Down-regulation of types I, II and III inositol 1,4,5-trisphosphate receptors is mediated by the ubiquitin/proteasome pathway

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Activation of certain phosphoinositidase-C-linked cell-surface receptors is known to cause an acceleration of the proteolysis of inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] receptors and, thus, lead to Ins(1,4,5) P_3 -receptor down-regulation. In the current study we have sought to determine whether the ubiquitin/proteasome pathway is involved in this adaptive response. The data presented show (i) that activation of phosphoinositidase-C-linked receptors causes Ins(1,4,5) P_3 -receptor ubiquitination in a range of cell types (AR4-2J cells, INS-1 cells and rat cerebellar granule cells), (ii) that the Ins(1,4,5) P_3 -receptor down-regulation induced by activation of these receptors is blocked by proteasome inhibitors, (iii) that all known Ins(1,4,5) P_3 receptors (types I, II

and III) are substrates for ubiquitination, (iv) that ubiquitination occurs while Ins(1,4,5) P_3 receptors are membrane-bound, (v) that Ins(1,4,5) P_3 -receptor ubiquitination and down-regulation are stimulated only by those agonists that elevate Ins(1,4,5) P_3 concentration persistently, and (vi) that a portion of cellular Ins(1,4,5) P_3 receptors (those that are not type-I-receptor-associated) can be resistant to ubiquitination and degradation. In total these data indicate that the ubiquitin/proteasome pathway mediates Ins(1,4,5) P_3 -receptor down-regulation and suggest that ubiquitination is stimulated by the binding of Ins(1,4,5) P_3 to its receptor.

Key words: adaptation, cell lines, degradation.

INTRODUCTION

Type-I, -II and -III inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] receptors are a family of proteins that form tetrameric ion channels in endoplasmic reticulum (ER) membranes [1,2]. Upon binding of Ins(1,4,5) P_3 , the channels open and Ca^{2+} stored within the ER flows into the cytoplasm [1,2]. Thus, Ins(1,4,5) P_3 receptors play a pivotal role in linking cell-surface-receptor-mediated Ins(1,4,5) P_3 formation to increases in cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$. Type-I, -II and -III receptors are 60–70 % similar at the amino acid level [1,2], are expressed at various levels in different tissues, are often co-expressed in the same cell type [3,4] and form heterotetramers [5–7].

Ins(1,4,5) P_3 receptors are down-regulated in response to activation of certain phosphoinositidase-C (PIC)-linked cell-surface receptors [3,8–11]. For example, activation of muscarinic receptors in SH-SY5Y human neuroblastoma cells causes a ≈ 90 % decline in type-I Ins(1,4,5) P_3 -receptor content with half-maximal effect at 0.5–1 h [9,12]. This adaptive response is mediated by an increase in the rate of receptor degradation [3,9], is specific, since other ER and signalling proteins are not affected simultaneously [3,9–12], and appears to exist to allow chronically stimulated cells to reduce the sensitivity of their Ca^{2+} stores to Ins(1,4,5) P_3 [8,10,11]. A major issue that remains, however, concerns the nature of the proteolytic mechanism responsible for Ins(1,4,5) P_3 -receptor degradation. On one hand, it has been shown that calpain, a member of a family of Ca^{2+} -dependent proteases [13], can degrade the type-I Ins(1,4,5) P_3 receptor in disrupted cells [12,14], and that *N*-acetyl-Leu-Leu-norleucinal (ALLN), a peptide inhibitor of calpain and other cysteine proteases [13], can inhibit Ins(1,4,5) P_3 -receptor down-regulation in intact SH-SY5Y cells [12]. These and other data (for review, see [12]) have led to the proposal that increases in $[Ca^{2+}]_i$ in the proximity of open Ins(1,4,5) P_3 receptor channels can locally raise calpain activity

and thus specifically degrade Ins(1,4,5) P_3 receptors [12]. On the other hand, evidence has been presented that ubiquitin, a 76-residue protein that marks certain cellular proteins for degradation [15–17], becomes associated with type-I and/or type-III Ins(1,4,5) P_3 receptors in angiotensin-II-stimulated WB rat liver epithelial cells [10]. Further, the specific proteasome inhibitor lactacystin [18,19] blocked angiotensin-II-stimulated Ins(1,4,5) P_3 -receptor down-regulation [10]. These data suggest that Ins(1,4,5) P_3 receptors can be degraded by the proteasome, a cytosolic 26 S multi-protein complex that recognizes and degrades poly-ubiquitinated proteins [20–22]. Indeed, as ALLN is now known to inhibit the proteasome in addition to calpain [19,22,23], those previous studies with ALLN that provided evidence for the involvement of calpain in down-regulation [12] can now be considered to provide evidence for involvement of the proteasome.

Whereas it was thought originally that the ubiquitin/proteasome pathway might be tailored towards the degradation of soluble cytosolic or nuclear proteins [15], it is becoming increasingly evident that ER and plasma membrane proteins can also be degraded by this pathway [24–28]. This realization has raised many complex issues [24,25]. For example, because the proteasome and the enzymes that ubiquitinate proteins are not found inside the ER [15,25,29,30], it is not yet clear how luminal ER proteins are degraded by this pathway [24,25]. Further, for transmembrane ER and plasma-membrane proteins, it is unclear whether they are ubiquitinated and degraded by the proteasome while membrane-bound, or whether they are first dislocated from the membrane and released into the cytosol [24,25]. Clearly, this question will pertain to Ins(1,4,5) P_3 receptors if they are indeed degraded by the proteasome.

To address these and other questions we have investigated the role of the ubiquitin/proteasome pathway in Ins(1,4,5) P_3 -receptor down-regulation in a range of cell types. In summary, we

Abbreviations used: Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PIC, phosphoinositidase C; ALLN, *N*-acetyl-Leu-Leu-norleucinal; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; CCh, carbachol; BBS, bombesin; SP, substance P; CCK, cholecystokinin; PACAP, pituitary adenyllyl-cyclase-activating peptide; HMG, 3-hydroxy-3-methylglutaryl.

