Function-specific calcium stores selectively regulate growth hormone secretion, storage, and mRNA level

JAMES D. JOHNSON,1 CHRISTIAN KLAUSEN,2 HAMID R. HABIBI,2 AND JOHN P. CHANG1
1Department of Biological Sciences, University of Alberta, Edmonton, T6G 2E9; and 2University of Calgary, Calgary, Alberta, Canada T2N 1N4

Received 26 January, 2001; accepted in final form 20 November, 2001

Johnson, James D., Christian Klausen, Hamid R. Habibi, and John P. Chang. Function-specific calcium stores selectively regulate growth hormone secretion, storage, and mRNA level. Am J Physiol Endocrinol Metab 282: E810–E819, 2002. First published November 27, 2001; 10.1152/ajpendo.00038.2001.—Ca2+ stores may regulate multiple components of the secretory pathway. We examined the roles of biochemically independent intracellular Ca2+ stores on acute and long-term growth hormone (GH) release, storage, and mRNA levels in goldfish somatotropes. Thapsigargin-evoked intracellular Ca2+ concentration ([Ca2+]i) signal amplitude was similar to the Ca2+-mobilizing agonist gonadotropin-releasing hormone, but thapsigargin (2 μM) did not acutely increase GH release, suggesting uncoupling between [Ca2+]i and exocytosis. However, 2 μM thapsigargin affected long-term secretory function. Thapsigargin-treated cells displayed a steady secretion of GH (2, 12, and 24 h), which decreased GH content (12 and 24 h), but not GH mRNA/production (24 h). In contrast to the results with thapsigargin, activating the ryanodine (Ry) receptor (RyR) with 1 nM Ry transiently increased GH release (2 h). Prolonged activation of RyR (24 h) reduced GH release, contents and apparent production, without changing GH mRNA levels. Inhibiting RyR with 10 μM Ry increased GH mRNA levels, production, and storage (2 h). Increasing [Ca2+]i independently of Ca2+ stores with the use of 30 mM KCl decreased GH mRNA. Collectively, these results suggest that parts of the secretory pathway can be controlled independently by function-specific Ca2+ stores.

CA2+ is an important regulator of many components of the protein-secretory pathway. It is well accepted that localized domains of high cytosolic Ca2+ concentration ([Ca2+]c) are the trigger for the membrane fusion and exocytosis that culminates this pathway (34). In addition, there is growing appreciation of the roles of both [Ca2+]c and luminal Ca2+ ([Ca2+]L) in the regulation of upstream components of the extended secretory pathway (i.e., from transcription to exocytosis; reviewed in Ref. 22). In neuroendocrine cells, [Ca2+]L has been implicated as a regulator of secretory vesicle/granule recruitment into the releasable pool (44), whereas secretory granule [Ca2+]L has been shown to modulate the condensation state of peptide hormones and the probability of exocytosis (1, 18, 42). Intracellular Ca2+ stores of the endoplasmic reticulum (ER) also have a dual function in the regulation of hormone release. On the one hand, Ca2+ release from the ER controls exocytosis in many nonexcitable, as well as some excitable, cell types (35, 38, 49). On the other hand, it has also been established that ER [Ca2+]L must remain high for the maintenance of normal secretory pathway function. The Ca2+ milieu of the ER lumen plays an important role in the posttranslational processing, sorting, and packaging of secreted proteins (2, 6, 11). In many cases, proteins are misfolded in Ca2+-depleted ER. Cells in which ER [Ca2+]L has been depleted with thapsigargin (Tg), a potent and selective inhibitor of ER Ca2+-ATPase (29, 47, 52), exhibit decreased levels of certain proteins within the ER. This phenomenon is due to a stimulation of proteolysis and/or increased transport out of the ER (54). Others have reported that Tg treatment inhibits the export of proteins from the ER, an effect probably related to errors in protein folding or other posttranslational modifications (11). Ca2+ has also been shown to regulate mRNA translation through multiple mechanisms. Depletion of ER Ca2+, with Tg or Ca2+-mobilizing agonist, leads to a rapid (within minutes) inhibition of translational initiation (5, 40). Interestingly, Preston and Berlin (39) noted that protein synthesis in HeLa cells was sensitive only to specific Ca2+-mobilizing treatments, suggesting a functional heterogeneity between multiple ER Ca2+ stores.

