this dynamic analytical tool, we pursued at the single cell level the following objectives: 1) to establish the dynamics (time course) and demographics of the PRL gene response to TRH; 2) to assess whether gender of the mammotrope donor, which greatly influences PRL release, affects responsiveness of the PRL gene; and 3) to explore the relationship between initial state of gene expression and the capacity of a given cell to mount a response after stimulation.

RESULTS

The major objective of this study was to assess expression responses of the PRL gene in individual, living mammotropes. To this end, we measured photonic emissions from pituitary cells transfected with a luciferase reporter plasmid (rPRL-LUC) under the control of 5′-regulatory sequences of the rat PRL gene (−2430 to +39). These measurements were made in a spatial and time-resolved manner. A reasonable degree of temporal resolution was provided by the short functional half-life (1 h) of the reporter enzyme in rat mammotropes (4). Figure 1 illustrates by representative example the experimental procedure. Here, a microscopic field containing transfected anterior pituitary cells was subjected to photon counting measurements for several hours before and after continuously perifusing the cells with TRH-containing medium. Photonic emissions were accumulated in 30-min bins, quantified, and converted into a pseudocolor image that corresponded to the ascending scale shown on the *right*. All cells that exhibited photonic emissions were considered mammotropes for reasons provided in detail later (see *Discussion*). In the first series of studies, we analyzed the responses of 152 individual mammotropes in primary cultures obtained from male (n = 47) and lactating female (n = 105) rats in 21 independent experiments. Representative profiles of individual cells treated continuously with TRH (n = 96) or vehicle (n = 56) are shown in Fig. 2. Consistent with our previous observations in cells from lactating animals (4, 14), the initial level of photonic activity reflective of PRL gene expression varied

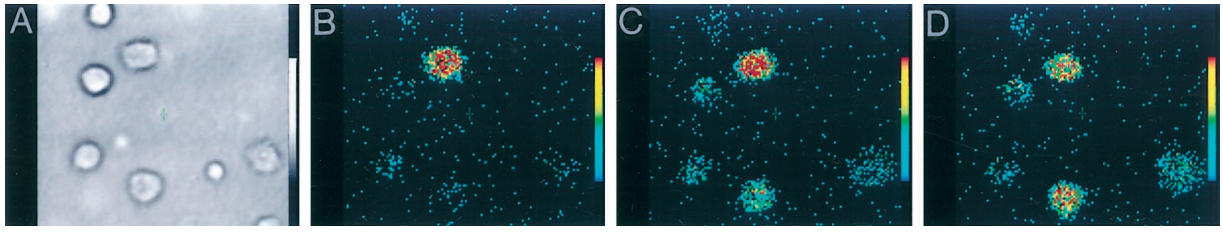

Fig. 1. Continuous Measurements of Reporter Activity from Individual Mammotropes in Primary Culture

Single pituitary cells were transfected by microinjection with a luciferase construct under control of the PRL promoter. One or two days later, cells were incubated for 4 h in luciferin-containing medium, transferred to a photon capture system, and perifused continuously with the same medium. After obtaining a bright field image of transfected cells for reference purposes (A), we then accumulated photonic emissions (reflective of PRL gene expression) in 30-min bins for several hours before and after treatment. This representative example shows accumulated, specific photonic emissions (signal-background) for the sampling period just before (B), 3 h after (C), or 6 h after (D) TRH (1 μM) addition. The rate of photonic activity is presented here as pseudocolors that progress in accordance with the ascending color scale shown. Note that mammotropes with the lowest basal level of reporter activity were the most responsive to TRH treatment.

