Ligand binding directly stimulates ubiquitination of the inositol 1,4,5-trisphosphate receptor

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Down-regulation of the Ins(1,4,5) P_3 receptor is an adaptive response to the activation of certain phosphoinositidase C-linked cell-surface receptors. It is manifested as a profound decline in cellular Ins(1,4,5) P_3 receptor content, occurs with a half-time of 0.5–2 h and is due to accelerated proteolysis. It has been shown that this process is mediated by the ubiquitin/proteasome pathway and is therefore initiated by Ins(1,4,5) P_3 receptor ubiquitination. To investigate the role of ligand binding in Ins(1,4,5) P_3 receptor ubiquitination, we expressed ‘exogenous’ wild-type and ligand-binding-defective mutant type I Ins(1,4,5) P_3 receptors in human neuroblastoma SH-SY5Y cells, in which muscarinic receptor activation elicits Ins(1,4,5) P_3 receptor down-regulation. We found (1) that exogenous wild-type Ins(1,4,5) P_3 receptors are efficiently ubiquitinated in response to muscarinic receptor stimulation, (2) that exogenous ligand

binding-defective mutant Ins(1,4,5) P_3 receptors are resistant to ubiquitination, (3) that this resistance is not caused by the removal of potential ubiquitin-conjugating sites in the mutated region, and (4) that in heterotetramers of exogenous mutant receptors and ‘endogenous’ receptors, only the latter are targeted for ubiquitination. These results indicate that the binding of Ins(1,4,5) P_3 directly stimulates ubiquitination of the Ins(1,4,5) P_3 receptor and that the targeting of Ins(1,4,5) P_3 receptors for ubiquitination is a highly specific process. We therefore propose that an Ins(1,4,5) P_3 -binding-induced conformational change in the receptor exposes a degradation signal that leads to ubiquitination.

Key words: degradation signal, muscarinic, neuroblastoma, ubiquitin.

INTRODUCTION

Ins(1,4,5) P_3 receptors [Ins(1,4,5) P_3 R] form tetrameric, Ins(1,4,5) P_3 -gated Ca^{2+} channels in endoplasmic reticulum membranes, and have a pivotal role in intracellular signalling [1–4]. By governing the release of Ca^{2+} stored in the endoplasmic reticulum, they allow extracellular ligands that activate phosphoinositidase C and elevate Ins(1,4,5) P_3 concentrations to increase cytoplasmic Ca^{2+} concentration [1–4]. Three Ins(1,4,5) P_3 R types, termed I, II and III, have been defined [4–8]. These proteins share moderate (60–70%) amino acid sequence identity and the same general structure [4–8], and can combine to form either homotetramers or heterotetramers [9–11].

Persistent phosphoinositidase C activation can cause Ins(1,4,5) P_3 R down-regulation [12–18]; Ins(1,4,5) P_3 R therefore represent a novel locus for the desensitization of phosphoinositidase C-mediated signalling. This down-regulation decreases the sensitivity of Ca^{2+} stores to Ins(1,4,5) P_3 [12,18], is due to accelerated receptor proteolysis [14,15], has a half-time of 0.5–2 h [14–18], can decrease Ins(1,4,5) P_3 R levels by as much as 90% [14,15], is evident with all three receptor types [15,17,18], and occurs in a variety of cells *in vitro* [14–18] and *in vivo* [19]. Although the mechanism of Ins(1,4,5) P_3 R proteolysis has yet to be defined, it might be executed through the ubiquitin/proteasome pathway [17–20], a pathway crucial to basal and regulated degradation of many cytosolic, nuclear, and membrane proteins [21–25].