Finally, the ER communicates with the nucleus through several routes to regulate gene transcription. As is the case for translation, perturbed ER [Ca2+]L homeostasis generates a signal, which acts independently of increased [Ca2+]c to control the expression of several genes. These genes include calreticulin and immunoglobulin heavy-chain-binding protein, which encode ER resident Ca2+ buffering proteins and chaperones (29). The are many examples of cytosolic Ca2+ signal that stimulate the expression of specific genes (e.g., Ref. 41). Both positive and negative transcription

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
factors have been identified (e.g., Refs. 7, 12, 43). However, the Ca$^{2+}$-dependent repression of gene expression seems to be less common. Collectively, the literature indicates that the regulation of multiple components of the extended secretory pathway by Ca$^{2+}$ is complex. The ubiquity of Ca$^{2+}$ as a signaling molecule inevitably leads to the question: how can all of these Ca$^{2+}$-dependent cellular functions be regulated independently?

Multiple function-specific Ca$^{2+}$ stores offer a solution to the problems associated with the independent regulation of multiple cellular functions by Ca$^{2+}$ (22). However, whether certain ER Ca$^{2+}$ stores regulate exocytosis whereas others control gene expression or protein processing is still poorly understood. Indeed, multiple ER Ca$^{2+}$ stores can be distinguished pharmacologically (22, 38). Most cell types contain agonist-sensitive Ca$^{2+}$ stores that are mobilized by inositol 1,4,5-trisphosphate (IP$_3$) and refilled by Tg-sensitive sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) Ca$^{2+}$-ATPases (38). An additional Ca$^{2+}$ store, which can be modulated by ryanodine (Ry), coexists with the IP$_{3}$-sensitive Ca$^{2+}$ pool(s) in a variety of cell types (e.g., Ref. 16). Some cell types possess at least three distinct intracellular Ca$^{2+}$ stores (reviewed in Ref. 22). Whether other classes of ER Ca$^{2+}$ stores, such as those sensitive to Ry, modulate early components (gene expression, protein synthesis, or protein processing) of the extended secretory pathway is poorly understood. There are no reports of the effects of pharmacologically distinct Ca$^{2+}$ stores on multiple components of the extended secretory pathway.

Goldfish somatotropes are an interesting subject for such studies, because they appear to contain several biochemical classes of intracellular Ca$^{2+}$ stores that may be attributable to the ER. The Ca$^{2+}$-dependent signal transduction pathways of two physiological growth hormone (GH)-releasing neuropeptides, salmon gonadotropin-releasing hormone (sGnRH) or chicken GnRH-II (cGnRH-II), have been characterized. Acute GH release evoked by both GnRHs is initiated entirely by rapidly exchanging intracellular Ca$^{2+}$ stores, which are sensitive to TMB-8 and caffeine (25, 55). Interestingly, although cGnRH-II-stimulated GH release can be abolished by 10 μM ryanodine (24), it is insensitive to Tg and other inhibitors of SERCA (25), suggesting the existence of additional, functionally independent Ca$^{2+}$ stores. In the present study, we examined the role of Tg-sensitive Ca$^{2+}$ stores in several cellular functions by use of Ca$^{2+}$ imaging, radioimmunoassay measurements of hormone secretion and cellular GH content, and Northern analysis. Although Tg-sensitive Ca$^{2+}$ stores did not regulate acute GH release or GH mRNA levels, they were involved in acute [Ca$^{2+}$], homeostasis as well as long-term GH secretion and storage. In contrast, inhibition of RyR led to a significant increase in GH mRNA. Increasing [Ca$^{2+}$], without emptying intracellular Ca$^{2+}$ stores led to a robust decrease in GH mRNA levels. This study provides novel evidence that certain classes of ER Ca$^{2+}$ stores may be uncoupled from specific components of the extended secretory pathway and imparts insights into the relative importance of cytosolic and luminal Ca$^{2+}$ signaling in these processes.

**MATERIALS AND METHODS**

**Reagents.** Stock solutions of sGnRH and cGnRH-II (Peninsula Laboratories, Belmont, CA) were made in distilled, deionized water. Depolarizing medium (30 mM KCl) was made by equimolar substitution of NaCl with KCl. High-purity Ry (99.5%; Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide (DMSO). Tg (Calbiochem) was dissolved in ethanol. Cyclohexamide (ICN, Aurora, OH) was dissolved directly into testing media. At the concentrations used in this study (final concentrations <0.1%), DMSO or ethanol did not affect [Ca$^{2+}$], (data not shown), GH release, or GH contents.

