Ubiquitination and Proteasomal Degradation of Endogenous and Exogenous Inositol 1,4,5-Trisphosphate Receptors in αT3-1 Anterior Pituitary Cells*

Richard J. H. Wojcikiewicz‡, Qun Xu, Jack M. Webster, Kamil Alzayady, and Chen Gao
From the Department of Pharmacology, State University of New York Upstate Medical University, Syracuse, New York 13210-2339

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In αT3-1 mouse anterior pituitary gonadotropes, chronic activation of gonadotropin-releasing hormone (GnRH) receptors causes inositol 1,4,5-trisphosphate (InsP₃) receptor down-regulation (Willars, G. B., Royall, J. E., Nahorski, S. R., El-Gehani, F., Everest, H. and McArdle, C. A. (2001) J. Biol. Chem. 276, 3123–3129). In the current study, we sought to define the mechanism behind this adaptive response. We show that GnRH induces a rapid and dramatic increase in InsP₃ receptor polyubiquitination and that proteasome inhibitors block InsP₃ receptor down-regulation and cause the accumulation of polyubiquitinated receptors. Thus, the ubiquitin/proteasome pathway is active in αT3-1 cells, and GnRH regulates the levels of InsP₃ receptors via this mechanism. Given these findings and further characterization of this system, we also examined the possibility that αT3-1 cells could be used to examine the ubiquitination of exogenous InsP₃ receptors introduced by cDNA transfection. This was found to be the case, since exogenous wild-type InsP₃ receptors, but not binding-defective mutant receptors, were polyubiquitinated in a GnRH-dependent manner, and agents that inhibited the polyubiquitination of endogenous receptors also inhibited the polyubiquitination of exogenous receptors. Further, we used this system to determine whether phosphorylation was involved in triggering InsP₃ receptor polyubiquitination. This was not the case, since mutation of serine residues 1588 and 1755 (the predominant phosphorylation sites in the type I receptor) did not inhibit polyubiquitination. In total, these data show that the ubiquitin/proteasome pathway is active in anterior pituitary cells, that this pathway targets both endogenous and exogenous InsP₃ receptors in GnRH-stimulated αT3-1 cells, and that, in contrast to the situation for many other substrates, phosphorylation does not trigger InsP₃ receptor polyubiquitination.

Hormone-induced secretion from anterior pituitary cells is modulated at many different levels, and among these is regulation of the activity and abundance of receptors involved in signal transduction (1, 2). Indeed, recent studies on the αT3-1 mouse gonadotrope cell line have indicated that the suppression of secretion from gonadotropes in patients treated chronically with gonadotropin-releasing hormone (GnRH) receptor agonists (2, 3) may result from a reduction in the expression of inositol 1,4,5-trisphosphate (InsP₃) receptors (2, 4, 5). InsP₃ receptors are a family of three proteins (termed type I, II, and III receptors) that form tetrameric ion channels in endoplasmic reticulum (ER) membranes, and upon binding of InsP₃, the channels open, and Ca²⁺ stored within the ER flows into the cytoplasm (6–8). Thus, InsP₃ receptors play a pivotal role in linking G-protein-coupled receptor (GPCR)-mediated InsP₃ formation to increases in cytoplasmic free Ca²⁺ concentration (9). A reduction in their expression (i.e. their down-regulation) would, therefore, be expected to suppress Ca²⁺ mobilization (4, 5, 10) and secretion (2).

InsP₃ receptor down-regulation in response to activation of certain GPCRs has also been seen in other cell types (11–15). This adaptive response is mediated by an increase in the rate of receptor degradation (11, 13); is specific, since other ER and signaling proteins are not simultaneously affected (11, 14); and appears to exist to allow chronically stimulated cells to reduce the sensitivity of their Ca²⁺ stores to InsP₃ (4, 5, 10, 14–16). The event that initiates receptor proteolysis appears to be InsP₃ binding, since only those GPCRs (e.g. GnRH, cholecystokinin, and muscarinic receptors) that persistently elevate InsP₃ levels cause InsP₃ receptor down-regulation (5, 12, 13), a binding-deficient mutant InsP₃ receptor resistant to down-regulation (17), and down-regulation in oocytes is elicited by microinjection of an InsP₃ analogue (18). Whether additional events (e.g. receptor phosphorylation) are required to trigger down-regulation remains to be resolved. Furthermore, it is not yet clear how InsP₃ receptors are degraded, and indeed, receptor proteolysis by calpain (12), caspase (19), and the ubiquitin/proteasome pathway (14, 15, 20, 21) have all been described.