The first step in the ubiquitin/proteasome pathway is the conjugation of ubiquitin, a 76-residue protein, to one or more lysine residues in the substrate destined for degradation [21–25]. This ubiquitination is sequentially catalysed by a ubiquitin-

activating enzyme (E1), a ubiquitin-conjugating enzyme (E2, or Ubc) and a ubiquitin ligase (E3) [21–25]. Because the newly attached ubiquitin can also be ubiquitinated, targeted proteins become tagged with ubiquitin chains [21–25]. Polyubiquitinated proteins are then recognized and degraded by the proteasome, a 26 S multi-subunit protease [26,27]. Thus ubiquitin conjugation has a central role, and seems to be the rate-limiting step, in directing proteins towards proteasomal degradation [21,26]. Several mechanisms [28–31] have been proposed as the stimulus or ‘degradation signal’ for ubiquitination but no information is yet available on what activates Ins(1,4,5) P_3 R ubiquitination, other than indirect evidence that Ins(1,4,5) P_3 is the trigger; this comes from studies showing that Ins(1,4,5) P_3 R ubiquitination is seen only with those agonists that persistently elevate Ins(1,4,5) P_3 concentration [20].

Here we describe studies aimed at defining the event that initiates Ins(1,4,5) P_3 R ubiquitination. We have focused on the ubiquitination of wild-type and ligand-binding-defective mutant Ins(1,4,5) P_3 R stably expressed in human neuroblastoma SH-SY5Y cells. This has allowed us to establish that ligand binding is the stimulus for Ins(1,4,5) P_3 receptor ubiquitination.

EXPERIMENTAL

Cell culture and cell lines

SH-SY5Y cells were grown as monolayers as previously described [15,32]. The generation and maintenance of SIns(1,4,5) P_3 RHA and ΔIns(1,4,5) P_3 RHA cells, which are transfected SH-SY5Y cells stably expressing haemagglutinin (HA) epitope-tagged mouse cerebellum wild-type type I receptor [Ins(1,4,5) P_3 RHA] and binding-defective mutant type I

Abbreviations used: ALLN, *N*-acetyl-Leu-Leu-norleucinal; HA, haemagglutinin; Ins(1,4,5) P_3 R, wild-type mouse type I Ins(1,4,5) P_3 receptor; Ins(1,4,5) P_3 RHA, HA epitope-tagged Ins(1,4,5) P_3 R; ΔIns(1,4,5) P_3 RHA, HA epitope-tagged binding-defective Ins(1,4,5) P_3 mouse type I Ins(1,4,5) P_3 receptor; K350RIns(1,4,5) P_3 RHA, Ins(1,4,5) P_3 RHA with a substitution of arginine for Lys-350.

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receptor [Δ Ins(1,4,5) P_3 RHA] respectively, have been described [32]. The HA epitope tag (YPYDVPDYA) was introduced at the receptor C-terminus [32]; the binding-defective mutant receptor lacked 37 residues (316–352) in the ligand-binding domain [33]. Culture medium was changed every 3 days and was replaced 1 day before experiments.

To replace Lys-350 (K350) with arginine (R) in Ins(1,4,5) P_3 RHA, pcWIHA, which carries the cDNA insert encoding Ins(1,4,5) P_3 RHA [32], was mutated; A¹³⁷⁷ was changed to G in the codon for K350 (A¹³⁷⁶AA¹³⁷⁸) by PCR-based site-directed mutagenesis. In brief, primers P1 (5'-ATGTCGTAACAACTCCGCCCATTTG-3') and P2 (5'-CCAGAGAGTATACATTCTTTCTTGCGCGTTTC-3') were used to amplify the region between nt 743 of the vector [32] and 1394 of the insert, and primers P3 (5'-GAAACGCGCAAGAAAGAAATGGTAT-CTCTCTGG-3') and P4 (5'-CTGCTTCTGCATGAAGCCA-AACTGC-3') were used to amplify the region between nt 1362 and 2080 of the insert. Thus mismatched nucleotides (underlined in P2 and P3) were introduced into the 1589 bp and 718 bp fragments produced. These two fragments were then fused via their overlapping regions through denaturing, annealing and DNA polymerization [32], yielding a 2135 bp sequence. After amplification with P1 and P4, the fusion product was digested with *KpnI* (New England Biolabs) and was substituted for the corresponding region of pcWIHA, yielding pcK350RWIHA, encoding K350RIns(1,4,5) P_3 RHA. The mutation and sequences derived from amplification were validated by DNA sequencing. Stable transfection of SH-SY5Y cells [32] with pcK350RWIHA was performed with LipofectAMINE (Life Technologies).

