Mechanism of Proteasomal Degradation of Inositol Trisphosphate Receptors in CHO-K1 Cells*

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**The activation of inositol 1,4,5-trisphosphate receptors (IP₃Rs)² by IP₃ initiates Ca²⁺ mobilization from the ER and triggers the Ca²⁺ signal that underlies alterations in cell function elicited by a diverse array of cell surface stimuli (1, 2). A commonly observed characteristic of cells is that they adapt their responses when chronically stimulated. In the case of cell surface receptors, this is usually the result of phosphorylation and/or internalization. Wojcikiewicz et al. (3, 4) were the first to note that chronic stimulation of cultured SHSY-5Y neuroblastoma cells with carbachol for 6 h causes 90% loss of type I IP₃R protein by a mechanism involving the marked acceleration of IP₃R degradation. Subsequently, similar effects on IP₃R degradation have been described in many different experimental systems with many different agonists (5–16). Down-regulation of IP₃R protein is associated with a decrease in the ability of IP₃ to mobilize Ca²⁺ (4, 8). More recently it has been shown that the frequency of elementary Ca²⁺ puffs that trigger propagating Ca²⁺ waves in cells is decreased by chronic agonist stimulation (14). The physiological significance of IP₃R down-regulation is unknown. However, down-regulation of receptors would be expected to inhibit the global Ca²⁺ signal elicited by all Ca²⁺ mobilizing agonists (heterologous desensitization). If this is the only component of the signal transduction system that is down-regulated, then an imbalance in signaling pathways would exist and may have profound consequences for cellular responses. A physiologically relevant example is the response of pituitary cells to GnRH receptor agonists (17). Mammalian GnRH receptors lack a C-terminal tail and therefore do not bind arrestin. Consequently these receptors desensitize and internalize only very slowly. However, sustained activation of GnRH receptors does lead to desensitization of gonadotrophin hormone secretion, which suggests the presence of adaptive mechanisms distal to the cell surface receptor. Treatment with GnRH receptor agonists produce a rapid and pronounced degradation of IP₃Rs, and this is associated with a marked reduction in the IP₃-mediated Ca²⁺ signal (15, 18).

The mechanism of IP₃R degradation has not been established. Previous studies have shown that chronic elevation of IP₃ and IP₃ binding to the receptor are required to facilitate IP₃R degradation (19, 20). It has been proposed that the sustained elevation of IP₃ causes the IP₃R to adopt a conformation that exposes sites that become ubiquitinated. The proteasome pathway then degrades the ubiquitinated IP₃R receptor. The regions of the IP₃R involved in ubiquitination have not been determined. Pretreatment of cells with the SERCA pump inhibitor thapsigargin has been found to inhibit agonist-mediated IP₃R degradation, suggesting that Ca²⁺ also plays a role in IP₃R degradation (4). In the present study we have examined IP₃R degradation in CHO-K1 cells stimulated with carbachol. This system has been used to examine the domains of IP₃Rs that are ubiquitinated and to further explore the role of Ca²⁺ in IP₃R degradation. The ability to readily transfect CHO-K1 cells with various IP₃R constructs has allowed an initial characterization of the substrate recognition properties of the IP₃R degradation system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbachol, acetylcholine, atropine, ALLN, and ALLM were purchased from Sigma. Lactacystin was purchased from Dr. E. J. Corey (Harvard University). ZL3VS was a kind gift of Dr. Mathew Bogoy (Stanford University). Stabilized acrylamide solution (Protogel) for the preparation of SDS gels was obtained from National Diagnostics (Atlanta, GA). Recombinant caspase-3 was purified from BL-21 bacterial lysates expressing the enzyme as a His₅-tagged fusion protein using previously described methods (21). The plasmid encoding caspase-3 was kindly given by Dr. E. S. Alnemri.

**Antibodies**—The type I polyclonal antibody was raised to unique amino acids 2731–2749 present at the C terminus of the rat type I IP₃R (CT-1) and has been previously characterized (22, 23). An N-terminal polyclonal antibody was raised to amino acids 326–341 of the type I
IP₃R (NT-1). This Ab was used after affinity purification using the immobilized antigenic peptide as a column matrix. The type III IP₃R monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Monoclonal Ab to ubiquitin was purchased from StressGen (Victoria, Canada). Myc monoclonal Ab (9E11) was obtained from the Cell Center of the University of Pennsylvania.

Expression Constructs—The cDNA encoding rat type I IP₃R SII(-)/SII(+) splice variant in pCMV3 was the kind gift of Dr. Thomas Sudhof (University of Texas Southwestern Medical Center) (24). The cDNA encoding the rat type I IP₃R SII (+) variant was the kind gift of Dr. Gregory Mignery (Loyola University Chicago) (25). The D2550A pore-defective mutant in SII (-) (26) was transferred to the SII (+) IP₃R using BstBI/XbaI restriction sites. The Myc-tagged constructs were made using PCR as described previously (27).