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find that all three $\text{Ins}(1,4,5)\text{P}_3$ -receptor types are ubiquitinated and degraded by the proteasome in response to PIC activation, that $\text{Ins}(1,4,5)\text{P}_3$ receptors are ubiquitinated and degraded while membrane-associated, that homomeric and heteromeric receptors are differentially susceptible to ubiquitination, and that a persistent increase in $\text{Ins}(1,4,5)\text{P}_3$ concentration appears to be the signal that mediates ubiquitination.

EXPERIMENTAL PROCEDURES

Materials

AR4-2J rat pancreatoma cells and neonatal rat cerebellar granule cells were cultured as described previously [3]. INS-1 rat insulinoma cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum/100 units/ml penicillin/100 µg/ml streptomycin/50 µM β -mercaptoethanol [31]. Rabbit polyclonal antisera CT1 and CT2 were raised against the C-termini of rat type-I and -II $\text{Ins}(1,4,5)\text{P}_3$ receptors, respectively, and were affinity purified and shown to be type-specific [3,7,9]. A type-III-receptor-specific mouse monoclonal antibody (TL3), raised against amino acids 22–230 of the human type-III receptor, was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit polyclonal anti-ubiquitin and mouse monoclonal anti-ubiquitin were purchased from the Dako Corporation (Carpinteria, CA, U.S.A.) and Chemicon International Inc. (Temecula, CA, U.S.A.), respectively. Peroxidase-conjugated antibodies, molecular-mass markers, silver-staining reagents, SDS, *N*-ethylmaleimide (NEM), Triton X-100, protease inhibitors and receptor agonists were obtained from Sigma (St. Louis, MO, U.S.A.); Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden); dithiothreitol (DTT) was from Bio-Rad (Hercules, CA, U.S.A.); lactacystin was from Biomol (Plymouth Meeting, PA, U.S.A.); ALLN and thapsigargin were from Alexis Corp. (San Diego, CA, U.S.A.); and chemiluminescence reagents were from Pierce (Rockford, IL, U.S.A.).

Electrophoresis and immunoblotting

Proteins in gel-loading buffer [50 mM Tris/HCl (pH 6.8)/100 mM DTT/2% SDS/0.1% Bromophenol Blue/10% glycerol] were subjected to 5% PAGE and were probed in immunoblots with primary antibodies (CT1, CT2, TL3 or rabbit polyclonal anti-ubiquitin) and then peroxidase-conjugated secondary antibodies. Immunoreactivity was detected with chemiluminescence and X-ray film [3,12] and was quantified by densitometric scanning of film. The molecular mass of immunoreactive bands was established by comparison with pre-stained standards of α -macroglobulin, β -galactosidase and fructose 6-phosphate kinase (molecular masses, 190, 108 and 89 kDa, respectively).

Measurement of $[\text{Ca}^{2+}]_i$ and $\text{Ins}(1,4,5)\text{P}_3$ formation

AR4-2J cells were detached from dishes with HBSE [155 mM NaCl/10 mM Hepes/0.7 mM EDTA (pH 7.4)], centrifuged (500 *g* for 2 min) and resuspended in 10–15 ml of Krebs/Hepes buffer [118 mM NaCl/4.7 mM KCl/25 mM NaHCO_3 /1.2 mM KH_2PO_4 /1.2 mM MgSO_4 /1.3 mM CaCl_2 /10 mM glucose/10 mM Hepes (pH 7.4)]. They were then centrifuged again (500 *g* for 2 min) and finally resuspended in Krebs/Hepes buffer at 3–6 mg of protein/ml. For $\text{Ins}(1,4,5)\text{P}_3$ quantification, cells were incubated at 37 °C for 50 min (to allow for cell recovery), were re-centrifuged (500 *g* for 2 min), resuspended in buffer at 6 mg of protein/ml, and 80 µl aliquots were dispensed into micro-centrifuge tubes containing 20 µl of stimuli (see the Results section for details). Incubations at 37 °C were terminated and the

$\text{Ins}(1,4,5)\text{P}_3$ concentration was measured with a radioreceptor assay as described in [32]. For measurement of $[\text{Ca}^{2+}]_i$, cells at 3 mg of protein/ml were incubated with 4 µM fura-2 acetoxymethyl ester at 37 °C for 50 min, were centrifuged (500 *g* for 2 min) and were finally resuspended in Krebs/Hepes buffer at 1.5 mg of protein/ml. $[\text{Ca}^{2+}]_i$ was then determined using a Perkin-Elmer LS 50B Luminescence Spectrometer and 0.15% Triton X-100/10 mM EGTA as calibrating agents [32].

Measurement of $\text{Ins}(1,4,5)\text{P}_3$ -receptor ubiquitination

In most experiments, control or stimulated cell monolayers were solubilized with ice-cold lysis buffer [50 mM Tris/150 mM NaCl/1% Triton X-100/1 mM EDTA/0.2 mM PMSF/10 µM leupeptin/10 µM pepstatin/0.2 µM soybean trypsin inhibitor (pH 8.0)] and were transferred to centrifuge tubes. NEM (2.5 mM) was then added to the lysate to inhibit de-ubiquitinating enzymes [33] followed, 1 min later, by 5 mM DTT, to neutralize the NEM. After 30 min on ice, the lysates were centrifuged (30000 *g* for 10 min at 4 °C). Supernatants (hereon referred to as 'cell extracts') were then collected and $\text{Ins}(1,4,5)\text{P}_3$ receptors were immunoprecipitated; cell extracts were incubated at 4 °C with CT1, CT2 or TL3 for 1 h and then for a further 1 h with Protein A-Sepharose CL-4B. Immune complexes were then isolated by centrifugation (500 *g* for 2 min), were washed 4 times with ice-cold lysis buffer and finally were resuspended in gel-loading buffer. The immunoprecipitates were then immunoblotted with CT1, CT2, TL3 or rabbit polyclonal anti-ubiquitin.