Measurement of Ins(1,4,5) P_3 R ubiquitination

Cells were grown in dishes 10 cm in diameter to confluence and, before exposure to agonist, were preincubated for 2 h with *N*-acetyl-Leu-Leu-norleucinal (ALLN), a potent proteasome inhibitor [34] that leads to the accumulation of ubiquitinated proteins within cells [17,20]. Agonist-stimulated or control cells were then disrupted for 20 min in ice-cold lysis buffer [150 mM NaCl/50 mM Tris base/1 mM EDTA/1% (v/v) Triton X-100/0.2 mM PMSF/1 mM dithiothreitol/10 μ M leupeptin/10 μ M pepstatin/0.2 μ M soybean trypsin inhibitor (pH 8.0); 1 ml per dish of cells] and were centrifuged (14000 *g* for 10 min at 4 °C). Supernatants were then incubated for 2 h at 4 °C with either 100 μ l of CT1 {an affinity-purified rabbit polyclonal antibody against the type I Ins(1,4,5) P_3 R [15]} or 2 μ l of HA11 (a mouse monoclonal antibody against the HA epitope [32]) and then for a further 2 h at 4 °C with 10 mg of Protein A-conjugated Sepharose CL-4B beads. The immunoprecipitates were then washed four times with 1 ml of lysis buffer and were finally resuspended in 80 μ l of gel-loading buffer [100 mM Tris/HCl (pH 6.8)/200 mM dithiothreitol/4% (w/v) SDS/0.2% Bromophenol Blue/20% (v/v) glycerol]. Samples and prestained molecular mass markers were then separated by SDS/PAGE [5% (w/v) gel], transferred to nitrocellulose membranes and probed with CT1, HA11 or polyclonal anti-ubiquitin as described [20]. Immunoreactivity was detected by enhanced chemiluminescence (Pierce) and X-ray film [15,20], and was quantified with Quantity One software (Bio-Rad), using the range in which band intensity was linearly related to the quantity of ubiquitinated receptors loaded. Molecular mass values and quantified chemiluminescence are expressed as means ($n = 3$) or means $\pm$ S.E.M. ($n = 3$).

To measure selectively the ubiquitination of HA-tagged Ins(1,4,5) P_3 Rs, a two-step immunoprecipitation procedure [20] was performed to concentrate ubiquitinated species. In brief, in step 1, HA11-derived immunoprecipitates were prepared, washed

three times with 1 ml of 150 mM NaCl/50 mM Tris base (pH 8.0)/1 mM EDTA/0.2% (v/v) Triton X-100 and heated for 3 min at 100 °C in 100 μ l of 150 mM NaCl/50 mM Tris base (pH 8.0)/1 mM EDTA/20 mM dithiothreitol/1% (w/v) BSA/1% (w/v) SDS. This denatured the immunoprecipitated proteins, causing disassembly of the immune complex and dissociation of Ins(1,4,5) P_3 R tetramers [20]. The samples were then centrifuged (500 *g* for 1 min at 20 °C) and supernatants were collected and diluted with 0.9 ml of ice-cold lysis buffer. In step 2, half of the diluted supernatants were incubated with 1.5 μ l of monoclonal anti-ubiquitin for 2 h and then with Protein A beads for 2 h at 4 °C to precipitate ubiquitinated receptors; the remainder, which served as a control, was processed identically, except that monoclonal anti-ubiquitin was omitted. After four washes with 1 ml of lysis buffer, the beads were resuspended in 80 μ l of gel-loading buffer and were probed in immunoblots with CT1, HA11 and polyclonal anti-ubiquitin.

Materials

Sources of materials for cell culture, immunoprecipitation, immunoblotting, transfection, and PCR were as defined previously [15,20,32].