Thus, we examined the mechanism of InsP₃ receptor down-regulation in GnRH-stimulated αT3-1 cells and showed that it occurs via the ubiquitin/proteasome pathway. In characterizing this adaptive response, we identified major differences in the properties of commonly used proteasome inhibitors and found that deubiquitination of InsP₃ receptors occurs rapidly and is...
likely to limit the accumulation of ubiquitinated receptors and that InsP₃ receptor ubiquitination is Zn²⁺-dependent. Importantly, we also used αT3-1 cells to develop conditions for the analysis of exogenous InsP₃ receptor ubiquitination, and by expressing mutant receptors, we show that InsP₃ binding is important in triggering this event but that phosphorylation is not.

**EXPERIMENTAL PROCEDURES**

**Materials**—αT3-1 cells were kindly provided by Dr. F. Mellon (University of California, San Diego, CA) and were cultured as monolayers in Falcon Integrid tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum; cells were subcultured every 5–7 days using 0.25% trypsin, 1 mM EDTA. Rabbit polyclonal antisera CT1h and CT1w were raised against the C terminus of the rat type I receptor and were affinity-purified and shown to specifically recognize endogenous type I InsP₃ receptors (13). CT1h immunoprecipitated both endogenous receptors and exogenous epitope-tagged type I receptors and was used in all immunoprecipitations. Surprisingly, however, this antiserum did not recognize epitope-tagged receptors in immunoblots. Thus, CT1w was used to probe for type I InsP₃ receptor expression in transfected cells, since this antiserum recognized both endogenous receptors and exogenous epitope-tagged receptors. Mouse monoclonal anti-ubiquitin (P4D1), which recognizes both mono- and polyubiquitinated proteins, was purchased from Affiniti Research Products Limited, anti-hemagglutinin (HA) epitope (HA11) was from Babco, and anti-c-Myc (9E10) was from Roche Molecular Biochemicals. Peroxidase-conjugated antibodies, molecular mass markers, SDS, Triton X-100, protease inhibitors, Nα,Nα,Nα-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), and receptor agonists were obtained from Sigma; Protein A-Sepharose CL-4B (Protein A) was from Amersham Biosciences; diithiothreitol was from Bio-Rad; lactacystin and MG-132 were from Biomol; oxomicin was a kind gift from Dr. C. Crews (Yale University, New Haven, CT).

**Electrophoresis and Immunoblotting**—Samples were subjected to 5% PAGE and were immunoblotted as described (20). Immunoreactivity was detected with chemiluminescence using reagents from Pierce and was digitally imaged and quantitated with a Genegene (Syngene), working within the nonsaturating range.

**Measurement of InsP₃ Receptor Down-regulation in αT3-1 Cells**—Cell suspensions were prepared by detaching adherent cells with HBSE (155 mM NaCl, 10 mM Hepes, 0.7 mM EDTA, pH 7.4) and vigorous pipetting in culture medium. Cells were then pipetted into wells of Falcon six-well plates (2 ml/well), were incubated with stimulants or inhibitors, were collected by centrifugation (750 × g for 3 min at 4 °C), and were solubilized by incubation for 30 min at 4 °C with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 1 mM EDTA, 0.2 mM Nα,Nα,Nα-tetrakis(2-pyridylmethyl) ethylenediamine, 10 μM leupeptin, 10 μM pepstatin, 0.2 mM soybean trypsin inhibitor, 1 mM diithiothreitol, pH 8.0). Following centrifugation (16,000 × g for 10 min at 4 °C), supernatants containing solubilized receptors were mixed with 2× gel loading buffer and were electrophoresed and immunoblotted with CT1h as described (20).

**Measurement of InsP₃ Receptor Ubiquitination by Immunoblotting**—Control or stimulated cells in suspension were collected by centrifugation (16,000 × g for 10 min at 4 °C) and were solubilized by incubation for 30 min at 4 °C with 1 ml of lysis buffer. Lysates were then centrifuged (16,000 × g for 10 min at 4 °C), supernatants were collected, and InsP₃ receptors were immunoprecipitated by incubation at 4 °C with CT1h for 1 h and then for a further 12–24 h with Protein A. Immune complexes were then isolated by centrifugation (500 × g for 2 min), were washed three times with ice-cold lysis buffer, and in most experiments were resuspended in 2× gel loading buffer and then immunoblotted with either CT1h or FK2. In additional experiments aimed at further characterizing the ubiquitinated species, washed immune complexes were resuspended from Protein A and denatured by incubation at 100 °C for 5 min in 100 μl of 50 mM Tris, 2% SDS, 2 mM diithiothreitol, pH 7.4, were centrifuged (16,000 × g for 1 min at 25 °C), were diluted to 4 ml with lysis buffer, were preincubated with Protein A to remove residual CT1h, and finally were immunoprecipitated with FK2 and Protein A for 12–24 h and resuspended in 2× gel loading buffer.