To determine whether homomeric type-II and -III receptors were ubiquitinated, AR4-2J and INS-1 cell extracts were first incubated with CT1 for 3 h and then Protein A-Sepharose CL-4B for 12 h to precipitate type-I-receptor-containing complexes (these relatively long incubations were employed to increase the efficiency of immunoprecipitation). The type-I-receptor-depleted extracts were then incubated for 1 h with CT2 or TL3 and then for 1 h with Protein A-Sepharose CL-4B to precipitate homomeric type-II and -III receptors, respectively.

To determine whether membrane-bound or perhaps soluble receptors were ubiquitinated, monolayers were rinsed with HBSE, were harvested in ice-cold homogenization buffer [10 mM Tris/1 mM EDTA/0.2 mM PMSF/10 µM leupeptin/10 µM pepstatin/0.2 µM soybean trypsin inhibitor/2.5 mM NEM (pH 8.0)] and were disrupted with 20 strokes of a glass/Teflon homogenizer. DTT, Tris and NaCl were then added to concentrations of 5, 50 and 150 mM, respectively, and the homogenate was centrifuged (100000 *g* for 30 min at 4 °C). The pellet was then re-homogenized in lysis buffer plus 2.5 mM NEM/5 mM DTT, and 1% Triton X-100 was added to the supernatant. Both the extracted pellet and the supernatant were then centrifuged (30000 *g* for 30 min at 4 °C), and the resulting supernatants were immunoprecipitated with CT2 and Protein A-Sepharose CL-4B.

Purification of ubiquitinated receptors

In some experiments, a two-step immunoprecipitation procedure was used to purify ubiquitinated receptors. In the first step, type-I-receptor-containing immune complexes were isolated from AR4-2J or INS-1 cell extracts with CT1 and Protein A-Sepharose CL-4B and were washed 4 times with ice-cold lysis buffer containing 0.2% Triton X-100. These complexes were then dissociated by heating (100 °C for 3 min) in 100 µl of Triton X-100-free lysis buffer supplemented with 1% SDS/20 mM DTT. The heated mixture was then centrifuged (500 *g* for 2 min) and the supernatant was removed and diluted with 1 ml of lysis buffer plus 1 mg of BSA. In the second step, ubiquitinated receptors

were purified by incubating half of the diluted supernatant with mouse monoclonal anti-ubiquitin for 3 h and then Protein A-Sepharose CL-4B for 12 h. As a control, the other half was incubated without antibody for 3 h and then Protein A-Sepharose CL-4B for 12 h. The immunoprecipitates were then immunoblotted with polyclonal rabbit anti-ubiquitin, CT1, CT2 or TL3.

Measurement of Ins(1,4,5) P_3 -receptor down-regulation

Cell monolayers were exposed to inhibitors and/or stimuli and membranes were prepared and probed as described [3]. To characterize the down-regulation of heteromeric and homomeric type-II receptors, control and cholecystokinin (CCK)-stimulated cells were solubilized with ice-cold lysis buffer containing 1 mM DTT, were incubated for 30 min on ice, were centrifuged (30000 *g* for 10 min at 4 °C) and supernatants were divided into two portions. One portion received only Protein A-Sepharose CL-4B and was centrifuged (500 *g* for 2 min), yielding supernatants that contained heteromeric and homomeric type-II receptors, termed 'whole lysate'. The other portion received CT1 and Protein A-Sepharose CL-4B and was centrifuged (500 *g* for 2 min), yielding supernatants containing only homomeric type-II receptors, termed 'type-I-Ins(1,4,5) P_3 -receptor-depleted lysate'. Both lysates were then mixed with 2 × gel-loading buffer and immunoblotted with CT1 or CT2.

RESULTS

Ins(1,4,5) P_3 -receptor down-regulation and intracellular signalling in AR4-2J cells

AR4-2J cells express predominantly type-II Ins(1,4,5) P_3 receptors (86 % of total) and smaller amounts of type-I and -III receptors (12 and 2 % of total, respectively) [3], and also express an array of PIC-linked cell-surface receptors [34,35]. We first wished to determine which of these PIC-linked receptors were able to elicit Ins(1,4,5) P_3 -receptor down-regulation and, thus, examined the effects of saturating concentrations of carbachol (CCh), bombesin (BBS), substance P (SP), pituitary adenylate cyclase-activating peptide (PACAP) and CCK. Figure 1(A) shows that BBS, PACAP and CCK, but not CCh or SP, markedly reduced type-I-, -II- and -III-receptor immunoreactivity and, interestingly, that whereas the reduction of type-I- and -III-receptor immunoreactivity was $\approx 80\%$, the decline in type-II-receptor immunoreactivity was only $\approx 60\%$.

We next examined the basis for the differences in the down-regulatory effects of the five stimuli. Clear differences in the ability of the stimuli to produce Ins(1,4,5) P_3 were evident (Figure 2A). Whereas each of the agonists produced significant increases at 10 s, from 10 min onwards only CCK, BBS and PACAP maintained Ins(1,4,5) P_3 concentration at significantly elevated levels (Figure 2A). Variation in the ability of PIC-linked receptors to catalyse phosphoinositide hydrolysis and generate Ins(1,4,5) P_3 has been described in detail elsewhere [34,36] and appears to result from differences in the extent to which these receptors are desensitized; some receptors (for example, CCK receptors) only partially desensitize and thus elevate Ins(1,4,5) P_3 levels persistently, whereas others (for example, SP receptors) completely desensitize and produce only a transient elevation.