RESULTS

Ins(1,4,5) P_3 R ubiquitination in human neuroblastoma SH-SY5Y cells

Before transfection of Ins(1,4,5) P_3 R cDNA species, we sought to determine whether 'endogenous' Ins(1,4,5) P_3 Rs in SH-SY5Y cells, which are predominantly (more than 99%) type I [15], are ubiquitinated and whether ubiquitination of these receptors is

Figure 1 Ins(1,4,5) P_3 R ubiquitination is stimulated by muscarinic receptor activation

SH-SY5Y cell monolayers were preincubated with ALLN (20 μ g/ml) for 2 h and then exposed for 1 h to vehicle (lane 1), 1 mM carbachol (lane 2), 10 μ M atropine (lane 3) or 1 mM carbachol plus 10 μ M atropine (lane 4). Ins(1,4,5) P_3 Rs were then immunoprecipitated with CT1, resolved by SDS/PAGE [5% (w/v) gel] and probed in immunoblots with either polyclonal anti-ubiquitin (upper panel) or CT1 (lower panel). The migration positions of molecular mass markers (in kDa) (lines), type I Ins(1,4,5) P_3 Rs (arrow) and ubiquitinated Ins(1,4,5) P_3 Rs (square bracket) are indicated. The arrowhead indicates the position to which unmodified type I Ins(1,4,5) P_3 Rs migrated. The images shown (the lower panel being only the approx. 190–380 kDa region of a gel) are representative of three independent experiments.

Figure 2 Concentration- and time-dependence of Ins(1,4,5) P_3 R ubiquitination

SH-SY5Y cell monolayers were preincubated with ALLN (20 μ g/ml) for 2 h and then exposed for 1 h to 0, 0.1, 1, 10, 100 or 1000 μ M carbachol (**A**, lanes 1–6) or to 10 μ M carbachol for 0, 10, 30, 60, 120 or 240 min (**C**, lanes 1–6). Ins(1,4,5) P_3 Rs were then immunopurified, probed and labelled as in Figure 1. The images shown (upper panels, approx. 190–420 kDa; lower panels, approx. 190–380 kDa) are representative of three independent experiments. (**B**, **D**) Quantified ubiquitin immunoreactivity is also expressed as a percentage of maximal chemiluminescence.

correlated with the characteristics of muscarinic agonist (carbachol)-induced Ins(1,4,5) P_3 R down-regulation described previously for this cell type [13–16]. Figure 1 shows that carbachol does indeed cause the association of ubiquitin immunoreactivity with immunoprecipitated type I receptors. The apparent molecular mass of the ubiquitinated species ranged from 285 to 383 kDa, was centred on 323 ± 6 kDa (Figure 1, upper panel, lane 2), and was considerably greater than that of unmodified type I Ins(1,4,5) P_3 Rs (approx. 260 kDa). This observation indicates that carbachol stimulates Ins(1,4,5) P_3 R polyubiquitination, and agrees with findings from agonist-stimulated WB rat liver epithelial cells [17], AR4-2J rat pancreatoma cells, INS-1 rat insulinoma cells and neonatal rat cerebellar granule cells [20], in which polyubiquitinated Ins(1,4,5) P_3 Rs migrated similarly. Ins(1,4,5) P_3 R down-regulation was not substantial in these experiments (Figure 1, lower panel, compare lanes 1 and 2), because the proteasome inhibitor ALLN was included in incubations. However, despite this blockade and the accumulation of ubiquitinated species, we were unable to detect CT1 immunoreactivity in the area in which ubiquitinated Ins(1,4,5) P_3 Rs migrated (Figure 1, lower panel, lane 2), indicating that the steady-state level of ubiquitinated Ins(1,4,5) P_3 Rs in stimulated cells remained low. This was consistent with findings in other cell types [17,20] and might reflect the activity of de-ubiquitinating

enzymes [20] or feedback inhibition of Ins(1,4,5) P_3 R ubiquitination by accumulating ubiquitinated species; it was not due to masking of the CT1 epitope by ubiquitin [20]. Figure 1 (upper panel, lane 4) also shows that the effects of carbachol were muscarinic-receptor-mediated, because atropine, a specific muscarinic receptor antagonist, totally blocked the appearance of ubiquitin immunoreactivity.

