Caffeine-stimulated GTH-II release involves Ca$^{2+}$ stores with novel properties

JAMES D. JOHNSON,* CALVIN J. H. WONG,* WARREN K. YUNKER, AND JOHN P. CHANG

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Received 9 February 2001; accepted in final form 2 November 2001

Johnson, James D., Calvin J. H. Wong, Warren K. Yunker, and John P. Chang. Caffeine-stimulated GTH-II release involves Ca$^{2+}$ stores with novel properties. Am J Physiol Cell Physiol 282: C635–C645, 2002. First published November 7, 2001; 10.1152/ajpcell.00044.2001.—Modulation of Ca$^{2+}$ stores with 10 mM caffeine stimulates robust secretion of gonadotropin (GTH-II) from goldfish gonadotropes. Although both endogenous forms of gonadotropin-releasing hormone (GnRH) utilize a common intracellular Ca$^{2+}$ store, sGnRH, but not cGnRH-II, uses an additional caffeine-sensitive mechanism. We examined caffeine signaling by using Ca$^{2+}$ imaging, electrophysiology, and cell-column perfusion. Although caffeine inhibited K$^{+}$ channels, this action appeared to be unrelated to caffeine-induced GTH-II release, because the latter was insensitive to tetraethylammonium. The effects of caffeine also were not mediated by the cAMP/protein kinase A pathway. Instead, caffeine-evoked GTH-II responses were Ca$^{2+}$ signal dependent because they were abolished by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid loading. Caffeine generated localized Ca$^{2+}$ signals that began near secretory granules. Surprisingly, caffeine-stimulated GTH-II release was insensitive to 100 μM ryanodine and, unlike GnRH action, was unaffected by inhibitors of voltage-gated Ca$^{2+}$ channels or sarco(endo)plasmic reticulum Ca$^{2+}$-ATPases. Collectively, these data indicate that caffeine-stimulated GTH-II release is not mediated by typical agonist-sensitive Ca$^{2+}$ stores found in endoplasmic reticulum, ryanodine; sarco(endo)plasmic reticulum calcium adenosine triphosphatase; potassium channels; voltage-gated calcium channels; protein kinase A; secretory granules.

Intracellular Ca$^{2+}$ stores are important components of the signal transduction of many neuroendocrine regulators. Although multiple Ca$^{2+}$ stores have been localized to the endoplasmic reticulum (ER), several other organelles, such as Golgi and secretory granules, contain considerable quantities of Ca$^{2+}$ (41) and may act as atypical agonist-sensitive Ca$^{2+}$ pools in secretory cells (13, 21, 25, 32, 36, 37, 39). Multiple independent intracellular Ca$^{2+}$ stores have been identified in a variety of cell types (15, 18). Intracellular Ca$^{2+}$ stores including, but not limited to, those sensitive to inositol 1,4,5-trisphosphate (IP$_3$), ryanodine (cADPribose sensitive), and nicotinic acid dinucleotide phosphate have been characterized biochemically (Refs. 3, 4, 12, 30; reviewed in Ref. 21). One of the most valuable agents for elucidating the roles of intracellular Ca$^{2+}$ stores in physiological processes has been caffeine because it is membrane permeant and activates ryanodine-sensitive Ca$^{2+}$ release channels by sensitizing them to ambient Ca$^{2+}$ levels (34, 41).

We have shown that 10 mM caffeine evokes robust secretion of maturational gonadotropin hormone (GTH-II) from cultured goldfish gonadotropes (23). Particularly, caffeine treatment abolished the GTH-II-releasing effect of one endogenous form of gonadotropin-releasing hormone (sGnRH) but not the other (cGnRH-II). Although the acute signaling cascades of both neuropeptides are entirely dependent on intracellular Ca$^{2+}$ stores (23), other studies have revealed that sGnRH, but not cGnRH-II, mobilizes depletion-resistant intracellular Ca$^{2+}$ stores and generates IP$_3$ (7, 19). The physiological relevance of these differences in signal transduction is underscored by the demonstration that these two closely related endogenous neuropeptides can generate quantitatively distinct Ca$^{2+}$ signals in identified gonadotropes (24) and differentially regulate GTH-II gene expression (27). Interestingly, over the course of the yearly reproductive cycle, the efficacy of caffeine-stimulated GTH-II release parallels that of sGnRH (20). Can caffeine be used as a probe for the selective study of sGnRH Ca$^{2+}$ signaling in goldfish gonadotropes? Before this question can be answered, a deeper understanding of the mechanisms of caffeine-stimulated GTH-II release must be ascertained. Thus we have examined the features of caffeine-dependent signal transduction in goldfish gonadotropes by using single-cell Ca$^{2+}$ imaging, patch-clamp electrophysiology, and cell-column perfusion of cultured goldfish pituitary cells.

