Rapid Down-regulation of the Type I Inositol 1,4,5-Trisphosphate Receptor and Desensitization of Gonadotropin-releasing Hormone-mediated Ca$^{2+}$ Responses in αT3–1 Gonadotropes*

Received for publication, September 29, 2000, and in revised form, November 6, 2000
Published, JBC Papers in Press, November 7, 2000, DOI 10.1074/jbc.M008916200

Gary B. Willars‡, Jean E. Royall‡, Stefan R. Nahorski‡, Faraj El-Gehani‡, Helen Everest¶, and Craig A. McArdle∥

From the ‡Department of Cell Physiology and Pharmacology, University of Leicester, Medical Sciences Building, P. O. Box 138, University Road, Leicester LE1 9HN, United Kingdom and the ¶University Neuroendocrine Unit, Department of Medicine, University of Bristol, Marlborough Street, Bristol BS2 8HW, United Kingdom

Despite no evidence for desensitization of phospholipase C-coupled gonadotropin-releasing hormone (GnRH) receptors, we previously reported marked suppression of GnRH-mediated Ca$^{2+}$ responses in αT3–1 cells by pre-exposure to GnRH. This suppression could not be accounted for solely by reduced inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) responses, thereby implicating uncoupling of Ins(1,4,5)P$_3$ production and Ca$^{2+}$ mobilization (McArdle, C. A., Willars, G. B., Fowkes, R. C., Nahorski, S. R., Davidson, J. S., and Forrest-Owen, W. (1996) J. Biol. Chem. 271, 23711–23717). In the current study we demonstrate that GnRH causes a homologous and heterologous desensitization of Ca$^{2+}$ signaling in αT3–1 cells that is coincident with a rapid (t$_{1/2}$ < 20 min), marked, and functionally relevant loss of type I Ins(1,4,5)P$_3$ receptor immunoreactivity and binding. Furthermore, using an αT3–1 cell line expressing recombinant muscarinic M$_3$ receptors we show that the unique resistance of the GnRH receptor to rapid desensitization contributes to a fast, profound, and sustained loss of Ins(1,4,5)P$_3$ receptor immunoreactivity. These data highlight a potential role for rapid Ins(1,4,5)P$_3$ receptor down-regulation in homologous and heterologous desensitization and in particular suggest that this mechanism may contribute to the suppression of the reproductive system that is exploited in the major clinical applications of GnRH analogues.

The decapeptide gonadotropin-releasing hormone (GnRH)$^1$ is released from the hypothalamus of mammals in a pulsatile manner to regulate the ectopic release of luteinizing hormone and follicle-stimulating hormone from pituitary gonadotropes. These hormones are central to the regulation of gonadal steroidogenesis and gamete maturation, and GnRH therefore plays a vital role in the control of vertebrate reproduction. GnRH acts on pituitary gonadotropes through a G-protein-coupled receptor that regulates phospholipase C (PLC) via G-proteins of the Go$_{q/11}$ family (1). GnRH-mediated activation of PLC results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate both inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) and diacylglycerol. These second messengers are able to mobilize Ca$^{2+}$ from intracellular stores and activate protein kinase C, respectively (2), thereby propagating a signaling cascade that accounts for the biological effects of GnRH.

In contrast to nearly all other known PLC-coupled G-protein-coupled receptors (GPCRs), the GnRH receptor does not undergo rapid (seconds to minutes) desensitization following exposure to agonist (3–8). Recent evidence has suggested that this lack of acute regulation is related to the lack of a C-terminal tail and the absence, therefore, of appropriate regulatory phospho-acceptor sites (8, 9). From a functional perspective, the resistance of the GnRH receptor to rapid desensitization may serve to maintain cellular sensitivity and responsiveness during events such as the pre-ovulatory gonadotropin hormone surge and allow the frequency-encoded pattern of the hypothalamic pulsatile GnRH release (10, 11) to be faithfully maintained at the level of the pituitary gonadotropes.

Despite the lack of acute regulation of the GnRH receptor, sustained exposure to GnRH is able to reduce GnRH-stimulated gonadotropin secretion, and this form of desensitization underlies the suppression of the reproductive system that is exploited in the major clinical applications of GnRH analogues (12). Given the importance of cytosolic Ca$^{2+}$ elevation in the mediation of GnRH-stimulated gonadotropin secretion (1, 13–15), we have previously explored the potential desensitization of this component of the GnRH receptor-mediated signaling pathway in an immortalized mouse pituitary cell line (αT3–1). Despite no evidence for rapid desensitization of the GnRH receptor in these cells, pre-exposure to GnRH can cause a marked suppression of subsequent GnRH-mediated elevations of [Ca$^{2+}$]$_i$. Both the spike phase of the response (which reflects Ins(1,4,5)P$_3$-dependent mobilization of intracellular Ca$^{2+}$) and the sustained phase of the response (which is dependent upon Ca$^{2+}$ entry across the plasma membrane through voltage-operated Ca$^{2+}$ channels) were attenuated by GnRH pretreatment (4, 5).