Analysis of the concentration dependence (Figures 2A and 2B) and the time course (Figures 2C and 2D) of ubiquitination revealed that the response had an EC_{50} of approx. 1 μ M carbachol and a half-time of approx. 30 min. These results are similar to those for the carbachol-induced down-regulation of the type I Ins(1,4,5) P_3 R in this cell type, which were approx. 0.1 μ M and approx. 1 h respectively [14]; the discrepancies might reflect the inclusion of ALLN in the present study. Thus Ins(1,4,5) P_3 R ubiquitination in SH-SY5Y cells occurs with characteristics consistent with its having a role in mediating Ins(1,4,5) P_3 R down-regulation.

Ins(1,4,5) P_3 R ubiquitination in SH-SY5Y cells transfected with Ins(1,4,5) P_3 R cDNA species

To define whether Ins(1,4,5) P_3 binding is important in initiating Ins(1,4,5) P_3 R ubiquitination, we generated SIns(1,4,5) P_3 RHA

Figure 3 Ins(1,4,5) P_3 R ubiquitination in cells expressing exogenous Ins(1,4,5) P_3 Rs

*S*_{vec} (lanes 1–4), *S*Ins(1,4,5) P_3 RHA (lanes 5–8), and Δ*S*Ins(1,4,5) P_3 RHA (lanes 9–12) cell monolayers were pretreated with ALLN (20 μ g/ml) for 2 h and then exposed to 1 mM carbachol for 0 h (lanes 1, 5, 9), 0.5 h (lanes 2, 6, 10), 2 h (lanes 3, 7, 11) or 4 h (lanes 4, 8, 12). Ins(1,4,5) P_3 Rs were then immunoprecipitated with either CT1 (**A**) or HA11 (**B**), resolved by SDS/PAGE [5% (w/v) gel] and probed in immunoblots with polyclonal anti-ubiquitin (upper panels) or CT1 (lower panels). The migration positions of type I Ins(1,4,5) P_3 Rs (arrow) and ubiquitinated Ins(1,4,5) P_3 Rs (square bracket) are indicated. The images shown (upper panels, approx. 190–420 kDa; lower panels, approx. 220–320 kDa) are representative of three independent experiments.

and Δ*S*Ins(1,4,5) P_3 RHA, SH-SY5Y cell lines that stably expressed HA-tagged wild-type and binding-defective mutant Ins(1,4,5) P_3 Rs respectively, at levels 2–4-fold those of endogenous type I receptors [32]. Previous work with these cells had shown (1) that the endogenous and 'exogenous' HA-tagged Ins(1,4,5) P_3 Rs assemble into heteromers and form functional Ins(1,4,5) P_3 -sensitive Ca^{2+} channels, (2) that carbachol-induced Ins(1,4,5) P_3 generation and Ca^{2+} mobilization in intact cells are essentially unaffected by the expression of either Ins(1,4,5) P_3 RHA or ΔIns(1,4,5) P_3 RHA, and (3) that CT1 recognizes and immunoprecipitates both [32].

Figure 3 depicts the ubiquitination of Ins(1,4,5) P_3 Rs in *S*Ins(1,4,5) P_3 RHA and Δ*S*Ins(1,4,5) P_3 RHA cells and in control cells (*S*_{vec}, SH-SY5Y cells transfected with empty vector [32]). To facilitate the comparison of the efficiencies of receptor ubiquitination, differences between the cell lines in receptor expression levels were compensated for by the loading of approximately equal amounts of immunopurified receptors for each cell line under control conditions (Figure 3A, lower panel, lanes 1, 5 and 9, and Figure 3B, lower panel, lanes 5 and 9). Consistent with SH-SY5Y cells (Figure 1) was the observation that carbachol stimulated the polyubiquitination of CT1-immunoprecipitable Ins(1,4,5) P_3 Rs in the three cell lines (Figure 3A, upper panel). Little difference in the time course and the extent of receptor ubiquitination was seen between *S*_{vec} and *S*Ins(1,4,5) P_3 RHA (lanes 1–4 and 5–8) cells, whereas ubiquitination was somewhat attenuated in Δ*S*Ins(1,4,5) P_3 RHA cells (lanes 9–12). These results suggest that, whereas Ins(1,4,5) P_3 RHA ubiquitination proceeds normally, ΔIns(1,4,5) P_3 RHA might be resistant to ubiquitination.