In contrast, and remarkably, however, the five stimuli mobilized Ca^{2+} very similarly (Figure 2B; results for PACAP and BBS not shown). For each agonist, the maximal 'peak phase' $[Ca^{2+}]_i$ was very similar (470–540 nM at ≈ 10 s) and was significantly higher than control values ($P < 0.01$), and there was no significant post-peak 'plateau-phase' elevation of $[Ca^{2+}]_i$ ($P > 0.01$). In fact, the only discernable difference between the five stimuli was that

Figure 1 Ins(1,4,5) P_3 -receptor down-regulation and inhibition of this process in AR4-2J cells

(A) Cell monolayers were exposed to CCh (1 mM), BBS (500 nM), SP (500 nM), PACAP (100 nM) or CCK (500 nM) for 1 h. Membranes were then prepared and type-I-, -II- and -III-Ins(1,4,5) P_3 -receptor content was probed with CT1, CT2 and TL3, respectively. (B) Cell monolayers were pre-incubated for 2 h without inhibitor or with 8 μ M lactacystin or 20 μ g/ml ALLN. Cells were then exposed to 500 nM CCK for 1 h, membranes were prepared and Ins(1,4,5) P_3 -receptor content was assessed. Data shown are means $\pm$ S.E.M. (A) or means $\pm$ range (B) of quantified type-I-, -II- and -III-receptor chemiluminescence (detected at ≈ 260 , 245 and 250 kDa, respectively) from three (A) or two (B) independent experiments expressed as a percentage of values from control (unstimulated) cells.

the SP-induced increase in $[Ca^{2+}]_i$ declined relatively rapidly (Figure 2B), consistent with the fact that SP produced only a short-lived increase in Ins(1,4,5) P_3 concentration (Figure 2A). Thus, in these cells, as in other cell types [37,38], the relationship between Ins(1,4,5) P_3 concentration and $[Ca^{2+}]_i$ is not proportional. Whereas the basis of this disparity was not examined in the present study, a likely explanation is that Ins(1,4,5) P_3 was over-produced; the relatively small amount of Ins(1,4,5) P_3 produced by CCh at 10 s may be sufficient to give maximal Ca^{2+} -store mobilization, and additional Ins(1,4,5) P_3 (for example, that produced by CCK) cannot raise $[Ca^{2+}]_i$ further. In total, these data show that, in AR4-2J cells, Ins(1,4,5) P_3 -receptor down-regulation is seen only with those agonists that stimulate persistently Ins(1,4,5) P_3 formation (CCK, BBS and PACAP), and does not correlate with changes in $[Ca^{2+}]_i$.

Figure 2 Agonist-induced Ins(1,4,5) P_3 formation and Ca^{2+} mobilization in AR4-2J cells

(A) Cells in suspension were exposed to CCK (500 nM), CCh (1 mM), BBS (500 nM), SP (500 nM) or PACAP (100 nM) for 10 s, 1 min, 10 min or 1 h and Ins(1,4,5) P_3 content was measured. Data shown are means $\pm$ S.E.M. of three independent experiments. Significance of differences from control values (*, $P < 0.01$) were assessed by unpaired *t*-test. (B) Fura 2-loaded cells in suspension were stimulated as in (A) at the point indicated by the arrowhead, and $[Ca^{2+}]_i$ was determined. Data shown are traces representative of four independent experiments. Means $\pm$ S.E.M. ($n = 4$) of 'peak-phase' and 'plateau-phase' $[Ca^{2+}]_i$ (recorded at ≈ 10 and 100 s) were, respectively, 127 ± 9 and 147 ± 11 nM (control), 468 ± 23 and 169 ± 10 nM (CCK), 514 ± 21 and 166 ± 7 nM (CCh), 536 ± 22 and 170 ± 11 nM (BBS), 541 ± 8 and 169 ± 11 nM (SP), and 542 ± 26 and 166 ± 10 nM (PACAP).

The ubiquitin/proteasome pathway mediates Ins(1,4,5) P_3 -receptor down-regulation in AR4-2J cells

First we examined the effects of lactacystin. This compound is a specific inhibitor of the proteasome, and has been used in other studies to probe for proteasome-mediated events [18]. Figure 1(B) shows that lactacystin blocks CCK-induced type-I-, -II- and -III-Ins(1,4,5) P_3 -receptor down-regulation, indicating that the proteasome mediates down-regulation of all Ins(1,4,5) P_3 -receptor types. Similar blockade was obtained with ALLN (Figure 1B), indicating that this less-specific proteasome inhibitor [19] also inhibits proteasome-mediated Ins(1,4,5) P_3 -receptor degradation. Control experiments without CCK showed that incubation with lactacystin and ALLN alone had no effect on immunoreactivity (results not shown).

We next sought to determine whether Ins(1,4,5) P_3 receptors are ubiquitinated. Figure 3(A; upper panel) shows that receptors immunoprecipitated from unstimulated cells cross-react

very weakly with anti-ubiquitin (lane 1), but that those from CCK-stimulated cells cross-react more strongly (lane 2). Figure 3(A; upper panel) also shows that inclusion of ALLN greatly enhances the amount of ubiquitin immunoreactivity recovered from stimulated cells (compare lanes 2 and 4), apparently because ALLN inhibits the proteasome and causes the accumulation of ubiquitinated receptors. Further, ubiquitination was only seen with those stimuli that cause down-regulation (lanes 4, 6 and 8). The molecular masses of the putative ubiquitinated receptors in control or stimulated cells was centred around 271 ± 6 kDa and ranged between ≈ 260 and 320 kDa (see Figure 3, legend). In contrast, the molecular mass of unmodified type-II receptors was 247 ± 4 kDa (Figure 3A, lower panel). The extent of the molecular-mass difference between unmodified and putative ubiquitinated type-II receptors was most clearly seen when samples were probed with a mixture of CT2 and anti-ubiquitin (Figure 3B). The size of this difference and the diffuse or smeared nature of the ubiquitin immunoreactivity indicates that a spectrum of poly-ubiquitinated receptors have been formed: high-molecular-mass smears of ubiquitin immunoreactivity are typically observed upon poly-ubiquitination of relatively large proteins, for example, growth-factor receptors [26], 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase [28] and sodium-channel subunits [39].