**Animals and cell preparation.** Animal use protocols were approved by the University of Alberta Animal Care Committee in accordance with national guidelines. Pituitaries from goldfish (Aquatic Imports, Calgary, AB, Canada) were dispersed using a trypsin-DNase protocol that has been described previously (8). Dispersed cells were cultured overnight on poly-L-lysine-coated coverslips (0.25 million cells/coverslip, 1-ml medium) for imaging experiments (24), preswollen Cytodex beads (1.5 million/dish, 6-ml medium) for cell column perfusion studies (8), or Primaria surface-modified tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ) for static incubation experiments designed to measure long-term hormone secretion/contents (24-well plates; 0.25 million cells/well, 1-ml medium) or GH mRNA levels (6-well plates; 2 million cells/well, 4-ml medium) in Medium 199 (M-199) with Earle’s salts (GIBCO, Grand Island, NY) supplemented with 2.2 g/l NaHCO$_3$, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, and 1% horse serum (pH adjusted to 7.2 with NaOH) at 28°C, 5% CO$_2$, and in saturated humidity.

**Cell identification and Ca$^{2+}$ imaging.** Morphologically identified somatotropes [95% accuracy (50)] that had been stably and evenly loaded with fura 2 by 35-min incubation in 10 μM of the acetoxymethyl ester form of the dye (Molecular Probes, Eugene, OR), were imaged for our laboratory as described previously (24). Cells were perfused with imaging medium (testing medium without phenol red) at room temperature. The ratio of emission intensity (at 510 nm) from alternate 340 nm and 380 nm excitation was converted to intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) estimates off-line using the formula of Grynkiewicz et al. (17) and empirically derived constants as detailed previously (24).

**Cellular GH content and hormone release experiments.** Prolonged cellular GH content and GH release were assayed in testing medium (GIBCO M-199 with Hanks’ salts, supplemented with 2.2 g/l NaHCO$_3$, 25 mM HEPES, 100,000 U/l penicillin, 100 mg/l streptomycin, 20 mg/l phenol red, and 0.1% BSA, pH adjusted to 7.2 with NaOH) under static incubation conditions [same as culture conditions described above (8)]. After the incubation period, the medium was aspirated, and cells were lysed and snap frozen. Prolonged exposures to the treatments used in this study did not reduce the cell number or the viability of the pituitary cell cultures as assessed by trypan blue exclusion. Furthermore, microscopic examination of treated cells did not reveal morphological signs of apoptosis. For dynamic measurements of hormone release, cells were perfused at 18°C with testing medium as described previously (8). Medium was collected in 5-min or 30-s fractions and stored at −26°C. GH content of samples from both static incubation and cell column perfu-
Hormone release measurements from static incubation studies were expressed as a percentage of controls. For studies of hormone secretion in perfusion conditions, values were normalized to the mean of the first five measurements (% pre-treatment). Where analyzed, net hormone release responses of perfused cell columns to treatments were quantified by integrating the area under the curve during the treatment period. Each time point was subtracted from the prepulse mean, defined as the average of the three time points before the treatment period.

Quantification of GH mRNA levels. RNA extraction and Northern blot analysis of dispersed gold pituitary cells (cultured as in Cellular GH content and hormone release experiments) were optimized from protocols designed for isolated pituitary fragments (20). Briefly, total RNA was isolated with TRIzol reagent (GIBCO), based on the acid-guanidinium thiocyanate phenol-chloroform method (10). RNA was quantified and checked for purity with a spectrophotometer (A260/A280). Treatments did not affect the amount of RNA isolated from individual wells. RNA (5 μg/lane) was loaded and resolved on a 1.2% agarose-formaldehyde gel before being transferred to a Hybond N+ membrane (Amersham, Arlington Heights, IL) by use of capillary action in the presence of 20× sodium chloride-sodium phosphate-EDTA [1× SSPE (in M) = 0.3 NaCl, 0.02 NaH2PO4, H2O, 0.002 EDTA, pH 7.4]. Membranes were prehybridized for 1 h and hybridized for 2 h at 60°C with a specific cDNA probe for goldfish GH (labeled with [α-32P]deoxyctydine 5’-triphosphate by means of the random primer method, 3,000 Ci/mM, Amersham) in rapid hybridization buffer (Amersham). Membranes were then subjected to a series of washes, with 0.1% SDS, of increasing stringency [0.1× standard sodium citrate (SSC); 1× SSC = 0.15 M NaCl, 15 mM sodium citrate]. Membranes were then exposed to film. Although ethidium bromide-stained gels were imaged to ensure equal loading of lanes, membranes were also stripped and rehybridized with a probe for 18S ribosomal RNA, which served as an internal standard. Treatments caused no observable change in 18S levels. Autoradiograms were scanned and quantified using the densitometry function of NIH Image 1.62 (National Institutes of Health, Bethesda, MD). GH mRNA values were normalized to the internal control (18S levels) for each lane.

Statistical analysis. Statistical analyses were performed using Student’s unpaired t-test or one-way ANOVA (followed by Fisher’s protected least significant difference test), where appropriate. Differences were considered to be significant when P < 0.05. Results are presented as means ± SE.