Note that the ubiquitin immunoreactivity detected in Figure 3(A) was derived from both endogenous and exogenous Ins(1,4,5) P_3 Rs, because CT1 interacts with both [32]. To examine more selectively the ubiquitination of exogenous Ins(1,4,5) P_3 Rs, we also used HA11, which interacts only with HA-tagged receptors [32], as an immunoprecipitating agent. Owing to receptor heteromerization, however, a minor proportion of the receptors recovered will be co-immunoprecipitating endogenous receptors [32]. Figure 3(B) shows that HA11 did not

immunoprecipitate material from *S*_{vec} cells (lanes 1–4), as expected, and that HA11-immunopurified receptors from *S*Ins(1,4,5) P_3 RHA cells cross-reacted with anti-ubiquitin in a similar fashion to CT1-immunopurified endogenous receptors (compare Figure 3B, upper panel, lanes 5–8, with Figure 3A, upper panel, lanes 1–4), suggesting again that Ins(1,4,5) P_3 RHA is ubiquitinated normally. Strikingly, a parallel analysis of Δ*S*Ins(1,4,5) P_3 RHA cells yielded much less ubiquitin immunoreactivity (Figure 3B, lanes 9–12), indicating that ΔIns(1,4,5) P_3 RHA ubiquitination was severely impaired; the residual ubiquitin immunoreactivity (lanes 9–12) might have originated from ubiquitinated endogenous Ins(1,4,5) P_3 Rs that co-immunoprecipitated with ΔIns(1,4,5) P_3 RHA [32]. It is therefore plausible that ΔIns(1,4,5) P_3 RHA is totally resistant to ubiquitination.

To confirm or refute this suggestion, equal quantities of HA11-immunopurified receptor complexes from stimulated *S*Ins(1,4,5) P_3 RHA and Δ*S*Ins(1,4,5) P_3 RHA cells (obtained as in Figure 3B) were disrupted by being heated in the presence of 1% (w/v) SDS, and then ubiquitinated receptors were isolated by immunoprecipitation. Figure 4(A) (lower panel, lane 1) shows, as expected, that receptors so isolated from *S*Ins(1,4,5) P_3 RHA cells cross-reacted strongly with polyclonal anti-ubiquitin. Furthermore, and in contrast with the findings in Figures 1, 2 and 3, these receptors were now also recognized by CT1 (Figure 4A, lane 1, top panel), apparently because of the enrichment of ubiquitinated species [20]. Importantly, HA11 also recognized these receptors (Figure 4A, middle panel, lane 1), confirming that ubiquitin was conjugated to Ins(1,4,5) P_3 RHA. Note also that immunoreactive bands were not detectable in control samples from this cell type (Figure 4A, middle panel, lane 3). When the same procedure was applied to Δ*S*Ins(1,4,5) P_3 RHA cells, only weak immunoreactivity to either anti-ubiquitin (Figure 4A, bottom panel, lane 2) or CT1 (top panel, lane 2) was identified and, strikingly, specific immunoreactivity against HA11 in the region corresponding to ubiquitinated receptors was not detected (middle panel, lane 2), indicating that ΔIns(1,4,5) P_3 RHA was totally resistant to ubiquitination. Note that a background of non-specific immunoreactivity is present in both lanes 1 and 2 of Figure 4(A) (middle panel), apparently because, in this case,

Figure 4 Ubiquitination of exogenous $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$