Cell Culture and Pretreatment—CHO-K1 cells were obtained from ATCC (catalog number CCL-61) and from Dr. John Pastorino. The cells were grown in Dulbecco’s minimal essential medium containing 5% fetal bovine serum and 1% penicillin/streptomycin. The confluent cells were grown in Dulbecco’s minimal essential medium containing 5% fetal bovine serum and then in HEPES buffer containing 0.25% bovine serum albumin and then in HEPES buffer containing 0.25% bovine serum albumin and 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science) (solubilization buffer). Insoluble material was removed by centrifugation for 10 min at 25,000 × g. 20 μg of protein was loaded on 5% SDS-PAGE, and the separated polypeptides were transferred to nitrocellulose. Repeated immunoblotting of the same nitrocellulose sheet was carried out after treating blots for 30 min at 60 °C in a stripping buffer containing 65 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol.

Measurement of Ca²⁺ Mobilization—Changes in cytosolic [Ca²⁺] in individual CHO cells plated on coverslips were measured by digital imaging fluorescence microscopy as previously described (28). Briefly, the cells were loaded with Fura-2 by incubating with 5 μM Fura-2AM in 0.03% pluronic F-127 in the presence of 100 μM sulfinpyrazone for 30 min at 37 °C. The coverslips were washed with HEPES buffer (10 mM HEPES, 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 10 mM glucose, pH 7.4) containing 2% bovine serum albumin and then in HEPES buffer containing 0.25% bovine serum albumin. Coverslips with Fura-2-loaded cells were transferred into a chamber with 1 ml of HEPES buffer with or without Ca²⁺ and mounted onto the stage of an inverted microscope thermostatically maintained at 37 °C. Fluorescence images with a 4-s delay were collected alternately at excitation wavelengths of 340 and 380 nm with an emission wavelength of 460–600 nm. [Ca²⁺], for each individual cell was calculated from the fluorescence values at each time point using the ratio method as described previously (28).

Measurement of IP₃R Ubiquitination—To measure ubiquitination CHO-K1 cells were pretreated for 1 h with or without proteasomal inhibitors and then treated for 4–6 h with 1 mM carbachol. The cells were then treated for 10 min at room temperature with phosphate-buffered saline containing 1 mM NEM and lysed in solubilization buffer supplemented with 1 mM NEM. The NEM was added to block deubiquitinating enzymes. The lysate was then immunoprecipitated overnight with CT-1 Ab. The immunoprecipitates were electrophoresed on 5% SDS-PAGE, transferred to nitrocellulose, and probed with a 1:5000 dilution of anti-ubiquitin Ab (StressGen). To resolve IP₃R domains that contained ubiquitin, we employed a Myc-tagged Ub plasmid (kindly given by Dr. Ron Kopito) that was transfected together with the type I IP₃R into 4 × 150-mm plates (20 μg of DNA each/plate) using LT-1 as a transfection reagent (Mirus Corp., Madison, WI). After 48 h the plates were treated for 1 h with a mixture of proteasomal inhibitors consisting of 10 μM lactacystin, 50 μM ZL3VS, and 50 μM ALLN. The plates were then treated with 1 mM carbachol, lysed as described above in NEM-containing medium, and immunoprecipitated overnight with CT-1 Ab. After washing twice in solubilization buffer, an aliquot of the immunoprecipitate (80%) was digested with caspase-3 in 200 μl of a buffer containing 50 mM HEPES (pH 7.2), 1 mM dithiothreitol, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 1 μg recombinant caspase-3. 20% of this sample was quenched with SDS-PAGE buffer and was used as the uncut control sample. The digested fragments were released from the PrA-Sepharose beads by 5 min of treatment at 95 °C with 0.35% s glycin (pH 2.8) and 0.1% Triton X-100. The sample was diluted in lysis buffer and reimmunoprecipitated with Myc-Ab to recover the ubiquitinated fragments. The uncut control sample and the Myc reimmunoprecipitated samples were processed on 10% SDS-PAGE and immunoblotted with IP₃R Abs to locate the ubiquitinated fragments.