RESULTS

Uncoupling of Tg-sensitive Ca2+ stores from acute GH release. The principle physiological regulators of GH release in goldfish somatotropes, sGnRH and cGnRH-II, act by mobilizing intracellular Ca2+ stores that are not sensitive to Tg (23). Previous studies have shown that Tg can generate Ca2+ signals, under extracellular Ca2+-free conditions, in goldfish pituitary cells (32). In the present study, we sought to confirm the presence of Tg-sensitive Ca2+ stores in single identified somatotropes and establish whether Ca2+ released from these pools can evoke GH secretion. In cells treated with 2 μM Tg, [Ca2+]i increased slowly and steadily before gradually decaying to a higher baseline value (Fig. 1); Tg-stimulated Ca2+ signals are presumably initiated as a result of the uncompensated passive leak from SERCA-containing intracellular Ca2+ stores and sustained by store-operated Ca2+ entry. Compared with Ca2+ signals evoked by Tg in the same cell, GnRH generated Ca2+ signals with distinct kinetics. Ca2+ signals evoked by 100 nM sGnRH (Fig. 1) or 100 nM cGnRH-II (data not shown) displayed a faster rate of rise and a rapid decay during the application period, consistent with a more “active” release of Ca2+ from intracellular stores (23). At the resolution used in the present study, the spatial pattern of Tg- and GnRH-evoked Ca2+ signals were not substantially different (Fig. 1).

Despite the fact that 2 μM Tg elicited Ca2+ signals with similar maximal amplitudes compared with endogenous secretagogues such as sGnRH and cGnRH-II (Figs. 1 and 2A, Ref. 23), Tg failed to stimulate the release of GH in “rapid fraction” cell column perfusion experiments (Fig. 2B). Thus Ca2+ signals generated by 2 μM Tg may be selectively uncoupled from the acute regulation of GH secretion in goldfish somatotropes. The inability of Tg treatment to cause acute GH release was also confirmed at 0.01–1 μM (data not shown).

Characterization of basal GH release and storage. In preparation for studies examining the effects of selective manipulations of Ca2+ stores on the long-term regulation of basal GH secretion and storage, we first characterized some features of these parameters in unstimulated goldfish somatotropes. Cellular GH contents of unstimulated cells, maintained in testing medium, remained stable for ≥24 h (results from a representative experiment are shown in Fig. 3A). Similarly, the rate of basal GH secretion did not change significantly between the time points (2 h: 5 h: ../../10.2203.32.2.onJune25,2017 http://ajpendo.physiology.org/ Downloaded from
370 ± 97 ng/h; 12 h: 263 ± 62 ng/h; 24 h: 271 ± 52 ng/h; pooled results from three separate experiments). Although the absolute amount of GH stored and released varied among cell cultures, the relative proportion of these two components was similar in different experiments. For this reason, GH values were normalized with respect to controls in subsequent analysis.

Endocrine cells are known to release proteins, including hormones, through multiple routes, including the regulated secretory pathway and the constitutive secretory pathway (27). The latter pathway is characterized by small vesicles of newly produced proteins that bud off from the trans-Golgi network, proceed directly to the plasma membrane without being stored, and immediately undergo exocytosis in the absence of a measurable global Ca2+ signal (total time from protein synthesis to exocytosis <1 h; Refs. 27, 51). To determine the relative involvement of constitutive and regulated secretory pathways in somatotropes, we monitored the effects of cyclohexamide on basal GH secretion and cellular contents. Treatment with the protein synthesis inhibitor cyclohexamide (100 μM) did not reduce GH release in 2-h static incubation studies (Fig. 3B). Likewise, long exposures of cyclohexamide did not affect basal GH release in perfusion experiments (Fig. 3C). These results suggest that the constitutive secretory pathway does not play a role in basal GH secretion (<2 h) from goldfish somatotropes.

The observation that exocytosis evoked by ionomycin-mediated Ca2+ influx is also unaffected by cyclohexamide indicates that somatotropes do not preferentially release newly synthesized GH through the regulated secretory pathway.

Although prolonged treatment (12 and 24 h) with cyclohexamide produced the expected decrease in cellular GH contents, a paradoxical increase in GH contents was observed after 2 h (Fig. 3B). At this time, we can only speculate as to the possible underlying mechanism of this response. Perhaps cyclohexamide inhibited the formation of a certain pool of proteins, with rapid turnover/synthesis rates, which are normally responsible for the degradation of some stored GH.