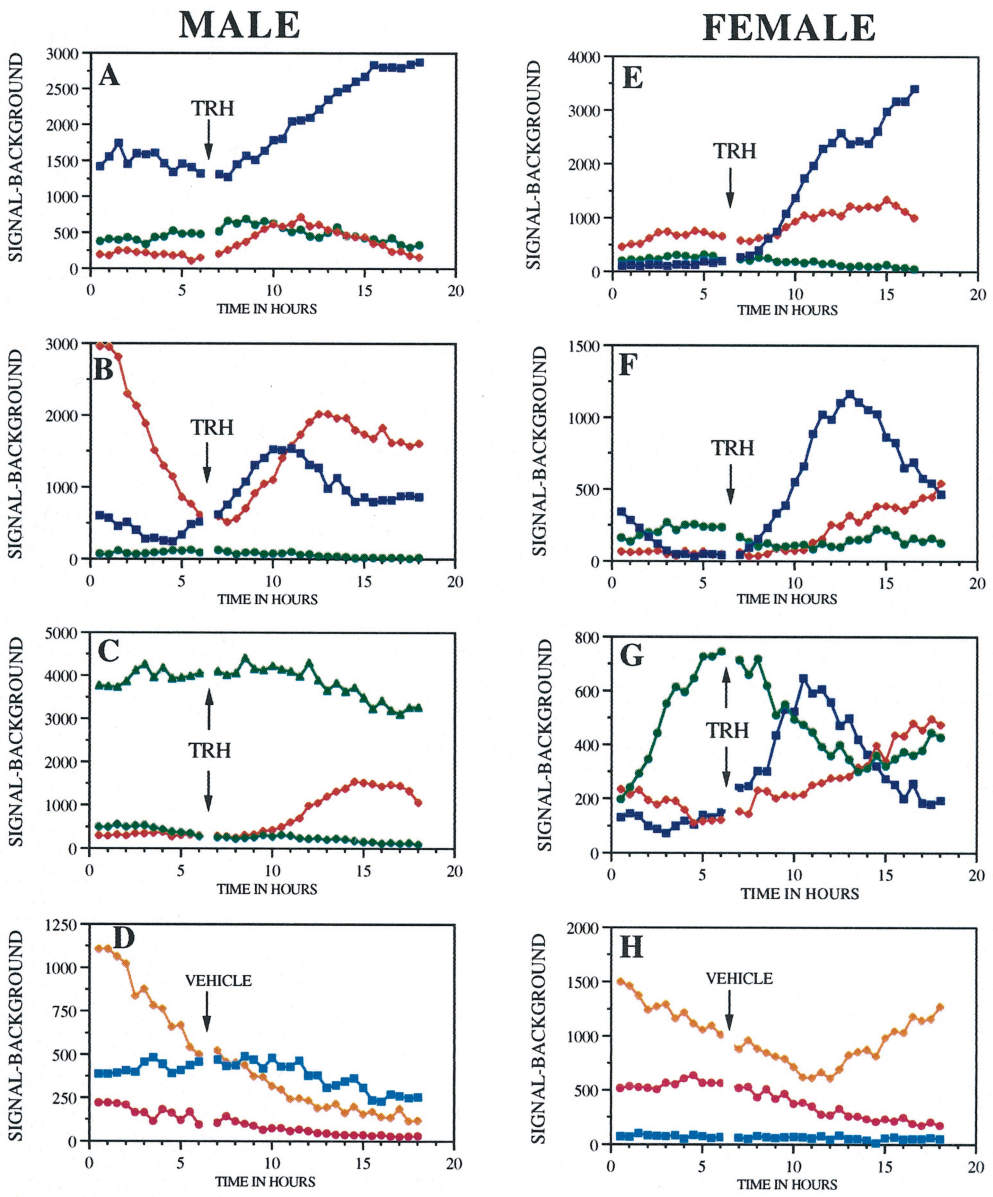

Fig. 2. Photonic Profiles of Individual Mammotropes Challenged with TRH

Individual transfected mammotropes from males (*left panels*) and lactating females (*right panels*) were continuously monitored, and specific photonic emissions were accumulated in 30-min bins for several hours before and after long-term exposure to TRH (1 μM) or vehicle. Profiles in each panel correspond to photonic emissions from selected cells present in the same microscopic field that were either responsive to TRH (*dark blue* and *red* traces) or not responsive (*green* traces). Each panel contains representative profiles from a single experiment.

greatly from cell to cell in cultures derived from both male and lactating female rats. After TRH addition, some cells (*blue* and *red* traces) exhibited clear increases in photonic emissions whereas others (*green* traces) did not show any apparent change after stimulation or exhibited a paradoxical decrease in their rate of photonic emissions. In addition, the dynamics of the changes evoked by TRH differed considerably from cell to cell. For example, some mammotropes (see *blue* traces in B, F, and G, in Fig. 2) exhibited striking increases of photonic activity that tended to decline toward basal values before the end of the sampling period. In contrast, other cells (*blue* trace in A and *red* traces in F and G, Fig. 2) responded in a more slow and sustained manner. Finally, in control experiments, we did not observe increases in photonic emissions reflective of PRL gene expression in cells perifused with vehicle instead TRH (panels D and H in Fig. 2). These data indicate that TRH-induced increases of reporter activity were not attributable to possible artifacts of the experimental protocol but rather to specific interactions with mammotropes that likely involved TRH receptors.

It is well established that mammotropes from lactating animals exhibit a much higher rate of PRL secretion than do their counterparts obtained from other pituitary donors, particularly males. To explore a possible relationship between PRL secretory status of the donor and gene expression, we averaged and compared photonic emission values for transfected mammotropes measured under resting conditions (just before stimulation with TRH). Surprisingly, we found that mammotropes derived from lactating females and males did not differ (on average) in the level of reporter activity measured under resting conditions (Fig. 3). Next, we evaluated, within the same set of data, possible gender-specific differences in responsiveness to TRH by assigning the cells treated with the secretagogue into one of two groups: those that were responsive to TRH and those that were not. [Responsive cells were defined as those that exhibited a clear increase in photonic emissions after TRH stimulation (*e.g. blue* and *red* traces in Fig. 2). Although seemingly arbitrary, such decisions were surprisingly clear-cut.] We found that 69% (n = 36) of mammotropes from males exhibited unequivocal increases of photonic emissions after TRH addition. For lactating females, however, the percentage of mammotrope responders was significantly lower (40%, n = 60, $P < 0.05$). To compare both the kinetics and magnitudes of the responses, we expressed each value obtained during the monitoring period as a percentage of the values obtained before TRH addition. The resulting normalized values were each pooled into three different subgroups: TRH-responsive cells, TRH-nonresponsive cells, and vehicle-treated cells (Fig. 4). On average, the stimulation of PRL promoter-driven reporter activity by TRH was considerably greater for female-derived mammotropes than for their male counterparts (6.4-fold *vs.* 3.3-fold, respectively, $P < 0.05$). The larger SEM observed for female mammotropes reflects the higher variability in responses. Taken together, these results demonstrate that although the endocrine (secretory) status of the pituitary donor does not influence the basal level of gene expression dramatically (estimated by reporter activity), it impacts greatly on both the capacity (proportional abundance) of mammotropes to mount a response after an evocative stimulus and their responsiveness (fold-increase).