**Measurement of InsP₃ Receptor Ubiquitination by Radiolabeling**—Cell monolayers in 75-cm² Falcon flasks were incubated for 48 h with 100 μCi of [³⁵S]cysteine (NEG022T; PerkinElmer Life Sciences) in αT3-1 cell culture medium supplemented with sufficient nonradioactive cysteine (200 μM) to allow for normal cell growth. Cells were then preincubated for 1 h with ALLN (20 μg/ml), stimulated for 1 h with GnRH (2 μM), and harvested in lysis buffer, and type I InsP₃ receptors were immunoprecipitated with CT1h and electrophoresed as described. Gels were stained with Coomasie Blue, and regions corresponding to unmodified and polyubiquitinated InsP₃ receptors were excised, homogenized, and assessed for radioactivity in 4 ml of scintillation fluid. Because ubiquitin does not contain cysteine, it does not become radio-labeled; thus, the percentage of receptor polyubiquitinated can be calculated from the amount of radioactivity migrating in the region corresponding to polyubiquitinated receptors relative to total receptor radioactivity.

**Transfection of αT3-1 Cells**—Cells were harvested using 0.25% trypsin/1 mM EDTA, were seeded into six-well Falcon plates at a density of 2 × 10⁶/well, and were transfected 24 h later by adding 1 ml of fresh culture medium containing a complex of DNA and 9 μl of Superfect (Qiagen), prepared according to the manufacturer’s instructions. The DNAs used were as follows: pCW7, which encodes His6-c-Myc epitope-tagged yeast ubiquitin (Myc-ubiquitin) and was a kind gift from Dr. R. R. Kopito (Stanford University) (22); pcDNA3 (empty vector); pc-WIHA (17), which encodes wild-type mouse type I InsP₃ receptor tagged at the C terminus with an HA epitope (InsP₃,RHA⁺)°; and pcWIHA (17), which encodes a binding-defective, HA-tagged mutant mouse type I receptor (InsP₃,RHA ᵃᵇ) with serine → alanine mutations at positions 1588 and 1775. This mutant was created using the QuickChange kit (Stratagene). In brief, pcWHA was first mutated to introduce alanine at position 1755 using primer pair 5'-GGAGAAGAGAACCGTTACGACGGTTGG-3' and 5'-CCAAAGCTGGTAAGGCCCTTCCTTCCTTC-3'. This mutant was then further mutated to introduce alanine at position 1588 using primer pair 5'-CCGAAGACGCTGATCCTTCTGCAGCCACTGACAGACTC-3' and 5'-GTAGCTGCTGATCCTTCTGCGCAGCCAGATCGGGC-3'. The first and second primer pairs also introduced HaeII and NotI sites, respectively, to facilitate screening. The correct introduction of the desired mutations into the polymerase-generated region was confirmed by sequencing. 48 h after transfection, the cells were exposed to stimulants or inhibitors and were harvested and solubilized by incubation for 30 min at 4 °C in 1 ml of lysis buffer. After centrifugation (16,000 × g for 10 min at 4 °C), type I InsP₃ receptors were immunoprecipitated with CT1h (to purify endogenous and exogenous receptors) or HA11 (to purify exogenous HA-tagged receptors only), and immunoprecipitates were immunoblotted with HA11, NE10, CT1h, or CT1w.

**Measurement of InsP₃ Concentration**—InsP₃ concentration in suspensions of αT3-1 cells incubated at 37 °C was measured with a radio-receptor assay exactly as described (20).

**Miscellaneous**—Data shown are representative of at least two independent experiments. Combined data are mean ± S.E. (n ≥ 3) or mean range (n = 2).

**RESULTS**

InsP₃ Receptor Down-regulation in αT3-1 Cells—Initial measurements of type I InsP₃ receptor levels in lysates from GnRH-stimulated cells showed that receptor down-regulation in response to GnRH (0.1 μM) was half-maximal at 15 ± 2 min (Fig. 1A). This is considerably more rapid than that seen in other cell types (11–15), most likely because GnRH receptors are refractory to desensitization and thus elevate InsP₃ concentration profoundly and persistently (2, 4).