**Differential involvement of Tg- and Ry-sensitive Ca2+ stores in proximal components of the GH secretory pathway.** Having ruled out a direct coupling to acute GH release, we asked whether Tg-sensitive Ca2+ stores regulate other aspects of the secretory pathway, such as long-term GH release, cellular GH contents, total GH (release + contents, an index of production), and GH mRNA levels at three time points. In addition, we compared the effects of Tg with those of Ry, a treatment that is known to modulate the function of a distinct subclass of ER Ca2+ stores in some cell types (e.g., Ref. 16). Ry has the advantageous property of locking the ryanodine receptor (RyR) Ca2+ release channel in an open subconductance state, thereby stimulating the release of Ca2+ at low (i.e., 1 nM) doses (Ref. 13 and J. D. Johnson and J. P. Chang, unpublished data). Ry blocks Ca2+ release from RyR at higher concentrations (i.e., 10 μM) and abolishes cGnRH-II-stimulated GH release (24). Although both Ry treatments selectively perturb Ca2+ flux from RyR-containing Ca2+ stores, only the former would be expected to deplete these Ca2+ pools.

Overall, the long-term effect of Tg treatment (2 μM) was the release of GH from the secretory pathway, without a concomitant maintenance of cellular GH contents (Fig. 4). In contrast, activation of RyR with 1 nM Ry produced a biphasic response. In the first 2 h, Ca2+ release from Ry-sensitive store is positively coupled to GH release, contents, and net production, whereas the net effect over 24 h was a decrease in all of these parameters. On the other hand, blocking RyR Ca2+ release channels with 10 μM Ry modulated GH cellular contents (through a slight increase in production), in a manner that was only transient. Further differences in the functional coupling of Tg-sensitive and Ry-sensitive Ca2+ stores to the extended GH secretory pathway are revealed when the data are examined at each time point. After 2 h, Tg treatment had initiated a sustained GH release response. Although 1 nM Ry also stimulated GH release over 2 h, this was accompanied by an increase in cellular GH contents. On the other hand, the inhibition of Ca2+ release from these stores with 10 μM Ry increased GH contents without affecting secretion. At the 12-h time point, Tg-treated cells continued to release stored GH without an apparent compensatory increase in hormone production. In the presence of Tg, cellular GH contents
were reduced to a level similar to that seen in experiments with cyclohexamide. In contrast, neither dose of Ry affected GH secretion or GH contents over 12 h. Over 24 h, the increase in GH release from Tg-treated cultures, although still significant, was at its lowest levels compared with controls. It is important to note that, because our measurements are cumulative, these latter determinations of GH release are still “contaminated” with the high responses seen after 2 h. Over the course of the entire experiment, the stimulatory dose of Ry had resulted in a reduction in basal GH secretion, presumably due to the failure to compensate with increases in GH contents and production. Nevertheless, the modest reduction in stored GH seen in 1 nM Ry-treated cultures was significantly different from the more robust decrease seen in Tg-treated cells during the same period ($P < 0.05$). On the other hand, blocking of RyR had no significant effect on these parameters. Taken together, these data suggest that manipulations of Tg-sensitive and Ry-sensitive Ca$^{2+}$ stores in goldfish somatotropes differentially control the long-term secretion and replenishment of GH.

Next, in parallel experiments, we assessed the role of these two intracellular Ca$^{2+}$ stores in the control of GH gene expression by Northern blot analysis of GH mRNA levels. Neither 2 µM Tg nor 1 nM Ry significantly altered GH mRNA levels at any of the time points tested (Fig. 5), despite their ability to evoke robust GH secretion during the first 2 h. Surprisingly, application of the blocking dose of Ry (10 µM) resulted in a significant, but transient, elevation in GH gene expression at 2 h, a time at which cellular GH contents and GH production were also increased. These results with 10 µM Ry suggest that perturbing Ry-sensitive Ca$^{2+}$ stores by blocking RyR can regulate GH biosynthesis. Although all of the treatments used in the present study were shown to modulate the apparent storage and production of GH, only the transient (2 h) effects of 10 µM Ry are correlated with changes in GH mRNA levels. It is unlikely that these effects of Tg and Ry are attributable to changes in cell viability, because the health and numbers of cells in these cultures were not different, as determined by the trypan blue exclusion test. The fact that similar amounts of protein and total mRNA (quantified spectrophotometrically) were obtained from all cell cultures also attests to a lack of cell loss.