Desensitization of voltage-operated Ca$^{2+}$ channels may account for the desensitization of the plateau phase of the GnRH-mediated response in αT3–1 cells (4), but the mechanism underlying attenuated mobilization of Ca$^{2+}$ from intracellular stores is unclear. Although pre-exposure to GnRH reduces both the number of plasma membrane GnRH receptors and the ability of GnRH to generate Ins(1,4,5)P$_3$, these effects are insufficient to account for the reduced release of intracellular Ca$^{2+}$ (5). Indeed, this desensitization is heterogeneous and therefore most probably reflects post-receptor modification(s). Because desensitization of GnRH-stimulated Ca$^{2+}$ mobiliza-
GnRH desensitizes Ca\(^{2+}\) requires several hours of agonist stimulation (17, 23), whereas indicated (\(\Delta\)) in buffer containing 100 nM GnRH, 1 mM methacholine, or no addition (control). The cells were then washed extensively and mounted on the microscope stage. During imaging the cells were stimulated as indicated with either 100 nM GnRH (a and c) or 1 mM methacholine (b). In panel c, showing the time course of homologous and heterologous desensitization of GnRH-mediated Ca\(^{2+}\) signaling, the GnRH-stimulated increase in [Ca\(^{2+}\)]\(_e\) (calculated by subtraction of pre-stimulation values from maximal post-stimulation values) is shown as a function of pretreatment time. Control responses to GnRH were determined at each time point but were not time-dependent and have, therefore, been pooled and plotted at the 0 time point for clarity. Each trace shows the mean ± S.E. derived from 3 separate experiments (a and b), 3–7 separate experiments (c), or >16 experiments (c, 0 h) with 20–50 cells imaged in each experiment.

FIG. 1. Homologous and heterologous desensitization of Ca\(^{2+}\) signaling in αT3–1/M3 cells. Cells were cultured, loaded with fura-2, and prepared for imaging as described under “Experimental Procedures.” Before imaging, they were pretreated for 6 h (a and b) or 30 min (c). A) [Ca\(^{2+}\)]\(_i\) time course for clarity. Each trace shows the mean ± S.E. derived from 3 separate experiments (a and b), 3–7 separate experiments (c), or >16 experiments (c, 0 h) with 20–50 cells imaged in each experiment.

Experimental Procedures

Materials and Cell Culture—Reagents of analytical grade were obtained from suppliers listed previously (5, 24–26), unless stated, or alternatively from Sigma. Antibodies against PLC isoforms and Go_{αq/11} were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), with the exception of PLCβ1, which was from Upstate Biotechnology (Lake Placid, NY). The αT3–1 gonadotrope cell line was originally a gift from Dr. P. Mellon, University of California, San Diego, CA, and in the current study we used a cell line (αT3–1/M3) derived from this, which also expresses the recombinant human muscarinic M3 receptor. Like the endogenously expressed GnRH receptor, this GPCR also couples to the activation of PLC in this cell line (7), and we have demonstrated that muscarinic M3 receptors are subject to rapid but partial desensitization, whereas the endogenously expressed GnRH receptors show no evidence of such regulation (7). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 10% (v/v) fetal calf serum. Cultures were maintained at 37 °C in 5% CO\(_2\), humidified air and passaged weekly. For experiments, cells were harvested with 10 mM HEPES, 154 mM NaCl, 10 mM NaHCO\(_3\), 5 mM Hepes, 10 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.1% bovine serum albumin, and incubated in a 5% CO\(_2\), humidified atmosphere at 37 °C, as described (14).

Western Blotting—Cells were grown on glass coverslips and were washed with ice-cold PBS and then resuspended in 50 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 0.1% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with isotype-specific Ins(1,4,5)P\(_3\) receptor antibodies. Immuno-reactive bands were detected using ECL reagents and exposure to Hyperfilm-ECL (Amersham Pharmacia Biotech). Where required, densitometric analysis of the resulting bands was performed using a Bio-Rad GS-670 imaging densitometer with Molecular Imagers version 1.2 software. For immune-detection of

Dynamic Video Imaging of Cytosolic Ca\(^{2+}\)—Video imaging of fura-2-loaded cells was performed as described previously (5). Briefly, cells grown on glass coverslips were loaded with the acetoxymethyl ester of fura-2 (2 μM) for 30 min at 37 °C in 1 ml of buffer (pH 7.4, composition (mM): NaCl 127, CaCl\(_2\) 1.8, KCl 5, MgCl\(_2\) 2, NaHPO\(_4\) 0.5, NaHCO\(_3\) 5, glucose 10, HEPES 10 with 0.1% bovine serum albumin). Cells were then washed several times and placed within a heated (37 °C) perfusion stage of a Nikon Diaphot inverted microscope. Image capture was performed using Mosaic hardware following alternate excitation at 340 and 380 nm with emission recorded at 510 nm. Values were averaged from 16 or 32 video frames, and background fluorescence was subtracted prior to ratioing. The ratio of fluorescence at 340 and 380 nm was calculated on a pixel-by-pixel basis using maximum and minimum values defined by treatment with 5 μM Ionomycin in medium with either 10 mM CaCl\(_2\) or 10 mM EGTA and assuming a dissociation constant of 225 nM for fura-2 and Ca\(^{2+}\) at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).

