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Desensitization of Gonadotropin-releasing Hormone Action in αT3–1 Cells Due to Uncoupling of Inositol 1,4,5-Trisphosphate Generation and Ca^{2+} Mobilization*

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Gonadotropin-releasing hormone (GnRH) acts via a G-protein coupled receptor on gonadotropes to increase cytosolic Ca^{2+} and stimulate gonadotropin secretion. Sustained exposure causes desensitization of these effects, but the GnRH receptor has no C-terminal tail and does not undergo rapid (<5 min) desensitization. Nevertheless, pretreatment of αT3–1 cells with GnRH reduced the spike Ca^{2+} response to GnRH and decreased the GnRH effect on inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) by 30–50%. Ca^{2+}-free medium with or without thapsigargin also decreased GnRH-stimulated $Ins(1,4,5)P_3$ generation, implying that attenuation of the Ca^{2+} response underlies the $Ins(1,4,5)P_3$ reduction rather than *vice versa*. Intracellular Ca^{2+} pool depletion cannot explain desensitization of the Ca^{2+} response because pool depletion and repletion were faster (half-times, <1 min) than the onset of and recovery from desensitization (half-times 10–20 min and 4–6 h). Moreover, 1-h GnRH pretreatment attenuated the spike Ca^{2+} response to GnRH but not that to ionomycin, and brief GnRH exposure in Ca^{2+}-free medium reduced the response to ionomycin more effectively in controls than in desensitized cells. GnRH pretreatment also attenuated the Ca^{2+} response to PACAP38. This novel form of desensitization does not reflect uncoupling of GnRH receptors from their immediate effector system but rather a reduced efficiency of mobilization by $Ins(1,4,5)P_3$ of Ca^{2+} from an intact intracellular pool.

Studies over recent years have revealed a general scheme for homologous desensitization of GPCRs[1] involving rapid uncoupling of receptors from their G-protein, subsequent sequestration of receptors from the plasma membrane, and internalization followed by proteolytic degradation (1). Mechanisms underlying rapid desensitization have been documented for a number of GPCRs, which involve rapid agonist-induced receptor phosphorylation and consequent uncoupling from the receptors effector system (2). Such phosphorylation may be mediated by protein kinases A or C or by specific G-protein coupled receptor kinases, and in many cases phosphorylation of specific amino acids within the C-terminal tail plays a crucial role (1–4). For example, specific residues in the C-terminal tail of adenylyl cyclase-activating β-adrenergic receptors have been identified that undergo agonist-induced phosphorylation by β-adrenergic receptor kinase. This phosphorylation permits association with β-arrestin, thereby inhibiting G-protein binding and adenylyl cyclase activation (1, 2). The C-terminal tail has also been found to be involved in internalization of a number of receptors of this superfamily (3, 4).

GnRH is a hypothalamic decapeptide that acts via GPCRs on gonadotropes to stimulate the exocytotic secretion of luteinizing hormone and follicle-stimulating hormone. Agonist occupancy of GnRH receptors causes a G-protein-mediated activation of PLC that hydrolyzes membrane phosphoinositides yielding diacylglycerol and inositol phosphates, including $Ins(1,4,5)P_3$, that respectively activate protein kinase C and mobilize Ca^{2+} from intracellular stores (5–11). GnRH also increases Ca^{2+} entry into gonadotropes, predominantly via voltage-operated Ca^{2+} channels, and the increase in cytosolic Ca^{2+} caused by GnRH is primarily responsible for the increase in exocytotic hormone release (7–10, 12, 13). Sustained exposure to GnRH reduces GnRH-stimulated gonadotropin secretion (homologous desensitization), and this underlies the suppression of the reproductive system, which is exploited in the major clinical applications of GnRH analogues (14, 15). Sustained treatment with GnRH also reduces the number of cell surface GnRH receptors (down-regulation), and this presumably contributes to desensitization but changes in GnRH receptor number can be uncoupled from those in gonadotrope responsiveness, demonstrating the involvement of additional mechanisms (16–20).

As with adenylyl cyclase coupled receptors, many PLC-coupled receptors clearly undergo rapid desensitization (21, 22), and evidence exists that similar regulatory mechanisms operates for receptors coupled to both effector system (21–23). However, GnRH receptors are unique amongst the currently known G-protein coupled receptors in that they lack the C-terminal cytoplasmic tail, which has proven to be so important for desensitization and/or internalization of other GPCRs (5–7), and recent work on the kinetics of GnRH-stimulated PLC activation in αT3–1 cells (a gonadotrope-derived cell line) indicates that the GnRH receptor does not undergo rapid (<10 min) homologous desensitization (24). We have shown, however, that pretreatment for 60 min with GnRH causes a pronounced reduction the GnRH-induced increase in cytosolic Ca^{2+} concen-

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[1] The abbreviations used are: GPCR, G-protein coupled receptor; GnRH, gonadotropin-releasing hormone; PACAP38, pituitary adenylyl cyclase activating polypeptide 1–38; PLC, phospholipase C; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate.

tration ($[Ca^{2+}]_i$) in these cells (25). Both the spike phase of the response (which reflects mobilization of intracellular Ca^{2+}) and sustained phase of the response (which is dependent upon Ca^{2+} entry across the plasma membrane) were attenuated by GnRH pretreatment (25). Here we have addressed the relationship between Ins(1,4,5)P_3 generation and Ca^{2+} mobilization in order to define more closely the cellular loci involved in desensitization of the spike response.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—Peptides were purchased from Peninsula Laboratories Europe Ltd. (Merseyside, UK), and culture media, sera, and plasticware were from Life Technologies, Inc. or Falcon (Becton Dickinson, Oxford, UK). Ionomycin and thapsigargin were from Calbiochem (Nottingham, UK), and fura 2/AM was from Molecular Probes Inc. (Eugene, OR). D-Ins(1,4,5)P_3 (K^+ salt) was from University of Rhode Island Foundation. D-*myo*-[^{3}H]Ins(1,4,5)P_3 (44 Ci/mmol) and *myo*-[2-^{3}H]inositol (14–16 Ci/mmol) was from Amersham International. All other reagents were from standard commercial suppliers. αT3–1 cells were cultured in serum-supplemented Dulbecco's modified Eagle's medium as described (25, 26). For experiments they were harvested by trypsinization and then incubated for 1–3 days in 12-well culture plates (2 ml of medium/well), which for Ca^{2+} imaging experiments contained untreated round glass coverslips.