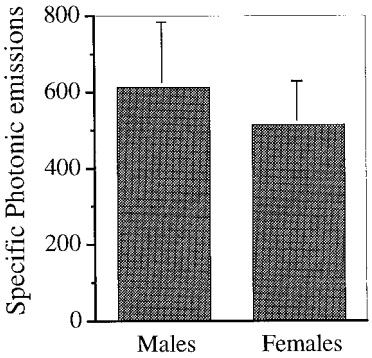

Fig. 3. Basal Levels of PRL Promoter-Driven Reporter Activity within Mammotropes Derived from Male and Lactating Female Rats

Illustrated here are the mean (±SEM) levels of specific photonic emissions measured during the 30 min immediately preceding stimulation with TRH. Data reflect measurements on 96 cells (36 for males, 60 for females) studied in 13 independent experiments. Differences between treatment groups were not significant ($P > 0.05$).

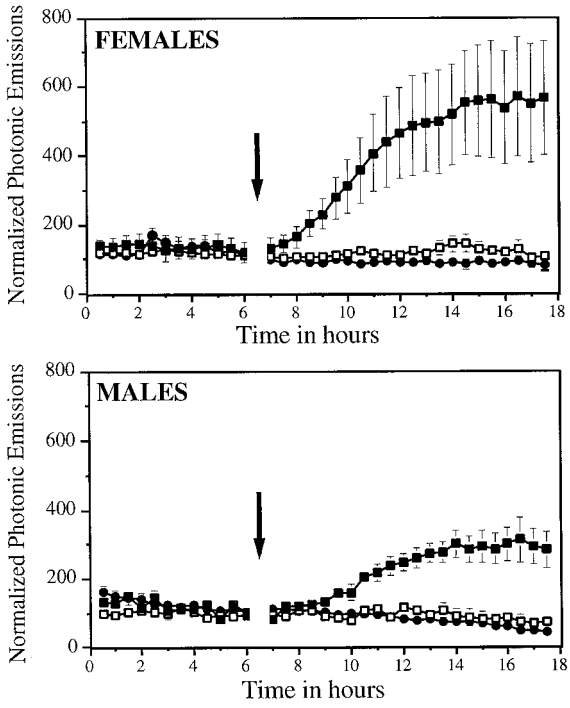

Fig. 4. Dynamic Analysis of Responsiveness to TRH

Photonic values for individual cells were normalized to the three baseline measurements obtained just before TRH (1 μM) addition. Mean (±SEM) values were then calculated for cells assigned to one of three specific groups on the basis of their response to TRH or vehicle. ■, TRH-responsive cells (n = 24 lactating, n = 25 male). □, Cells not responsive to TRH (n = 36 lactating, n = 11 male). ●, Cells treated with vehicle (n = 45 lactating, n = 11 male). The *arrow* shows the time at which the perifusion medium was switched to one containing either TRH or vehicle alone.

Having established gender-specific differences in the dynamics and demographics of mammotrope responsiveness, we questioned whether the initial, resting level of expression might also influence responsiveness. To address this question, we used raw data from the same set of experiments and compared pretreatment levels of photonic emissions for TRH-responsive cells and TRH-nonresponsive cells (Fig. 5A). Interestingly, those mammotropes that proved to be TRH nonresponsive exhibited pretreatment levels of reporter activity that were 4- to 6-fold higher than the corresponding values for TRH-responsive cells (Fig. 5A), and this was true regardless of the gender of the pituitary donor. Thus, only those mammotropes with low to moderate levels of PRL promoter-driven re-

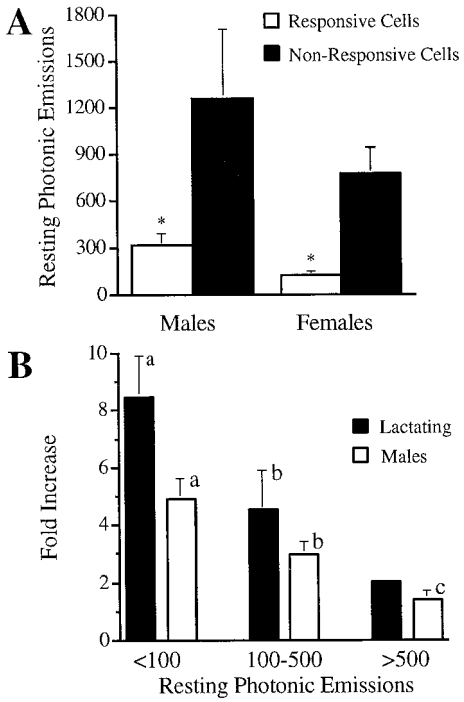

Fig. 5. Relationship between Initial PRL Promoter-Driven Reporter Activity within Individual Mammotropes and Responsiveness to TRH