Western Blotting—Cells were grown to confluence in 6-well multwell dishes. Medium was removed, and the cells were washed (2 × 1 ml) with medium containing 0.1% bovine serum albumin and incubated in a further 1 ml. GnRH was then added at the appropriate concentration, and the cells were incubated at 37 °C in 5% CO\(_2\), humidified air. For immuno-detection of Ins(1,4,5)P\(_3\) receptors, medium was aspirated after the required time, the cell monolayers were washed several times, and the cells were incubated at 37 °C, as described (14).
other proteins (Go/t and PLC isoforms), Western blotting was carried out as above with the exception that the cell monolayers were solubilized in 200 μl of solubilization buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml iodoacetamide, 100 μg/ml benzamidine) for 30 min on ice before being processed as described above.

Details and use of the polyclonal antibody against the type I Ins(1,4,5)P3 receptor was quantified and expressed as a percentage of that under basal (no agonist treatment) conditions. The data are the mean ± S.E.; n = 3.

Western blot is shown (d); below the blot are the mean densitometric data (e). The density of the band representing the type I Ins(1,4,5)P3 receptor was quantified and expressed as a percentage of that in the absence of agonist (c). Data are mean ± S.E.; n = 3.

Other antibodies were at dilutions according to the instructions of the suppliers.

Determination of Ins(1,4,5)P3 Receptor Density by Binding of [3H]Ins(1,4,5)P3—Ins(1,4,5)P3 receptor binding was determined in membranes of aT3–1 cells as described (16). Incubations were for 45 min at 4 °C in 200 μl of incubation buffer with 2–10 μg of membrane protein, 10 nCi of [3H]Ins(1,4,5)P3, and 0 or 10−2–10−5 M unlabeled Ins(1,4,5)P3. The incubations were terminated by centrifugation and removal of supernatants by aspiration. Pellets were then solubilized in NaOH and transferred to scintillant for β-counting.

Ins(1,4,5)P3-mediated Release of 45Ca2+ from Intracellular Stores of Permeabilized Cells—45Ca2+ release assays were performed in cytosol-

RESULTS

Pretreatment of aT3–1/M3 cells for 1 h with maximal concentrations (7) of either GnRH (1 μM) or methacholine (1 mM) resulted in both homologous and heterologous desensitization of agonist-mediated Ca2+ signaling (Fig. 1, a and b). The homologous and heterologous loss of Ca2+ signaling as a result of GnRH or methacholine pretreatment were maximal following...
GnRH-mediated Down-regulation of Ins(1,4,5)P₃ Receptors

**Fig. 5.** The effect of thimerosal on mobilization of Ca²⁺ by GnRH in control and GnRH-pretreated aT3–1/M₃ cells. Cells were pretreated with 0 (a and c; Control) or 1 μM GnRH (b and d; GnRH pretreated) for 60 min and then prepared for Ca²⁺ imaging as described in the legend to Fig. 1. During imaging, the cells were transferred to Ca²⁺-free medium (first vertical arrow) and then to Ca²⁺-free medium containing 0 or 1 μM thimerosal (second vertical arrow) as indicated. Finally, the cells were stimulated with 5 μM GnRH (a and b) or 1 μM GnRH Pretreatment (Fig. 1c). During imaging, the cells were transferred to Ca²⁺-free medium (first vertical arrow) and then to Ca²⁺-free medium containing 0 or 1 μM thimerosal (second vertical arrow) as indicated. Finally, the cells were stimulated with 5 μM GnRH (a and b) or 1 μM GnRH pretreatment (Fig. 1c). During imaging, the cells were transferred to Ca²⁺-free medium (first vertical arrow) and then to Ca²⁺-free medium containing 0 or 1 μM thimerosal (second vertical arrow) as indicated. Finally, the cells were stimulated with 5 μM GnRH (a and b) or 1 μM GnRH pretreatment (Fig. 1c).

Expression of type I, II, and III Ins(1,4,5)P₃ receptors was examined by Western blotting of solubilized aT3–1/M₃ cells using isoform-specific antibodies (26). Expression of all three isoforms was detected, although bands representing type II and III Ins(1,4,5)P₃ receptors were faint and then only apparent after exposure of the Western blot to film for longer periods of time (data not shown). Although we are unable to quantitate precisely the relative levels of the different types of Ins(1,4,5)P₃ receptors, the predominant expression of type I is consistent with that in the pituitary, which expresses type I, II, and III Ins(1,4,5)P₃ receptors at 73, 24, and 3% of the total receptor population, respectively (19).

Challenge of aT3–1/M₃ cells with 1 μM GnRH resulted in a rapid and marked loss of type I Ins(1,4,5)P₃ receptor immunoreactivity (Fig. 2, a and c), which had a half-time of −20 min, was maximal by 60 min (<20% of immunoreactivity remaining), and sustained for at least 24 h in the continued presence of agonist (Fig. 2, a and c). Comparable data were obtained using 100 nM GnRH (Fig. 2c). Challenge of aT3–1/M₃ cells with 1 μM methacholine also resulted in a marked loss of type I Ins(1,4,5)P₃ receptor immunoreactivity, albeit with a rate and magnitude that was less than that observed with GnRH (Fig. 2, b and c). In contrast to treatment with 1 μM GnRH, there was some recovery of type I Ins(1,4,5)P₃ receptor immunoreactivity during 24-h treatment with methacholine (Fig. 2, b and c). The GnRH-mediated loss of type I Ins(1,4,5)P₃ receptor immunoreactivity was concentration-dependent, with an EC₅₀ of 9.9 ± 0.61 (log₁₀ M; n = 4; 0.12 μM) (all data with errors are mean ± S.E.) at 60 min of treatment (data not shown).

