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Proteolysis of type I inositol 1,4,5-trisphosphate receptor in WB rat liver cells

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A comparison of the basal degradation of type I InsP_3Rs [D-*myo*-inositol 1,4,5-trisphosphate receptor], measured by pulse–chase analysis or by analysis of immunoreactive InsP_3Rs after cyclo-heximide addition, indicated that the small pool of newly synthe-sized radioactive InsP_3Rs degraded relatively rapidly compared with the large pool of mature InsP_3Rs. An antibody (Ab) against a peptide sequence within the IL-3 (third intraluminal loop) of the receptor (IL-3 Ab) was used to identify protected proteolytic fragments that may accumulate in cells. The IL-3 Ab recognized a 56 kDa fragment in both WB rat liver cells and A7R5 smooth-muscle cells. Gel filtration experiments indicated that the 56 kDa fragment was monomeric and, based on reactivity to other Abs, was missing the cytosol-exposed N- and C-terminal segments of the receptor. The addition of the lysosomal protease inhibitor chloroquine resulted in the rapid disappearance of the 56 kDa band. This effect was mimicked by the cysteine protease in-hibitors leupeptin, *N*-acetyl-L-leucyl-L-leucyl-L-methioninal and *N*-acetyl-leucyl-leucyl-norleucinal. Lactacystin and NH$_4$Cl were less effective. A second fragment of 16 kDa containing the C-terminus accumulated only when the cells were treated with NH$_4$Cl, and not with any of the other inhibitors tested. No N-ter-minal-reactive fragments were observed. We propose that mature InsP_3R tetramers dissociate into monomers and that the 56 kDa fragment is a cleavage intermediate of the monomer representing the six transmembrane domains. Angiotensin-II-stimulated down-regulation of InsP_3Rs in WB cells has been shown to involve the ubiquitin/proteasome pathway. Angiotensin-II treatment of WB cells neither resulted in the accumulation of any new frag-ments nor increased the levels of the 56 or 16 kDa fragments. We conclude that basal and agonist-stimulated degradations of InsP_3Rs occur by different pathways. The agonist-mediated path-way involves the concerted removal and proteolysis of the entire receptor molecule from the endoplasmic reticulum membrane without the appearance of intermediate intraluminal fragments.

Key words: calcium-release channel, endoplasmic reticulum pro-tein turnover, inositol 1,4,5-trisphosphate, InsP_3 receptor.

INTRODUCTION

Inositol 1,4,5-trisphosphate receptors (InsP_3Rs) are a family of intracellular Ca^{2+} release channels that play an essential role in evoking Ca^{2+} signals triggered by the occupation of numerous types of cell-surface receptors that are coupled with enhanced inositol–lipid turnover [1]. Three different InsP_3R isoforms have been identified and most cells appear to express multiple isoforms [2,3]. InsP_3R channels are tetramers and previous studies have shown that both homo- and heterotetramers are formed in cells [4–6]. Considerable research had focused on characterizing the channel properties of InsP_3Rs and their regulation in an effort to understand the complex spatial and temporal dynamics of Ca^{2+} signalling. However, very little is known regarding the biosyn-thesis and degradation pathways of InsP_3R channels that ulti-mately determine the expression level of these signalling proteins.

Pulse–chase studies of the type I InsP_3R have indicated a half-life of 8–11 h for turnover [7,8]. The pathway(s) involved in the basal degradation of InsP_3Rs and the mechanism by which this large complex protein is disassembled and degraded have not yet been investigated. An additional pathway for InsP_3R degradation is activated in response to the chronic administration of a Ca^{2+}-mobilizing stimulus. Agonist-mediated InsP_3R down-regulation has been demonstrated in many cell types stimulated with many Ca^{2+}-mobilizing agonists [8–15]. Further studies have led to the proposal that sustained elevation of InsP_3 induces a con-formational change in the InsP_3R, which promotes ubiquitination and renders the protein susceptible to degradation by the pro-teasomal pathway [9,16]. The detailed mechanism by which this is accomplished is poorly understood. InsP_3Rs contain six TM (transmembrane) domains located in the C-terminal portion of the protein, and the current topological model of this ER (endoplasmic reticulum) resident protein indicates that the bulk of the N-terminus and a small portion of the C-terminal 'tail' are exposed to the cytosol. One possible model of agonist-mediated degradation is that the InsP_3R is extricated from the membrane by a retrograde translocation that utilizes the same machinery originally used to insert the protein into the ER. This would be similar to the mechanism utilized for the ER degradation of a number of misassembled or misfolded proteins such as CFTR (cystic fibrosis transmembrane conductance regulator) [17] or T-cell receptor α-subunit [18]. In this model, the processive degradation of the entire molecule by the proteasome would not be expected to generate any sizeable fragments, other than small peptides. An alternative model envisages that the cytosol-exposed N- and C-terminal portions of the InsP_3R are cleaved by the proteasome but the TM domains are removed by an alternative pathway. In this model, there is a possibility that fragments containing the TM domain and their intraluminal loops could accumulate during agonist-mediated degradation. In principle, different fragments of InsP_3Rs could also accumulate as intermediates in the pathway of basal degradation.