RESULTS

Basic Characterization of IP₃R Down-regulation in CHO-K1 Cells—Our previous studies on IP₃R degradation were carried out in WB rat liver epithelial cells stimulated with angiotensin II (8, 30). These cells have proved very difficult to transiently transfected with IP₃R constructs, and stable WB cell lines lose their IP₃R degradation response to angiotensin II (data not shown). We therefore examined other cells types that were more amenable to transfection for an agonist-mediated IP₃R degradation response. Fig. 1A shows that carbachol (Cch) addition to CHO-K1 cells stimulated IP₃R down-regulation. Similar Cch effects were seen when the plots were reprocured for the type III IP₃R isoform (data not shown). The effect was specific to Cch because ATP, which also elevates Ca²⁺ in these cells (cf. Fig. 5), did not induce significant IP₃R degradation. To further characterize these effects we examined time course and dose-response relationships (Fig. 1, B and C). Densitometric analysis of immunoblots showed that IP₃R down-regulation was detected within 2 h and peaked between 4 and 6 h of Cch stimulation (Fig. 1B). This pattern of response is comparable with what has been observed for agonist-mediated IP₃R down-regulation in other systems (8). Fig. 1C shows the dose response for IP₃R down-regulation using carbachol, methacholine, and acetylcholine as agonists. The dose response for the carbachol effect was complex and showed two distinct components with half-maximal inhibitory effects at ~20 and ~80–100 μM. Methacholine was somewhat more potent than carbachol (IC₅₀ = ~10 μM). Acetylcholine was the least potent agonist with half-maximal effects at 50 μM and only a 40% down-regulation even at 1 mM. These data, together with the finding that the carbachol effects on down-regulation are completely blocked by atropine (Fig. 2), suggest that IP₃R down-regulation is mediated by endogenous muscarinic receptors in CHO-K1 cells.

The role of different proteolytic pathways in IP₃R degradation was investigated using various protease inhibitors (Fig. 2). The most pronounced inhibition of Cch-mediated IP₃R degradation was observed with the proteasomal inhibitor lactacystin. Rather surprisingly, 50 μM of the peptide aldehyde inhibitor ALLN, which is effective at preventing proteasomal degradation of IP₃Rs in WB cells (8), exerted only a small inhibitory effect on Cch-mediated IP₃R degradation. A small inhibitory effect of 100 μM chloroquine was also observed. This is probably an indirect effect and unlikely to reflect the involvement of the lysosomal pathway because 5 mM NH₄Cl was without effect. Similarly the lack of

[Image 234x26 to 262x38]
effects of ALLM, DEVD-CHO, and calpain inhibitor would rule out the involvement of general cysteine proteases, caspases, and calpains, respectively. Thus the data would suggest that the major proteolytic system involved in Cch-mediated IP$_3$R degradation is the proteasomal pathway.

Ubiquitination Domains in IP$_3$R—We first carried out experiments to confirm that the proteasomal degradation of IP$_3$Rs was accompanied by ubiquitination. CHO cell lysates were treated with Cch in the presence or absence of lactacystin, and the immunoprecipitated IP$_3$R was probed for ubiquitin with anti-Ub Ab (Fig. 3A). The data show that ubiquitinated IP$_3$R accumulated only when the cells were treated with a combination of Cch and lactacystin (Fig. 3A, lane 3). There are 159 cytosol-exposed lysines in the primary sequence of the type I IP$_3$R that could potentially serve as sites for ubiquitination. Our limited objective in the present study was to identify domains in the IP$_3$R that may serve as targets of ubiquitination. The strategy we chose to employ was to digest the ubiquitinated IP$_3$R with a protease that would cleave the IP$_3$R but not ubiquitin. A protease that satisfies this criteria is caspase-3, which is known to cleave IP$_3$Rs (31, 32). Initial experiments to detect endogenous Ub attached to caspase-3-cleaved IP$_3$R fragments were limited by the poor sensitivity of the available anti-Ub Abs. For this reason we chose to transfect the CHO cells with Myc-tagged Ub. However, transfection with Myc-tagged Ub alone did not significantly label endogenous receptors immunoprecipitated from CHO cells treated with Cch and lactacystin (Fig. 3B, lane 1). Initial experiments to detect endogenous Ub attached to caspase-3-cleaved IP$_3$R fragments were limited by the poor sensitivity of the available anti-Ub Abs. For this reason we chose to transfect the CHO cells with Myc-tagged Ub. However, transfection with Myc-tagged Ub alone did not significantly label endogenous IP$_3$Rs immunoprecipitated from CHO cells treated with Cch and lactacystin (Fig. 3B, lane 1). At high exposures of the blot it was possible to detect a single band at the expected migration of IP$_3$R, indicating that endogenous receptors may become monoubiquitinated under these conditions (Fig. 3B, lane 3). A weak labeling of endogenous receptors by Myc-tagged Ub has also been observed in H9251/T3–1 anterior pituitary cells (18). In part, this may be related to the low transfection efficiency of our cells, which was estimated at 10–20% by indirect immunofluorescence measurements (data not shown). To maximize the signal we cotransfected CHO cells with both Myc-tagged Ub and IP$_3$R cDNA. Under these conditions a significant increase in Myc ubiquitinated IP$_3$R was observed when IP$_3$R was immunoprecipitated from CHO cells treated with Cch and lactacystin (Fig. 3B, lane 2).