In experiments designed to examine the rate of recovery of type I Ins(1,4,5)P₃ receptor immunoreactivity, cells were first treated with 1 μM GnRH for 1 h to induce down-regulation. Agonist activation of GnRH receptors was then stopped by washing the cell monolayer and continuing the incubation in the presence of the GnRH receptor antagonist, antide (1 μM). Type I Ins(1,4,5)P₃ receptor immunoreactivity was then examined over the subsequent 24 h. The 1-h pretreatment with GnRH resulted in a marked loss of type I Ins(1,4,5)P₃ receptor immunoreactivity that was further reduced after 1 h of "recovery" time (Fig. 2, d and e). Levels of receptor immunoreactivity then increased back to basal levels by 24 h (Fig. 2, d and e). Similar results were obtained when cells were treated with 1 μM GnRH and washed, but antagonist was not added (data not shown).

In binding experiments, pretreatment of intact cells for 60 min with 1 μM GnRH reduced the binding of [³²P]Ins(1,4,5)P₃ to αT3–1/M₃ membranes to 51 ± 7% (n = 4) of control without measurably altering the Kᵦ (Fig. 3).

Permeabilization of αT3–1/M₃ cells with β-escin allowed intracellular Ca²⁺ stores to be loaded with tracer ⁴⁵Ca²⁺, which could then be released by the addition of Ins(1,4,5)P₃. This exogenous Ins(1,4,5)P₃ was able to release a maximum of −60% of the ⁴⁵Ca²⁺ that had been loaded over a 15-min period, with an EC₅₀ of −6.8 ± 0.2 (log₁₀ M; n = 4; 0.16 μM) (Fig. 4). Treatment of cells for 1 h with 1 μM GnRH significantly reduced the magnitude of Ins(1,4,5)P₃-mediated release of ⁴⁵Ca²⁺ from the intracellular stores (p < 0.001, two way analysis of variance) but had no significant effect on the EC₅₀ for Ins(1,4,5)P₃, which was −6.5 ± 0.3 (log₁₀ M; n = 4; 0.32 μM) (Fig. 4).

Thimerosal has been reported to increase Ins(1,4,5)P₃ receptor sensitivity (29). In naive αT3–1/M₃ cells, 100 μM thimerosal increased the spike [Ca²⁺], response to a sub-maximal (5 nM) (Fig. 5a) but not maximal (1 μM) (Fig. 5c) concentration of GnRH when cells were challenged in the absence of extracellular Ca²⁺ (to assess the effects on Ca²⁺ release only). This suggests that the Ins(1,4,5)P₃ receptor does not limit the magnitude of the Ins(1,4,5)P₃-mediated Ca²⁺ release in naive cells stimulated with a maximal concentration of GnRH but can limit the response to submaximal agonist concentrations. When cells were pretreated for 1 h with 100 μM GnRH (in the presence of extracellular Ca²⁺), thimerosal had little effect on the subsequent response (again in the absence of extracellular Ca²⁺) to a submaximal concentration of GnRH but markedly potentiated the response to a maximal concentration (Fig. 5, b and d), suggesting that, in GnRH-pretreated cells, Ins(1,4,5)P₃ receptor activation is rate-limiting for GnRH-stimulated Ca²⁺ mobilization.

Although challenge of cells with 1 μM GnRH resulted in a dramatic reduction in type I Ins(1,4,5)P₃ receptor immunoreactivity (see above), exclusion of Ca²⁺ from the extracellular buffer prevented the GnRH-mediated loss of Ins(1,4,5)P₃ receptors but had no significant effect on the basal (nonagonist-stimulated) levels over a 1-h period (Fig. 6a). Furthermore, the absence of extracellular Ca²⁺ partially prevented the homologous desensitization of GnRH-mediated spike and plateau Ca²⁺ signaling (Fig. 6, b and c). Incubation of cells for 4 h with the cysteine protease (and proteasome) inhibitor N-acetyl-Leu-Leu-norleucinal (ALLN, 100 μg/ml) (20, 22, 23) prior to and during a 1-h incubation with 1 μM GnRH also markedly attenuated the agonist-induced loss of type I Ins(1,4,5)P₃ receptor immunoreactivity (Fig. 7a). The effects of ALLN on the desensitization of the agonist-mediated Ca²⁺ response were, however, difficult to interpret. Thus, even in the absence of GnRH pretreatment, ALLN markedly inhibited the spike and plateau Ca²⁺ responses to GnRH (data not shown), and we therefore used the more specific proteasome inhibitor lactacystin (10 μM, 4 h) (22). Lactacystin also markedly protected type I Ins(1,4,5)P₃ receptor immunoreactivity against GnRH-mediated down-regulation (control (untreated), 100%; 1 h, 1 μM GnRH, 44.6 ± 4.5%; lactacystin, 80.6 ± 23.0%; 1 h, 1 μM GnRH...
show the mean inmunoreactivity of those antibodies that did not detect proteins challenged with GnRH (1 m, 1 h) in Krebs/HEPES buffer with either 1.3 mM Ca$^{2+}$ or no added Ca$^{2+}$. Western blotting for the type I Ins(1,4,5)P$_3$ receptor was then performed. The density of the bands representing the type I Ins(1,4,5)P$_3$ receptor was quantified and expressed as a percentage of that in the presence of extracellular Ca$^{2+}$ but in the absence of GnRH. The data are the mean ± S.E.; n = 4. For determination of [Ca$^{2+}$]i, signaling (b and c), cells were pretreated with 0 (b) or 1 μM GnRH (c) for 60 min in buffer (filled symbols) or Ca$^{2+}$-free buffer (open symbols), then washed in normal buffer, and prepared for Ca$^{2+}$ imaging as described in the legend to Fig. 1. During imaging, the cells were transferred to Ca$^{2+}$-free buffer (vertical arrow) and then stimulated with 100 nM GnRH (still in Ca$^{2+}$-free buffer) as indicated by the horizontal arrows. Each trace shows the mean ± S.E. derived from three separate experiments, with 20–50 cells imaged in each experiment.