The present study was undertaken to examine the pathway of InsP_3R turnover under basal conditions and to locate InsP_3R fragments using several domain-specific InsP_3R antibodies (Abs). This included an Ab directed at an intraluminal epitope, expected to be protected during agonist-mediated degradation. Distinct fragments derived from the C-terminus of type I InsP_3R were observed under basal conditions, but not during agonist-mediated InsP_3R degradation. We have characterized these C-terminal fragments and have utilized this information to gain some insights into the mechanism of InsP_3R proteolysis.

EXPERIMENTAL

Materials

CHAPS, Triton X-100, ALLM (*N*-acetyl-L-leucyl-L-leucyl-L-methioninal), ALLN (*N*-acetyl-leucyl-leucyl-norleucinal), leupeptin, NH$_4$Cl and chloroquine were purchased from Sigma (St. Louis, MO, U.S.A.). Lactacystin was purchased from Dr E. J. Corey (Harvard University, Cambridge, MA, U.S.A.). Richter's modified minimal essential medium was obtained from Irvine Scientific (Santa Ana, CA, U.S.A.). Fugene transfection reagent was from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.).

Antibodies

The CT-1 Ab (C-terminal Ab), which is specific to the C-terminal residues (2731–2749) of the type I InsP_3R, has been characterized previously [19]. The IL-3 (third intraluminal loop) Ab was raised against a peptide sequence from the intraluminal loop of the rat type I InsP_3R, corresponding to amino acids 2499–2516 (see Figure 6A). Additional Abs were raised against peptide sequences located on the cytosolic side of TM-1 (amino acids 2252–2266) and TM-6 (amino acids 2613–2628). Peptides were synthesized with an N-terminal cysteine by Research Genetics (Huntsville, AL, U.S.A.) coupled with keyhole limpet haemocyanin. Antibodies were raised in rabbits by Cocalico Biologicals (Reamstown, PA, U.S.A.) and were affinity-purified using a peptide, coupled with Ultralink beads, by procedures described by the manufacturer (Pierce, Rockford, IL, U.S.A.).

Cell culture and membrane isolation

Early passage WB cells were kindly given by Dr R. Whitson (Beckman Research Institute of the City of Hope, Duarte, CA, U.S.A.). The cells were grown in 150 mm dishes in Richter's minimal essential medium supplemented with 10 % foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml gentamicin. The cells were maintained at 37 °C in 5 % CO$_2$ atmosphere. The cells were serum-deprived for 24 h and then treated with various protease inhibitors for 16 h. At the end of the treatment period, the medium was aspirated and the plates were washed twice in ice-cold PBS. The cells were scraped into a buffer containing 150 mM NaCl, 50 mM Tris/HCl (pH 7.8), 1 % (w/v) Triton X-100, 1 mM EDTA, 0.5 mM PMSF, and 5 μg/ml each of aprotonin, soya-bean trypsin inhibitor and leupeptin (WB solubilization buffer). Insoluble material was removed by centrifugation for 10 min at 25 000 *g*, and the soluble lysate was quenched in SDS/PAGE sample buffer [50 mM Tris, pH 6.8/1 % SDS/0.01 % Bromophenol Blue/0.14 M 2-mercaptoethanol/5 % (v/v) glycerol].

Metabolic labelling and immunoprecipitation

For labelling WB cells, Richter's medium was replaced by methionine-free Dulbecco's modified Eagle's medium for 1 h.

The cells were labelled for 30 min with 200 μCi/ml Tran^{35}S-label (ICN Radiochemicals) and then chased with 1 mM methionine for various times. Cell lysates were prepared as described above and immunoprecipitated with CT-1 Ab and Protein A–Sepharose overnight. Immune complexes were washed three times in WB solubilization buffer and separated on either 5 % (w/v) poly-acrylamide gels or 4–15 % polyacrylamide gradient gels (Bio-Rad, Melville, NY, U.S.A.) unless otherwise stated. The separated polypeptides were transferred to nitrocellulose which was auto-radiographed and then immunoblotted with CT-1 Ab to locate the InsP_3R. Quantitative analysis of the autoradiographs and immunoblots was performed using Imagemaster software (Amersham Biosciences). Repeated immunoblotting of the same nitro-cellulose sheet was performed after treating blots for 30 min at 60 °C in a stripping buffer, containing 65 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS and 100 mM 2-mercaptoethanol.