Dynamic Video Imaging of Cytosolic Ca^{2+}—Video imaging of fura 2-loaded αT3–1 cells was performed as described (27, 28). Cells were washed and pretreated for 60 min at 37 °C in 0.9 ml of PSS (127 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM glucose, 0.1% bovine serum albumin, and 10 mM HEPES, pH 7.4) containing 0 (control) or 0.1 μM GnRH with fura 2 (100 μl of 20 μM fura 2/AM) added 30 min before the end of this incubation. They were then washed several times in PSS, and the coverslips were loaded into a holder that was fitted into a heating chamber at 37 °C. Image capture was typically performed within 10–25 min of loading in approximately 500 μl of PSS or in PSS with 100 μM EGTA and no $CaCl_2$ (Ca^{2+}-free medium) using MagiCal hardware, Tardis software, and a Nikon Diaphot microscope. The cells were excited alternately at 340 and 380 nm, and emitted light was collected at 510 nm averaging the data from 8 or 16 video frames and subtracting background values before ratioing. The ratio of fluorescence at 340 and 380 nm was calculated on a pixel-by-pixel basis and used to determine the Ca^{2+} concentration assuming a dissociation constant of 225 nM for fura 2 and Ca^{2+} at 37 °C. Calibration was performed as described (27, 28).

Quantification of Intracellular D-Ins(1,4,5)P_3—D-Ins (1,4,5)P_3 mass was determined using a modification of a radioreceptor assay previously validated for stereospecificity and positional specificity (29, 30). Briefly, αT3–1 cells were grown to approximately 80% confluence and then washed and incubated for 30 min in 1 ml of Krebs/HEPES at 37 °C. The medium was aspirated and replaced with 150 μl of buffer with the indicated concentration of GnRH. Incubations were performed in triplicate and were terminated at 5–300 s by addition of 150 μl of ice-cold 1 M trichloroacetic acid. For zero time points, trichloroacetic acid was added before the GnRH. The D-Ins(1,4,5)P_3 was then extracted as described (30) using duplicate aliquots for each of the triplicate samples and standards (0.1 nM to 3 μM). Protein content of the wells was determined in 0.1 M NaOH digests of control cells using bovine serum albumin as standard.

Statistical Analysis and Data Presentation—The figures show data from a single experiment (representative of four experiments) or the mean ± S.E. of data pooled from n independent experiments (raw data or data normalized as described in the figure legends). Data are reported in the text as mean ± S.E., and statistical analysis was by Student's t test, accepting $p < 0.05$ as statistically significant. EC_{50} values were estimated by nonlinear regression using Graphpad Prism (San Diego, CA). For Ca^{2+} measurements, image analysis was used to quantify the mean ionized Ca^{2+} in all of the cells in each field of view (which typically contained 10–50 cells) as well as in individual cells. The figures show the mean (with or without S.E.) of data pooled from the indicated number of fields of view. Where spike and plateau Ca^{2+} values are reported these were defined arbitrarily as the maximum response within 10 s of stimulation and the response after 1 min, respectively.

RESULTS

In the first series of experiments Ca^{2+} imaging was performed with αT3–1 cells pre-treated with 0 or 0.1 μM GnRH for 60 min and then after extensive washing stimulated with GnRH during image capture. As shown (Fig. 1, *main panel*) 0.1 μM caused the expected biphasic $[Ca^{2+}]_i$ increase in control cells from a basal value of 52 ± 9 nM to a spike of 556 ± 76 nM followed by a plateau of 165 ± 13 nM (mean ± S.E., $n = 7$). In cells pre-treated for 60 min with GnRH basal $[Ca^{2+}]_i$ was unaffected (52 ± 7 nM, $n = 7$), but the spike and plateau responses to GnRH were attenuated (to 127 ± 16 and 124 ± 12 nM, respectively, $p < 0.05$). Further studies characterizing the time course of desensitization of the spike phase indicated a significant ($p < 0.05$) reduction after only 10 min of pretreatment with GnRH (from 605 ± 80 to 308 ± 73 nM) and that the spike response to GnRH was maximally attenuated following a 20 min pretreatment (Fig. 1, *inset*).

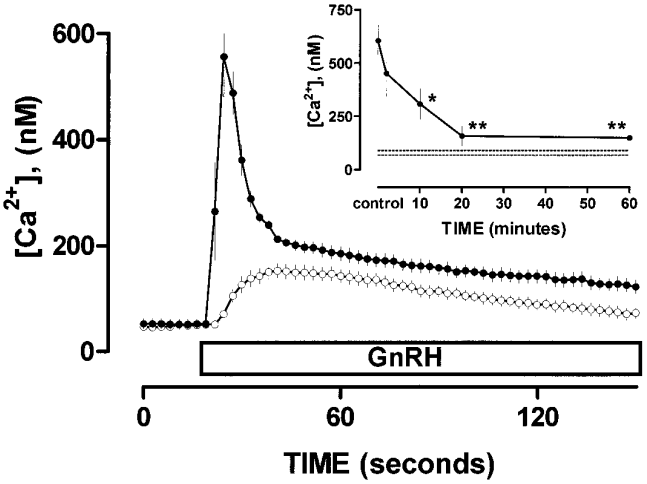

FIG. 1. **Homologous desensitization of the GnRH effect on $[Ca^{2+}]_i$ in αT3–1 cells.** *Main figure*, cells were pre-treated for 60 min with medium containing 0 (●) or 0.1 μM (○) GnRH (fura 2/AM present for the last 30 min of pretreatment) and then washed extensively, mounted on the microscope stage, and stimulated for the indicated period with 0.1 μM GnRH in Ca^{2+}-containing medium. Each curve shows the mean ± S.E. of seven separate experiments. *Inset*, cells labeled for 30 min with fura 2 were washed, mounted on the microscope stage, and incubated for the indicated period in medium with 0.1 μM GnRH (control cells received no GnRH pretreatment) and then washed extensively, and stimulated with 0.1 μM GnRH in Ca^{2+}-containing medium. The mean ± S.E. of maximal spike-phase responses in three to six experiments is shown for each time point, and the *dotted lines* show the upper and lower S.E. limits for basal Ca^{2+} pooled for all experiments. *, $p < 0.05$, **, $p < 0.01$ by Student's t test.