$\text{SIns}(1,4,5)\text{P}_3\text{RHA}$ (lanes 1 and 3), $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ (lanes 2 and 4) and $\text{SK350RIns}(1,4,5)\text{P}_3\text{RHA}$ (lanes 5 and 6) cell monolayers were pretreated with ALLN (20 $\mu\text{g}/\text{ml}$) for 2 h and then exposed to 1 mM carbachol for 1 h. $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ were then immunoprecipitated with HA11 and disrupted by being boiled in the presence of 1% (w/v) SDS. The dissociated receptors were then immunoprecipitated with monoclonal anti-ubiquitin to concentrate ubiquitinated receptors (lanes 1, 2 and 5). As controls, duplicate samples were processed identically except that monoclonal anti-ubiquitin was omitted (lanes 3, 4 and 6). After separation by SDS/PAGE [5% (w/v) gel], samples were probed with CT1 (top panel), HA11 (middle panel) or polyclonal anti-ubiquitin (bottom panel). The migration positions of ubiquitinated $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ are denoted by square brackets. Images (approx. 220–420 kDa) shown are representative of three (A) or two (B) independent experiments.

HA11 was used both as the immunoprecipitating antibody and the probing antibody; the band corresponding to ubiquitinated receptors in lane 1 is superimposed on this background. The lack of immunoreactivity to HA11 (Figure 4A, middle panel, lane 2) also indicates that the weak immunoreactivity to anti-ubiquitin and CT1 was derived from ubiquitinated co-immunoprecipitating endogenous receptors [32], and that the low level of ubiquitin immunoreactivity seen in Figure 3(B) (upper panel, lanes 10–12) had the same origin. Remarkably, therefore, these results also reveal that individual subunits in a tetrameric channel can be differentially targeted for ubiquitination.

Mutation of K350 does not affect $\text{Ins}(1,4,5)\text{P}_3\text{R}$ ubiquitination

Although the total resistance of $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ to ubiquitination (Figure 4A) most probably reflects its inability to bind $\text{Ins}(1,4,5)\text{P}_3$, the possibility also exists that the 316–352 deletion in $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ might have removed lysine residues that were ubiquitin conjugation sites. Indeed, the deletion did remove one lysine residue, K350. Therefore, to define the importance of K350 to receptor ubiquitination, we replaced it with arginine, to which ubiquitin cannot be conjugated, and stably expressed this mutant [$\text{K350RIns}(1,4,5)\text{P}_3\text{RHA}$] in SH-SY5Y cells.

Figure 4(B) (lane 5) shows that in response to carbachol, immunopurified $\text{K350RIns}(1,4,5)\text{P}_3\text{RHA}$ was recognized by CT1, HA11 and anti-ubiquitin, indicating that it was ubiquitinated and that the loss of K350 could not account for the resistance of $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ to ubiquitination. Consistent with this observation was our finding that carbachol caused $\text{Ins}(1,4,5)\text{P}_3\text{R}$ down-regulation in $\text{SK350RIns}(1,4,5)\text{P}_3\text{RHA}$ cells (results not shown) to an extent indistinguishable from that seen in $\text{SIns}(1,4,5)\text{P}_3\text{RHA}$ cells [32], indicating that the K350R mutation did not affect $\text{Ins}(1,4,5)\text{P}_3\text{R}$ down-regulation.

DISCUSSION

In the present study we have demonstrated (1) that muscarinic receptor stimulation activates type I $\text{Ins}(1,4,5)\text{P}_3\text{R}$ ubiquitination in SH-SY5Y cells and that this correlates well with the characteristics of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ down-regulation in this cell type, (2) that ligand-binding-defective mutant $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ are resistant to ubiquitination, (3) that K350, which is missing from the mutant $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$, is not required for $\text{Ins}(1,4,5)\text{P}_3\text{R}$ ubiquitination, and (4) that individual $\text{Ins}(1,4,5)\text{P}_3\text{R}$ subunits in a tetrameric channel are differentially targeted for ubiquitination (only those subunits with ligand-binding activity are ubiquitinated). We therefore conclude that $\text{Ins}(1,4,5)\text{P}_3$ binding is directly responsible for initiating $\text{Ins}(1,4,5)\text{P}_3\text{R}$ ubiquitination and that the targeting of $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ for ubiquitination is a highly specific process. Importantly, these results can account for our previous findings [32] that $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ is resistant to carbachol-initiated down-regulation and strengthen the causal link between ubiquitination and receptor degradation. They also agree with findings [32] that only the endogenous $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ in $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ -containing heterotetramers are degraded in response to carbachol.