In view of this concordance with previous studies, we were surprised that our data differed from those of Bokkala and Joseph [10], who found a 'ladder' of high-molecular-mass species in WB cells that were taken to be poly-ubiquitinated Ins(1,4,5) P_3 receptors. This result came from studies in which ubiquitinated species were immunoprecipitated with the same rabbit polyclonal anti-ubiquitin used to probe immunoblots in the present study. Thus to investigate this difference we repeated the previously described immunoprecipitation [10] and, surprisingly, also detected a ladder of high-molecular-mass immunoreactivity (results not shown). Remarkably, however, a control immunoprecipitation with rabbit polyclonal anti-ubiquitin alone produced the same pattern (Figure 3E), indicating that this preparation contained high-molecular-mass contaminants that produce signals in immunoblots. Comparison of the bands seen in Figure 3(E) with those that were previously designated poly-ubiquitinated Ins(1,4,5) P_3 receptors [10] reveals a remarkable degree of similarity. Thus the previously described ladder [10] appears not to be a measure of poly-ubiquitinated Ins(1,4,5) P_3 receptors. Rather, Ins(1,4,5) P_3 -receptor poly-ubiquitination appears to be reflected by a smear of immunoreactivity.

In view of this conclusion we were surprised initially that CT2 appeared to recognize only unmodified type-II receptor in the immunoprecipitates (Figure 3A, lower panel). The apparent inability of CT2 to recognize the high-molecular-mass ubiquitinated species in lanes 2, 4, 6 and 8 was not due to masking of the CT2 epitope by ubiquitin, since we show below that purified ubiquitinated type-II receptor does cross-react with CT2. Further, Figure 3(C) shows that the 1 h incubations used in Figure 3(A) were sufficient to achieve maximal ubiquitination. Rather, these data indicate that only a relatively small proportion of cellular type-II Ins(1,4,5) P_3 receptors is ubiquitinated at any given time, even in the presence of ALLN; this proportion appears to be too small to be detected by CT2, but can be detected by anti-ubiquitin, presumably because the receptors contain multiple ubiquitin residues.

Whereas it is to be expected that ubiquitinated receptors will be rare in the absence of ALLN, since they will be degraded by the proteasome, it is puzzling that they remain rare in the presence of ALLN, when the proteasome is blocked (Figure 3A, lanes 3–8). Possible reasons for this were examined. First, we

Figure 3 Ubiquitin is associated with Ins(1,4,5) P_3 receptors immunoprecipitated from AR4-2J cells

(A–D) Cells were pre-incubated for 2 h with 20 μ g/ml ALLN (except for lanes 1 and 2 in A) and were then stimulated as indicated; cells were exposed to CCK (500 nM), CCh (1 mM), BBS (500 nM), SP (500 nM) or PACAP (100 nM) for 1 h (A and B), 500 nM CCK for up to 1 h and then CCK-free medium for various times (C), or 500 nM CCK in the absence or presence of 1 μ M thapsigargin for 1 h (D). Ins(1,4,5) P_3 receptors were then immunoprecipitated with CT₂ and samples were probed with rabbit polyclonal anti-ubiquitin, CT₂ or rabbit polyclonal anti-ubiquitin plus CT₂. Data shown are images of chemiluminescence from the $\approx$ 220–380 kDa regions of gels (representative of two independent experiments), or are quantified chemiluminescence (means $\pm$ S.E.M. or means $\pm$ range, $n \geq 2$). The migration positions of unmodified type-II receptor (arrows, A and B; 247 $\pm$ 4 kDa) and of putative ubiquitinated Ins(1,4,5) P_3 receptor [square brackets, A, B and D; 259 ($\pm$ 2)–319 ($\pm$ 15) kDa] are indicated. (E) Rabbit polyclonal anti-ubiquitin alone was processed in a control immunoprecipitation and was probed with rabbit polyclonal anti-ubiquitin. The $\approx$ 220–380 kDa region of a representative gel is shown. The white arrowheads indicate the migration positions of high-molecular-mass immunoreactive contaminants. The black arrowhead indicates where unmodified type-II receptor would migrate to.

examined whether the free ubiquitin concentration declines and might limit ubiquitination in CCK-stimulated cells incubated in the presence of ALLN, but found this not to be the case (results not shown). We also examined whether de-ubiquitination was occurring, since this process [40] would counter the accumulation of ubiquitinated receptors. Figure 3(C) shows that withdrawal of CCK from cells maintained in the presence of ALLN resulted in a rapid decline in the level of ubiquitinated Ins(1,4,5) P_3 receptors. Thus, de-ubiquitinating enzymes [40] are active in AR4-2J cells, and these may suppress the build-up of ubiquitinated Ins(1,4,5) P_3 receptors during cell stimulation. De-ubiquitination may also explain another perplexing aspect of this work; that ALLN blocks the CCK-induced decline in the levels of unmodified receptor (Figure 3A, lower panel, lane 4), rather than simply causing ubiquitinated receptors to accumulate. In the presence of ALLN, de-ubiquitination will return receptors that have been ubiquitinated to their unmodified form and thus the level of unmodified receptor will remain relatively constant. In total, these data indicate that Ins(1,4,5) P_3 receptors are poly-ubiquitinated in response to stimuli that persistently activate PIC, and indicate that several factors govern the cellular level of ubiquitinated receptors.

Finally, Figure 3(D) shows that ubiquitination is blocked by the ER Ca^{2+} -ATPase inhibitor thapsigargin [41], which has been shown previously to block Ins(1,4,5) P_3 -receptor down-regulation

in SH-SY5Y cells without affecting agonist-induced Ins(1,4,5) P_3 formation [9]. Thapsigargin similarly blocked CCK-induced Ins(1,4,5) P_3 -receptor down-regulation in AR4-2J cells without affecting Ins(1,4,5) P_3 formation (results not shown).