In control cells, GnRH caused a rapid increase in Ins(1,4,5)P_3 from basal values of 24 ± 8 to 246 ± 72 pmol/mg protein at 20 s and Ins(1,4,5)P_3 levels were then maintained (Fig. 2). Pretreatment for 60 min with 0.1 μM GnRH attenuated this effect of GnRH at all time points (Fig. 2). A similar inhibitory effect was seen when the concentration-response curve for the GnRH effect on Ins(1,4,5)P_3 was determined (Fig. 3). Stimulation for 5 s with GnRH caused a concentration-dependent increase in Ins(1,4,5)P_3 with a maximum increase from 15 ± 4 to 344 ± 37 pmol/mg protein (mean ± S.E., $n = 5$) in control cells. GnRH pretreatment (0.1 μM GnRH for 60 min) attenuated maximal GnRH-stimulated Ins(1,4,5)P_3 accumulation to approximately 65% of control. Although the EC_{50} for this effect of GnRH was increased from 85 to 134 nM by GnRH pretreatment, this difference was not statistically significant ($\log_{10}$ EC_{50} values ± S.E. were −7.07 ± 0.07 and −6.87 ± 0.14, respectively).

Although the effect of GnRH pretreatment on GnRH-stimulated Ins(1,4,5)P_3 production may contribute to desensitization of the Ca^{2+} response, PLCs are Ca^{2+}-dependent (31), so it is equally possible that desensitization of the Ca^{2+} response underlies the attenuated Ins(1,4,5)P_3 response. To address this, we assessed the effects of manipulations that alter the

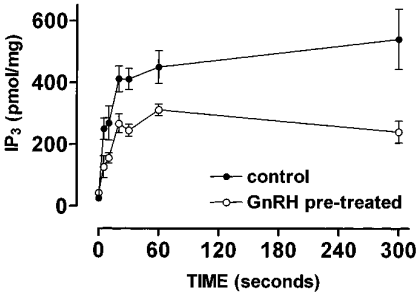

FIG. 2. **Time course of Ins(1,4,5)P_3 elevation by GnRH in control and GnRH pre-treated αT3–1 cells.** Cells were pre-treated for 60 min in Krebs/HEPES buffer with 0 or 0.1 μM GnRH and then washed extensively and stimulated for the indicated period with 0.1 μM GnRH before processing for Ins(1,4,5)P_3 measurement. The data shown are the means ± S.E. (n = 3) of data from a single representative experiment.

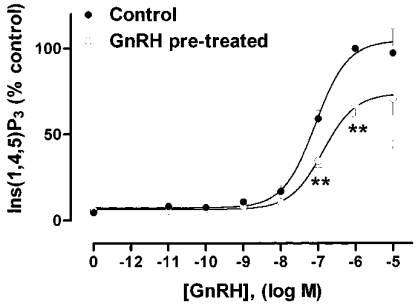

FIG. 3. **Dose dependence of Ins(1,4,5)P_3 elevation by GnRH in control and GnRH pre-treated αT3–1 cells.** Cells were pre-treated for 60 min in Krebs/HEPES buffer with 0 or 0.1 μM GnRH and then washed extensively and stimulated for 5 s with the indicated concentration of GnRH before processing for Ins(1,4,5)P_3 measurement. The data are pooled from five separate experiments (each having triplicate observations for each time point) normalized as a percentage of internal control responses (the responses of control cells to 1 μM GnRH), which were 344 ± 37 pmol/mg protein.

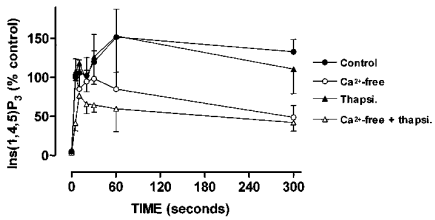

FIG. 4. **Ca^{2+} dependence of Ins(1,4,5)P_3 elevation by GnRH in αT3–1 cells.** Cells were washed and preincubated for 10 min in normal Ca^{2+}-containing medium (*filled symbols*) or in Ca^{2+}-free medium (*open symbols*) in the absence (*circles*) or the presence (*triangles*) of 2 μM thapsigargin and then stimulated as indicated in the same medium with 0.1 μM GnRH, before processing for Ins(1,4,5)P_3 measurement. The data are from three separate experiments (each having triplicate observations for each time point) normalized as a percentage of internal control responses (values observed at 5 s), which were 325 ± 79 pmol/mg protein.

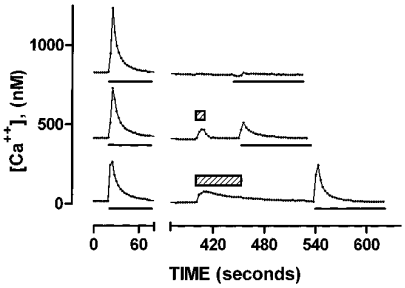

FIG. 5. **Depletion and refilling of the GnRH-mobilizable intracellular Ca^{2+} pool in αT3–1 cells.** Cells were loaded with fura 2, then washed, mounted on the microscope stage, and transferred to Ca^{2+}-free medium 1 min before the start of image capture. They were then stimulated twice with 0.1 μM GnRH in Ca^{2+}-free medium as indicated by the *bars*. The break in the horizontal axis represents a period during which the cells were washed extensively in Ca^{2+}-free medium to remove GnRH. Between the two periods of GnRH stimulation, cells were exposed to normal Ca^{2+}-containing medium (*hatched bars*) for approximately 60 s (*lower trace*) or 10 s (*middle trace*) or were maintained in Ca^{2+}-free medium throughout (control, *upper trace*). Each curve shows the mean ± S.E. of three to six separate experiments. The *middle* and *upper traces* are offset by 400 and 800 nM, respectively, and the S.E. bars are omitted for clarity.

effect of GnRH on $[Ca^{2+}]_i$ In normal Ca^{2+}-containing medium GnRH caused the expected rapid and sustained increase in Ins(1,4,5)P_3, from 16 ± 4 at 0 s to 325 ± 79 pmol/mg protein at 5 s (Fig. 4). This response was not measurably altered by 2 μM thapsigargin but was decreased in Ca^{2+}-free medium and in Ca^{2+}-free medium with thapsigargin (to 38 ± 11 and 33 ± 9% of control respectively, at 300 s). The effect of Ca^{2+}-free medium was most pronounced at the later time points (1 and 5 min), and only the combined treatment with thapsigargin in Ca^{2+}-free medium reduced the initial response (to 42 ± 10% of control at 5 s).