We have demonstrated that caffeine-evoked GTH-II release is resistant to blockade of extracellular Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels (VGCC) but is completely dependent on the ability of caffeine to generate Ca$^{2+}$ signals. Together with the characteristics of caffeine-stimulated GTH-II release, the features of caffeine-evoked Ca$^{2+}$ signals suggest the possibility...
that caffeine mobilizes $\text{Ca}^{2+}$ from novel, localized stores in or around the secretory granules. Unlike the situation in other cells types, caffeine-stimulated hormone release was independent of the modulation of $K^+$ current, ryanodine receptors (RyR), or the sarco(endo)plasmic reticulum $\text{Ca}^{2+}$-ATPases (SERCA). Thus, caffeine may activate an intracellular $\text{Ca}^{2+}$ store that is novel in both subcellular location and pharmacology in goldfish gonadotropes.

**METHODS**

**Reagents.** High-purity ryanodine (99.5%), 1,2-bis(2-amino-phenyloxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM), N-[2-(p-bromocinnamamido)ethyl]-5-isoquinolinesulfonamide (H-89), and 2,5-di-[(t-butyl)-1,4-hydroquinone (BHQ) from Calbiochem (La Jolla, CA) and dantroline from RBI (Natick, MD) were dissolved in di-methyl sulfoxide (DMSO). Caffeine (RBI) was dissolved directly into media. At the concentration used in this study (0.1%), DMSO does not affect GTH-II release, intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$), or ionic currents (not shown).

**Animals and cell preparation.** Animal use protocols were approved by the University of Alberta Biological Sciences Animal Care Committee in accordance with national guidelines. Goldfish from Aquatic Imports (Calgary, Canada) were maintained as described previously (5). Animals were anesthetized in 0.05% tricaine methanesulfonate before decapitation and removal of the pituitary. Cells were dispersed enzymatically and cultured overnight on Cytodex beads (for perfusion), 24-well surface-modified (Falcon Primaria line) tissue culture plates at a density of 0.25 million cells/well (for 2-h static incubation), or polylysine-coated coverslips at a density of 0.25 million cells/well (for $\text{Ca}^{2+}$ imaging and electrophysiology) in medium 199 (M199) 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, and cultured overnight at 28ºC, 5% CO$_2$, and saturated humidity. Additional details are published elsewhere (5, 24).

**Cell identification.** Gonadotropes were identified under Nomarski differential interference contrast (DIC) microscopy optics by using a validated set of morphological criteria, which previously was shown to allow the correct identification of GTH-II-containing and GnRH-sensitive cells with $>$95% accuracy (24, 50). The morphologically identifiable subpopulation of gonadotropes represents approximately half of all GTH-II-containing cells in the dispersed goldfish pituitary (24). Before imaging, the location of visible landmarks, such as the reniform nucleus located against one side of the cell and groups of large globules and secretory granules generally found opposite the nucleus, were carefully mapped out.

**Fura 2 cytosolic $\text{Ca}^{2+}$ imaging.** Cells were evenly loaded with fura 2 by 35-min incubation in imaging medium [M199 with Hanks’ salts (GIBCO), without phenol red, 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] and cultured overnight at 28ºC, 5% CO$_2$, and 100% humidity. Additional details are published elsewhere (5, 24).

**Hormone release.** For dynamic measurements of hormone secretion, cells grown on Cytodex beads (1.5 million/column) were perifused at 18ºC with testing medium (M199 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) as described previously (5). Media were collected in 100 µl aliquots every 10 s, to minimize photobleaching. After the whole cell configuration was obtained (17), the extracellular solution was switched over to testing medium containing TTX (100 nM) and Cd$^{2+}$ (50 µM) to block Na$^+$ and Ca$^{2+}$ conductances, respectively. The liquid junctional potential (~10 mV) between the pipette and the bath solution was corrected for using post hoc methods (2). Currents were recorded with a Dagan 3900 patch-clamp amplifier, a Digidata 1200 acquisition board, and pCLAMP 5.2 (Axon Instruments, Foster City, CA). Leak and capacitive currents were subtracted using the P/4 procedure. Data were analyzed by using custom-written macros in Igor Pro (WaveMetrics, Lake Oswego, OR) or Excel (Microsoft, Seattle, WA).