A, The average (mean ± SEM) level of specific photonic emissions just before TRH stimulation is shown for TRH-responsive cells (□) and those not responsive to the secretagogue (■). Other details as in Fig. 4. *, $P < 0.05$ *vs.* non-responsive cells. B, Distribution of responses as a function of basal reporter activity. Values for individual cells were assigned to one of the three subgroups according to the mammotrope's basal level of specific photonic emissions. <100 (n = 20 lactating, 14 male); 100–500, (n = 22 lactating, 11 male); >500 (n = 18 lactating, 11 male). The maximum fold-increase in photonic emissions induced by TRH was then calculated for each responsive cell only and averaged for each group. The numbers adjusted for only the responsive cells are as follows: <100, 12 female, 10 male; 100–500, 11 female, 9 male; >500, 1 female, 6 male. Within each gender, subgroups with different letters are significantly different at $P < 0.05$.

porter activity at the time of challenge were capable of responding to an evocative stimulus. In addition, the basal level of reporter activity served as an excellent predictor of the magnitude of the TRH response. More specifically, we found that the fold-increase of reporter activity evoked by TRH in those responsive mammotropes was inversely proportional to the pretreatment level of such activity (Fig. 5B). As before, gender of the pituitary donor had no influence on this inverse relationship between the resting level of reporter activity and fold-induction of the response. When viewed as a whole, these findings indicate that the initial level of PRL gene expression has a striking impact on both the ability of an individual mammotrope to respond to a transcriptional stimulus and the magnitude of the response.

In a previous study, we subjected the same transfected mammotropes to photonic measurements in 10-min windows on 2 consecutive days and found that the resting level of reporter activity changed spontaneously, sometimes dramatically, from one day to the next (4). This observation, coupled with our aforementioned discovery of an inverse relationship between the basal state of PRL gene expression and responsiveness, invited speculation that a particular cell's response to a transcriptional challenge should also fluctuate over time. As a first step toward testing this line of reasoning, we subjected transfected cells to continuous measurements of photonic emissions for 24 h and found (Fig. 6) that a majority of mammotropes did indeed exhibit spontaneous, random changes in the rate of photonic emissions reflective of PRL gene expression. In fact, 58% of the mammotropes studied (n = 24; four independent experiments) exhibited at least a 2-fold change (ranging up to 50-fold) in the photonic emissions rate over the course of the day-long measurement sessions.

After demonstrating that individual mammotropes do undergo dynamic, short-term changes of basal PRL promoter-driven reporter activity, we next focused on the issue of whether responsiveness might likewise change over time. Our experimental strategy was to expose single, transfected mammotropes to successive, transient TRH challenges. The concentration of secretagogue was the same as that used in the continuous perifusion experiments, and challenges (of 10 min duration) were separated by 8 h. The rationale here was that if the basal rate of expression dictates the direction as well as the magnitude of response, then those cells in which expression was elevated by an initial TRH challenge should exhibit a diminished response when presented with a second challenge 8 h later. Figure 7 provides representative examples of cells that were subjected to single (*left* and *right* columns) or double (*center* column) TRH challenges. As shown, the initial stimulus evoked a spectrum of responses consistent with our findings illustrated earlier (Fig. 2). Individual mammotropes were then pooled into two subpopulations on the basis of whether or not they responded to the initial, transient presentation of TRH. To facilitate comparisons between these groups, all photonic values were then normalized to those obtained before stimulation (Fig. 8). As hypothesized, those mammotropes that responded positively to the first TRH challenge exhibited a pronounced diminution of reporter activity when presented with a second challenge. [Specifically, only 12% of the cells exhibited a diminished response after the first challenge whereas the value rose to 82% for the second challenge.] This failure to respond cannot be attributed to the possibility that the cells were already at a maximal level of activity. This is evidenced by comparison of the data in panels A and B of Fig. 8, which reveals that the stimulatory response to the first challenge would have continued to rise were if not for the second TRH pulse. Moreover, the relative expression values (fold-

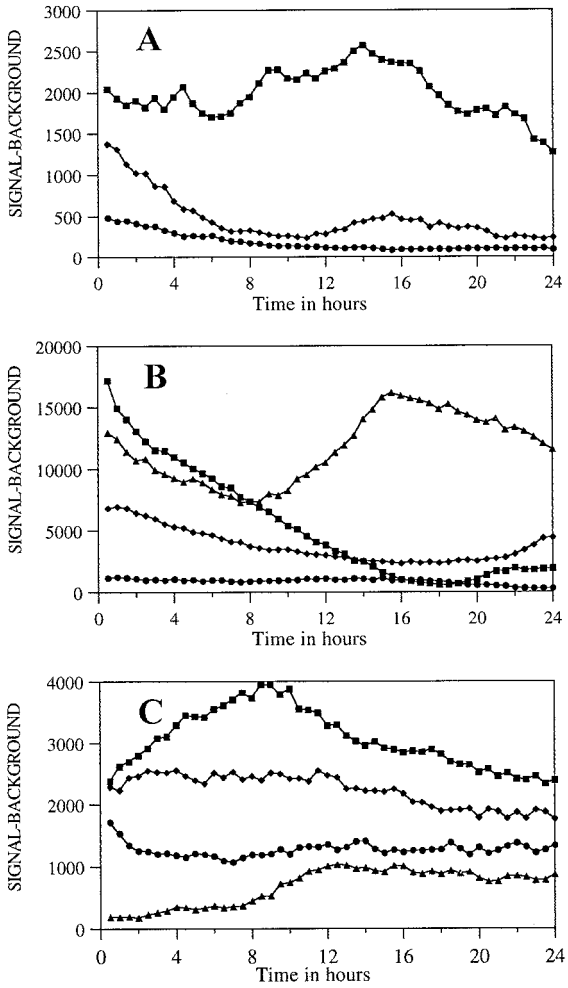

Fig. 6. Individual Mammotropes Exhibit Spontaneous Fluctuations in the Resting Level of Photonic Emissions