We next assessed whether depletion of the GnRH-mobilizable Ca^{2+} pool underlies desensitization of the Ca^{2+} response. We have shown that treatment with GnRH in Ca^{2+}-free or Ca^{2+}-containing medium can deplete the hormone-mobilizable Ca^{2+} pool (33), but the imaging experiments described herein were not performed until at least 10 min after the GnRH pretreatment (the time required to wash the cells and prepare for imaging), and we suspected that pool refilling may well have occurred. To assess the time required for pool refilling, αT3–1 cells were stimulated first for 1 min with 0.1 μM GnRH in Ca^{2+}-free medium (in order to deplete the GnRH-mobilizable pool). They were then washed extensively (>5 min) in Ca^{2+}-free medium and then either stimulated with 0.1 μM GnRH in Ca^{2+}-free medium or exposed briefly (5–60 s) to Ca^{2+}-containing medium before being returned to Ca^{2+}-free medium and stimulated again with 0.1 μM GnRH. In cells incubated throughout in Ca^{2+}-free medium, the second exposure failed to increase $[Ca^{2+}]_i$ (Fig. 5, *upper trace*). However, brief exposure (5–60 s) to Ca^{2+}-containing medium both increased the $[Ca^{2+}]_i$ and enabled GnRH to cause a spike of $[Ca^{2+}]_i$ elevation during the subsequent (Fig. 5, *middle* and *lower traces*), indicating that pool refilling had occurred. In cells exposed to Ca^{2+}-containing medium for 1 min, the response to the second GnRH challenge was comparable with that seen in the first, and the half-time for pool refilling was estimated to be 5–20 s. In parallel studies the influence of such manipulations on GnRH-stimulated Ins(1,4,5)P_3 accumulation was also addressed. As shown (Fig. 6), GnRH caused a rapid increase in Ins(1,4,5)P_3 levels in control cells (pool intact) stimulated in Ca^{2+}-free medium. This response was attenuated in cells that had been pre-treated for 1 min with 0.1 μM GnRH in Ca^{2+}-free medium in order to deplete the intracellular Ca^{2+} pool (pool emptied) and was returned to control values by 1 min of exposure to normal Ca^{2+}-containing medium in order to allow pool refilling after the GnRH pretreatment (pool emptied and refilled).

Although the experiments described above indicated that rapid refilling of the GnRH-mobilizable Ca^{2+} pool had occurred, recovery from desensitization was much slower (Fig. 7). Indeed, αT3–1 cells pre-treated for 60 min with 0.1 μM GnRH did not recover from such desensitization until at least 4 h after pretreatment. In order to test the possible involvement of pool depletion more directly, we compared the effects of GnRH and the Ca^{2+} ionophore ionomycin on $[Ca^{2+}]_i$ in control cells (Fig. 8, *left panel*) and in cells pre-treated for 60 min with 0.1 μM GnRH (Fig. 8, *right panel*). In control cells stimulated in Ca^{2+}-free

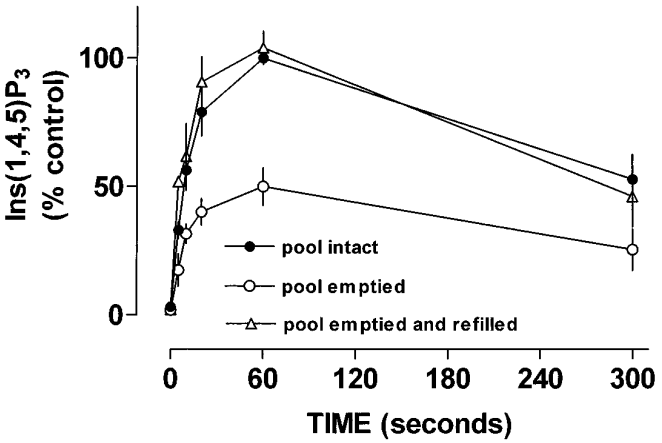

FIG. 6. **Influence of depletion and refilling of the GnRH-mobilizable intracellular Ca^{2+} pool $Ins(1,4,5)P_3$ responses to GnRH in αT3–1 cells.** Cells were pre-treated for 1 min in Ca^{2+}-free medium with 0 (pool intact) or 0.1 μM (pool emptied) GnRH and then washed and maintained in Ca^{2+}-free medium for a period of 5 min during which one group of pool-depleted cells were exposed to Ca^{2+}-containing medium for 1 min before being returned to Ca^{2+}-free medium (pool emptied and refilled). The cells were then stimulated as indicated in Ca^{2+}-free medium with 0.1 μM GnRH before processing for $Ins(1,4,5)P_3$ measurement. The data are from three separate experiments normalized as a percentage of internal control responses (the maximum responses seen in pool intact cells) of 48.2 ± 8.9 pmol/well.