This selectivity argues against the suggestion [16] that mobilized Ca^{2+} might stimulate $\text{Ins}(1,4,5)\text{P}_3\text{R}$ degradation, perhaps by activating ubiquitination, a possibility given credence by observations that domains of high Ca^{2+} concentration exist near the mouth of open $\text{Ins}(1,4,5)\text{P}_3\text{R}$ channels [2]. If this were so, then ubiquitination and degradation of all subunits in a tetramer should be accelerated simultaneously whether or not they bound $\text{Ins}(1,4,5)\text{P}_3$, because they would all be exposed to the high Ca^{2+} concentrations.

Rather, our results point to two possible mechanisms by which $\text{Ins}(1,4,5)\text{P}_3$ binding could initiate $\text{Ins}(1,4,5)\text{P}_3\text{R}$ ubiquitination. First, ubiquitination could result from a modification of interactions between tetramerized receptor subunits. The degradation signal that mediates ubiquitination in each subunit could be shielded by other subunits of the tetrameric complex; $\text{Ins}(1,4,5)\text{P}_3$ -induced subunit reorganization could expose this region. This type of mechanism has been observed for the yeast transcription factors $\alpha 2$ and $\alpha 1$, both of which, when expressed alone, are short-lived proteins that are degraded through the ubiquitin/proteasome pathway [35,36]. However, when co-expressed they heterodimerize, their degradation signals become masked, and they are stable [35,36]. Although theoretically possible, it is, however, hard to envisage how only the degradation signal of endogenous receptors, and not mutant receptors, could be exposed after $\text{Ins}(1,4,5)\text{P}_3$ -driven conformational changes to a tetramer. Second, and perhaps more likely, is the possibility that conformational changes within individual subunits account for receptor ubiquitination. In this model, the degradation signal of each receptor subunit is buried intramolecularly and exposure of this signal is triggered by $\text{Ins}(1,4,5)\text{P}_3$ -binding-induced conformational changes. Thus ligand-occupied endogenous $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ in $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ -containing channels are ubiquitinated, whereas $\Delta\text{Ins}(1,4,5)\text{P}_3\text{RHA}$ subunits are not. This mechanism would be analogous to the ubiquitin-dependent destruction of misfolded proteins in the endoplasmic reticulum, in which the exposure of normally buried hydrophobic motifs seems to have a role [28,37]; $\text{Ins}(1,4,5)\text{P}_3$ -induced conformational changes might similarly cause the exposure of hydrophobic regions.

It is important to consider that these models are valid only if the 316–352 deletion has not removed a ubiquitin conjugation site or the putative degradation signal. K350, which is conserved in types I, II and III receptors [4–8], is indeed removed by this

deletion; however, Ins(1,4,5) P_3 Rs with a substitution of arginine for K350 are ubiquitinated and down-regulated normally, indicating that K350 is not required for Ins(1,4,5) P_3 R ubiquitination. Furthermore, region 316–352 is not compatible with known degradation signals; this sequence is highly hydrophilic [5], whereas degradation signals are often stretches of hydrophobic residues [31,35]. In addition, as all three Ins(1,4,5) P_3 Rs are ubiquitinated in response to phosphoinositidase C activation [17–20], it would be expected that the 316–352 region would be highly conserved between receptors if it were the degradation signal. This, however, is not so: the 316–352 region is poorly conserved in types I, II and III receptors (only 16% similarity in this region).

Taken together, our results show that Ins(1,4,5) P_3 binding initiates Ins(1,4,5) P_3 R ubiquitination, possibly by causing a conformational change that exposes a degradation signal. The ubiquitinated receptors are then translocated to proteasomes for degradation. To define the putative degradation signal and the enzymes that mediate Ins(1,4,5) P_3 R ubiquitination is a challenging goal for future studies.

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