RESULTS

Newly synthesized and mature InsP_3Rs have different turnover rates

To measure the basal degradation of the type I InsP_3R, we pulse-labelled WB cells for 30 min with Tran^{35}S and then chased the cells with excess methionine for periods up to 24 h in the presence or absence of cycloheximide (Figure 1). Total cell lysates were immunoprecipitated with an Ab specific to the C-terminus of the type I InsP_3R (CT-1 Ab). The progressive decrease in labelled InsP_3R during the chase was consistent with previous estimates of 8–11 h for the half-life of the InsP_3R [7,8]. An alternative method to estimate the degradation rate of the receptor is to measure the decrease in receptor levels when biosynthesis is prevented by the addition of cycloheximide. In many instances, both methods gave similar results (e.g. CFTR [20]). The same samples used for estimating radioactive InsP_3R were used to measure InsP_3R expression by immunoblotting with CT-1 Ab. Surprisingly, there was no significant decrease in InsP_3R levels over a 24 h period in response to cycloheximide addition (Figure 1). In contrast, cells chased in the presence of cycloheximide showed a decrease in radioactive receptor, not significantly different from cells chased in the absence of the inhibitor. The difference in the behaviour of labelled and unlabelled InsP_3Rs in response to cyclo-heximide suggests that the newly synthesized labelled InsP_3Rs and the mature pool of InsP_3Rs measured by immunoblotting have quite different turnover rates. The results are consistent with a small pool of newly synthesized InsP_3Rs being degraded comparatively faster ($t_{1/2} = 8–11$ h), whereas the major pool of mature InsP_3Rs have a much slower turnover rate ($t_{1/2} > 24$ h).

Some indication of the pathways responsible for the disposal of newly synthesized InsP_3Rs was obtained by examining the effects of proteolysis inhibitors added during the chase period (Figure 2). The proteasomal inhibitor lactacystin did not affect the turnover rate of newly synthesized InsP_3Rs. However, the cysteine protease inhibitors ALLN and ALLM inhibited the decrease in radioactivity during the chase period. The two lysosomal protease inhibitors tested had different effects on this system. Only chloroquine inhibited the turnover of labelled InsP_3Rs, whereas NH$_4$Cl had either no effect or, in some experiments, actually enhanced InsP_3R degradation (cf. Figure 2A).

Recognition properties of the IL-3 Ab

We utilized several domain-specific Abs to examine the presence of proteolytic intermediates of InsP_3Rs. In particular, an Ab was raised to the IL-3 Ab, since fragments containing intraluminal segments would be expected to be protected from cytoplasmic
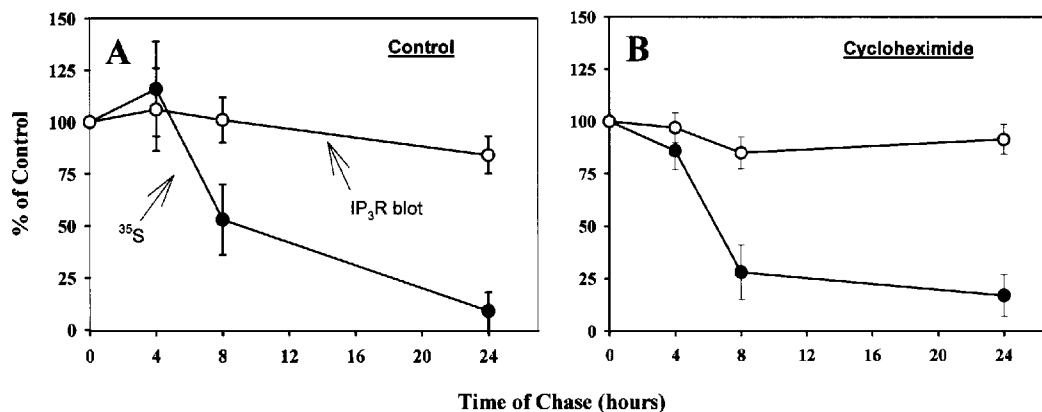
Figure 1   Effect of cycloheximide on the turnover rate of ³⁵S-labelled and unlabelled pools of InsP₃R in WB cells