+ lactacystin, 102.5 ± 34.1%). Furthermore, lactacystin attenuated, but did not completely prevent, GnRH-mediated desensitization of [Ca$^{2+}$]i mobilization (Fig. 7, b and c). There was also some inhibition of GnRH-mediated spike [Ca$^{2+}$]i signaling in the presence of lactacystin (Fig. 7b), although the plateau was unaffected (data not shown).

Using commercially available antibodies against the PLC isoforms β$_{1–4}$, γ$_{1–2}$, and δ$_{1–2}$, the expression of PLCβ$_1$, β$_2$, γ$_1$, and γ$_2$ was demonstrated in αT3–1/M$_3$ cells (Fig. 8). The immunoreactivity of those antibodies that did not detect proteins in αT3–1/M$_3$ cells was confirmed using extracts from either SH-SY5Y neuroblastoma cells or rat brain (data not shown). Given that GnRH receptor-mediated responses are via Go$_q/11$ and most likely, therefore, via PLCβ isoforms, we examined the influence of GnRH or methacholine treatment on the expression of Go$_q/11$, PLCβ$_1$, and β$_3$. Exposure of αT3–1/M$_3$ cells for up to 1 h with maximal concentrations of either GnRH (1 μM) or methacholine (1 mM) had no consistent effects on the levels of Go$_q/11$ or the PLC isoforms β$_1$ and β$_3$ (Fig. 8).

**DISCUSSION**

It has been known for over two decades that sustained stimulation of gonadotropes with GnRH causes desensitization of GnRH-stimulated gonadotropin secretion (30). For determination of Ins(1,4,5)P$_3$ receptor immunoreactivity, (a), cells were challenged with GnRH (1 μM, 1 h) in Krebs/HEPES buffer with either 1.3 mM Ca$^{2+}$ or no added Ca$^{2+}$.

**FIG. 6.** The influence of extracellular Ca$^{2+}$ on GnRH-mediated down-regulation of type I Ins(1,4,5)P$_3$ receptor immunoreactivity and GnRH-mediated [Ca$^{2+}$]i signaling in αT3–1/M$_3$ cells. For quantification of type I Ins(1,4,5)P$_3$ receptor immunoreactivity (a), cells were challenged with GnRH (1 μM, 1 h) in Krebs/HEPES buffer with either 1.3 mM Ca$^{2+}$ or no added Ca$^{2+}$. Western blotting for the type I Ins(1,4,5)P$_3$ receptor was then performed. The density of the bands representing the type I Ins(1,4,5)P$_3$ receptor was quantified and expressed as a percentage of that in the presence of extracellular Ca$^{2+}$ but in the absence of GnRH. The data are the mean ± S.E.; n = 4. For determination of [Ca$^{2+}$]i, signaling (b and c), cells were pretreated with 0 (b) or 1 μM GnRH (c) for 60 min in buffer (filled symbols) or Ca$^{2+}$-free buffer (open symbols), then washed in normal buffer, and prepared for Ca$^{2+}$ imaging as described in the legend to Fig. 1. During imaging, the cells were transferred to Ca$^{2+}$-free buffer (vertical arrow) and then stimulated with 100 nM GnRH (still in Ca$^{2+}$-free buffer) as indicated by the horizontal arrows. Each trace shows the mean ± S.E. derived from three separate experiments, with 20–50 cells imaged in each experiment.
reaches maximal levels within 20–30 s (7). This clearly implies
ated down-regulation of type I Ins(1,4,5)P3 immunoreactivity
the mean
Ins(1,4,5)P3 receptor were quantified and expressed as a percentage of
then performed. The densities of the bands representing the type I
mM methacholine) (7). However, the muscarinic M3 receptor
increase in Ins(1,4,5)P3 levels in these cells (100 nM GnRH, 1
these cells and occur even when the concentrations of GnRH
undergoes a partial rapid homologous desensitization and
reception to a sustained plateau after a peak at 10 s, whereas the
GnRH receptor does not rapidly desensitize and therefore
expressed as a percentage of
that under basal conditions (no ALLN or agonist). The data shown are
mean ± S.E.; n = 4. For determination of [Ca2+]i signaling (b and
cells were pretreated for 3 h in buffer with 0 (control, filled symbols)
or 10 μM (open symbols) lactacystin with 0 (b) or 100 nM GnRH (c) added
to the final 60 min of the pre-incubation and fura-2-acetoxymethyl
erster present for the final 30 min. The cells were then washed in normal buffer and prepared for Ca2+
imaging as described in the legend to Fig.
1. During imaging, the cells were transferred to Ca2+-free buffer and then stimulated with 100 nM GnRH. Each trace shows the mean ± S.E. derived from three separate experiments, with 20–50 cells imaged in
each experiment.