FIGURE 1. Time course and dose response of IP$_3$R down-regulation by carbachol in CHO cells. A, cells were serum-deprived overnight and treated with ATP (1 mM) or Cch (1 mM). The cell lysates were electrophoresed on a 5% gel, transferred to nitrocellulose, and probed with type I IP$_3$R Ab. The upper panel shows a representative blot. The lower panel shows quantitation of the blots by densitometric analysis of three to five independent experiments. The data are expressed as percentages of IP$_3$R levels in untreated control samples. B, CHO cells were incubated with 1 mM carbachol for the indicated time periods and were analyzed for type I IP$_3$R by immunoblotting. C, cells were incubated with different concentrations of carbachol ( ), acetylcholine ( ), or methacholine ( ) for 4 h and were analyzed for type I IP$_3$R immunoreactivity. The data in both panels are expressed as percentages of the control untreated sample and are the means ± S.E. of three experiments.

FIGURE 2. The effect of protease inhibitors on carbachol-mediated IP$_3$R degradation. CHO cells were incubated with 1 mM carbachol for 6 h in the absence or presence of protease inhibitors and were analyzed for type I IP$_3$R by immunoblotting. The concentration of drugs used were as follows: atropine (100 μM), ALLN (50 μM), ALLM (50 μM), lactacystin (10 μM), caspase inhibitor DEVD-CHO (100 μM), chloroquine (1 mM), and calpain inhibitor MDL-28170 (20 μM). None of the agents changed IP$_3$R levels when added alone with the exception of NH$_4$Cl and chloroquine, which increased levels by 44 ± 14 and 35 ± 7.9%, respectively. *, p > 0.05 significant from carbachol alone. The data are the means ± S.E. for three to five experiments.
fragment and a 215-kDa N-terminal fragment. IP$_3$R immunoprecipitates were prepared from CHO cells cotransfected with Myc-Ub and type I IP$_3$R plasmids that were then treated with Cch in the presence of proteasomal inhibitors. IP$_3$R was immunoprecipitated with Myc-Ab as described under "Experimental Procedures." Both uncut and Myc reimmunoprecipitated samples were processed on 10% SDS-PAGE buffer and used as the uncut control sample (lanes 1, 3, and 5). The remaining 80% was digested with caspase-3 and reimmunoprecipitated with Myc Ab as described under "Experimental Procedures." Both uncut and Myc reimmunoprecipitated samples were processed on 10% SDS-PAGE and consecutively immunoblotted with the indicated Abs. The brackets and arrows indicate the putative polyubiquitinated and monoubiquitinated species, respectively. In lanes 7 and 8 nonubiquitinated lysates were immunoprecipitated with CT-1 Ab, and control and caspase-3-digested samples were probed by immunoblotting with NT-1 Ab to locate the N-terminal cleavage fragment (arrowhead).

**FIGURE 4. Localization of ubiquitination sites in the type I IP$_3$R using Myc-tagged Ub sites of ubiquitination in type I IP$_3$R.** A, the schematic diagram shows the location of eight putative caspase-3 cleavage sites in the type I IP$_3$R based on the presence of a DXXD consensus sequence. The calculated size of the C-terminal fragment (indicated in bold type) and the N-terminal fragment (italics) for cleavage at each of the sites is shown. The open arrows indicate the locations of the epitopes of the CT-1 and NT-1 Abs used in this study. The locations of the splice sites are also shown. B, lysates were prepared from CHO cells transfected with Myc-tagged Ub and type I IP$_3$R and then treated with 1 mM Cch for 6 h. The lysates were immunoprecipitated overnight with CT-1 Ab, and 20% of this sample was quenched with SDS-PAGE buffer and used as the uncut control sample (lanes 1, 3, and 5). The remaining 80% was digested with caspase-3 and reimmunoprecipitated with Myc Ab as described under "Experimental Procedures." Both uncut and Myc reimmunoprecipitated samples were processed on 10% SDS-PAGE and consecutively immunoblotted with the indicated Abs. The brackets and arrows indicate the putative polyubiquitinated and monoubiquitinated species, respectively. In lanes 7 and 8 nonubiquitinated lysates were immunoprecipitated with CT-1 Ab, and control and caspase-3-digested samples were probed by immunoblotting with NT-1 Ab to locate the N-terminal cleavage fragment (arrowhead).