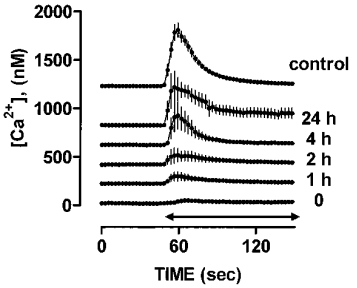

FIG. 7. **Recovery from desensitization.** Cells were pre-treated for 1 h with 0 (control) or 0.1 μM GnRH and then washed extensively and maintained without GnRH for the indicated period before being stimulated as indicated (*arrow*) during imaging with 0.1 μM GnRH. Fura 2 loading was always in the 30 min immediately before imaging. The data shown are the means ± S.E. of three separate experiments except for the control curve, which shows the mean ± S.E. for 15 experiments (three at each time point).

medium, both GnRH (0.1 μM, *upper trace*, *left hand panel*) and ionomycin (2 μM, *lower trace*, *left hand panel*) transiently increased $[Ca^{2+}]_i$. Stimulation with 0.1 μM GnRH almost abolished the subsequent response to ionomycin, whereas 1 nM GnRH caused a submaximal increase in $[Ca^{2+}]_i$ and reduced but did not prevent the subsequent response to ionomycin (*middle trace*, *left panel*). These data indicate that the two stimuli mobilize Ca^{2+} from a shared intracellular pool and that the ionomycin response can therefore be used as a measure of the filling state of the GnRH-mobilizable intracellular Ca^{2+} pool (see also Ref. 32). In cells pre-treated for 60 min with 0.1 μM GnRH, ionomycin alone caused an increase in $[Ca^{2+}]_i$, comparable with that seen in control cells (compare lower traces in *left* and *right panels*), indicating the retention of this intracellular pool. Stimulation with 1 nM GnRH in Ca^{2+}-free medium did not increase $[Ca^{2+}]_i$ or reduce the subsequent response to ionomycin. Stimulation with 0.1 μM GnRH caused only a modest increase in $[Ca^{2+}]_i$ and reduction of the subsequent ionomycin response. The data shown in Fig. 8 are from a series of experiments from which concentration response curves for the increase in $[Ca^{2+}]_i$ caused by GnRH and the reduction in ionomycin response due to GnRH were constructed (Fig. 9). As shown, GnRH caused a dose-dependent increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium with an EC_{50} of 6 nM and also attenuated the ionomycin response to approximately 15% of control, with an IC_{50} of 33 pM. In GnRH pre-treated cells, the EC_{50} for the increase in Ca^{2+} could not be calculated but is apparently greater than 1 μM. In these cells GnRH decreased the ionomycin response to approximately 50% of control with an IC_{50} of 4.2 nM.

Finally, we compared the effects of GnRH pretreatment on Ca^{2+} response with GnRH and to PACAP38, another peptide that acts via GPCRs to activate PLC in αT3–1 cells (36, 37). As expected, pretreatment for 60 min with 0.1 μM GnRH abolished the spike response to GnRH seen in Ca^{2+}-free medium (Fig. 10, *upper panel*). PACAP38 (0.1 μM) caused a comparable spike-type increase in Ca^{2+} to GnRH, and this effect was also greatly diminished by GnRH pretreatment (Fig. 10, *lower panel*).

DISCUSSION

Gonadotropin-releasing hormone is released from the hypothalamus in a pulsatile manner and elicits pulsatile gonadotropin secretion. This stimulation pattern maintains responsiveness to GnRH, whereas continuous stimulation causes desensitization and attenuates gonadotropin secretion (16, 38). Recent studies (24) have revealed, however, that GnRH-stimulated $[^3H]IP_{total}$ accumulation (an indicator of PLC activity measured in cells labeled with [^{3}H]inositol and stimulated in the presence of LiCl) remains linear for 10 min after stimulation. These data argue against rapid agonist-induced uncoupling of the GnRH receptor from its effector system (G-proteins activating PLC), possibly because it lacks the C-terminal tail, which has been implicated in homologous desensitization of other GPCRs and rhodopsin (39, 40). Desensitization of GnRH-induced Ca^{2+} responses can, however, be demonstrated within an intermediate time frame. Pretreatment for 60 min with GnRH causes a dose-dependent reduction in the ability of GnRH to increase Ca^{2+} in αT3–1 cells (25) and comparable effects are seen in primary cultures of rat pituitary cells[2] and with GnRH receptors transfected into HEK-293 cells (34). This pretreatment caused desensitization of both spike and plateau phases of the Ca^{2+} response to GnRH in αT3–1 cells and also decreased the effect of KCl on $[Ca^{2+}]_i$ (25), supporting the suggestion that desensitization occurs at the level of voltage operated Ca^{2+} channels in gonadotropes and αT3–1 cells (25, 41) but leaving the mechanism underlying desensitization of the spike Ca^{2+} response unresolved.

In addition to attenuation of the effect of GnRH on $[Ca^{2+}]_i$, pretreatment with GnRH has been shown to decrease GnRH-stimulated $[^3H]IP_{total}$ accumulation by approximately 25% (25), but in these experiments total $[^3H]IP_{total}$ accumulation was measured in cells labeled with [^{3}H]inositol. This has the possible disadvantages that PLC activity directed against phospholipids other than PIP_2 may be quantified (21) and that the GnRH pretreatment may have altered the specific activity of the [^{3}H]inositol-labeled phospholipid substrate pool. Accordingly, we have now examined the relationship between GnRH effects on $Ins(1,4,5)P_3$ mass levels and on $[Ca^{2+}]_i$ in control and GnRH pre-treated cells. We show here that pretreatment for 60 min with 0.1 μM GnRH causes desensitization of both spike and plateau phases of the Ca^{2+} response to GnRH (Fig. 1, see also Ref. 25) and that desensitization of the spike response is relatively rapid in onset (half-time 10–20 min) but is only slowly reversed (half-time 4–6 h). Desensitization of the Ca^{2+} response was associated with a reduction in the effect of GnRH on

[2] C. A. McArdle and W. Forrest-Owen, unpublished observations.