Ins(1,4,5)P3 receptors for proteasomal degradation, as demonstrated by the fact that proteasome inhibitors can prevent GPCR-mediated down-regulation (22, 22, 23). Our data are in
accord with this model, because we have found that GnRH-mediated down-regulation of type I
Ins(1,4,5)P3 receptor immunoreactivity is prevented in Ca2+-free medium and by the two protease inhibitors ALLN and lactacystin. Interestingly, we have found that the down-regulation of type I
Ins(1,4,5)P3 receptors caused by GnRH is more rapid, more pronounced, and more slowly reversed than that caused by methacholine (muscarnic M3 receptor activation). This is despite the fact that both stimuli cause comparable increases in [Ca2+]i, in these cells and occur even when the concentrations of GnRH and methacholine are matched to give comparable maximal increases in Ins(1,4,5)P3 levels in these cells (100 nM GnRH, 1 mM methacholine) (7). However, the muscarinic M3 receptor undergoes a partial rapid homologous desensitization and therefore causes a transient increase in Ins(1,4,5)P3 mass, reducing to a sustained plateau after a peak at 10 s, whereas the
GnRH receptor does not rapidly desensitize and therefore causes a sustained increase in Ins(1,4,5)P3 mass, which reaches maximal levels within 20–30 s (7). This clearly implies that the
Ins(1,4,5)P3 receptor down-regulation is sensitive not just to the magnitude of the Ins(1,4,5)P3 response but also to its duration, precisely as expected if it is the Ins(1,4,5)P3 occupied (active) receptor conformation that is sensitive to proteolysis

FIG. 7. The influence of protease inhibition on GnRH-mediated down-regulation of type I Ins(1,4,5)P3 immunoreactivity and homologous desensitization of [Ca2+]i signaling in αT3–1/M3 cells. For determination of Ins(1,4,5)P3 receptor immunoreactivity (a), cells were incubated with (+) or without (−) the cysteine protease inhibitor ALLN (100 μg/ml) for 4 h. Agonist (1 μM GnRH or 1 mM methacholine) was then added, and the incubation was continued for an additional 1 h. Western blotting for the type I
Ins(1,4,5)P3 receptor was then performed. The densities of the bands representing the type I
Ins(1,4,5)P3 receptor were quantified and expressed as a percentage of
that under basal conditions (no ALLN or agonist). The data shown are
mean ± S.E.; n = 4. For determination of [Ca2+]i signaling (b and
cells were pretreated for 3 h in buffer with 0 (control, filled symbols)
or 10 μM (open symbols) lactacystin with 0 (b) or 100 nM GnRH (c) added
to the final 60 min of the pre-incubation and fura-2-acetoxymethyl
erster present for the final 30 min. The cells were then washed in normal buffer and prepared for Ca2+
imaging as described in the legend to Fig.
1. During imaging, the cells were transferred to Ca2+-free buffer and then stimulated with 100 nM GnRH. Each trace shows the mean ± S.E. derived from three separate experiments, with 20–50 cells imaged in
each experiment.

FIG. 8. Lack of effect of acute agonist treatment (≤1 h) on the expression of GnRH, PLCβ1, and PLCβ3 in αT3–1/M3 cells. Western blotting for the PLC isoforms β1, γ1, and δ3 demonstrated the expression of PLCβ1, β2, γ1, and γ2 in αT3–1/M3 cells. Agonist treatment for up to 1 h had no effect on the expression levels of GnRH, PLCβ1, and PLCβ3. This was also reflected in the densitometric scan data from three separate experiments (data not shown).

(22). Thus, the lack of GnRH receptor desensitization may contribute to the unusual rapidity of Ins(1,4,5)P3 receptor down-regulation in these cells. Typically, Ins(1,4,5)P3 receptor
down-regulation occurs with a half-time of 4–24 h (17, 23), as compared with <20 min in GnRH-stimulated αT3–1/M3 cells (Fig. 2). Presumably with other PLC-activating GPCRs, receptor
desensitization attenuates Ins(1,4,5)P3 responses and thereby reduces the rapidity and/or magnitude of Ins(1,4,5)P3 receptor down-regulation. It should be noted, however, that
bombesin and cholecystokinin reduce Ins(1,4,5)P3 receptor levels with a half-time of <30 min in AR4–2J cells (19) and that methacholine caused increased
Ins(1,4,5)P3 receptor down-regulation with a half-time of <60 min in αT3–1/M3 cells, demonstrating that relatively rapid down-regulation can occur, even with receptors that do desensitize.