WB cells were pulse-labelled with Tran³⁵S (200 μCi/ml) for 30 min and then chased in the presence of 1 mM methionine (**A**). Cycloheximide (50 μg/ml) was present during the chase in the experiments shown in (**B**). Cell lysates obtained at the indicated times were immunoprecipitated with type I InsP₃R Ab. The samples were separated by SDS/PAGE (5 % gel) and transferred on to nitrocellulose, which was autoradiographed to quantify the radioactive pool of InsP₃R (●). The same nitrocellulose sheet was immunoblotted with type I InsP₃R Ab to measure the total pool of type I InsP₃R (○). Autoradiographs and immunoblots were quantified by densitometry. Results are the means $\pm$ S.E.M. of three separate experiments. IP₃R, InsP₃R.
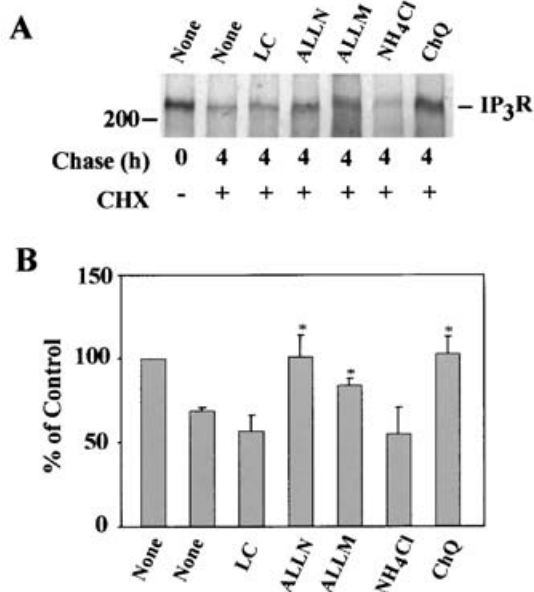

Figure 2   Effect of protease inhibitors on the degradation of ³⁵S-labelled InsP₃R

WB cells were pulse-labelled as described in Figure 1 and the indicated inhibitors were applied to the cells together with cycloheximide (50 μg/ml) during a 4 h chase with 1 mM methionine. Final concentrations of the inhibitors were as follows: lactacystin (LC, 10 μM), ALLN (50 μM), ALLM (50 μM), NH₄Cl (5 mM) and chloroquine (ChQ, 200 μM). Samples were separated by SDS/PAGE (5 % gel) and transferred on to nitrocellulose, which was subjected to autoradiography. The results of a typical experiment are shown in (**A**). The data pooled from 3 to 8 separate experiments are shown in (**B**).*Significantly different from the level of labelled receptor observed after 4 h chase in the absence of any protease inhibitors ($P < 0.05$). CHX, cycloheximide.

proteases. The peptide sequence selected (CTSPAPKEELLPV-EET) was the same epitope as used by Michikawa et al. (1ML1 Ab) [21]. The recognition properties of the affinity-purified IL-3 Ab are shown in Figure 3(A). In WB cell lysates, the IL-3 Ab recognized the full-length 235 kDa InsP₃R and also three prominent additional bands having molecular masses of 79, 56 and 28 kDa (Figure 3A, lane 1). Recognition of all four bands was

prevented by pre-blocking the Ab with the peptide antigen (Figure 3A, lane 2). The 79, 56 and 28 kDa bands were also observed in A7R5 smooth-muscle cell lysates (Figure 3A, lane 3), but were absent from cerebellum microsomes (Figure 3A, lane 4). None of the additional bands was recognized by an Ab raised against the C-terminus of the type I InsP₃R (Figure 3A, lane 5). Proteinase K cleavage of the InsP₃R in cerebellum microsomes generated an approx. 20 kDa protected fragment that was recognized by IL-3 Ab (Figure 3B). On the basis of the amino acid sequence, the calculated mass of the protected fragment encompassed by the TM-5 and TM-6 domains would be 16.7 kDa. There is reasonable agreement in the masses of the calculated and observed fragments if glycosylation at the two N-glycosylation sites (approx. 6 kDa) is also taken into account. These results confirm that the IL-3 Ab recognizes an intraluminal epitope in type I InsP₃R.