**Electrophysiology.** Gonadotropes were morphologically identified as described in Cell identification. After 24-h incubation, cells were rinsed three times in testing medium and then perfused on the stage of an inverted microscope for 20 min before being recorded at room temperature (~20ºC) as described previously for our laboratory (48). Fire-polished pipettes were filled with (in mM) 120 K$^+$-aspartate, 20 KCl, 20 HEPES, 2 MgCl$_2$, 1 CaCl$_2$, 10 EGTA, 2.5 Na$_2$ATP, and 0.4 Na$_2$GTP, with pH adjusted to 7.2 with 1 M KOH. Bath solution was standard testing medium. Open-tip resistance was 3–5 MΩ. Series resistance (10–20 MΩ) was compensated ~80%. After the whole cell configuration was obtained (17), the extracellular solution was switched over to testing medium containing TTX (100 nM) and Cd$^{2+}$ (50 µM) to block Na$^+$ and Ca$^{2+}$ conductances, respectively. The liquid junctional potential (~10 mV) between the pipette and the bath solution was corrected for using post hoc methods (2). Currents were recorded with a Dagan 3900 patch-clamp amplifier, a Digidata 1200 acquisition board, and pCLAMP 5.2 (Axon Instruments, Foster City, CA). Leak and capacitive currents were subtracted using the P/4 procedure. Data were analyzed by using custom-written macros in Igor Pro (WaveMetrics, Lake Oswego, OR) or Excel (Microsoft, Seattle, WA).

**Hormone release.** For dynamic measurements of hormone secretion, cells grown on Cytodex beads (1.5 million/column) were perifused at 18ºC with testing medium (M199 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) as described previously (5). Media were collected in 100 µl aliquots every 10 s, to minimize photobleaching. After the whole cell configuration was obtained (17), the extracellular solution was switched over to testing medium containing TTX (100 nM) and Cd$^{2+}$ (50 µM) to block Na$^+$ and Ca$^{2+}$ conductances, respectively. The liquid junctional potential (~10 mV) between the pipette and the bath solution was corrected for using post hoc methods (2). Currents were recorded with a Dagan 3900 patch-clamp amplifier, a Digidata 1200 acquisition board, and pCLAMP 5.2 (Axon Instruments, Foster City, CA). Leak and capacitive currents were subtracted using the P/4 procedure. Data were analyzed by using custom-written macros in Igor Pro (WaveMetrics, Lake Oswego, OR) or Excel (Microsoft, Seattle, WA).

**Hormone release.** For dynamic measurements of hormone secretion, cells grown on Cytodex beads (1.5 million/column) were perifused at 18ºC with testing medium (M199 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) as described previously (5). Media were collected in 100 µl aliquots every 10 s, to minimize photobleaching. After the whole cell configuration was obtained (17), the extracellular solution was switched over to testing medium containing TTX (100 nM) and Cd$^{2+}$ (50 µM) to block Na$^+$ and Ca$^{2+}$ conductances, respectively. The liquid junctional potential (~10 mV) between the pipette and the bath solution was corrected for using post hoc methods (2). Currents were recorded with a Dagan 3900 patch-clamp amplifier, a Digidata 1200 acquisition board, and pCLAMP 5.2 (Axon Instruments, Foster City, CA). Leak and capacitive currents were subtracted using the P/4 procedure. Data were analyzed by using custom-written macros in Igor Pro (WaveMetrics, Lake Oswego, OR) or Excel (Microsoft, Seattle, WA).
denoted as percentages of pretreatment (%pretreatment). Net hormone release responses of perifused cell columns to caffeine were quantified by integrating the area under the curve during the caffeine treatment (25 min). This was subdivided into “peak” response (first 10 min of caffeine treatment) and “plateau” response (last 15 min of caffeine treatment). Each time point was subtracted from the prepulse mean, defined as the average of the three time points before the caffeine challenge. Statistical analyses were performed with ANOVA, followed by Fisher’s protected least significant difference post hoc test. Differences were considered significant when \( P < 0.05 \). Results are presented as means ± SE.