The major question raised by our data is whether down-regulation of Ins(1,4,5)P3 receptors contributes to or underlies desensitization of Ca2+ mobilization. Our investigations of
response kinetics are entirely compatible with this possibility because we have found a) that the time-course of Ins(1,4,5)P3 receptor down-regulation in response to GnRH is comparable with that for the onset of desensitization (Figs. 2 and 1c, respectively) and b) that both effects are maintained as GnRH
pretreatment is extended to 24 h. Further support for the possible causal relationship is provided by the demonstrations a) that the GnRH-mediated Ins(1,4,5)P3 receptor loss and desensitization of Ca2+ signaling are associated with reduced
Ins(1,4,5)P3-stimulated mobilization of 45Ca2+ from permeabilized cells (directly establishing the functional significance of Ins(1,4,5)P3 receptor regulation in this system), b) that Ca2+-
free medium prevents and attenuates GnRH-mediated
Ins(1,4,5)P3 receptor down-regulation and desensitization of Ca2+ mobilization, respectively, c) that lactacystin prevents and attenuates GnRH-mediated
Ins(1,4,5)P3 receptor down-regulation and desensitization of Ca2+ mobilization, respectively, and d) that thimerosal partially reverses desensitization of Ca2+ mobilization. Because thimerosal increases the affinity of
Ins(1,4,5)P3 receptors for Gαq/11, PLCβ1, and PLCβ3 in αT3–1/M3 cells.

Fig. 8. Lack of effect of acute agonist treatment (≤1 h) on the expression of GnRH, PLCβ1, and PLCβ3 in αT3–1/M3 cells. Western blotting for the PLC isoforms β1, γ1, and δ3 demonstrated the expression of PLCβ1, β2, γ1, and γ2 in αT3–1/M3 cells. Agonist treatment for up to 1 h had no effect on the expression levels of GnRH, PLCβ1, and PLCβ3. This was also reflected in the densitometric scan data from three separate experiments (data not shown).
precisely what would be expected if Ins(1,4,5)P$_3$ receptor loss leaves the desensitized cells with insufficient Ins(1,4,5)P$_3$ receptors for efficient mobilization of Ca$^{2+}$ even in the face of sufficient GnRH-stimulated Ins(1,4,5)P$_3$ levels.

Although our data are largely consistent with the possibility that GnRH-mediated Ins(1,4,5)P$_3$ receptor down-regulation underlies desensitization of Ca$^{2+}$ mobilization, several lines of evidence might argue against this interpretation. Thus, stimulation of muscarinic receptors results in a time course of heterologous desensitization of GnRH-mediated [Ca$^{2+}$]$_i$ elevation similar to the homologous desensitization caused by GnRH pretreatment. This is despite the finding that GnRH causes a more rapid and greater loss of Ins(1,4,5)P$_3$ receptor immunoreactivity than muscarinic receptor stimulation. Furthermore, the retention of ~50% of Ins(1,4,5)P$_3$ receptors (Fig. 3) and the fact that maximal Ins(1,4,5)P$_3$-stimulated 45Ca$^{2+}$ mobilization is only reduced by ~42% (Fig. 4) stand in contrast to the almost complete loss of the spike phase [Ca$^{2+}$]$_i$ response to GnRH in desensitized cells (Fig. 1). Similarly, complete inhibition of type I Ins(1,4,5)P$_3$ receptor down-regulation by pretreatment in Ca$^{2+}$-free medium, or in the presence of ALLN or lactacystin, contrasts to only partial inhibition, or no measurable inhibition, of desensitization. These apparent inconsistencies could reflect contributions from other as yet unidentified mechanisms of desensitization or may reflect differences in the relative contributions of Ins(1,4,5)P$_3$ receptor subtypes, or of receptors in different cellular locations, to the end points quantified. Thus, type II Ins(1,4,5)P$_3$ receptors, which are relatively resistant to down-regulation in other systems (19), may contribute disproportionately to the 45Ca$^{2+}$ mobilization response, and local down-regulation of Ins(1,4,5)P$_3$ receptors in the immediate vicinity of GnRH receptors may be more extreme than that revealed by global measurements of all Ins(1,4,5)P$_3$ receptors. Alternatively, it is possible that the 50% loss of Ins(1,4,5)P$_3$ receptors and the consequent increase in mean distance between functional Ins(1,4,5)P$_3$ receptors are sufficient to prevent propagation of Ca$^{2+}$ mobilization by calcium-induced calcium release (34) and therefore have a disproportionately large effect on Ca$^{2+}$ responses in intact cells (as compared with permeabilized cells or membrane preparations). It is equally possible, however, that other modifications of Ins(1,4,5)P$_3$ receptors (e.g. phosphorylation, ATP binding, ubiquitination) inhibit Ins(1,4,5)P$_3$ receptor signaling in the desensitized cells without altering immunoreactivity or radioligand binding in membrane preparations. Some of these modifications, particularly ubiquitination, appear to be involved in the targeting of Ins(1,4,5)P$_3$ receptors for degradation (22). That such targeting occurs is demonstrated by our finding that Ins(1,4,5)P$_3$ receptor immunoreactivity continues to decline following removal of GPCR activation (Fig. 2).

Whereas a number of studies have demonstrated the principle of agonist-induced Ins(1,4,5)P$_3$ receptor down-regulation (16–23), the current study provides evidence of a setting in which such regulation may be functionally relevant. Thus, loss of Ins(1,4,5)P$_3$ receptors following either pre-ovulatory surges in GnRH or, in particular, the clinical use of GnRH agonists may play a part in the suppression of gonadotrope function. It should be noted that such a mechanism would also result in a compromised function of other Ins(1,4,5)P$_3$-dependent, Ca$^{2+}$-mobilizing receptors expressed on pituitary cells (e.g. pituitary adényl cyclase-activating polypeptide receptors). Such heterologous loss of function by this mechanism may be less apparent in other systems in which GPCR desensitization may serve to limit the down-regulation of signaling components shared with other receptors.