Immunoprecipitation of WB cell lysates with IL-3 Ab recovered only the 79 kDa band and the full-length InsP₃R (results not shown). Edman microsequencing of the immunoprecipitated 79 kDa band yielded a sequence that matched the N-terminus of ezrin, a protein involved in linking membranes to the cytoskeleton [22]. An anti-ezrin Ab, raised against amino acids 479–498 of human ezrin, recognized the 79 kDa band but did not recognize the full-length InsP₃R in WB or A7R5 cells. There was no indication that ezrin and InsP₃R were associated in WB cell lysates from co-immunoprecipitation experiments using anti-ezrin or CT-1 Abs (results not shown). Therefore we conclude that the recognition of the 79 kDa ezrin band by IL-3 Ab must reflect the presence of a shared epitope in the peptide antigen used to raise the IL-3 Ab. A possible candidate is the sequence KEEL, which is found at amino acids 399–402 in the ezrin sequence.

It has been shown previously that COS-7 cells contain undetectable levels of endogenous type I InsP₃R [3,23] and therefore provide a good negative control for testing the IL-3 Ab. Immunoblotting of COS-7 lysates with IL-3 Ab shows the presence of the 79 kDa ezrin band and the 28 kDa band (Figure 3C, lane 1). However, the full-length InsP₃R and the 56 kDa band were not observed. Accumulation of the 56 kDa band was observed only when the COS cells were transfected with type I InsP₃R (Figure 3C, lane 2). From these results, we conclude that only the IL-3-reactive 56 kDa is derived from the type I InsP₃R. We have therefore focused solely on this fragment in subsequent experiments.
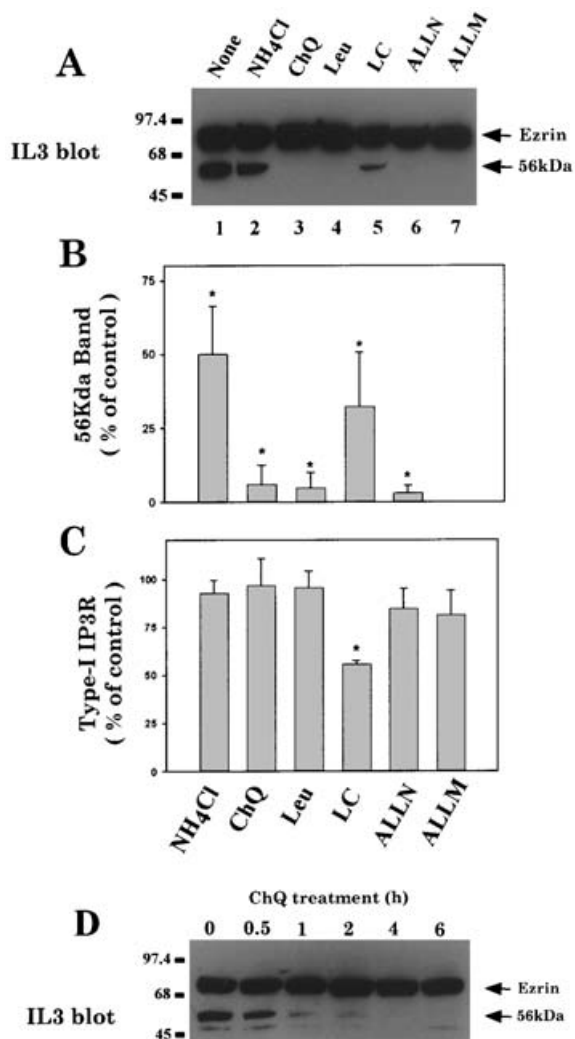
Figure 3   Characterization of anti IL-3 Ab

(**A**) WB cell lysates were subjected to SDS/PAGE on a 4–15 % gradient gel. The proteins were transferred on to nitrocellulose membranes and probed with either IL-3 Ab (lane 1) or IL-3 Ab pretreated with 100 μg/ml IL-3 peptide (lane 2). Lysates from A7R5 smooth-muscle cells (lane 3) or rat cerebellum microsomes (lane 4) were processed for immunoblotting with IL-3 Ab. The full-length InsP_3R recognized by the CT-1 in WB and cerebellum microsome lysates is shown in lanes 5 and 6 respectively. (**B**) The protease protection assays on rat cerebellum microsomes (1 mg/ml), which were incubated with two different concentrations of proteinase K (200 μg/ml, lane 2; 400 μg/ml, lane 4) in an incubation buffer containing 120 mM KCl, 1 mM EDTA, 20 mM Tris/HCl (pH 7.8) and 1 mM dithiothreitol. Samples were incubated with and without 1 % Triton X-100 for 1 h at room temperature (22 °C) and then processed further for immunoblotting using IL-3 Ab. (**C**) COS cells were transfected with pcDNA3.1 vector (lane 1) or wild-type I InsP_3R DNA (lane 2) using Fugene transfection reagent. After 48 h transfection, the cells were lysed, subjected to SDS/PAGE and immunoblotted with IL-3 Ab. Lane 3 shows a control sample from untransfected WB cells. Molecular-mass markers (in kDa) are indicated.