Ins(1,4,5) P_3 receptors are ubiquitinated while membrane-bound

We next examined whether ubiquitination occurs when the receptors are membrane-bound or whether it might require their prior translocation to the cytosol. Thus control and stimulated AR4-2J cells were homogenized, cytosol and membrane fractions were prepared and the distribution of ubiquitinated Ins(1,4,5) P_3 receptors was determined. Figure 4 (lower panel) shows that ubiquitinated receptors are present in the membrane fraction (lane 6) but not the cytosol fraction (lane 4) and, indeed, that essentially all of the ubiquitin immunoreactivity present in the whole-cell extract can be recovered in the membrane fraction (compare lanes 2 and 6). Consistent with these findings, Ins(1,4,5) P_3 -receptor immunoreactivity did not appear in the cytosol after CCK stimulation (Figure 4, upper panel, lane 4). The absence of Ins(1,4,5) P_3 receptors, ubiquitinated or otherwise, from the cytosol of CCK-stimulated cells, shows that the receptors are not released into the cytosol prior to or after ubiquitination.

Figure 4 Ins(1,4,5) P_3 receptors are ubiquitinated while they are membrane-associated

Cells were pre-incubated with 20 μ g/ml ALLN for 2 h and were then incubated without or with 300 nM CCK for 30 min. Total cell extract (lanes 1 and 2) or cytosol and membrane fractions obtained from homogenized cells (lanes 3–6) were then prepared and immunoprecipitated with CT2. Samples were then electrophoresed and probed with either CT2 (upper panel) or rabbit polyclonal anti-ubiquitin (lower panel). Data shown are representative of two independent experiments and presented as in Figure 3(A).

Subtype specificity of Ins(1,4,5) P_3 -receptor ubiquitination

To establish that immunoprecipitated ubiquitin is truly Ins(1,4,5) P_3 receptor-associated and also to determine the type-specificity of ubiquitination, we employed a two-step immunoprecipitation procedure, involving a primary immunoprecipitation with Ins(1,4,5) P_3 -receptor antibody (CT1) to purify Ins(1,4,5) P_3 receptors, followed by a secondary immunoprecipitation with anti-ubiquitin to concentrate ubiquitinated species (mouse monoclonal anti-ubiquitin was used to avoid the contaminants present in rabbit polyclonal anti-ubiquitin). Figure 5 (lane 2) shows the results of this procedure applied to CCK-stimulated AR4-2J cells (lane 1 is a control secondary immunoprecipitation in which anti-ubiquitin was omitted). As expected, lane 2 shows that the secondary immunoprecipitate contains much ubiquitin (Figure 5, upper panel). Significantly though, CT1 and CT2 also recognized the ubiquitin-containing band (middle panels), indicating that type-I and -II receptors are directly associated with ubiquitin. Cross-reactivity of TL3 with the ubiquitin-containing band was also evident but was weak (lower panel), presumably because of the paucity of type-III receptors in AR4-2J cells. It is important to note that lane 2 also contains bands that co-migrated with, and appear to be, unmodified type-I and -II receptors (middle panels). However, these also appear in the control immunoprecipitation (lane 1) and thus were not immunoprecipitated by anti-ubiquitin. Rather, it appears that some CT1 and heteromeric Ins(1,4,5) P_3 receptors survived the heat denaturation at the end of the primary step and were carried over into the secondary step where they associated with Protein A–Sepharose CL-4B and were precipitated.

To determine unequivocally that type-III receptors are ubiquitinated we applied the same procedure to INS-1 cells, which contain approximately equal amounts of type-I and -III receptors, but no type-II receptors, and in which CCh stimulates Ins(1,4,5) P_3 -receptor down-regulation (R. J. H. Wojcikiewicz, unpublished work). In CCh-stimulated INS-1 cells (Figure 5, lane 4), the secondary immunoprecipitate again contained much

Figure 5 Type-I, -II and -III receptors are ubiquitinated

AR4-2J and INS-1 cells were preincubated with 20 μ g/ml ALLN for 2 h and were incubated for a further 1 h with 300 nM CCK or 1 mM CCh, respectively. Cell lysates were then immunoprecipitated with CT1 and Protein A–Sepharose CL-4B and immune complexes were dissociated by heating. The solubilized immune complexes were then secondarily immunoprecipitated with mouse monoclonal anti-ubiquitin and Protein A–Sepharose CL-4B (lanes 2 and 4). As controls for the secondary immunoprecipitation, duplicate samples were incubated with Protein A–Sepharose CL-4B alone (lanes 1 and 3). Samples were then electrophoresed and probed with rabbit polyclonal anti-ubiquitin, CT1, CT2 or TL3. Data shown (representative of two independent experiments) are presented as in Figure 3(A). The migration positions of type-I, -II and -III receptors (arrows) and of ubiquitinated Ins(1,4,5) P_3 receptors (square brackets) are indicated.

ubiquitin, and CT1 and TL3 cross-reacted with the ubiquitin-containing band. Again, lane 3 is a control secondary immunoprecipitation and shows the extent to which the signal in lane 4 is due to carry-over. Thus in INS-1 cells both type-I and -III receptors are clearly ubiquitinated. In total, these experiments show that concentration of ubiquitinated species makes it possible to demonstrate that ubiquitin becomes coupled to type-I, -II and -III receptors in stimulated cells.

Homomeric type-II and -III receptors are not ubiquitinated

Ins(1,4,5) P_3 receptors form both homomeric and heteromeric complexes in cells that express multiple receptor types [6]. To determine whether both homomeric and heteromeric type-II receptors are ubiquitinated in AR4-2J cells we attempted to separate the two populations on the basis that almost all of the heteromeric type-II receptors would be type-I-receptor-associated (since AR4-2J cells contain $\approx 86\%$ type-II receptors and $\approx 12\%$ type-I receptors). Figure 6A (upper panel, lane 1) indicates that CT1 immunoprecipitated approximately equal amounts of type-I and -II receptors from AR4-2J cell lysate (see the upper and lower bands, respectively) [3], and that subsequent immunoprecipitation of the same lysate with CT2 (lane 2) isolated essentially pure type-II receptors. These conclusions are supported by probing with CT1 and CT2 (Figure 6A, middle panels). Probing with rabbit polyclonal anti-ubiquitin showed surprisingly that ubiquitin was abundant only in the CT1