**Negative coupling of [Ca$^{2+}$]$_c$ to GH gene expression.** To further evaluate the functional importance of specific Ca$^{2+}$ signals, we examined the role of [Ca$^{2+}$]$_c$...
signals generated independently of intracellular Ca\(^{2+}\) stores in the regulation of GH gene expression. To this end, we treated cells with 30 mM KCl for 30 min to activate voltage-gated Ca\(^{2+}\) channels (VGCCs) and measured GH mRNA 12 h later. It has previously been shown that KCl-stimulated Ca\(^{2+}\) signals in populations of goldfish pituitary cells are completely dependent on the influx of extracellular Ca\(^{2+}\) (21). A 30-min treatment protocol was chosen because prolonged exposure to KCl (6–24 h) resulted in the detachment of cells in our cultures (J. P. Chang, unpublished observations). The results of this experiment clearly show that GH mRNA levels were reduced as a result of KCl treatment (Fig. 6A), although the expected relationship between [Ca\(^{2+}\)]\(_c\) and hormone secretion is positive in somatotropes (Fig. 6, B and C). This result suggests that elevated [Ca\(^{2+}\)]\(_c\) initiates a signal that is inhibitory to GH gene expression in the goldfish.

DISCUSSION

**Uncoupling of Tg-stimulated Ca\(^{2+}\) signals from acute GH release.** We have hypothesized that cells use function-specific Ca\(^{2+}\) stores to generate a complex array of Ca\(^{2+}\) signals capable of controlling different components of the extended secretory pathway independently (23). A prediction of such a hypothesis is that Ca\(^{2+}\) signals from certain intracellular stores would fail to modulate certain Ca\(^{2+}\)-dependent processes. The present study clearly indicates that such functional specificity occurs. No GH release was seen at a dose of Tg that evoked increases in [Ca\(^{2+}\)]\(_c\) of similar maximal amplitude to those generated by GnRH, suggesting that one class of ER Ca\(^{2+}\) stores is functionally uncoupled from the acute control of exocytosis. Different degrees of uncoupling between Ca\(^{2+}\) signals and hormone release have been reported for cell lines derived from the mammalian pituitary [e.g., αT3–1 cells (48)]. In the present study, the comparison between Tg and GnRH is valid, because acute GnRH-stimulated hormone release relies exclusively on the release intracellular Ca\(^{2+}\) stores, as is the case in mammalian and fish gonadotropes (25, 47). Together with the present data, we have shown that GnRH signaling involves Ca\(^{2+}\) stores that are completely independent of those modulated by Tg (23), in contrast to the situation in many other cell types (e.g., Ref. 49).

![Fig. 4. Differential coupling of endoplasmic reticulum Ca\(^{2+}\) stores sensitive to Tg or ryanodine (Ry) to GH secretion, contents, and production (secretion + contents) at 3 timepoints. GH values from cultures treated with 2 μM Tg (n = 36), 1 nM Ry (n = 16), or 10 μM Ry (n = 16) are normalized to time-matched controls (n = 48). See Fig. 3A for typical absolute GH values (pre-normalization). *Significant difference compared with time-matched controls.](http://ajpendo.physiology.org/)

![Fig. 5. Differential effects of Tg and Ry on GH mRNA levels at 3 timepoints. Results are presented as relative change from untreated cultures at each time point (n = 4). GH mRNA was quantified using densitometry and normalized to an internal standard (18S ribosomal RNA). *Significant difference from time-matched control cultures.](http://ajpendo.physiology.org/)
Instead, acute GnRH-stimulated GH release depends on a rapidly depleting intracellular Ca\textsuperscript{2+} pool that is sensitive to 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester and caffeine (23, 55). Recently, we have identified the Ry-sensitive Ca\textsuperscript{2+} stores as an essential mediator of cGnRH-II-stimulated GH release (24). Thus somatotropes contain multiple agonist-sensitive intracellular Ca\textsuperscript{2+} stores, as we have shown to be the case in goldfish gonadotropes (24, 25).

**Tg-stimulated [Ca\textsuperscript{2+}]\textsubscript{L} depletion induces the prolonged release of GH from the secretory pathway.** What is the role of Tg-sensitive Ca\textsuperscript{2+} stores in goldfish somatotropes if not to directly regulate acute GH release or mediate agonist signaling? We suggest that prolonged Tg treatment, which would deplete Tg-sensitive ER Ca\textsuperscript{2+} stores, led to a long-term emptying of stored GH. This response was not accompanied by a compensatory increase in GH production (assessed by net hormone present or GH mRNA levels). The net result was a reduction in the cellular GH contents. Although the decrease in cellular GH contents induced by Tg is quantitatively similar to that seen in cyclohexamide-treated cells, the observation that Tg does not affect GH production or mRNA levels suggests that Tg and cyclohexamide have different mechanisms of action. Therefore, Tg must reduce cellular contents at a site that is distal to protein synthesis on the extended secretory pathway. Although the exact mechanism of Tg action remains to be determined, we hypothesize that Tg accelerates GH traffic through the distal parts of the secretory pathway. In light of the findings that Tg does not stimulate acute exocytosis directly, we propose that the prolonged secretion of GH in Tg-treated cultures results from an increased amount of GH released through the “basal” mechanism. In some endocrine cell types, basal secretion has been attributed to a portion of secretory granules, which undergo random fusion in the steady state (51). We are not aware of studies in other model systems that have measured similar reductions in hormone contents in Tg-treated cells.