Effect of protease inhibitors on proteolytic fragments of InsP_3Rs

Figure 4(A) shows the effect on the levels of the 56 kDa fragment on treating WB cells with various protease inhibitors for 16 h. The accumulation of 56 kDa was almost completely prevented by the lysosomal protease inhibitor chloroquine, and this effect was mimicked by leupeptin, ALLN and ALLM. All three of the latter group of inhibitors are known to inhibit serine and cysteine proteases, including several that are located in lysosomes. Surprisingly, NH$_4$Cl, a well-characterized lysosomotropic agent, was much less effective than chloroquine in decreasing accumulation of the 56 kDa protein. Inhibition of proteasomes with lactacystin also decreased the levels of 56 kDa, albeit incompletely. The levels of 56 kDa were unaffected by treatment of the cells with 50 μM caspase inhibitors DEVD or YVAD (results not shown). With the exception of lactacystin, the remaining inhibitors produced very little change in the levels of the full-length type I InsP_3R measured at 16 h (Figure 4B). The reason for the decrease in full-length type I InsP_3R with lactacystin treatment is not known, but may have contributed to the decrease in levels of the 56 kDa band. When chloroquine was combined with either lactacystin or NH$_4$Cl, the 56 kDa band behaved similar to the cells treated with chloroquine alone (results not shown).

The time course of the effect of chloroquine is shown in Figure 4(C). The results indicate that the effects of chloroquine in promoting the disappearance of the 56 kDa fragment occur within 1–2 h, suggesting that the fragment itself turned over relatively rapidly. The pharmacological profile of the effects of the inhibitors observed in Figure 4(A) would be consistent with the formation of the 56 kDa fragment being mediated by a serine or cysteine protease located in an acidic compartment. However, these experiments do not exclude the possibility that the inhibitors affect the 56 kDa metabolism indirectly through effects on intermediary proteins or by modulating membrane-trafficking events.

Accumulation of a 16 kDa C-terminal fragment in NH$_4$Cl-treated cells

The lack of reactivity of the 56 kDa fragment with CT-1 Ab indicates that this fragment does not retain the extreme C-terminus of the InsP_3R (Figure 3A, lane 5). However, when the samples obtained from inhibitor-treated cells were analysed by immunoblotting, it was evident that a 16 kDa fragment, reactive with CT-1 Ab, was formed in the presence of NH$_4$Cl, but not with any of the other inhibitors, including chloroquine (Figure 5A). The

Figure 4   Treatment of WB cells with various proteosomal and lysosomal inhibitors

(**A**) WB cells were treated with NH₄Cl (5 mM), chloroquine (200 μM), leupeptin (250 μM), lactacystin (5 μM), ALLM (50 μM) and ALLN (50 μM) for 16 h. Lysates were subjected to SDS/PAGE on 4–20 % gradient gels and processed for immunoblotting with IL-3 Ab. (**B**) Quantification of the 56 kDa band by densitometry for 3–5 separate experiments. (**C**) Levels of the full-length type I InsP₃R were also quantified from densitometry of samples processed by SDS/PAGE (5 % gel) and immunoblotted with CT-1 Ab. In (**B**, **C**) the asterisk indicates that the value is significantly different from the control value of 100 % ($P < 0.05$). (**D**) Time course of the decrease in 56 kDa polypeptide on treatment with 200 μM chloroquine. In (**A**, **D**), molecular-mass markers (in kDa) are indicated on the left.

Figure 5   Accumulation of a 16 kDa C-terminal fragment after incubation with NH₄Cl

(**A**) WB cells were treated with the inhibitors as described in Figure 3(A) and cell lysates were immunoblotted with a specific Ab raised against the C-terminus of the type I InsP₃R (CT-1 Ab). (**B**) The time course of the changes in full-length type I InsP₃R (5 % SDS/polyacrylamide gel) and the 16 kDa fragment (4–20 % gradient SDS/polyacrylamide gel) was measured after incubation of WB cells with 5 mM NH₄Cl. (**C**) WB cells were scraped into a buffer, containing 250 mM sucrose, 1 mM EDTA, 4 mM MgCl₂, and were subjected to 35 strokes of a Dounce homogenizer. Intact cells and nuclei were removed by centrifugation at 1500 **g** for 10 min. The supernatants were centrifuged at 100 000 **g** for 30 min. The membrane pellets were resuspended in the above buffer. Aliquots of the supernatants and resuspended membranes (50 μg) were subjected to SDS/PAGE and probed with either CT-1 or anti-ezrin Abs. For the analysis of the 16 kDa fragment, the WB cells were first treated with 5 mM NH₄Cl for 16 h. Molecular-mass markers (in kDa) are indicated on the left.