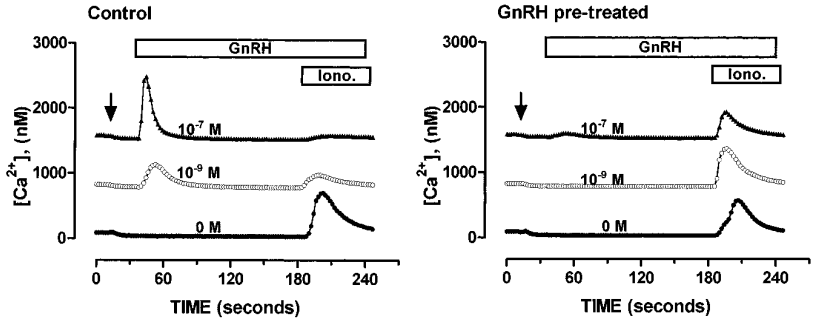

FIG. 8. **Effect of GnRH pretreatment on elevation of $[Ca^{2+}]_i$ by GnRH and ionomycin in Ca^{2+}-free medium.** Cells were pre-treated for 60 min with medium containing 0 (control, *left panel*) or 0.1 μM (GnRH pre-treated, *right panel*) GnRH and then washed, mounted on the microscope stage, transferred to Ca^{2+}-free medium (*vertical arrow*), and maintained in Ca^{2+}-free medium for the duration of the experiment. The cells were stimulated with 0, 1 nM, or 0.1 μM GnRH (*long bar*) and after approximately 2 min with 2 μM ionomycin in medium with the same concentration of GnRH (*short bar*). Each curve shows the mean of three to six experiments. To improve clarity, S.E. bars are omitted, and the data for cells stimulated with 1 nM and 0.1 μM GnRH are offset by 750 and 1500 nM, respectively.

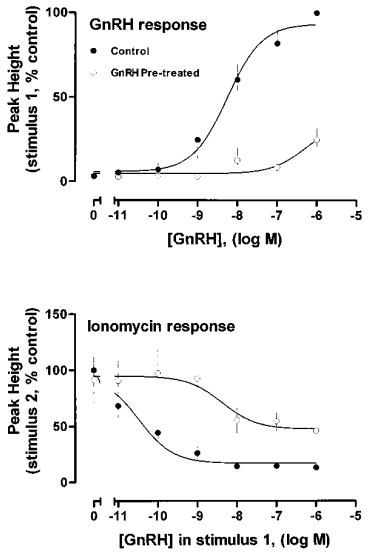

FIG. 9. **Dose dependences of the effects of GnRH on elevation of $[Ca^{2+}]_i$ and depletion of the ionomycin mobilizable Ca^{2+} pool in control and GnRH pre-treated cells in Ca^{2+}-free medium.** Cells were pre-treated and stimulated exactly as described in the legend to Fig. 8 (data from the same series of experiments were used). Peak heights were calculated without background subtraction for the responses to GnRH (stimulus 1, *upper panel*) and to ionomycin (stimulus 2, *lower panel*) and expressed as a percentage of internal control responses of cells receiving no pretreatment with GnRH. The $\log_{10}$ molar EC_{50} value for the GnRH response (*upper panel*) in control cells was -8.255 ± 0.154, and IC_{50} values for inhibition of the ionomycin response by GnRH (*lower panel*) were -10.48 ± 0.164 and -8.373 ± 0.649 (control and GnRH pre-treated cells, respectively).

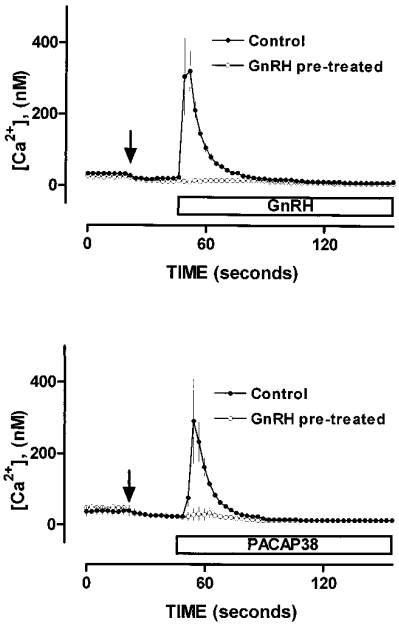

FIG. 10. **Homologous and heterologous desensitization of GnRH and PACAP38 effects on $[Ca^{2+}]_i$ in αT3–1 cells.** Cells were pre-treated for 60 min with medium containing 0 or 0.1 μM GnRH (with fura 2/AM present during the last 30 min of the pretreatment) and then washed extensively and mounted on the microscope stage. The cells were then either stimulated as indicated with 0.1 μM GnRH or PACAP38 in normal Ca^{2+}-containing medium (*left panels*) or transferred first to Ca^{2+}-free medium (*vertical arrows*) and then stimulated with GnRH or PACAP38 in Ca^{2+}-free medium (*right panels*) as indicated. Each curve shows the mean ± S.E. of data from three to six separate experiments.

$Ins(1,4,5)P_3$ levels (to between 50 and 80% of control) without any measurable change in EC_{50}.

Although pretreatment with GnRH clearly reduced the effect of GnRH on $Ins(1,4,5)P_3$ levels, this effect alone cannot explain the desensitization of the Ca^{2+} response because the $Ins(1,4,5)P_3$ responses to 0.1 μM GnRH in control cells and to 1 μM GnRH in GnRH pre-treated cells were indistinguishable, yet 1 μM GnRH caused very little increase in $[Ca^{2+}]_i$ in GnRH pre-treated cells, whereas the $[Ca^{2+}]_i$ response to 0.1 μM GnRH is maximal in control cells (Figs. 3 and 9). An alternative possibility is that the attenuated $[Ca^{2+}]_i$ increase in the desensitized cells is the cause of the decrease in $Ins(1,4,5)P_3$ because in other systems Ca^{2+} mobilization supports agonist stimulated $Ins(1,4,5)P_3$ generation, and depletion of intracellular Ca^{2+} pools can underlie desensitization of $Ins(1,4,5)P_3$ responses (42, 43). The demonstration that the GnRH effect on $Ins(1,4,5)P_3$ levels is attenuated in Ca^{2+}-free medium supports this interpretation. Interestingly, the immediate (5 s) effect of GnRH on $Ins(1,4,5)P_3$ was only reduced by thapsigargin in Ca^{2+}-free medium (Fig. 4). Thapsigargin specifically blocks the ATPase, which sequesters Ca^{2+} into rapidly releasable pools in many cells (32), and as a consequence of continuous leak of Ca^{2+} from these pools causes their depletion. Thapsigargin, thereby prevents mobilization of intracellular Ca^{2+} by many ligands acting via PLC-linked receptors, including GnRH in αT3–1 cells (33–35). Inhibition of the immediate effect of GnRH on $Ins(1,4,5)P_3$ levels by thapsigargin in Ca^{2+}-free medium implies that the maximal $Ins(1,4,5)P_3$ response requires Ca^{2+} elevation and the lack of effect of thapsigargin in Ca^{2+}-containing medium or of Ca^{2+} -free medium alone suggests that the Ca^{2+} requirement can be met either by mobilization or entry. Similarly, depletion of the hormone mobilizable Ca^{2+} pool by brief pretreatment with GnRH reduced the immediate effect of GnRH on $Ins(1,4,5)P_3$ in Ca^{2+}-free medium, and this inhibition