**FIGURE 3. Ubiquitination of IP$_3$Rs in CHO cells.** A, CHO cells were treated with Cch for 4 h with and without a preincubation for 30 min with 10 mM lactacystin (LC). The cells were lysed in buffer containing 1 mM NEM, and endogenous type I IP$_3$Rs were immunoprecipitated (IP) with CT-1 Ab. The immunoprecipitates were processed on 5% SDS-PAGE and immunoblotted (IB) with Anti-Ub Ab. B, CHO cells were transfected with Myc-Ub plasmid alone (lane 1) or together with type I IP$_3$R DNA (lanes 2–4). The cells were treated as in A except that proteasomal inhibitors (50 mM ZL3VS and ALLN) were included together with the LC. Where indicated Cch (1 mM) was added for 5 h. The lysates were immunoprecipitated with CT-1 Ab and then cleaved with caspase-3. The fragments formed were reimmunoprecipitated with Myc-Ab to bring down the Myc-tagged cleavage fragments (Fig. 4B). Analysis of the fragments by immunoblotting with Myc Ab showed a prominent single band at 95 kDa and a smeared signal that extended from 119 to 230 kDa (Fig. 4B, lane 2). The 95-kDa band was cross-reactive with the CT-1 Ab and corresponds to the expected C-terminal caspase-3 cleavage product (Fig. 4B, lane 4). Because it appears as a single band, we propose that the C-terminal segment of IP$_3$Rs can be monoubiquitinated. The smeared signal seen in the Myc Ab blots was cross-reactive with the N-terminal Ab (lane 6) but not the C-terminal Ab (lane 4). We conclude that polyubiquitination is confined to the N-terminal segments of the IP$_3$R. When caspase-3-cleaved IP$_3$R was examined with N-terminal Ab, a fragment of ~95 kDa was observed rather than the expected 215-kDa fragment (Fig. 4B, lanes 7 and 8). This suggests that in vitro caspase may cleave at other sites in addition to aspartate 1892. The size of the 95-kDa N-terminal fragment is compatible with cleavage at aspartate 820, which would locate the polyubiquitination sites proximal to this amino acid. The spread of the Myc-Ub signal would suggest heterogeneity in the size of the polyubiquitin chains and/or the use of multiple attachment sites in the N-terminal domain.

**The Role of Ca$^{2+}$ in Carbachol-mediated IP$_3$R Degradation—**Fig. 5A shows the Ca$^{2+}$ transients recorded in Fura-2-loaded CHO-K1 cells in response to a maximal dose of Cch that triggers IP$_3$R degradation. Cch addition elicited a Ca$^{2+}$ signal that remained elevated for a prolonged period (>30 min). By contrast a Ca$^{2+}$-mobilizing stimulus such as ATP, which did not cause IP$_3$R degradation, produced a smaller and more transient Ca$^{2+}$ signal. The ATP responses observed in cells that were pretreated with Cch for 6 h was substantially blunted as would be anticipated if IP$_3$Rs were degraded by Cch pretreatment (Fig. 5B). To further examine the role of Ca$^{2+}$, we pretreated the cells for 30 min with thapsigargin (to empty intracellular stores) or EGTA (to remove extracellu-
The effect of buffering the cytosolic Ca\textsuperscript{2+} degradation, in agreement with previous studies on other cell types (4).

Fig. 6. The effect of inhibitors of Ca\textsuperscript{2+} mobilization on Cch-mediated IP\textsubscript{3}R degradation. A, CHO cells were incubated with 1 mM Cch for 4 h with or without pretreatment with 2 \mu M thapsigargin or 1 mM EGTA for 30 min. The lysates were analyzed for type I IP\textsubscript{3}R by immunoblotting. B shows the effect of BAPTA-AM (100 \mu M) addition at the indicated times after the addition of 1 mM carbachol. In all cases the cells were lysed after 6 h of treatment with agonist and analyzed for type I IP\textsubscript{3}R by immunoblotting. The Cch-mediated Ca\textsuperscript{2+} transient was completely inhibited within 5 min of addition of BAPTA-AM to the cells (data not shown). C, CHO cells were exposed to 1 mM Cch for 15, 30, 60, and 90 min prior to the addition of 0.1 mM atropine followed by an additional 4-h incubation. The levels of type I IP\textsubscript{3}R were compared with cells continuously exposed to Cch for 4 h.

The interpretation of the finding that thapsigargin, EGTA, and BAPTA-AM inhibit IP\textsubscript{3}R degradation is complicated by the known Ca\textsuperscript{2+} sensitivity of phospholipase C (PLC) in CHO cells (33). Because it has been established that chronic elevation of IP\textsubscript{3} and IP\textsubscript{3} binding to the IP\textsubscript{3}R are prerequisite for obtaining IP\textsubscript{3}R degradation (19, 20), the perturbing effect of Ca\textsuperscript{2+} chelating and mobilizing agents could reflect an inadequate PLC-dependent generation of IP\textsubscript{3}. To distinguish between Ca\textsuperscript{2+}-dependent and IP\textsubscript{3}-dependent effects, we carried out the experiments shown in Fig. 7. Extracellular Ca\textsuperscript{2+} was chelated with EGTA, and the cells were stimulated with Cch. This resulted in a Ca\textsuperscript{2+} elevation that was transient and returned to base line within 5 min (Fig. 7A). This transient elevation of Ca\textsuperscript{2+} is clearly insufficient to sustain IP\textsubscript{3}R degradation (Fig. 6A). Depletion of intracellular stores is known to activate Ca\textsuperscript{2+} entry channels in the plasma membrane. This can be experimentally monitored as a marked elevation of cytosolic Ca\textsuperscript{2+} when extracellular Ca\textsuperscript{2+} is added back to store-depleted cells (Fig. 7A). Using this protocol we noted that the addition of Ca\textsuperscript{2+} also caused marked IP\textsubscript{3}R degradation (Fig. 7B) and occurred even when Ca\textsuperscript{2+} was added back 1 h after carbachol. The effect of Ca\textsuperscript{2+} was not mimicked by the addition of Mn\textsuperscript{2+}, Ba\textsuperscript{2+}, or Sr\textsuperscript{2+} (Fig. 7C). There was an absolute requirement for receptor stimulation because Ca\textsuperscript{2+} addition in the absence of Cch did not cause IP\textsubscript{3}R degradation (Fig. 7C, lane 8) or promote Ca\textsuperscript{2+} entry (Fig. 7A). The effect of Ca\textsuperscript{2+} addition was completely blocked by pretreatment with proteasomal inhibitors (Fig. 7D).