**Inhibition of Ry-sensitive Ca\textsuperscript{2+} stores transiently stimulates GH production.** It has been reported that Tg-mobilizes only a fraction of stored Ca\textsuperscript{2+}, equivalent to between −40 and 60%, depending on the cell type (14, 37, 46). Thus we examined whether other components of the ER are functionally important in somatotropes. Previous studies have shown that Ry-sensitive Ca\textsuperscript{2+} stores participate in acute agonist Ca\textsuperscript{2+} signaling in mammalian somatotropes (19, 36). Despite the fact that RyR is found in many tissues (45), the modulation of gene expression and protein synthesis by Ry-sensitive Ca\textsuperscript{2+} stores is not well characterized in nonmuscle cells. We report, for the first time, evidence of the involvement of RyR in the long-term functioning of somatotropes. Stimulation of ER Ca\textsuperscript{2+} release with 1 nM Ry evoked significant GH secretion after 2 h. This response was transient and eventually resulted in the inhibition of GH release over 24 h, unlike the prolonged secretory activity of Tg. On the other hand, the inhibitory dose of Ry did not affect the release of GH.

Likewise, preliminary experiments have shown that GnRH modulation of GH mRNA levels is also transient (C. Klausen, J. P. Chang, and H. R. Habibi, unpublished observations). Collectively, these data suggest that GH release is positively coupled to Ca\textsuperscript{2+} release from Ry-sensitive stores, at least initially.

Interestingly, whereas both Ry treatments elevated GH contents at 2 h, only 10 μM Ry increased GH mRNA. This result suggests (indirectly) that part of the increase in total GH seen in cells treated with 1 nM Ry over 2 h results from some posttranscriptional enhancement of GH production. On the other hand, the increased GH contents in 10 μM Ry-treated cells can be accounted for by the significant increase in GH mRNA expression. We propose that the inhibition of normal Ca\textsuperscript{2+} homeostasis, and perhaps an elevated [Ca\textsuperscript{2+}]\textsubscript{L}, rather than a cytosolic Ca\textsuperscript{2+} signal, mediates acute changes in GH gene expression. Although others have elegantly shown the importance of ER [Ca\textsuperscript{2+}]\textsubscript{L} in the control of ER regulatory genes (29), this is the first time such a mechanism has been considered as a reg-
ulator of hormonal mRNA in cultured pituitary cells. A complete test of this hypothesis awaits the measurement of [Ca\textsuperscript{2+}]\textsubscript{i}, in the Ry-sensitive ER of intact somatotropes. Nevertheless, our results clearly demonstrate that ER Ca\textsuperscript{2+} stores sensitive to Ry, but not Tg, can be coupled to GH mRNA expression. This finding does not rule out the possibility that Tg-sensitive Ca\textsuperscript{2+} pools control the expression of other genes in somatotropes. Indeed, the independent modulation of multiple gene sets would offer additional levels of signaling specificity to the cell.

**Role of [Ca\textsuperscript{2+}]\textsubscript{i} in GH production.** We directly examined the relationship between [Ca\textsuperscript{2+}]\textsubscript{i} and GH mRNA expression by use of depolarizing concentrations of KCl to generate Ca\textsuperscript{2+} signals mediated by VGCC (but presumably not intracellular stores). Surprisingly, Ca\textsuperscript{2+} signals mediated by VGCC strongly inhibited GH mRNA expression, although they were positively coupled to GH release. In other experiments, ionomycin, which is also a potent GH secretagogue, dose-dependently decreased GH cellular contents and production within 2 h (M. Volk and J. P. Chang, unpublished results). Thus these effects of KCl on goldfish somatotropes are not likely due to the preferential signaling by VGCC-mediated Ca\textsuperscript{2+} signals, as others have reported for some neurons and pituitary cells (3, 33), but may be generalized to elevation of [Ca\textsuperscript{2+}]\textsubscript{i}. A comparable dissociation between GH release and GH synthesis has been noted in studies of KCl-treated mammalian somatotropes. Treating rat somatotropes with high KCl concentration for 1 h had no effect on GH mRNA levels, despite a robust secretory response (4). Similarly, some treatments that would be expected to increase cellular Ca\textsuperscript{2+} dramatically, such as replacing Ca\textsuperscript{2+}-free medium with high-Ca\textsuperscript{2+} medium, fail to stimulate increases in GH mRNA expression in GH\textsubscript{3} cells and normal rat somatotropes [although prolactin synthesis was stimulated in GH\textsubscript{3} cells (15, 53)].