**RESULTS**

Caffeine-stimulated GTH-II release is \( Ca^{2+} \) dependent. Perfusion of dispersed goldfish pituitary cells with 10 mM caffeine resulted in a very large GTH-II release response (Fig. 1A). A second application of caffeine (45 min after the first) evoked only the plateau portion of the GTH-II release response (Fig. 1B). Significant GTH-II secretion was measured at 1 and 10 mM caffeine in static incubation experiments (Fig. 1C); 10 mM caffeine was used for the rest of the experiments. It is notable that caffeine is much less powerful in this assay of prolonged hormone release, suggesting the importance of a rapidly depleting or desensitizing component. Obvious candidates for such a “depletable” component are intracellular \( Ca^{2+} \) stores, which 10 mM caffeine is known to mobilize in many cell types.

In accordance with the idea that caffeine acts by mobilizing intracellular \( Ca^{2+} \) stores, caffeine generated \( Ca^{2+} \) signals in single identified gonadotropes (Fig. 2A). Caffeine-stimulated \( Ca^{2+} \) signals and GTH-II release responses were abolished by loading cells with the high-affinity \( Ca^{2+} \) chelator BAPTA, accomplished by preincubation with 50 \( \mu \)M BAPTA-AM (Fig. 2C). Although the possibility of nonspecific BAPTA actions cannot be totally ruled out, these data suggest that caffeine-stimulated GTH-II release is not mediated by a parallel \( Ca^{2+} \)-independent signaling cascade. In preliminary experiments, caffeine-stimulated GTH-II release was significantly inhibited when cells were subjected to prolonged nominally extracellular \( Ca^{2+} \)-free conditions that would be expected to deplete intracellular \( Ca^{2+} \) pools by preventing capacitative \( Ca^{2+} \) entry (\( n = 3 \), data not shown).

Although acute caffeine-elicited GTH-II responses were severalfold greater than those induced by GnRH, \( Ca^{2+} \) signals evoked by caffeine were of similar maximal amplitude to those stimulated by sGnRH or cGnRH-II (24). Peak [\( Ca^{2+} \)], measured over the bulk cytosol, rose only 157.5 ± 33.6 nM above baseline in the presence of 10 mM caffeine. It is important to note that the actual [\( Ca^{2+} \)], in some regions of the cell is likely misrepresented by these bulk measurements, because the peak of the caffeine-evoked \( Ca^{2+} \) signal was highly localized (Fig. 2B). In all cells tested, caffeine generated \( Ca^{2+} \) signals that were initially focused in the region opposite the nucleus, which is known to be rich in secretory granules (50), but subsequently spread across the cell. Collectively, these data suggest that caffeine-stimulated GTH-II release requires \( Ca^{2+} \) signals initiated by spatially localized \( Ca^{2+} \) stores.

Protein kinase AlcAMP, \( K^+ \) channels, and VGCC do not mediate caffeine-stimulated GTH-II release. Xanthines, such as caffeine, have been shown to modulate the cAMP pathway through inhibition of phosphodiesterases. The actions of cAMP are mediated through H-89-sensitive protein kinase A (PKA) in goldfish gonadotropes (6). In the present study, we investigated whether actions on the cAMP/PKA system underlie the GTH-II-releasing effect of caffeine. Alone, H-89 (10 \( \mu \)M) reduced GTH-II release by ~50%, suggesting a role for the cAMP/PKA pathway in the maintenance of basal release in this cell type. However, H-89 had no significant effect on caffeine-evoked GTH-II release (Fig. 3). This result agrees with the observation that the massive hormone release response to caffeine is not mimicked, in previous experiments, by IBMX, a more specific phosphodiesterase inhibitor (8). Together, these data indicate that caffeine signaling in goldfish gonadotropes does not have a prominent cAMP/PKA component and that basal and agonist-evoked GTH-II release may be controlled independently.

Caffeine has been reported to inhibit \( K^+ \) conductances in cell lines derived from mammalian pituitary cells (see e.g., Ref. 1). To investigate this possibility, we recorded from identified gonadotropes in the whole cell configuration. As has been shown previously (48), depolarizing steps in the presence of TTX and \( Cd^{2+} \) evoked a biphasic outward \( K^+ \) current (Fig. 4A). Caffeine treatment rapidly decreased the amplitude of both the peak and plateau portions of this current by ~40% in a voltage-independent and reversible manner.