The addition of thapsigargin prior to Ca\textsuperscript{2+} should prevent Ca\textsuperscript{2+} entry into the ER lumen without preventing the elevation of Ca\textsuperscript{2+} in the cytosol or the consequent activation of PLC. Thapsigargin addition was found to inhibit the IP\textsubscript{3}R degradation caused by Ca\textsuperscript{2+} addition, indicating that Ca\textsuperscript{2+} entry into the lumen of the ER is necessary for initiating IP\textsubscript{3}R degradation (Fig. 7E). The importance of luminal ER Ca\textsuperscript{2+} is further re-enforced by the experiment shown in Fig. 7F where CHO cells were incubated for 30 min with 10 mM Ca\textsuperscript{2+} to load the intracellular stores before removal of extracellular Ca\textsuperscript{2+} with EGTA. Under these conditions EGTA treatment failed to block Cch-induced IP\textsubscript{3}R degradation.

Degradation of Exogenously Transfected IP\textsubscript{3}R Constructs—The ability of Cch to induce the degradation of epitope-tagged IP\textsubscript{3}Rs was examined in transiently transfected CHO cells (Fig. 8). Previous studies have shown that stably transfected C-terminally hemagglutinin-tagged type I
IP$_3$Rs were degraded more slowly than wild-type IP$_3$Rs in SH-SY5Y cells (19) or were not degraded at all in transiently transfected /H9251 T3–1 cells (18). In the latter study it was shown that the tagged construct was still a substrate for ubiquitination. We confirmed that the C- and N-terminal Myc-tagged constructs were not degraded in transiently transfected CHO cells (Fig. 8A). However, untagged IP$_3$R was also not degraded (Fig. 8B, closed circles). This was observed over a wide range of expression levels as determined by blotting with CT-1 Ab, which would detect both endogenous and overexpressed receptors. The inability to detect even the degradation expected for endogenous receptors may suggest that the untagged receptor exerts a dominant negative effect in these experiments. The type I IP$_3$R contains 3 sites that are alternatively spliced (Fig. 4A). All of the IP$_3$R constructs used so far in these studies are derived from the rat cDNA and correspond to the SI(−),SII(+),SIII(−) splice variant (24). Fig. 8 (A and B) shows that the untagged SI(+),SII(+),SIII(−) variant could be degraded when transiently transfected into CHO cells. To be able to specifically recognize the transfected construct without using epitope tags, we made use of the observation that only neuronal cells contain the SII(SII) splice variant, whereas the SII(SIII) form is present in all peripheral tissues (35). We utilized an Ab to a sequence within the SII(SII) splice domain (36) and confirmed that it preferentially recognized the IP$_3$R from cerebellum but not the endogenous receptor in CHO cells (Fig. 8, C and D). The SII Ab was used to detect the transiently transfected SI splice variants in CHO cells and confirmed our observation that only the SI(SII) splice variant was degraded (Fig. 8D).

An explanation for this preference for the SI(+ ) form may be that it reflects the endogenously occurring form of the IP$_3$R in CHO cells. To test this we cleaved the IP$_3$R in microsomes from CHO cells with trypsin...
and compared the size of the N-terminal tryptic fragment with the corresponding fragments from cerebellum microsomes and COS-7 cell microsomes prepared from SI(H11001) and SI(H11002) transfected cells (Fig. 8E). Cerebellum microsomes generate a doublet of bands at 38 and 43 kDa corresponding to the presence of both SI(H11001) and SI(H11002) forms in this tissue (36). CHO cells contained only a single band corresponding to the SI(H11001) form.