was reversed by brief exposure to normal medium in order to replenish the Ca^{2+} pool. Together these observations demonstrate that Ca^{2+} mobilized from intracellular stores during the spike phase of the response to GnRH exerts a positive feedback effect on Ins(1,4,5)P_3 production, probably reflecting the Ca^{2+} dependence of PLC (31). Accordingly, the data suggest that desensitization of the Ca^{2+} response contributes to the observed reduction in Ins(1,4,5)P_3 rather than *vice versa*. Confirmation of the fact that this uncoupling does not occur at the level of the GnRH receptor is provided by the demonstration that GnRH pretreatment causes comparable inhibition of the effects of GnRH and of PACAP on $[Ca^{2+}]_i$ in these cells (Fig. 10).

The observations above raise the question of why Ins(1,4,5)P_3 failed to increase $[Ca^{2+}]_i$ in GnRH pre-treated cells, and we have addressed the possibility that this simply reflects depletion of the hormone mobilizable Ca^{2+} pool as seen in other systems (44). In Ca^{2+}-free medium GnRH caused only a transient increase in $[Ca^{2+}]_i$ presumably because the hormone-mobilizable Ca^{2+} pool became rapidly depleted. This is verified by the fact that after extensive washing, a second stimulation with GnRH does not increase $[Ca^{2+}]_i$ unless the cells are first exposed to normal Ca^{2+}-containing medium to enable pool refilling (Fig. 5, see also Ref. 32). Using this protocol, and assuming the maximal increase in $[Ca^{2+}]_i$ caused by GnRH to be directly proportional to the filling state of the GnRH mobilizable Ca^{2+} pool, we estimate that pool refilling occurs with a half-time of 5–20 s (in Ca^{2+}-containing medium without GnRH), which is in stark contrast to the slow recovery from desensitization of the spike effect of GnRH on $[Ca^{2+}]_i$ (half-time 4–6 h). In all of the Ca^{2+} imaging experiments described here, cells were removed from the pretreatment solution and then washed extensively in Ca^{2+}-containing medium before transfer to the microscope stage for imaging. Imaging was therefore not started until at least 10 min after termination of the pretreatment, and it is most unlikely that pool depletion had been maintained for this time. Comparison of the rates of onset of desensitization and of pool depletion also argue against a causal relationship, because desensitization of the spike phase of the Ca^{2+} response to GnRH occurred with a half-time of 10–20 min (Fig. 1), whereas depletion of the GnRH-mobilizable Ca^{2+} pool was complete within 1 min (Fig. 5).

To more directly test the involvement of Ca^{2+} pool depletion, we exploited the fact that GnRH and ionomycin mobilize Ca^{2+} from shared intracellular Ca^{2+} pools so that responses to ionomycin in Ca^{2+}-free medium can be used to measure the filling state of the hormone-mobilizable pool (33). In control cells GnRH and ionomycin caused comparable transient increase of $[Ca^{2+}]_i$ in Ca^{2+}-free medium, and 0.1 μM GnRH dramatically diminished the $[Ca^{2+}]_i$ increase on subsequent treatment with ionomycin. In cells pre-treated for 60 min with 0.1 μM GnRH, ionomycin caused comparable increases in $[Ca^{2+}]_i$ to that seen in control cells indicating that the intracellular Ca^{2+} pool was intact at the time of ionomycin addition. In contrast, in GnRH-desensitized cells, 0.1 μM GnRH caused only a minor increase in Ca^{2+} and very little reduction of the subsequent response to ionomycin (Fig. 8), suggesting that in these cells, GnRH is unable to increase Ca^{2+} because it fails to mobilize this extant intracellular Ca^{2+} pool. Comparison of concentration response curves from such experiments support this interpretation (Fig. 9). In control cells the brief exposure to GnRH in Ca^{2+}-free medium reduced the subsequent effect of ionomycin to <15%, indicating that the vast majority of the ionomycin mobilizable pool is accessible to GnRH and did so with an IC_{50} of 33 pM. In contrast, in the GnRH-desensitized cells the brief GnRH treatment only reduced the ionomycin response to 50%, with an IC_{50} of 4200 pM. Comparison of EC_{50} values for GnRH-stimulated Ins(1,4,5)P_3 accumulation (Fig. 3) and inhibition of the ionomycin effect on $[Ca^{2+}]_i$ provides an indication of the efficiency with which Ins(1,4,5)P_3 mobilizes Ca^{2+}. In control cells GnRH is at least 2000 times more potent at mobilization of Ca^{2+} than at elevation of Ins(1,4,5)P_3 (EC_{50} values of 33 pM and 83 nM, respectively), whereas for the GnRH pre-treated cells the difference is less than 40-fold (EC_{50} values of 4.2 and 134 nM, respectively). Thus desensitization of the $[Ca^{2+}]_i$ response to GnRH reflects both a reduction in the proportion of the intracellular ionomycin mobilizable Ca^{2+} pool, which can be released by GnRH, and a reduction in the efficiency of such mobilization.

In summary, we have found no evidence for uncoupling of GnRH receptors from their immediate effector system (PLC activated by $G_{q/11}$) even after a relatively protracted period of stimulation with GnRH. Instead, GnRH pretreatment apparently impairs the efficiency with which Ins(1,4,5)P_3 mobilizes Ca^{2+} from intracellular stores in αT3–1 cells. Although the reason for this reduction is unknown, we are currently investigating the possible involvement of Ins(1,4,5)P_3 receptor regulation and cellular compartmentalization of the effector system. We suggest that this effect, together with desensitization of voltage-operated Ca^{2+} channels, may underlie desensitization of GnRH-stimulated gonadotropin secretion and that αT3–1 cells provide a valuable model system for investigation of the post-receptor regulation of cellular responsiveness to PLC activating ligands.