---

**Fig. 1.** Characteristics of caffeine-stimulated gonadotropin (GTH-II) release. A: goldfish pituitary cells were treated with 10 mM caffeine (open horizontal bar) in a cell-column perfusion chamber. Samples were collected every 60 s to determine the kinetics of the GTH-II response (\( n = 6 \)). B: sequential challenges with 10 mM caffeine (open horizontal bars) display desensitization (\( n = 4 \)). Horizontal scale for B is the same as for A. All perfusion data are normalized to the pretreatment GTH-II levels in each column. Caff, caffeine. C: dose-response relationship of caffeine-stimulated GTH-II release was assessed in 2-h static incubation studies (\( n = 12 \)). ★Significant difference compared with control.
Although these data indicate that caffeine blocks both the A current (\(I_{K(A)}\)) and the delayed rectifier current, they do not show that the blockage of \(K^+\)/

H11001 currents mediates the secretagogue actions of caffeine. To evaluate this possibility, we examined whether caffeine-stimulated GTH-II release is sensitive to tetraethylammonium (TEA), a broad-specificity inhibitor of \(K^+\)/

H11001 currents in many cell types. In goldfish gonadotropes, 5 mM TEA is known to block the majority of \(K^+\)/

H11001 currents (48). Surprisingly, caffeine-stimulated GTH-II release was not reduced by 5 mM TEA (Fig. 5). These results indicate that although caffeine may modulate ionic currents in the plasma membrane, these events are dissociated from the signaling cascades that mediate caffeine stimulation of exocytosis.

Although acute GnRH-stimulated GTH-II release evoked by the two GnRHs is independent of extracellular Ca\(^{2+}\) (23), influx through VGCCs is important in the maintenance of a prolonged secretory response, especially to cGnRH-II (7). Thus we have hypothesized a role for VGCC in the refilling of the rapidly exchanging Ca\(^{2+}\) pools sensitive to both GnRHs. Goldfish gonadotropes possess a single class of VGCCs, which are high-voltage activated, inactivation resistant, and completely blockable by micromolar Cd\(^{2+}\) (48). Blockade of VGCCs with 50 \(\mu\)M Cd\(^{2+}\) did not block either...
phase of the caffeine-evoked GTH-II release response (Fig. 6A), even when given up to 35 min before the application of caffeine (Fig. 6B), suggesting that caffeine-sensitive Ca\(^{2+}\) stores are not maintained through the activity of VGCCs. Treatment with 50 \(\mu\)M Ca\(^{2+}\) alone caused an increase in GTH-II release of variable amplitude (Fig. 6A). Like the large secretagogue response of gonadotropes when conditions are switched to Ca\(^{2+}\)-free medium, we have previously attributed this to the homeostatic release of Ca\(^{2+}\) from intracellular stores (23).

Independence from typical RyR and SERCA-containing Ca\(^{2+}\) stores of the ER. The effects of 10 mM caffeine are most commonly attributed to the activation of RyR. In the present study, we used a high dose of ryanodine and a two-pulse protocol to test whether caffeine-stimulated GTH-II release involves RyR. This protocol was selected because ryanodine binds to the RyR in its open conformation (11, 43) and, therefore, may exert a block that is use dependent in some systems. Our results clearly demonstrate that 100 \(\mu\)M ryanodine has no effect on either caffeine pulse, compared with untreated controls (Fig. 7, A and B). The GTH-II response evoked by caffeine also was not inhibited by 50 \(\mu\)M dantrolene, another inhibitor of RyR (Fig. 7C). The potentiation of hormone release by dantrolene might have resulted from a shunting of Ca\(^{2+}\) into a caffeine-sensitive pool. Collectively, these data indicate that caffeine-stimulated GTH-II release is not mediated through activation of the RyRs, which are presumed to be components of ER Ca\(^{2+}\) stores (3, 41).