To confirm that persistent GnRH receptor activation and InsP₃ formation were needed for type I InsP₃ receptor down-regulation, we utilized the GnRH receptor antagonist antide, which blocks GnRH-induced InsP₃ formation when added simultaneously with or after GnRH (Fig. 3B). As expected, antide blocked down-regulation when added simultaneously with GnRH (Fig. 1B, lane 3). However, we also observed that the down-regulation seen after a 60-min exposure to GnRH (lane 2) was not mimicked by exposure to GnRH alone for 5 min, followed by a further 55-min incubation in antide-supplemented medium (lane 5). This shows that acute GnRH receptor activation is not sufficient to program the cells to subsequently down-regulate InsP₃ receptors and is consistent with the view (13, 20) that persistent elevation of InsP₃ concentration is a prerequisite for down-regulation.

**Proteasome Inhibitors Block Down-regulation and Cause the
Inositol 1,4,5-Trisphosphate Receptor Ubiquitination

Accumulation of Ubiquitinated Receptors—In order to determine whether or not GnRH-induced InsP$_3$ receptor down-regulation is via the ubiquitin/proteasome pathway, we exposed αT3-1 cells to GnRH in the absence or presence of a range of proteasome inhibitors and monitored type I InsP$_3$ receptor levels and associated ubiquitin immunoreactivity. Three of these inhibitors, ALLN, MG-132, and lactacytin, are widely employed, the first two being peptides that are reversibly acting transition state analogues and the latter being a structurally different pseudosubstrate that covalently modifies the active site (23). The remaining inhibitor used, epoxomicin, is a novel, highly potent, irreversible inhibitor (24). In the absence of inhibitor, incubation with GnRH for 1 h caused InsP$_3$ receptor down-regulation (Fig. 2A, lane 2, lower panel) but did not cause the accumulation of ubiquitinated species (Fig. 2A, lane 2, upper panel). In contrast, when the cells were preincubated with inhibitors for 2 h, GnRH-induced InsP$_3$ receptor down-regulation was blocked, and a parallel increase in the level of ubiquitin immunoreactivity associated with InsP$_3$ receptors was observed (Fig. 2A, lanes 3–10). Whereas they exhibited different potencies (see legend to Fig. 2A), the four inhibitors were equally efficacious in causing the accumulation of ubiquitinated species and completely blocked down-regulation at maximal concentration (Fig. 2, A and E, lanes 2, 4, 6, and 8). Control experiments (e.g. Fig. 2D, lane 3) showed that the inhibitors alone did not increase basal InsP$_3$ receptor levels or cause the accumulation of ubiquitinated species. Additional controls showed that the ubiquitinated species were indeed modified type I receptors, since when purified, they were clearly immunoreactive with type I receptor antisera (Fig. 2B, lower panel, lane 2). The inability of the same antiserum to detect ubiquitinated receptors in crude type I receptor immunoprecipitates (Fig. 2A, lower panel) is most likely explained by the low abundance of ubiquitinated receptors relative to unmodified receptors. To address this issue, the proportion of type I InsP$_3$ receptors ubiquitinated was defined in experiments in which cells were radiolabeled with $^{35}$S)cysteine, and in maximally stimulated cells it was found to be $9 \pm 1\%$ of total ($n = 3$). The ubiquitinated receptors migrated as a "smear" (275–380 kDa) (Fig. 2A, upper panel, and Fig. 2B) slightly less rapidly than unmodified type I receptor (260 kDa) (Fig. 2A, lower panel), indicative of the formation of a spectrum of polyubiquitinated species and typical of the migration of other polyubiquitinated proteins (25, 26). In total, the finding that the four structurally and mechanistically different proteasome inhibitors all have the same effect shows that the ubiquitin/proteasome pathway mediates InsP$_3$ receptor down-regulation in αT3-1 cells. This conclusion is supported by findings that specific inhibitors of other candidate proteolytic pathways (20 μM benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone, a caspase inhibitor, and 20 μM PD150606, a calpain inhibitor) did not block GnRH-induced InsP$_3$ receptor down-regulation.$^2$

Consistent with this conclusion and the rapidity of down-regulation (Fig. 1A), analysis of the time dependence of polyubiquitination (Fig. 2C) revealed that in the absence of ALLN, polyubiquitinated receptors accumulated very rapidly (peaking at 5 min) and were detectable only transiently, presumably because they are degraded rapidly by the proteasome; this also explains why polyubiquitinated receptors were not detected after incubation with GnRH alone for 1 h (Fig. 2A, lane 2). In contrast, when ALLN was present, polyubiquitinated receptor accumulation peaked at ~20 min and thereafter did not decline (Fig. 2C). Surprisingly, ALLN also suppressed the initial rate of receptor polyubiquitination (Fig. 2C). This was not due to a reduction in the potency of GnRH, which was half-maximally effective at ~5 nM in the absence or presence of ALLN,$^2$ and indicates that as well as inhibiting the degradation of polyubiquitinated species, proteasome inhibitors may also reduce the rate of polyubiquitination.