Figure 6 Homomeric type-II and -III receptors are not ubiquitinated

AR4-2J (**A**), INS-1 (**B**) and cerebellar granule (**C**) cells were pre-incubated with 20 μ g/ml ALLN for 2 h and were then incubated for 30 min with 300 nM CCK (AR4-2J cells) or 1 mM CCh (INS-1 and cerebellar granule cells). Cell lysates were then immunoprecipitated with CT1 to remove type-I-receptor-containing complexes (lanes 1) and AR4-2J and INS-1 cell lysates were then immunoprecipitated with CT2 or TL3, respectively, to isolate the remaining type-II or -III-receptor homomers (lanes 2). Samples were then electrophoresed and either silver stained or probed with CT1, CT2, TL3 or rabbit polyclonal anti-ubiquitin. Data shown (representative of two independent experiments) are presented and labelled as in Figure 5.

immunoprecipitate (lane 1). Thus, the homomeric type-II receptors in AR4-2J cells (lane 2) appear not to have been ubiquitinated. Figure 6(B) shows that a similar situation existed in INS-1 cells. In these cells, CT1 co-immunoprecipitated type-I and -III receptors that contained ubiquitin (lane 1), and subsequent immunoprecipitation with TL3 yielded type-III receptor homomers that did not contain ubiquitin (lane 2). In total, these data show that AR4-2J and INS-1 cells contain populations of homomeric type-II and -III receptors that are resistant to ubiquitination.

To examine the possibility that all homomers might be resistant to ubiquitination, we determined whether homomeric type-I receptors were ubiquitinated. Thus, type-I receptors were immunoprecipitated from cerebellar granule cells that express only type-I receptors [42] and in which CCh causes Ins(1,4,5) P_3 -receptor down-regulation [9,42]. CT1 immunoprecipitates from CCh-stimulated cells contained type-I receptors only and a considerable amount of ubiquitin immunoreactivity (Figure 6C). Thus, type-I receptor homomers can be ubiquitinated.

Homomeric type-II receptors are resistant to down-regulation

We next sought to determine whether the ubiquitination-resistant homomers detected in Figure 6 were resistant to down-regulation and chose to do these experiments in AR4-2J cells, in which evidence already exists that type-II receptors are somewhat resistant to down-regulation (Figure 1 and [3]). Figure 7(A) shows the effects of CCK on type-II receptor immunoreactivity in 'whole lysate' (containing heteromeric plus homomeric type-II receptors) and in 'type-I-receptor-depleted lysate' (containing only homomeric type-II receptors). Note that ALLN was absent from these experiments and thus that receptor down-regulation occurred. Type-II receptor immunoreactivity in the whole lysate was markedly reduced at 1 h, whereas that in the type-I-receptor-

depleted lysate did not change during the first 1 h and declined only slowly thereafter (Figure 7A). These data suggest that only those type-II receptors that are type-I-receptor-associated are rapidly down-regulated and, thus, that homomeric type-II receptors are resistant to degradation. The data shown in Figure 7(B) confirm that the type-I-receptor content of whole lysate was dramatically reduced by CCK treatment and demonstrate the effectiveness of type-I-receptor depletion.

The occurrence of degradation-resistant type-II receptor homomers is consistent with previous studies in AR4-2J cells [3] showing that type-I, -II and -III receptors are initially down-regulated at similar rates, but that prolonged exposure to CCK maximally down-regulates type-II receptors by only $\approx 60\%$. Confirmation that these homomers are not ubiquitinated is shown in Figure 7(C); after a 2 h exposure to CCK, CT2 immunoprecipitated essentially pure type-II receptor homomers with very little associated ubiquitin (lane 2). In contrast, when cells were stimulated for 15 min with CCK, CT2 immunoprecipitated a type-I- and -II-receptor mixture that was relatively strongly ubiquitinated (lane 1).

DISCUSSION

In total, the data presented indicate that the ubiquitin/proteasome pathway mediates type-I-, -II- and -III-Ins(1,4,5) P_3 -receptor down-regulation and that ubiquitination is mediated by Ins(1,4,5) P_3 formation. The essential features of the ubiquitin/proteasome pathway are the 26 S proteasome, which is composed of a central 20 S proteolytic core and two 19 S 'cap' structures [20–22], and three enzyme families (E1, E2 and E3) that catalyse the attachment of ubiquitin to proteins destined for degradation [15–17]. Once a protein is poly-ubiquitinated, it appears to be recognized and unfolded by the 19 S cap and then enters the 20 S core where it is cleaved [20–22]. Our findings that

Figure 7 Effects of CCK on heteromeric and homomeric type-II receptors

AR4-2J cells were incubated with 300 nM CCK for 0–4 h and were solubilized. (A and B) Lysates were processed to yield either 'whole lysates' or 'type-I-receptor-depleted lysates', were electrophoresed and then probed with either CT2 (A) or CT1 (B). Data shown are chemiluminescence from three independent experiments expressed as a percentage of that in whole lysates from unincubated cells (means $\pm$ S.E.M.). (C) Lysates were immunoprecipitated with CT2 and the immune complexes were probed with CT1, CT2 or rabbit polyclonal anti-ubiquitin. Data shown (representative of two independent experiments) are presented and labelled as in Figure 5.

Ins(1,4,5) P_3 receptors are ubiquitinated in AR4-2J, INS-1 and cerebellar granule cells can thus be taken as evidence for the involvement of the ubiquitin/proteasome pathway in Ins(1,4,5) P_3 -receptor down-regulation. This argument is confirmed by the findings that lactacystin and ALLN inhibit down-regulation and cause the accumulation of ubiquitinated receptor in stimulated cells.