Another hallmark of typical agonist-sensitive ER Ca\(^{2+}\) stores is the activity of SERCA pumps, which refill ER Ca\(^{2+}\) pools in all cell types studied to date (41). Treatment with 10 \(\mu\)M BHQ, a concentration that blocks SERCA (33) and that inhibits the Ca\(^{2+}\) stores common to both GnRHs in goldfish gonadotropes (23), had no effect on caffeine-stimulated...
GTH-II release (Fig. 8). GTH-II release responses to caffeine also are not blocked by 2 μM thapsigargin (Johnson JD and Chang JP, unpublished results). Collectively, these findings strongly suggest that caffeine-sensitive Ca\(^{2+}\) stores that modulate GTH-II release may be largely independent of classic ER Ca\(^{2+}\)-ATPases.

**DISCUSSION**

**Relationship between caffeine- and sGnRH-sensitive Ca\(^{2+}\) stores.** Caffeine-sensitive Ca\(^{2+}\) stores are highly coupled to GTH-II release in cultured goldfish gonadotropes. These Ca\(^{2+}\) stores also are of particular interest, because they represent a clear difference between the signaling cascades of the major endogenous GTH-II secretagogues, sGnRH and cGnRH-II. Acute GTH-II stimulation by both GnRHs depends on the mobilization of intracellular Ca\(^{2+}\) stores that are sensitive to TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethylamino)octoyl ester] and SERCA inhibitors. This common store seems to be highly sensitive to the manipulation of extracellular Ca\(^{2+}\) levels and probably requires VGCCs for refilling (23). Recent work from our laboratory (23) has shown that, between the two neuropeptides, only sGnRH signaling involves caffeine-sensitive mechanisms. This correlated well with the observation that GTH-II release evoked by sGnRH, but not cGnRH-II, requires an IP\(_3\)-sensitive Ca\(^{2+}\) store that is resistant to depletion under conditions that reduce the availability of extracellular Ca\(^{2+}\) (6, 7, 19, 23). Caffeine has been reported to inhibit several aspects of the IP\(_3\) signaling system (11, 45). The Ca\(^{2+}\) dependence and the Ca\(^{2+}\) insensitivity of caffeine-stimulated GTH-II release, revealed in the present study, provide further evidence that caffeine may act on the same depletion-resistant Ca\(^{2+}\) store as sGnRH. The apparent slow Ca\(^{2+}\) exchange of the caffeine-sensitive Ca\(^{2+}\) pool may result from a lack of SERCA pumps (see Novel properties of caffeine-mediated Ca\(^{2+}\) release leading to exocytosis).

The difference in magnitude between GTH-II release responses evoked by caffeine when compared with sGnRH can be explained if caffeine modulates multiple Ca\(^{2+}\) pools. Perhaps the secretagogue effects of caffeine are not manifested through the same Ca\(^{2+}\) stores that mediate the effects of caffeine on sGnRH signaling.