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REFERENCES

1. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem.* **60,** 653–688
2. Lefkowitz, R. J., Hausdorff, W. P., and Caron, M. G. (1990) *Trends Pharmacol. Sci.* **11,** 190–194
3. Nussenzveig, D. R., Heinflink, M., and Gershengorn, M. C. (1993) *J. Biol. Chem.* **268,** 2389–2392
4. Rodriguez, M. C., Xie, Y.-B., Wang, H., Collison, K., and Segaloff, D. L. (1992) *Mol. Endocrinol.* **6,** 327–336
5. Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, C. A., Dong, K., Gillo, B., and Sealfon, S. C. (1992) *Mol. Endocrinol.* **6,** 1163–1169
6. Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C., and Neill, J. D. (1992) *Biochem. Biophys. Res. Commun.* **189,** 289–295
7. Stojilkovic, S. S., Reinhart, J., and Catt, K. J. (1994) *Endocr. Rev.* **15,** 462–499
8. Conn, P. M., (1995) in *Reproductive Endocrinology, Surgery and Technology* (Adashi, E. Y., Rock, J. A., and Rosenwaks, Z., eds) Vol 1, pp. 163–179, Lippincott-Raven Publishers, Philadelphia, PA
9. Naor, Z. (1990) *Endocr. Rev.* **11,** 326–353
10. Davidson, M. J., van der Merwe, P. A., Wakefield, I. K., and Millar, R. P. (1991) *Mol. Cell. Endocrinol.* **76,** 33–38
11. Berridge, M. J. (1993) *Nature* **361,** 315–325
12. Hille, B., Tse, A., Tse, F. W., and Bosma, M. M. (1995) *Recent Prog. Horm. Res.* **50,** 75–95
13. McArdle, C. A., and Poch, A. (1992) *Endocrinology* **130,** 3567–3574
14. Conn, P. M., and Crowley, W. F., Jr. (1991) *N. Engl. J. Med.* **324,** 93–103
15. Barbieri, R. L. (1992) *Trends Endocrinol. Metab.* **3,** 30–34
16. Dekoning, J., van Dieten, J. A. M. J., and van Rees, G. P. (1978) *J. Endocrinol.* **79,** 311–318
17. Jinnah, H. A., and Conn, P. M. (1985) *Am. J. Physiol.* **249,** E619–E625
18. Jinnah, H. A., and Conn, P. M. (1986) *Endocrinology* **118,** 2599–2604
19. McArdle, C. A., Gorospe, W. C., Huckle, W. R., and Conn, P. M. (1987) *Mol. Endocrinol.* **1,** 420–429
20. McArdle, C. A., Huckle, W. R., Johnson, L., and Conn, P. M. (1988) *Endocrinology* **122,** 1905–1914
21. Wojcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1993. *Trends Pharmacol. Sci.* **14,** 279–285
22. Tobin, A. B., Lambert, D. G., and Nahorski, S. R. (1992) *Mol. Pharmacol.* **42,** 1042–1048
23. Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 9161–9164
24. Davidson, J. S., Wakefield, I. K., and Millar, R. P. (1994) *Biochem. J.* **300,** 299–302
25. McArdle, C. A., Forrest-Owen, W., Willars, G., Davidson, J., Poch, A., and Kratzmeier, M. (1995) *Endocrinology* **136,** 4864–4871
26. Windle, J. J., Weiner, R. I., and Mellon, P. L. (1990) *Mol. Endocrinol.* **4,** 597–603

27. Akerman, S. N., Zorec, R., Cheek, T. R., Moreton, R. B., Berridge, M. J., and Mason, W. T. (1991) *Endocrinology* **129,** 475–488
28. McArdle, C. A., Bunting, R., and Mason, W. T. (1992) *Mol. Cell. Neurosci.* **3,** 124–132
29. Challis, R. A. J., Chilvers, E. R., Willcocks, A. L., and Nahorski, S. R. (1990) *Biochem. J.* **265,** 421–427
30. Willars, G., and Nahorski, S. R. (1995) *Brit. J. Pharmacol.* **114,** 1133–1142
31. Rhee, S. G., and Choi, K. D. (1992) *J. Biol. Chem.* **267,** 12393–12396
32. Thastrup, O., Dawson, A. P., Scarff, O., Foder, B., Culen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B., and Hanley, M. R. (1989) *Agents Actions* **27,** 17–23
33. McArdle, C. A., Forrest-Owen, W., Davidson, J. S., Fowkes, R., Bunting, R., Mason, W. T., Poch. A., and Kratzmeier, M. (1996) *J. Endocrinol.* **149,** 155–169
34. Anderson, L., Milligan, G., and Eidne, K. A. (1993) *J. Endocrinol.* **136,** 51–58
35. Merelli, F., Stojilkovic, S. S., Iida, T., Krsmanovic, L. Z., Zheng, L., Mellon, P. L., and Catt, K. J. (1992) *Endocrinology* **131,** 925–932
36. Schomerus, E., Poch, A., Bunting, R., Mason, W. T., and McArdle, C. A. (1994) *Endocrinology* **134,** 315–323
37. Rawlings, S. R., and Hezareh, M. (1996) *Endocr. Rev.* **17,** 4–29
38. Knobil, E. (1980) *Recent Prog. Horm. Res.* **36,** 53–88
39. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) *FASEB J.* **4,** 2881–2889
40. Lorenz, W., Inglese, J., Palczewski, K., Onorato, J. J., Caron, M. G., and Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U. S.A.* **88,** 8715–8719
41. Stojilkovic, S. S., Rojas, E., Stutzin, A., Izumi, S., and Catt, K. J. (1989) *J. Biol. Chem.* **264,** 10939–10942
42. Willars, G. B., and Nahorski, S. R. (1995) *Mol. Pharmacol.* **47,** 509–516
43. Wojcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1994) *J. Neurochem.* **63,** 177–185
44. McDonough, P. M., Eubanks, J. H., and Brown, J. H. (1988) *Biochem. J.* **249,** 135–141

Desensitization of Gonadotropin-releasing Hormone Action in αT3-1 Cells Due to Uncoupling of Inositol 1,4,5-Trisphosphate Generation and Ca^{2+} Mobilization

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