REFERENCES

1. Stojilkovic, S. S., Reinhart, J., and Catt, K. J. (1994) Endocrinology 133, 462–499
2. Berridge, M. J. (1993) Nature 361, 315–325
3. Duckworth, J. S., Wakefield, I. K., and Millar, R. P. (1994) Biochem. J. 306, 299–302
4. McArdle, C. A., Forrest-Owen, W., Willars, G., Davidson, J. S., Poch, A., and Kratzmeter, M. (1995) Endocrinology 136, 4864–4871
5. McArdle, C. A., Willars, G. B., Fewkes, R. C., Nahorski, S. R., Davidson, J. S., and Forrest-Owen, W. (1996) J. Biol. Chem. 271, 23711–23717
6. McArdle, C. A., Davidson, J. S., and Willars, G. B. (1999) Mol. Cell. Endocrinol. 151, 129–136
7. Willars, G. B., McArdle, C. A., and Nahorski, S. R. (1998) Biochem. J. 333, 391–398
8. Heding, A., Vredel, M., Bopold, M., Sellar, R., Taylor, P. L., and Eidne, K. A. (1998) J. Biol. Chem. 273, 11472–11477
9. Willars, G. B., Heding, A., Vredel, M., Sellar, R., Blomenrohr, M., Nahorski, S. R., and Eidne, K. A. (1999) J. Biol. Chem. 274, 30146–30153
10. Moenter, S. M., Caraty, A., Locattelli, A., and Karsch, F. J. (1991) Endocrinology 129, 1175–1182
11. Moenter, S. M., Brand, R. M., Midgley, A. R., and Karsch, F. J. (1992) Endocrinology 130, 503–510
12. Barbi, R. L. (1992) Trends Endocrinol. Metab. 3, 30–34
13. Hansen, J. R., McArdle, C. A., and Conn, P. M. (1987) Mol. Endocrinol. 1, 808–815
14. McArdle, C. A., and Poch, A. (1992) Endocrinology 130, 3567–3574
15. Tse, F. W., Tse, A., Hille, B., Horstmann, H., and Almers, W. (1997) Neuron 18, 121–132
16. Wojcikiewicz, R. J. H., and Nahorski, S. R. (1991) J. Biol. Chem. 266, 22234–22241
17. Wojcikiewicz, R. J. H., Furuichi, T., Nakade, S., Mikoshiba, K., and Nahorski, S. R. (1994) J. Biol. Chem. 269, 7963–7969
18. Simpson, P. B., Challiss, R. A. J., and Nahorski, S. R. (1994) J. Neurochem. 63, 2369–2372
19. Wojcikiewicz, R. J. H. (1995) J. Biol. Chem. 270, 11678–11683
20. Wojcikiewicz, R. J. H., and Oberdorf, J. A. (1996) J. Biol. Chem. 271, 16652–16655
21. Oberdorf, J., Valliano, M. L., and Wojcikiewicz, R. J. H. (1997) J. Neurochem. 69, 1897–1903
22. Oberdorf, J., Webster, J. M., Zhu, C. C., Luo, S. G., and Wojcikiewicz, R. J. H. (1999) Biochem. J. 339, 453–461
23. Sijpina, H., Deelman, L., De Smedt, H., Missiaen, L., Parsy, J. B., Vanlingen, S., Henning, R. H., and Casteels, R. (1998) Cell Calcium 23, 11–21
24. Jenkins, S., Nahorski, S. R., and Challiss, R. A. J. (1994) Mol. Pharmacol. 46, 1138–1148
25. Willars, G. B., Nahorski, S. R., and Challiss, R. A. J. (1998) J. Biol. Chem. 273, 5037–5046
26. Mackrill, J. I., Wilcox, R. A., Miyawaki, A., Mikoshiba, K., Nahorski, S. R., and Challiss, R. A. J. (1996) Biochem. J. 318, 873–878
27. Wilcox, R. A., Faug, A., Kuzikowski, A. P., and Nahorski, S. R. (1997) FEBS Lett. 402, 241–245
28. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3460
29. Bloch, P. E., Plant, T. M., Nakai, Y., Keogh, J. E., and Knoblo, E. (1978) Science 202, 631–633
30. Shah, B. H., and Milligan, G. (1994) Mol. Pharmacol. 46, 1–7
31. Garrel, G., McArdle, C. A., Henning, R. H., and Casteels, R. (1997) Endocrinology 138, 2209–2216
32. Harris, D., Reiss, N., and Nasr, Z. (1997) J. Biol. Chem. 272, 13534–13540
33. Bootman, M. D., Berridge, M. J., and Lipp, P. (1997) Cell 91, 367–373
Rapid Down-regulation of the Type I Inositol 1,4,5-Trisphosphate Receptor and Desensitization of Gonadotropin-releasing Hormone-mediated Ca\textsuperscript{2+} Responses in α-T3-1 Gonadotropes

Gary B. Willars, Jean E. Royall, Stefan R. Nahorski, Faraj El-Gehani, Helen Everest and Craig A. McArdle

*J. Biol. Chem.* 2001, 276:3123-3129.
doi: 10.1074/jbc.M008916200 originally published online November 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008916200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 18 of which can be accessed free at http://www.jbc.org/content/276/5/3123.full.html#ref-list-1