**Features of caffeine-evoked Ca\(^{2+}\) signals.** In the present study, experiments including the use of BAPTA suggest that the ability of caffeine to generate Ca\(^{2+}\) signals is essential for caffeine to stimulate GTH-II exocytosis. However, given the disparity in the magnitude of the relatively small global Ca\(^{2+}\) signals over the course of caffeine treatment and the large hormone release response, we speculate that caffeine evokes highly localized Ca\(^{2+}\) release from intracellular stores that are in close proximity to sites of GTH-II secretion. Consistent with this hypothesis, caffeine generated Ca\(^{2+}\) signals that began as highly localized events near a region of the membrane opposite the nucleus. Previous morphological studies have suggested that goldfish gonadotropes exhibit a weak polarity in culture and that this region opposite the nucleus is often rich in secretory granules and globules (50). The location of these events was similar in all cells tested, at least with respect to the ultrastructural landmarks (nucleus and globules) that are visible using DIC optics. One major advantage of our morphological identification protocol is that cells are selected for a specific orientation, thereby ensuring that they are imaged on roughly the same focal plane relative to the nucleus. However, beyond rough estimates of the localization of Ca\(^{2+}\) signals, the low spatial and temporal resolution of our data do not permit extrapolation to the level of Ca\(^{2+}\) microdomains, which are known to be important in the regulation of exocytosis and other localized cellular functions, in many cell types (3, 21, 35).
Novel properties of caffeine-mediated Ca\(^{2+}\) release leading to exocytosis. In rat gonadotropes, Tse et al. (47) have suggested that the strong coupling of GnRH signaling to GTH-II exocytosis results from the close proximity of agonist-sensitive ER stacks to secretory granules (and presumably exocytotic protein machinery). In the rat model, acute GnRH signaling follows a classic pathway and is mediated by a single ER Ca\(^{2+}\) pool that is IP\(_3\) sensitive, rapidly exchanging, and refilled by SERCA pumps (44, 46). Caffeine-sensitive Ca\(^{2+}\) stores, if present, have not been assigned a significant role in the generation of basal or GnRH-evoked Ca\(^{2+}\) signals in mammalian gonadotropes. However, a large body of work (6, 7, 24) has demonstrated many differences in GnRH signal transduction and, specifically, Ca\(^{2+}\) signaling between rats and goldfish. Indeed, the characteristics of caffeine-stimulated GTH-II release in the present study differ substantially from what we would expect of typical agonist-sensitive ER Ca\(^{2+}\) pools. First, on the basis of hormone release experiments with Cd\(^{2+}\), it appears that caffeine-sensitive Ca\(^{2+}\) stores may not be rapidly exchanging. Second, caffeine action is independent of RyR and SERCA pumps, which are known to mediate Ca\(^{2+}\) flux from caffeine-sensitive ER of many cell types (41). Caffeine-stimulated GTH-II release also is unlikely to be mediated by mitochondria, because it is not blocked by 10 \(\mu\)M carbonyl cyanide \(m\)-chlorophenylhydrazone \((n = 4, \text{ data not shown}), a \text{ mitochondrial uncoupler shown to modulate the shape of Ca}^{2+}\text{ signals in mammalian gonadotropes (26). Interestingly, a recent report (18) indicated that the involvement of cyclopiazonic acid (CPA)-sensitive SERCA pumps in caffeine-evoked Ca}^{2+}\text{ release may be tissue dependent. Thapsigargin-}
Insensitive Ca\textsuperscript{2+} stores are found in many cell types, including several pituitary cell lines. However, these have not been described to be agonist or caffeine sensitive (40). Nevertheless, these features suggest the possible involvement of several classes of non-ER Ca\textsuperscript{2+} stores, including acidic compartments such as the secretory granules, in caffeine-stimulated GTH-II release and, by association, suggest a possible role in sGnRH signaling.

Given the localization of caffeine-evoked Ca\textsuperscript{2+} signals and the pharmacological evidence suggesting a role for non-ER Ca\textsuperscript{2+} stores, we speculate that caffeine may act on targets close to or on the secretory granules themselves. Secretory granules contain very large quantities of Ca\textsuperscript{2+}. In several cell types, Ca\textsuperscript{2+} accumulation into granules is SERCA independent; instead they are filled by H\textsuperscript{+}/Ca\textsuperscript{2+} exchange or possibly as a result of endocytosis (32, 37). The slow Ca\textsuperscript{2+} uptake was a primary reason why acidic organelles were rejected as candidates for the prototypical agonist-sensitive store, subsequently attributed to the ER (41). Nevertheless, there is growing evidence that the secretory granules may constitute a physiologically relevant IP\textsubscript{3} (agonist)-sensitive Ca\textsuperscript{2+} store in a number of experimental systems (21, 37). Although the most unequivocal evidence has come from studies of exocrine cell types (13, 32, 36), there also is evidence of novel Ca\textsuperscript{2+} stores associated with the secretory granules in endocrine cells (25). Future work, including the direct measurement of Ca\textsuperscript{2+} levels inside the secretory granules of intact goldfish gonadotropes, is required to confirm this proposal.