Transfected mammotropes from lactating rats were subjected to continuous measurement of photonic emissions in 30-min bins for 24 h. Shown here are the profiles of individual cells that were in the same microscopic fields (A, B, and C). Data are representative of 24 individual mammotropes studied in four independent experiments.

increases) achieved 19 h after a TRH pulse were roughly half the maximal values measured after continuous TRH infusion (compare the responders in Fig. 8A to those in Fig. 4, *upper panel*). These results show that when a TRH challenge is superimposed upon an elevated baseline, the response is not just attenuated, but reversed. As such, they provide experimental evidence that a mammotrope's capacity to respond to a transcriptional stimulus is influenced largely by its initial level of expression.

DISCUSSION

In this study, we demonstrated the feasibility and utility of continuously monitoring the dynamics of gene expression within individual, living mammotropes in primary culture. This goal was achieved by quantifying photonic emissions, reflective of endogenous gene expression, from anterior pituitary cells transfected with a PRL promoter-driven luciferase reporter plasmid. By using this approach, we were able to record, in a time-resolved manner, an indirect measure of gene expression under basal conditions and stimulus-expression coupling in response to TRH. The validity of this single-cell approach as a paradigm for monitoring expression dynamics is evidenced by our earlier observation that the vast majority of transfected mammotropes exhibited predictable photonic responses to secretagogues known to either increase (epidermal growth factor) or decrease (dopamine) PRL gene transcription within entire populations of pituitary cells (4). Additional supportive evidence is that expression of the same reporter construct was highly specific for rat mammotropes. This was revealed by coupling photonic analysis with measurements of PRL storage (immunocytochemistry), release (reverse hemolytic plaque assay), and mRNA content (*in situ* hybridization cytochemistry) (4). Indeed, we could not detect any pituitary cell transfected with the rPRL-LUC reporter that emitted photons and subsequently proved to be something other than a mammotrope. Given the rapid decay of firefly luciferase activity in rat mammotropes ($t_{1/2}$ = 1 h, Ref. 14), this system comprises a highly responsive and valid tool for estimating (albeit indirectly) the dynamics of gene expression within living, primary mammotropes.

Armed with this tool, we characterized first the dynamic response to TRH, a physiologically relevant transcriptional stimulus of the PRL gene. We found that not all mammotropes responded to TRH with a robust augmentation of reporter activity. In fact, a significant fraction of transfected mammotropes exhibited no stimulation whatsoever. Why did some mammotropes from the same pituitary gland exhibit responses to TRH whereas others did not? Although available evidence will not support an unequivocal answer to this question, the modulation of the concentration of free intracellular calcium ($[Ca^{2+}]_i$) is probably involved in the regulatory mechanism. Indeed, cytosolic Ca^{2+} plays a crucial role in the control of basal PRL gene transcription (15) and is a requisite mediator of the transcriptional response of mammotropes to TRH (16). Interestingly, only a subpopulation of primary mammotropes was found to exhibit an increase of $[Ca^{2+}]_i$ after exposure to TRH (17, 18). Therefore, heterogeneity in the distribution of functional TRH receptors (*i.e.* those linked to Ca^{2+} mobilization) may very well account for the failure of some mammotropes to mount an acute response to TRH. Another possible explanation for the selective response to TRH is that the decay characteristics of the luciferase protein and mRNA may have compromised our ability to detect very rapid and transient changes of PRL gene expression in the majority of nonresponders. Indeed, results from nuclear run-on assays