It has been suggested that Ca\textsuperscript{2+}/H11001 translocation through a functional IP\textsubscript{3}R channel and the local accumulation of Ca\textsuperscript{2+}/H11001 may play roles in IP\textsubscript{3}R degradation (5, 18). We have previously shown that the point mutation D2550A in the pore domain of the IP\textsubscript{3}R is inactive as a Ca\textsuperscript{2+}/H11001 channel (26, 27). The data in Fig. 8F show that this mutant construct expressed in the SI(H11001) background was degraded in response to Cch stimulation. We conclude that there is no requirement for a functional channel in degradation, presumably because the Ca\textsuperscript{2+} requirement for degradation is supplied by intraluminal Ca\textsuperscript{2+} and by active endogenous receptors.

**DISCUSSION**

In the present study we have investigated various aspects of the mechanism of IP\textsubscript{3}R degradation including sites of receptor ubiquitination, Ca\textsuperscript{2+} dependence of IP\textsubscript{3}R degradation, and splice variant specificity of the degradation process. All of these studies were performed using CHO-K1 cells and Cch as an agonist. Previous studies have shown that 1 mM Cch for 18 h can effectively degrade type I IP\textsubscript{3}Rs in CHO cells stably overexpressing M1 or M3 muscarinic subtypes that are coupled to phospholipase C but not in CHO cells overexpressing M2 muscarinic receptors, which are coupled to adenylate cyclase inhibition (4). In the present study we used the parental (untransfected) CHO-K1 cells,
which are normally considered to lack endogenous muscarinic receptors as measured by radioligand binding studies (37). However, in our experiments these cells displayed a robust mobilization of Ca\(^{2+}\) in response to Cch stimulation (Figs. 5 and 7) as well as an IP\(_3\)R degradation response. Others have reported that Cch addition to parental CHO-K1 results in the generation of nitric oxide, even though muscarinic receptors cannot be detected by radioligand binding assays (37). This suggests that low levels of endogenous muscarinic receptors in the parental CHO-K1 cells are still capable of eliciting functional responses when stimulated by the relatively high concentrations of Cch employed in the present study. Although Cch-mediated Ca\(^{2+}\) signals and IP\(_3\)R degradation were blocked by atropine, we have not attempted to identify the exact muscarinic subtype(s) involved. CHO-K1 cells stably expressing the M1 receptor subtype also showed Cch-mediated IP\(_3\)R degradation, but surprisingly the dose response for Cch was not significantly different from the parental CHO-K1 cells (data not shown).

A common feature of agonist-mediated IP\(_3\)R degradation is that it is accompanied by ubiquitination (8, 16, 18, 19). Recent studies have shown that IP\(_3\)R ubiquitination involves Ubc7 (38), an ER-associated E2-ligase, that also plays a role in the ER degradation of other substrates (39, 40). In the present study we localized the IP\(_3\)R domains that become ubiquitinated and found that both monoubiquitin and polyubiquitin become attached at different regions of the receptor. The monoubiquitination site is in the C-terminal region of the protein. This site becomes monoubiquitinated even when ligand-binding defective mutants are transfected in COS cells together with Myc-tagged Ub (data not shown). Because polyubiquitination and agonist-mediated IP\(_3\)R degradation are prevented in ligand-binding defective mutants (19), this suggests that monoubiquitination is unrelated to agonist-mediated IP\(_3\)R degradation. However, the attachment of monoubiquitin plays a role in the endocytic targeting/degradation of a number of cell surface receptors (41) and could potentially play a similar role in the basal turnover of IP\(_3\)Rs (42). Polyubiquitination appeared to be confined to an N-terminal segment of the receptor containing the ligand-binding domain. It is possible that the attachment of long Ub chains at lysines adjacent to the ligand-binding site may disrupt the structure of the site or sterically interfere with access of IP\(_3\), thereby inactivating the receptor as a prelude to degradation.