The observation that caffeine action is ryanodine insensitive is unusual but not unprecedented. For example, the intracellular Ca\textsuperscript{2+} release component of Ca\textsuperscript{2+} transients in the presynaptic terminals of goldfish retinal bipolar cells is sensitive to caffeine but not to ryanodine (28). It has been proposed for mouse pancreatic islets that a caffeine-sensitive intracellular Ca\textsuperscript{2+} store modulated glucose-stimulated Ca\textsuperscript{2+} oscillations, an effect that was not mimicked by ryanodine (42). There also are several accounts in the literature of RyR that are caffeine insensitive (9, 14, 29). Further work, including direct measurements of Ca\textsuperscript{2+} within organelles, is required to characterize the putative novel class of Ca\textsuperscript{2+} stores in goldfish gonadotropes.
Differential coupling of signaling systems to GTH-II secretion. Another interesting conclusion from this study is that one part of the caffeine signal transduction circuit is dissociated from GTH-II release. Treatments with K+ channel blockers such as TEA or 4-aminopyridine (4-AP) are known to depolarize gonadotropes (Wong CJH and Change JP, unpublished observations), which would presumably activate VGCCs. Nonetheless, although caffeine inhibits K+ channels, caffeine-stimulated GTH-II release does not result from the inhibition of K+ channels, as indicated by the lack of effect of TEA. Similarly, caffeine-stimulated GTH-II release is not affected by 4-AP, an inhibitor of I_{K(A)} in goldfish gonadotropes (48) (Wong CJH and Chang JP, unpublished observations). The reason for this uncoupling remains to be elucidated. Direct activation of VGCC with high KCl or BAY K 8644 generates Ca^{2+} signals (23) (Van Goor F and Chang JP, unpublished observations) and have been reported to stimulate GTH-II release (49). In contrast, TEA treatment failed to increase GTH-II release in the present study, suggesting that the site of this functional “uncoupling” may be situated downstream of K+ channels but upstream of VGCC. Accordingly, preliminary experiments have shown that 5 mM TEA evokes weak Ca^{2+} signals in single gonadotropes (Wong CJH and Chang JP, unpublished). Together, these data suggest the existence of selective uncoupling between events that modulate electrical excitability of the plasma membrane and hormone release in this cell type.

Comparison with goldfish somatotropes. The intracellular mechanisms mediating the control of hormone release by sGnRH and cGnRH-II differs dramatically between goldfish gonadotropes and somatotropes (6, 7). In somatotropes, growth hormone release evoked by both GnRHs is mediated by caffeine-sensitive Ca^{2+} stores that are largely SERCA independent (22). However, unlike the agonist-specific Ca^{2+} stores described in the present work, caffeine/GnRH-sensitive Ca^{2+} stores in somatotropes are rapidly depleting (<5 min), partially sensitive to ryanodine, and dependent on cAMP signaling (22, 52). Comparisons of the mechanisms of caffeine-stimulated hormone release between gonadotropes and somatotropes may lead to important insights into how these two closely related cell types are differentially controlled by neuroendocrine regulators, including the GnRHs.

Physiological relevance. In the present report, we have explored some pharmacological properties of signaling cascades linking an agonist-specific Ca^{2+} store to the control of hormone exocytosis. It is tempting to speculate that caffeine-sensitive Ca^{2+} stores may be involved in the differential control of gonadotrope function by sGnRH and cGnRH-II (27). Caffeine and sGnRH have similar seasonal profiles with respect to their maximum efficacy in releasing GTH-II in vitro, with both being most efficient in between the stages of gonadal recrudescence and maturation (20, 31). Compared with the kinetics of cGnRH-II-stimulated GTH-II release, responses to sGnRH become very extended during this period (31), raising the possibility that the prolonged component may be due to the recruitment of caffeine-sensitive intracellular Ca^{2+} stores. Ca^{2+} signals generated by sGnRH also have slower rates of rise and more often exhibit a prolonged monophasic waveform compared with those of cGnRH-II (24). Although it has been elegantly demonstrated in a few cell types (e.g., Ref. 10; reviewed in Ref. 21), the functional specificity of Ca^{2+} signals has not been demonstrated within single pituitary cells from any species. Despite the fact that multiple GnRHs have been discovered in species from all vertebrate classes and in man (51), the signal transduction of two GnRHs has been extensively compared only in goldfish. These comparative analyses are providing novel insights into the diversity of Ca^{2+} signaling systems.

We acknowledge the support of the National Sciences and Engineering Research Council of Canada for operating grant OGP121399 to J. P. Chang, postdoctoral fellowships to C. J. H. Wong and J. D.
Johnson, and a postgraduate studentship to W. K. Yunker, J. D. Johnson received support from a Province of Alberta Graduate Fellowship and the Andrew Stewart Memorial Prize.

Present address of J. D. Johnson: 815 Yalem Bldg., Renal Division, Mailstop: 9032648, Washington University Medical Center, BJH North, 216 South Kingshighway Blvd., St. Louis, MO, 63110.

REFERENCES


38. Pinton P, Pozzan T, and Rizzuto R. The Golgi is an inositol 1,4,5-trisphosphate-sensitive Ca2+ store, with functional properties distinct from those of the endoplasmic reticulum. EMBO J 17: 5298–5308, 1998.