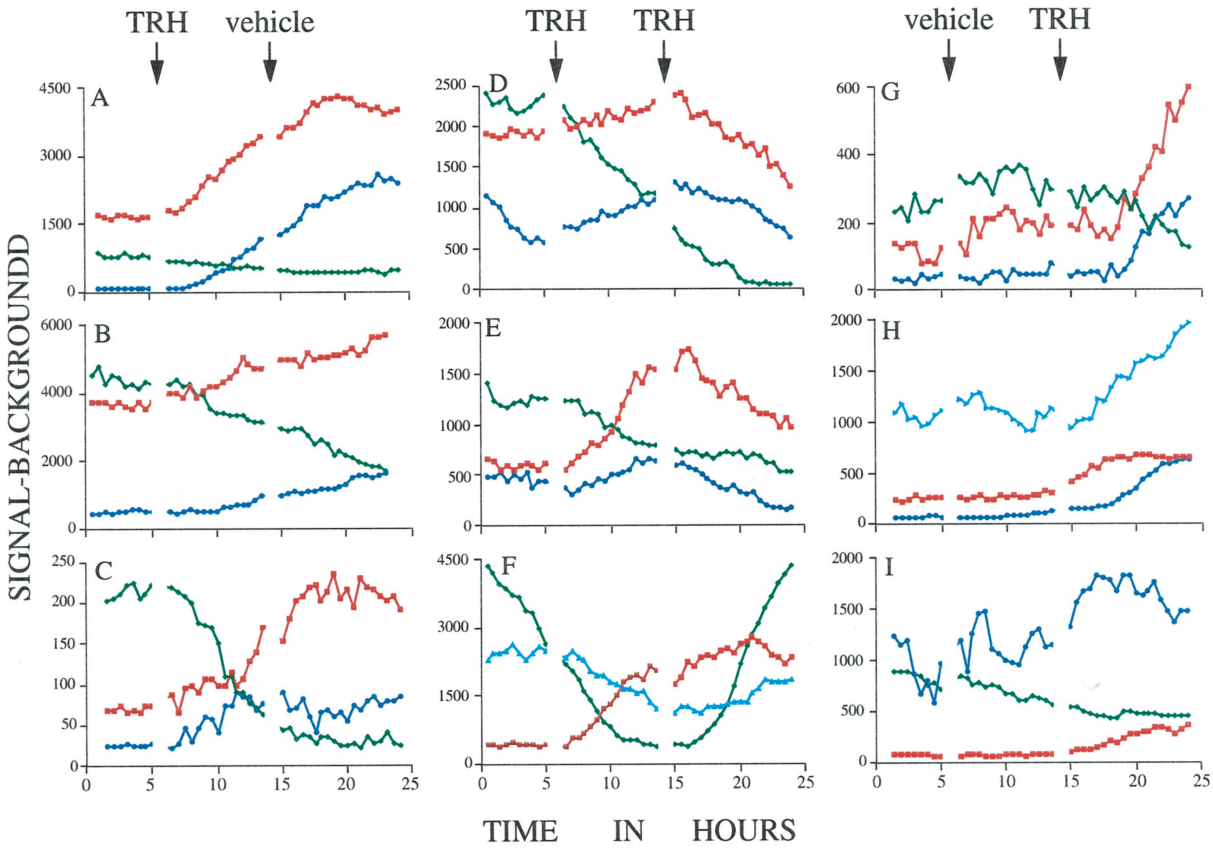

Fig. 7. Representative Profiles of Mammotropes Subjected to Successive TRH Challenges

Transfected mammotropes from lactating rats were subjected to photonic emission measurements before and after a transient (10 min) stimulation with TRH or vehicle. After 8 h, the same cells were subjected to a second challenge with TRH or vehicle. Profiles in each panel represent measurements of photonic emissions made on representative, individual mammotropes present in the same microscopic field. Data are representative of 34 (*left panels*), 66 (*central panels*), and 33 (*right panels*) individual mammotropes studied in 4, 8, and 3 independent experiments, respectively. *Red* and *blue* traces represent those cells that exhibited clear increases of PRL promoter-driven reporter activity after the first stimulation with TRH. The selected cells that exhibited no change or a reduction in the rate of photonic emissions after TRH are presented as *green* traces.

show that changes of PRL gene expression can be detected within just a few minutes, but available evidence does not support the idea that such changes are transient (19, 20). Therefore, while we cannot discount this alternative possibility, we deem it remote.

In pursuit of our second objective, we explored the relationship between the secretory status of the pituitary donor and the level of PRL promoter-driven gene expression. For this purpose, we compared the resting reporter activity values of mammotropes from male and lactating female rats because the secretory capacity of mammotropes from the former group was reported to pale in comparison with that of the latter group (21). Interestingly, we failed to find a gender-specific difference in basal reporter activities suggesting that other variables might contribute to differences in secretory capacity. On the other hand, we found that the secretory status of the pituitary donor did have a striking influence on the proportional abundance of TRH-responsive mammotropes; the fraction for males was almost 2-fold greater than that for females (69% *vs.* 40%, respectively). Collectively, these results raise an interesting and provocative question: If striking, gender-specific differences in basal expression are not obvious, and the proportional abundance of TRH-responsive mammotropes favors males, why do females secrete more PRL than males? The answer might derive from at least two considerations. First, the percentage of all pituitary cells that secrete PRL is 2-fold greater for lactating females than for males (~55% *vs.* 30%, respectively). Thus, the absolute numbers of TRH-responsive mammotropes are very similar for both genders of pituitary donor. Second, and perhaps equally important, the magnitude of response to TRH is greater (again by ~2-fold) for mammotropes derived from females as opposed to males. The net result, then, is that both the dynamics and demographics of TRH responsiveness favor a higher level of PRL output by pituitary cells from females. Of course, this conclusion must be tempered by the fact that we did not measure PRL secretion in parallel in these particular experiments, and that our inability to detect a gender-specific difference in basal expression cannot be interpreted unequivocally to mean that