time course of this effect, shown in Figure 5(B), indicates that the 16 kDa fragment accumulated only after prolonged incubation of the cells with 5 mM NH₄Cl with maximal effects being observed after 18 h. In Figure 5(C), we examined membrane and cytosol fractions of WB cells treated for 16 h with NH₄Cl. Surprisingly, all the 16 kDa cytosol-exposed C-terminal fragments remained associated with the membrane fraction. This may indicate that the 16 kDa proteolytic fragment is non-covalently attached to other membrane-associated fragments, as observed for proteolytic fragments obtained after treatment of the InsP₃R with trypsin [24,25] *in vitro*. The 56 kDa fragment was also found in the pellet fraction as expected for a segment of the protein containing TM domains (Figure 5C).

The effect of NH₄Cl on the accumulation of the 16 kDa fragment was mimicked by methylamine (results not shown), suggesting that the action of NH₄Cl is unrelated to its metabolism and is probably associated with its effects on cellular pH. We utilized a fluorimetric method utilizing 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein [26] to measure the cytosolic pH in WB cells exposed to 5 mM NH₄Cl or 200 μM chloroquine. The fluorescence emission ratio (490 nm/440 nm) in control, NH₄Cl and chloroquine-treated cells was 4.73 ± 0.43, 5.80 ± 0.12 and 4.53 ± 0.18 ($n = 3$). Using a nigericin-based calibration method
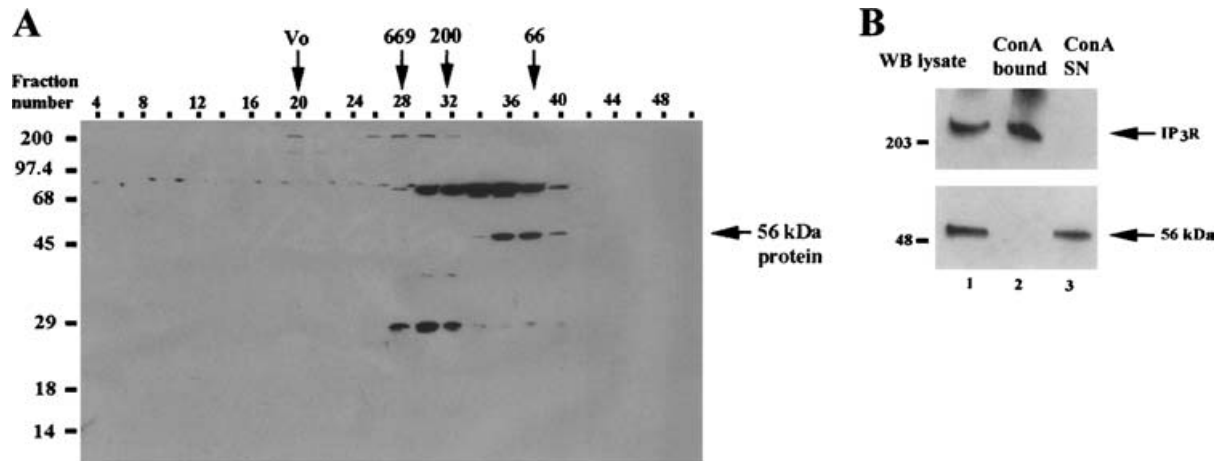
Figure 6   Gel filtration analysis of the 56 kDa band

(**A**) Triton extracts from WB cells were subjected to FPLC on a Superose 6 column. Fifty fractions of 0.5 ml were collected. Alternative fractions were subjected to SDS/PAGE (10 % gel). Immunoblotting was done using IL-3 Ab. The peak elution positions of the void volume (V_0) of thyroglobulin (669 kDa), myosin (200 kDa) and BSA (66 kDa) are shown. (**B**) WB microsomal membranes were prepared as described in Figure 5(C) and solubilized in WB solubilization buffer: 100 μg of lysate protein was incubated with 50 μl of concanavalin A–Sepharose (ConA) beads (50 % slurry) and 5 mM $CaCl_2$ for 2 h at 4 °C. The beads were then washed three times in WB solubilization buffer. The presence of full-length $InsP_3R$ and 56 kDa polypeptide in the ConA pellets (lane 2) and in 20 μg of the original extract (lane 1) or the ConA supernatant (lane 3) was determined by immunoblotting. Molecular-mass markers (in kDa) are indicated on the left.