We have investigated the role of Ca\(^{2+}\) in IP\(_3\)R degradation. Studies with pleckstrin homology domain probes suggest that PLC generation of IP\(_3\) is Ca\(^{2+}\)-dependent in CHO cells (33). The experimental evidence suggests that sustained elevation of IP\(_3\) is a prerequisite for initiating IP\(_3\)R degradation (19, 20). Thus the absence of agonist-mediated IP\(_3\)R degradation when Ca\(^{2+}\) is removed may be the consequence of a limited generation of IP\(_3\) rather than an effect of Ca\(^{2+}\) on the degradation process. We show in the present study that the readdition of Ca\(^{2+}\) to CHO cells, incubated in a Ca\(^{2+}\)-free medium, initiates IP\(_3\)R degradation provided the cells have been exposed to Cch. Although this Ca\(^{2+}\) readdition is expected to stimulate IP\(_3\) generation, this cannot be the sole factor responsible for IP\(_3\)R degradation because the effect of Ca\(^{2+}\) addition was blocked by thapsigargin. Under these conditions thapsigargin does not interfere with the activation of PLC (4, 33) or the elevation of cytosolic Ca\(^{2+}\) but would prevent accumulation of Ca\(^{2+}\) into the lumen of the ER. The inhibition of IP\(_3\)R degradation seen when Ca\(^{2+}\) is removed can be prevented by first overloading the intracellular stores with Ca\(^{2+}\) (Fig. 7F). This suggests that there may be a minimal level of ER intraluminal Ca\(^{2+}\) that is necessary to facilitate degradation. From our data we conclude that both a sustained IP\(_3\) elevation and intraluminal Ca\(^{2+}\) are necessary for agonist-mediated IP\(_3\)R degradation. Pretreatment with thapsigargin has been shown to block agonist-mediated IP\(_3\)R ubiquitination (18). This supports the idea that Ca\(^{2+}\) is required for initiating the early steps of IP\(_3\)R degradation, although this observation does not distinguish between a requirement for cytosolic or luminal Ca\(^{2+}\). Intraluminal Ca\(^{2+}\) may promote interactions of IP\(_3\)Rs with ER-resident chaperones or act as a permissive factor allowing IP\(_3\)Rs to adopt a degradation-sensitive conformational state upon prolonged IP\(_3\) binding. Elevated cytosolic Ca\(^{2+}\) promotes large conformational changes in the receptor (43), which may expose ubiquitination sites. Both cytosolic and luminal Ca\(^{2+}\) could therefore play regulatory roles at different stages of IP\(_3\)R degradation. A recently published study has examined the role of Ca\(^{2+}\) in the ubiquitination and degradation of IP\(_3\)Rs in gonadotrophin (GnRH)-responsive aT3–1 cells (44). The authors found that the combination of 25 \(\mu\)M BAPTA-AM and 1 \(\mu\)M nifedipine did not prevent ubiquitination of wild-type IP\(_3\)Rs. These conditions did not entirely eliminate the Ca\(^{2+}\) signal mediated by GnRH, and it was suggested that local increases in cytosolic Ca\(^{2+}\) are sufficiently large, even with BAPTA-AM and nifedipine, to facilitate ubiquitination. In our hands a 30-min preincubation of CHO cells with 100 \(\mu\)M BAPTA-AM was sufficient to entirely eliminate the carbachol-mediated Ca\(^{2+}\) signal and the accompanying IP\(_3\)R degradation (data not shown). The D2550A pore-inactive mutant stably expressed in aT3–1 cells was ubiquitinated and down-regulated in response to GnRH, albeit with a slightly reduced efficiency. Overall, the study in Ref. 44 and the present report are in general agreement that both Ca\(^{2+}\) and IP\(_3\) binding to the IP\(_3\)R are required for IP\(_3\)R degradation.

Transient transfection experiments in CHO cells with exogenous IP\(_3\)R constructs have provided insights into the specificity of the degradation process. We noted that only the SI(+) splice variant of the type 1 IP\(_3\)R was degraded and that this was also the only form detectable endogenously in CHO cells. The 15 amino acids of the SI splice site are located in the ligand-binding domain of the receptor. Functional studies comparing the SI(−) and SI(+) forms of the type 1 IP\(_3\)R have not revealed any marked differences in ligand binding or channel function (25, 45), although a recent study noted differences in the Ca\(^{2+}\) sensitivity of the two variants (29). We have also observed that the exposure of highly reactive surface thiol groups in the ligand-binding domain is different for the SI(+) and SI(−) variants (34). By contrast the SI splice site does not appear to be critical because both exogenous SI(+)/SI(+) and endogenous SI(+)/SI(−) forms were degraded. Interestingly, the only previous studies demonstrating the degradation of exogenous receptors used the mouse SI(+) clone transfected into SH-SY5Y neuroblastoma cells (19) or aT3–1 anterior pituitary cells (18). However, the SI splice status of the endogenous receptor in these cells has not been determined. The preference for the SI(+) splice variant is unlikely to be related to ubiquitination because the SI(−) variant was readily ubiquitinated (Fig. 4). Nevertheless, only a crude analysis of IP\(_3\)R ubiquitination domains was carried out in the present study, and detailed differences in ubiquitination sites between splice constructs cannot be excluded. Previous studies by others have noted that insertion of a C-terminal tag has a strong inhibitory effect on IP\(_3\)R degradation, although ubiquitination of the tagged receptor was not noticeably affected (18, 19). After ubiquitination, the large tetrameric IP\(_3\)R complex in the ER membrane is presumably subject to complex unfolding and disassembly steps. These processes may also have strict structural requirements that are disrupted by the insertion of epitope tags or inappropriate SI splice variants. The presence of a Cch-sensitive IP\(_3\)R degradation system in CHO cells and the ability to transfec these cells with mutant IP\(_3\)Rs should allow us to further probe the structural specificity of the IP\(_3\)R proteasomal degradation pathway.
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