Given the mechanistic differences between the proteasome inhibitors, we also analyzed their kinetics. Fig. 2D shows that the effects of ALLN are very rapid in onset; ALLN was maximally effective with a preincubation time of 1 h or more (lanes 5–8) and was close to being maximally effective when added simultaneously with GnRH (lane 4). Fig. 2E shows that when used at maximally effective concentrations (5–10 times higher than the IC$_{50}$ values defined in the legend to Fig. 2A), MG-132 (lanes 4 and 5), like ALLN (lanes 2 and 3), acted rapidly, being similarly effective with 0- or 2-h preincubation. In contrast, epoxomicin (lanes 8 and 9) and particularly lactacytin (lanes 6 and 7) were slower acting, being much less effective when added simultaneously with GnRH as compared with 2-h preincubation.

InsP$_3$ Receptor Deubiquitination—Since proteasome inhibitors completely block GnRH-induced InsP$_3$ receptor down-regulation, it would be expected that a large proportion of cellular InsP$_3$ receptors would accumulate as polyubiquitinated species when the proteasome was inhibited. However, this was not the case, since only 9 $\pm 1\%$ of receptors were polyubiquitinated in the presence of ALLN plus GnRH, and maximal accumulation of polyubiquitinated receptors in the presence of ALLN was only approximately twice that seen in its absence (Fig. 2C). Thus, we examined whether deubiquitination might be counteracting the accumulation of polyubiquitinated receptors. Antide was used for these studies, since it blocks InsP$_3$ formation and InsP$_3$ receptor polyubiquitination when added simultaneously with GnRH (Fig. 3B, left panel, and Fig. 3A, lane 6) and rapidly (within 10 min) returns InsP$_3$ concentration to basal levels when added to GnRH-stimulated cells (Fig. 3B, right). Fig. 3A (lanes 1–5)

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$^2$R. J. H. Wojcikiewicz and Q. Xu, unpublished results.
down-regulation occurred at 4 panels, pol
maximal accumulation of polyubiquitinated species and inhibition of indicated and were then exposed to GnRH for 1 h (lanes 3
275 et
ets mark the respective positions of unmodified type I receptor (lanes 1–5). Ubiquitin immunoreactivity associated with immunoprecipitated type I InsP3 receptors was then assessed as in Fig. 2, B, effects of antide on InsP3 formation. Cells in suspension were incubated for 20 min with 0.1 μM GnRH alone or with 3 μM antide plus 0.1 μM GnRH (left panel), for 30 min with 0.1 μM GnRH alone, or for 20 min with 0.1 μM GnRH followed by a further 10-min incubation after 3 μM antide addition (right panel). Data shown are mean ± S.E. or range of replicate samples.

shows that the addition of antide to ALLN-preincubated, GnRH-stimulated cells results in a rapid decline in the level of polyubiquitinated InsP3 receptors, indicating that they are being deubiquitinated. Thus, deubiquitinating enzymes (27, 28) are active in αT3-1 cells and appear to participate in suppressing the build-up of polyubiquitinated InsP3 receptors.

Thapsigargin, TPEN, and Glycerol Inhibit Polyubiquitination and Down-regulation—The effects of potential inhibitors that might provide insight into the mechanism of polyubiquitination and down-regulation were also tested (Fig. 4). Thapsigargin inhibits Ca2+-ATPases that pump Ca2+ into the ER, reduces intraluminal Ca2+ concentration, and disrupts ER function (29). Fig. 4 (A, lane 4, and B, lane 2) shows that thapsigargin inhibits GnRH-induced InsP3 receptor down-regulation and polyubiquitination without affecting InsP3 formation (Fig. 4C), suggesting that Ca2+ binding to intraluminal regions of the type I InsP3 receptor (6–8) or to other ER proteins that interact with the type I InsP3 receptor (29–31) is required for this process. TPEN chelates Zn2+ with high affinity and has been shown to inhibit the activity of purified RING domain-containing E3 ubiquitin-protein ligases, presumably by removing the Zn2+ that is normally complexed with the RING domain (28, 32–34). Fig. 4 (A, lane 6, and B, lane 3) shows that TPEN inhibits InsP3 receptor down-regulation and polyubiquitination, and Fig. 4C shows that this is not due to inhibition of InsP3 formation.3 These data suggest that a RING