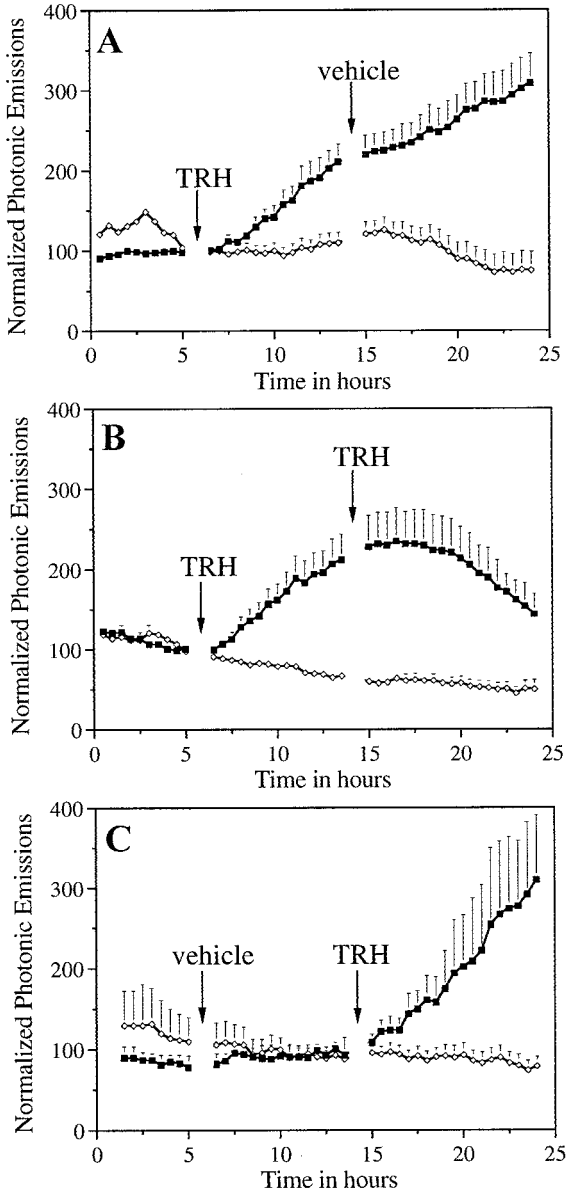

Fig. 8. Elevation of Basal Reporter Activity by an Initial TRH Challenge Renders Mammotropes Unresponsive to a Subsequent Challenge

Photonic emission values for each individual mammotrope studied in the experiment described in Fig. 7 were normalized to baseline values obtained before the first TRH or vehicle challenge. After normalization, cells were assigned to one of two subgroups: those that responded positively to the initial TRH challenge and those that did not. It is noteworthy that the fraction of positive responders to a transient (10-min) TRH challenge was only 29% *vs*. 40% for cells continuously perifused with the peptide. Data for TRH-responsive cells are the mean (±SEM) of 10 (panel A), 17 (B), and 11 (C) individual mammotropes studied in 4, 8, and 3 independent experiments, respectively. Data for cells not responsive to TRH are presented as the mean of 24 (A), 49 (B), and 22 (C) individual mammotropes studied in the same sets of experiments. As shown, a single, transient presentation of TRH evoked, in responsive cells, a long-term elevation of reporter activity (A), and this effect was independent of the time at which the transient challenge was applied (compare panels A and B to C). However, presentation of a second TRH pulse (B), elicited on average a clear reduction in the rate of photonic emissions reflective of gene activity.

one does not exist. The latter consideration notwithstanding, our studies reveal fundamental differences in the way mammotropes from males and females respond to an evocative stimulus of PRL gene transcription.

Our final objective was to evaluate the intriguing possibility that the basal level of expression might influence the capacity of a mammotrope to mount a response after stimulation with TRH, and three types of analysis were employed for this purpose. First, we screened the profiles of individual mammotropes exposed to TRH and observed (Fig. 2) that elevated pretreatment levels of reporter activity were generally associated with poor TRH responsiveness and *vice versa*. The existence of such an inverse relationship between basal activity and magnitude of stimulation was confirmed more quantitatively when we plotted the averaged magnitudes of response against the resting rate of photonic emissions (Fig. 5B) and found a negative association between the two parameters. Finally, we conducted an experiment in which we were able to reverse (positive to negative) TRH responsiveness by pharmacologically elevating the pretreatment level of PRL promoter-driven reporter activity (Fig. 8). The results of all three analyses support the same conclusion: that responsiveness to TRH is dictated largely by the cell's level of expression at the time of challenge.

When considered collectively, our findings invite speculation that mammotropes are able to sense their expression state and send a feedback signal conveying information as to whether an individual cell need respond to TRH, and if so, the magnitude of the response. The physiological implications of such a servo-mechanistic model are rather obvious: mammotropes could be induced by an appropriate stimulus to convert between two expression states — responsive and nonresponsive. In this manner, PRL gene expression could be tightly regulated to satisfy, but not exceed, physiological requirements for production of the corresponding hormone. While the identity of the putative, autocrine (or intracrine) feedback agent remains to be established, there are already some candidate molecules to subserve such a role. These include cytoplasmic Ca^{2+} (for reasons detailed earlier) and PRL itself [owing to the well established autocrine feedback effects of the hormone on its own secretion (22), and the presence of PRL receptors within the nucleus (23)]. Other candidates deserving of special consideration are the so-called lumicrine peptides for which RESP18 is a prototype in neuroendocrine cells (24). Induction of RESP18 expression initiates a signaling pathway that conveys a signal from the lumen of the endoplas-

mic reticulum to the nucleus to regulate expression of responsive genes.

During the course of completing the present studies on PRL gene expression, and subsequent to our prior publication of a system for making continuous measurements of gene expression in single, living cells (11), Takasuka *et al.* (25) reported the results of a study in which they made multiple measurements (30-min windows at 3-h intervals) of gene expression on cells from a rat PRL-secreting cell line stably transfected with a human PRL promoter-driven luciferase construct. They observed with intermittent measurements on transformed cells (transfected with a heterologous promoter), as we did in the present study with normal mammotropes (transfected with a homologous promoter), that the basal level of PRL gene expression is not constant but can vary in the same cell over the course of several hours. Collectively, these results confirm and provide an explanation for our earlier observation (4) that individual, transfected mammotropes sampled photonically in 10-min windows at 24-h intervals exhibited what appeared to be spontaneous, random fluctuations of PRL gene expression. These same investigators also made intermittent measurements of PRL promoter-driven reporter activity before and after treatment with TRH. On the basis of six single-cell profiles (no quantitative data were provided), they proposed that the gene expression response to TRH was heterogeneous, a conclusion supported by a more rigorous, quantitative analysis in our present study.