The finding that elevated [Ca\textsuperscript{2+}]\textsubscript{i} is negatively coupled to mRNA expression is compatible with the observed differential effects of 1 nM Ry and 10 \mu M Ry on GH mRNA levels. The major presumed difference between the two Ry treatments is that only the stimulatory dose activates the RyR to release Ca\textsuperscript{2+}, whereas both treatments would be expected to perturb the normal cycling of Ca\textsuperscript{2+} through the Ry-sensitive component of the ER. One might speculate that 1 nM Ry generates two Ca\textsuperscript{2+} signals, one luminal and one cytosolic, which have opposing effects on GH mRNA levels, thus resulting in no net change in GH mRNA accumulation. Additional work is needed to establish signaling cascades coupling Ry-sensitive Ca\textsuperscript{2+} stores to GH gene expression.

Future studies will also be required to determine whether this unique relationship between [Ca\textsuperscript{2+}]\textsubscript{i} and GH mRNA plays a role in the long-term signaling of the many endogenous agonists and antagonists that regulate the function of goldfish somatotropes. sGnRH, cGnRH-II, dopamine, and pituitary adenylate cyclase-activating polypeptide are all known to require VGCC activation to sustain long-term (2 h) evoked GH release (9). Recent preliminary experiments have shown that sGnRH and cGnRH-II stimulated GH mRNA accumulation in vitro and in vivo over the same time period when it was inhibited by KCl (28). Therefore, it seems likely that VGCC-independent postreceptor pathways are important in the long-term effects of GnRH on GH gene expression.

**Physiological significance and comparison with other goldfish pituitary cell types.** The fact that goldfish somatotropes are regulated by multiple agonists suggests that functional specificity of Ca\textsuperscript{2+} signaling must be sufficiently complex to ensure the precise independent control of specific cellular functions by specific extracellular signals. There has been a recent appreciation for the range of organelles participating in intracellular Ca\textsuperscript{2+} signaling, including the ER, nucleus, Golgi, secretory granules, and mitochondria. This has paralleled a growing understanding of the diversity of intracellular Ca\textsuperscript{2+}-release channels and associated second messengers, such as IP\textsubscript{3}, cADP-ribose, and nicotinic acid adenine dinucleotide phosphate, which make up a multitude of pharmacological classes of Ca\textsuperscript{2+}-signaling systems (reviewed in Ref. 22). Future studies should include the examination of novel classes of intracellular Ca\textsuperscript{2+} stores and more mRNA species involved in different aspects of the secretory pathway.

Like somatotropes, goldfish gonadotropes are also regulated by multiple agonists, including both GnRHs. However, Ca\textsuperscript{2+}-dependent signaling cascades differ considerably between these cell types (9, 23–26). It is likely that the actions of Tg may be cell type specific. For example, studies of goldfish gonadotropes in which Tg also evokes a reduction in cellular hormone contents have indicated that Tg significantly reduces GTH-II mRNA levels at 12 and 24 h (J. D. Johnson, C. Klausen, H. R. Habibi, and J. P. Chang, unpublished results). By comparing the functional roles of intracellular Ca\textsuperscript{2+} stores between these two cell types, we may learn important details about pituitary physiology.

It is important to note that pharmacological treatments used in the present study cannot replicate the complexities of agonist stimulation. However, the observations that specific Ca\textsuperscript{2+}-mobilizing treatments can be uncoupled from specific Ca\textsuperscript{2+}-dependent cellular functions is likely to have relevance to how pituitary function is controlled by multiple agonists. Similarly, the observation that specific components of the extended secretory pathway can be modulated independently may eventually lead to strategies targeting specific cellular functions in other cell types.

In conclusion, the present results provide new insights into the regulation of multiple aspects of the extended secretory pathway by two intracellular Ca\textsuperscript{2+} stores and by cytosolic Ca\textsuperscript{2+} signals. Moreover, our data support a model whereby both [Ca\textsuperscript{2+}]\textsubscript{i} signals and [Ca\textsuperscript{2+}]\textsubscript{c} participate in the control of hormonal gene expression. We propose that function-specific Ca\textsuperscript{2+} stores may represent a novel mechanism for the independent control of cellular functions by multiple agonists.

**AJP-Endocrinol Metab • VOL 282 • APRIL 2002 • www.ajpendo.org**

**Downloaded from [http://ajpendo.physiology.org/](http://ajpendo.physiology.org/) by 10.220.33.2 on June 25, 2017**
REFERENCES


E818