3 Surprisingly, TPEN significantly raised InsP3 concentration, most likely due to relief of inhibitory effects of Zn2+ on phosphoinositidase C activity.
domain-containing E3 mediates InsP₃ receptor ubiquitination. Finally, glycerol has been proposed to act as a “chemical chaperone,” acting to enhance the proper folding and suppress the degradation of either misfolded ER-associated proteins or peptides destined for ER-associated degradation (35, 36). Glycerol did inhibit down-regulation and polyubiquitination of InsP₃ receptors (Fig. 4, A, lane 8, and B, lane 4). However, it also completely inhibited InsP₃ formation (Fig. 4C), making it impossible to draw conclusions related to its action as a chaperone.

GnRH-induced Ubiquitination of Exogenous InsP₃ Receptors in Transfected Cells—Having characterized the polyubiquitination of endogenous receptors (Figs. 1–4), we next examined whether exogenous receptors, introduced by transient transfection, could be polyubiquitinated in a GnRH-dependent manner, since this would provide a system for the analysis of mutant receptors. Pilot studies utilizing cDNA encoding green fluorescent protein and a variety of transfected and were probed with CT1h as in Fig. 1. Co-expression of a Myc-immunoreactive band very similar in size to unmodified InsP₃RHAwt (Fig. 5C), which hereafter is referred to as “monoubiquitinated” receptor. Importantly, however, exposure of these cells to GnRH led to a large increase in Myc-polyubiquitination (upper panel, lanes 5 and 6), confirming that exogenous InsP₃RHAwt is polyubiquitinated. In contrast, in cells expressing InsP₃RHAΔ, which does not bind InsP₃ (17), the level of Myc-ubiquitination was unaffected by GnRH (upper panel, lanes 7–9), indicating that InsP₃RHAΔ is not polyubiquitinated. Parallel analysis of HA-tagged receptor content (lower panel) showed that both InsP₃RHAwt and InsP₃RHAΔ were expressed and that InsP₃RHAΔ (~265 kDa) migrated slightly more rapidly than InsP₃RHAwt (~270 kDa). To demonstrate that only the HA-tagged receptors were purified and that endogenous receptors did not co-immunoprecipitate, we probed the HA11-derived immunoprecipitates with CT1w, which recognizes both HA-tagged and endogenous receptors in immunoblots, and with CT1h, which recognizes only endogenous receptors in immunoblots. Fig. 5C (upper panel, lane 2) shows that InsP₃RHAwt migrates more slowly than endogenous receptor (lane 5), as previously described (17), and that no endogenous receptor was co-immunoprecipitated. This was confirmed by the observation that CT1h (lower panel) did not immunoreact with lane 2. Likewise, CT1h did not immunoreact with immunoprecipitated InsP₃RHAΔ (lane 3), showing again that endogenous receptors

Fig. 4. Effects of inhibitors on InsP₃ receptor down-regulation and polyubiquitination. Cells in suspension were incubated without or with 0.1 μM GnRH in the absence or presence of 1 μM thapsigargin, 100 μM TPEN, or 10% glycerol as indicated. A, inhibition of down-regulation. Cells were incubated for 60 min and were probed with CT1h as in Fig. 1. B, inhibition of polyubiquitination. Cells preincubated with 20 μg/ml ALLN for 2 h were incubated for 20 min, and ubiquitin immunoreactivity was assessed as in Fig. 2. C, effects on InsP₃ formation. Cells were incubated for 20 min, and InsP₃ concentration was assessed as in Fig. 3. Data shown are mean ± S.E. of triplicate samples (*, p < 0.02).
Inhibitory effects of 1/H9262
pared. InsP3 receptors were immunoprecipitated (the conditions indicated. Cells were then harvested, lysates were pre-
molayers were transfected with 0.5/H9262 ubiquitin) and 0.05/H9262 of immunoprecipitates from unstimulated transfected cells (4
 tors (17). The basis for this discrepancy was not examined, but it may reflect differences in the rate of receptor synthesis resulting from the two modes of receptor expression.