In summary, we have developed and refined a model for monitoring, in a time-resolved manner, an indirect measure of stimulus-expression coupling within single, living mammotropes. We have used this paradigm to gain several interesting and unexpected insights about the *dynamics* of PRL gene activation. These include the finding that expression responses are highly heterogeneous and are impacted greatly by the secretory status of the pituitary donor and the presentation of the stimulus. Moreover, we found that these responses are entrained by the initial level of PRL gene expression. Inasmuch as the basal level of PRL gene expression can change spontaneously over time, it seems reasonable to propose that responsiveness of a given cell to a transcriptional stimulus might also vary over time as a function of its expression state.

MATERIALS AND METHODS

Cell Dispersion and Microinjection

Anterior pituitaries from male (250 g) and primiparous, lactating female (days 6–10 postpartum) rats (Sprague-Dawley Harlan, Madison, WI) were dispersed with trypsin as reported elsewhere (4). All the rats used were of a comparable age at the time of pituitary collection. Monodispersed cells were plated on gridded coverslips previously coated with poly-L-lysine 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, Inc., Gaithersburg, MD, in which L-valine had been replaced by D-valine) and supplemented with 0.1% BSA, insulin-transferrin-selenium Premix, and antibiotics. Cells were allowed to attach for about 1 h, covered with 2 ml of defined medium supplemented with 10% FBS, and incubated in a humidified atmosphere of 5% CO_2-95% air. After 2 days in culture, cells within a particular grid were microinjected with a reporter plasmid (rPRL-LUC, 0.2 μg/μl in 10 mM PBS) in which 2.5 kbp of the 5′-flanking region of the rat PRL gene were placed upstream of the coding sequence for firefly luciferase. Cell microinjection was performed as described previously (4) to ensure the delivery of the same amount of plasmid among cells. After microinjection, cells were washed twice and cultured for 24 or 48 h in phenol red-free DMEM (Life Technologies, Inc.) supplemented with 10 mM HEPES, 10% FBS, 0.1% BSA, and antibiotics.

Continuous Monitoring of PRL Gene Expression in Single, Living Cells

For monitoring of photonic emissions, reflective of PRL gene expression, microinjected cells were incubated in phenol-free DMEM supplemented with 0.1% BSA, 10% FBS, 10 mM HEPES, and antibiotics for 24 or 48 h. Four hours before measurements of reporter activity, we incubated the cells with the same medium containing 0.1 mM luciferin (Sigma, St. Louis, MO). [This step was taken to ensure the equilibration of intracellular luciferin stores before imaging and to allow stabilization of a diminution of photonic activity that occurs in some cells during the first few hours after exposure to low concentrations of luciferin]. Then, coverslips were assembled in Sykes-Moore chambers that were subsequently filled with a culture medium of the same composition as before except that it was devoid of BSA and bicarbonate, and supplemented with 10% FBS and 0.1 mM luciferin. [Preliminary experiments established this to be a saturating dose of luciferin for perfusion studies. Higher concentrations did not influence the average rate of photonic emissions]. The chamber bearing the coverslip was next transferred to the heated (37 C) stage of a Zeiss Axioscope (Carl Zeiss, Jena, Germany) located in a dark room. Transfected cells were then reidentified with the help of the numbered/lettered grid, and a bright field image was captured for reference purposes. Photonic emissions from cells in single grids on three separate coverslips were generally captured consecutively, and the coverslip supporting the most photon-emitting cells was chosen for long-term monitoring of reporter activity. This was achieved by accumulating photonic emissions in 30-min bins for 18 h. During this period, cells were perifused continuously with the same medium at a very low rate (10 μl/min) to ensure replenishment of nutrients and substrate. After the first 6 h, cells were perifused for 10 min with the same medium containing either TRH (1 μM) or vehicle at a rate of 1 ml/min (to accelerate exchange of chamber contents), and then the flow was returned to the lower rate with the same treatments. For administration of transient TRH pulses, cells were infused (1 ml/min) with TRH (or vehicle) for 10 min after which they were infused again with vehicle (1 ml/min) for another 10 min in order to remove the secretagogue before returning to the normal flow rate.

For photonic emission measurements, we used a photon capture system comprised of a Hamamatsu VIM photonic camera and an Argus 50 image processor (Hamamatsu Photonics, Bridgewater, NJ). To quantify reporter activity in living cells, we accumulated images of photonic emissions and superimposed them over the corresponding bright field image of cells. The number of photonic events within a window of fixed area corresponding to the position of each transfected cell was then calculated. Photonic measurements made in at least 10 adjacent areas devoid of cells were used to compute a background value that was subsequently subtracted from the total accumulation to calculate specific pho-

tonic emissions from each cell. Other details of this procedure have been reported previously (4, 14).

Comparisons between treatment groups were made with a two-tailed, Student's *t* test, and results were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

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