These results are broadly in agreement with those of Bokkala and Joseph [10], who provided evidence that either one of or both type-I and -III receptors were ubiquitinated in WB cells in response to angiotensin II and that these receptors were then degraded by the proteasome. In the present study we have characterized Ins(1,4,5) P_3 -receptor ubiquitination in detail and have shown that all of the known Ins(1,4,5) P_3 -receptor types can be ubiquitinated. This has implications for the mechanism of ubiquitination, since type-I, -II and -III receptors are only

60–70% similar [1,2]: it is now apparent that the lysine residue or residues that is/are ubiquitinated [15–17] and the sequences that interact with ubiquitin-conjugating enzymes are conserved in the three receptors. This information provides insight into which sequences should be examined to define these sites. It is, however, noteworthy that, in contrast to the findings in WB cells [10], we did not detect a 'ladder' of ubiquitinated species, but rather a smear of ubiquitin immunoreactivity centred at $\approx$ 270 kDa, $\approx$ 25 kDa greater than unmodified receptors. Our examination of this discrepancy indicated that the previously detected ladder [10] was due to the presence of contaminants in the immunoprecipitating antibody. Evidence that the high-molecular-mass smear that we detect reflects poly-ubiquitinated receptors comes from studies in which poly-ubiquitination of other proteins yields a very similar smear of ubiquitin immunoreactivity [26,28,39]. Interpretation of the $\approx$ 25 kDa molecular-mass shift in terms of the number of ubiquitin residues added is difficult because of the unknown effect of introducing, perhaps at multiple sites, $\approx$ 8 kDa ubiquitin residues on receptor mobility in a denaturing gel. However, given the diffuse nature of the smear it appears that many residues may have been added. Finally, our data indicate that ubiquitinated receptors are substrates for de-ubiquitinating enzymes [40] that regenerate unmodified receptors. This may explain why the steady-state level of ubiquitinated receptors remains relatively low, even in the presence of proteasome inhibitors (this study and [10]).

Recently, there has been an explosion of interest in the possibility that ER proteins (both luminal and transmembrane) can be degraded by the proteasome, and that this might account for 'ER degradation' [43], a process by which inappropriately folded or oligomerized ER proteins are cleaved [24,25]. How aberrant ER proteins are delivered to the proteasome is currently being investigated intensively [24,25], and it is too early to judge how much of what is being learnt from these studies can be applied to native ER proteins such as Ins(1,4,5) P_3 receptors. Nevertheless, it is noteworthy that our data show that Ins(1,4,5) P_3 receptors become ubiquitinated, and indeed are degraded, while they are membrane-bound. This suggests that either Ins(1,4,5) P_3 receptors do not need to leave the ER membrane to be degraded by the proteasome, or that translocation from the membrane and proteasome-mediated degradation are tightly coupled processes. Interestingly, these findings parallel those from studies on other membrane proteins cleaved by the ubiquitin/proteasome pathway, for example, HMG-CoA reductase [28] and membrane transporters [44] in yeast.

Another major question concerns the signal that causes Ins(1,4,5) P_3 receptors to become ubiquitinated. Our data suggest that it is receptor activation by Ins(1,4,5) P_3 itself since, in AR4-2J cells, only those agonists that persistently elevate Ins(1,4,5) P_3 concentration cause ubiquitination and down-regulation. Of course, the possibility exists that activation of protein kinases C by the diacylglycerol that is generated simultaneously with Ins(1,4,5) P_3 , or increases in $[Ca^{2+}]_i$, could mediate the ubiquitination. However, previous studies have shown that activation of protein kinases C with phorbol ester does not elicit down-regulation [8,10] and the present study rules out increases in $[Ca^{2+}]_i$. Whereas it remains possible that the stimulus for ubiquitination is provided by activation of another receptor-initiated signalling pathway [45], the most rational explanation of our data is that a conformational change in the Ins(1,4,5) P_3 receptor induced by Ins(1,4,5) P_3 binding [46] facilitates ubiquitination. Support for this view comes from studies showing that mutant Ins(1,4,5) P_3 receptors that are unable to bind Ins(1,4,5) P_3 are not down-regulated [47] or ubiquitinated (C. C. Zhu and R. J. H. Wojcikiewicz, unpublished work) and is particularly attractive,

as it would explain why Ins(1,4,5) P_3 receptors are specifically down-regulated [3,9–12]. How a conformational change might lead to ubiquitination can only be speculated upon, however, since details of how ubiquitin-conjugating enzymes select their targets are largely unknown [16,17]. In this regard, it is noteworthy that thapsigargin blocks ubiquitination, indicating that this process requires ER Ca^{2+} stores to be intact. Intriguingly, proteasomal degradation of ER proteins in yeast has the same requirement [48].

We have also discovered a surprising degree of subtlety in the susceptibility of Ins(1,4,5) P_3 receptors to ubiquitination, since our data suggest that homomeric type-II receptors in AR4-2J cells and type-III receptors in INS-1 cells are resistant to ubiquitination, whereas type-II and -III receptors in association with type-I receptors are readily ubiquitinated. This was particularly evident in AR4-2J cells where, based on the estimate that 86% of receptors are type II [3], a polynomial equation [10,49] and the assumption that type-I, -II and -III receptors associate randomly, it can be calculated that $\approx 55\%$ of the cell's receptor complement will be type-II-receptor homotetramers. The resistance of these homotetramers to ubiquitination may explain the reduced sensitivity of type-II receptors to down-regulation as compared with type-I and -III receptors (Figure 1 and [3]). Why homomeric type-II and -III receptors should be resistant to ubiquitination was not examined in the present study, but may be related to their location within the cell. Evidence supporting this view comes from immunofluorescence studies in AR4-2J cells, showing that exposure to CCK reduces type-II receptor levels in general, but leaves a substantial fraction of type-II receptors located in punctate structures that are resistant to down-regulation [50]. Perhaps these type-II receptors are located in such a way that they are not susceptible to ubiquitination.

In summary, the data presented provide clear evidence that extracellular stimuli that activate PIC can cause Ins(1,4,5) P_3 -receptor degradation via the ubiquitin/proteasome pathway. Ins(1,4,5) P_3 receptors can thus be added to the growing list of proteins that are selectively destroyed in this manner. It will now be fascinating to define the elements that link cell-surface receptor activation to Ins(1,4,5) P_3 -receptor ubiquitination.

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