This lack of co-immunoprecipitation indicates that the exogenous and endogenous receptors do not associate in transiently transfected cells. This contrasts with the situation in stably transfected cells, where some heterotetramer formation and co-immunoprecipitation was observed (17). The basis for this discrepancy was not examined, but it may reflect differences in the rate of receptor synthesis resulting from the two modes of receptor expression.

polyubiquitination of transiently expressed exogenous receptors is mediated by InsP3 binding, indicating that they interact appropriately with InsP3 and are subject to the same regulatory processes as stably expressed receptors (37). Furthermore, their processing paralleled that of endogenous receptors, since GnRH-induced Myc-polyubiquitination of InsP3RHAwt was inhibited by thapsigargin and antide (Fig. 5D).

However, it must be noted that the processing of exogenous and endogenous receptors was not identical, since exogenous receptors were “monoubiquitinated.” This was not dependent on GnRH stimulation or InsP3 binding, since both InsP3RHAwt and InsP3RHA^A were modified (Fig. 5B), was not blocked by thapsigargin or antide, and, significantly, became more prominent at InsP3 receptor cDNA levels >0.1 μg, indicating that it may result from the overexpression of exogenous protein. Nevertheless, by expressing relatively low amounts of exogenous InsP3 receptor, the contribution of monoubiquitination to the overall ubiquitination signal could be minimized, and it was possible to use transient receptor expression to probe the events that trigger polyubiquitination.

The Role of Phosphorylation in Ubiquitination—Phosphorylation has been shown to trigger the polyubiquitination of many proteins (27, 28) and could contribute to triggering InsP3 receptor polyubiquitination, since the type I InsP3 receptor is phosphorylated by protein kinase A (PKA) (6–8) under conditions that lead to down-regulation (38). PKA-mediated phosphorylation of the mouse type I receptor occurs at serine residues 1588 and 1755 (39); thus, we created and analyzed a phosphorylation-resistant mutant receptor (InsP3RHA^A/A) in which both sites are converted to alanine. Fig. 5B shows that exogenous InsP3RHA^A/A was Myc-polyubiquitinated in an identical manner to InsP3RHAwt (lanes 4–6), indicating that PKA-dependent phosphorylation does not contribute to the triggering of InsP3 receptor polyubiquitination.

**DISCUSSION**

In summary, the data presented show that GnRH-induced InsP3 receptor down-regulation in αT3-1 cells is mediated by the ubiquitin/proteasome pathway, that transiently expressed exogenous InsP3 receptors are polyubiquitinated similarly to endogenous receptors, and that InsP3 binding, but not PKA-mediated InsP3 receptor phosphorylation, is a key event in the process that leads to polyubiquitination. In addition, we demonstrate that deubiquitination limits the accumulation of polyubiquitinated InsP3 receptors and that both thapsigargin and TPEN inhibit polyubiquitination.

Importantly, to the best of our knowledge, this study repre-
sents the first analysis of the ubiquitin/proteasome pathway in anterior pituitary cells and the first demonstration that a hypothalamic releasing factor, such as GnRH, can utilize this pathway to regulate protein levels. Indeed, ubiquitin/proteasome pathway-mediated InsP3 receptor down-regulation is likely to contribute to the mechanism by which long term administration of GnRH and its analogues to patients suppresses luteinizing hormone/follicle-stimulating hormone secretion and produces a hypogonadal state (2, 3). Further, these data raise the possibility that other proteins, perhaps those involved signal transduction (26, 40, 41), might also be targeted by the ubiquitin/proteasome pathway in anterior pituitary cells upon GPCR activation.

These studies also show that it is the ubiquitin/proteasome pathway, and not other candidate proteolytic systems (12, 19), that accounts for GnRH-mediated InsP3 receptor down-regulation. Thus, in response to GnRH receptor activation, InsP3 receptors are targeted by members of the E2/E3 enzyme family
that conjugate ubiquitin to proteins (27, 28). In analyzing this response in αT3-1 cells, we have extended our understanding of InsP3 receptor polyubiquitination and the ubiquitin/proteasome pathway in general in several ways. First, our data show that the accumulation of polyubiquitinated InsP3 receptors in the presence of proteasome inhibitors is countered by deubiquitination. Currently, virtually all work on deubiquitination has been done on purified proteins or disrupted cells (27, 42), and very little is known about the role of this activity in intact cells, apart from a recent study showing that a novel enzyme specifically deubiquitinates and stabilizes p53 (43). Whereas the nature of the activity that deubiquititates InsP3 receptors in intact cells was not examined, its existence explains why the accumulation of polyubiquitinated species in proteasome inhibitor-treated cells is relatively minor, amounting to only 9 ± 1% of the total receptor complement. It also suggests that in cells not exposed to proteasome inhibitors, polyubiquitinated InsP3 receptors will be subject to the competing effects of deubiquitinating enzymes (causing stabilization) and the proteasome (causing degradation). These and other findings (43) raise the possibility that this situation is the norm for all polyubiquitinated proteins. Second, with regard to the mechanism of ubiquitination, the ability of TPEN to inhibit InsP3 receptor polyubiquitination implicates aRING domain-containing E3 in this process, perhaps one of the recently identified E3s involved in the degradation of ER proteins (36, 44). Mechanistic insight was also obtained using thapsigargin, which depletes ER Ca2+ (29, 30) and completely inhibited InsP3 receptor polyubiquitination. This indicates that intraluminal Ca2+ plays a role in InsP3 receptor polyubiquitination. Intriguingly, ubiquitin/proteasome pathway-mediated processing of other ER proteins is also inhibited by depletion of ER Ca2+ (45, 46). Thus, InsP3 receptor polyubiquitination appears to be via a pathway common to all ER proteins targeted via the ubiquitin/proteasome pathway that is dependent on the normal storage of Ca2+ in the ER and perhaps on Ca2+ binding to one or more of the many Ca2+-binding ER proteins (29–31). Finally, comparison of the effects of different proteasome inhibitors showed that they varied considerably in their rate of action, with ALLN and MG-132 acting much more rapidly than lactacytin and epoxomicin. This kinetic variation most likely reflects the mechanistic differences between the inhibitors (23, 24) and clearly should be taken into account when these inhibitors are used.

We have also been able to show that exogenous transiently expressed receptors are polyubiquitinated in response to GPCR activation. This is significant, because in order to study the triggering and site specificity of polyubiquitination, it will be necessary to assess a large number of mutant receptors, which can be easily accomplished by expressing them transiently. In summary, we found that exogenous InsP3, RHA WT was processed in response to GnRH receptor activation similarly to endogenous InsP3 receptor but with the exception that it was also constitutively “monoubiquitinated” (modified with one or a very small number of Myc-ubiquitin residues). Significantly, whereas InsP3, RHA WT was both poly- and monoubiquitinated, InsP3, RHA A, which does not bind InsP3 (17), was only monoubiquitinated, indicating that only polyubiquitination occurs in response to InsP3 binding. Furthermore, InsP3, RHA WT polyubiquitination, but not monoubiquitination, was blocked by thapsigargin and antibody. Thus, monoubiquitination appears to be a process mechanistically discrete from that which mediates polyubiquitination and may be a response to the overexpression of exogenous receptors, a view supported by the observation that the prominence of monoubiquitination increased as exogenous receptor expression increased. Thus, it is possible that the capacity of αT3-1 cells to accommodate and correctly process transiently expressed exogenous InsP3 receptors is relatively limited, and above that capacity, the receptors are monoubiquitinated. The fact that exogenous receptors did not associate to a detectable extent with endogenous receptors lends credence to this view. An alternative explanation is that InsP3 receptor monoubiquitination is a normal cellular event that has been revealed because of the high sensitivity of the Myc epitope raised. Indeed, it has recently been shown that other receptors and their associated proteins can be monoubiquitinated (40, 48) as a prelude to their trafficking to lysosomes. It is an intriguing possibility that InsP3 receptors could be similarly processed.

With regard to events that trigger type I InsP3 receptor polyubiquitination, the analysis of InsP3, RHA WT shows that InsP3 binding, and presumably the conformational changes that result from this binding (49), cause the receptor to become polyubiquitinated. The possibility that phosphorylation might be involved in triggering polyubiquitination was also examined, since the type I InsP3 receptor is phosphorylated stoichiometrically in response to PKA activation in intact cells (6–8) and, indeed, in response to activation of the Gq-linked GPCRs that lead to InsP3 receptor down-regulation (38). However, PKA-dependent phosphorylation of the receptor was clearly not required for polyubiquitination, since InsP3, RHA A/A was polyubiquitinated equivalently to InsP3, RHA WT.

In conclusion, our studies show that the ubiquitin/proteasome pathway is active in αT3-1 anterior pituitary cells and mediates InsP3 receptor degradation in response to activation of GnRH receptors. Since transiently expressed exogenous receptors are polyubiquitinated similarly to endogenous receptors, use of this cell type will allow for the analysis of a range of mutant receptors and the dissection of the molecular events that lead to InsP3 receptor polyubiquitination.

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Inositol 1,4,5-Trisphosphate Receptor Ubiquitination

947

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Richard J. H. Wojcikiewicz, Qun Xu, Jack M. Webster, Kamil Alzayady and Chen Gao

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