[26], this translated into cytosolic pH values of 7.4, 7.9 and 7.3 respectively. The results suggest that the prolonged increase in cytosolic pH by NH_4Cl may be the factor underlying its selective effect on the generation of the 16 kDa C-terminal $InsP_3R$ fragment.

Gel filtration analysis and boundaries of the 56 kDa polypeptide

Triton X-100 extracts from WB cells were analysed on an FPLC Superose 6 column. Functional $InsP_3Rs$ exist as tetrameric proteins (molecular mass > 10^3 kDa), and elute in the void volume of this column. A significant fraction of disassembled subunits could also be observed under our experimental conditions (Figure 6A). However, the 56 kDa polypeptide eluted as a single peak at the position expected of a monomer, suggesting that this fragment may be derived from the proteolysis of disassembled monomeric $InsP_3Rs$. The intact full-length type I $InsP_3R$ contained two sites that were glycosylated with core oligosaccharides binding concanavalin A [2]. In contrast, the 56 kDa polypeptide in lysates from WB microsomal membranes did not bind to concanavalin A–Sepharose (Figure 6B). This indicated that disassembly of the $InsP_3R$ tetramer was accompanied by deglycosylation or modification of the sugar residues on the 56 kDa polypeptide.

The location of the IL-3 epitope and the mass of the 56 kDa polypeptide were consistent with the fragment containing all six of the TM segments (Figure 7A). The calculated molecular mass of the region encompassing only the six TM domains was 35 kDa, implying that additional sequences may be present in the 56 kDa fragment. To explore the boundaries of the fragment, two additional Abs were raised against peptide epitopes on the cytosolic side of TM-1 and TM-6 domains. Both these Abs recognized full-length $InsP_3R$ in WB cell lysates (results not shown). Although the TM-1 Ab recognized the 56 kDa band, the TM-6 Ab did not (Figure 7B). This suggested that the 56 kDa polypeptide contained approx. 21 kDa of sequence derived from a region proximal to TM-1 and truncated close to the end of TM-6. Neither TM-1 nor TM-6 Ab recognized additional pieces that would be consistent with the presence of shorter TM domain fragments (results not shown). The portion of the $InsP_3R$ C-terminus that extended into the cytosol from the TM-6 domain had
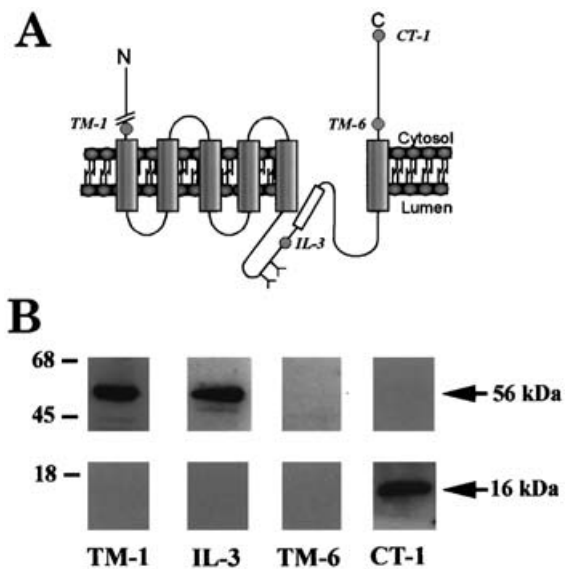

Figure 7   Boundaries of the 56 kDa fragments

(**A**) The topology of the TM domains located in the C-terminal region of $InsP_3Rs$ is schematically shown together with the location of the peptide epitopes of the IL-3, TM-1 and TM-6 Ab used to define the boundaries of the 56 kDa fragment as shown in (**B**). Only TM-1 and IL-3 Ab reacted with the 56 kDa polypeptide. Although TM-6 Ab did not recognize the 56 kDa band, it showed reactivity with the full-length $InsP_3R$ (results not shown). For the analysis of the 16 kDa fragment, the WB cells were first treated with 5 mM NH_4Cl for 16 h. As expected, only CT-1 Ab reacted with the 16 kDa fragment.

a calculated molecular mass of 18.8 kDa. The 16 kDa C-terminal fragment that accumulated in the presence of NH_4Cl was also not reactive with TM-6 Ab (Figure 7B), suggesting that the 16 kDa fragment was missing (or was cleaved within) the epitope for TM-6 Ab.

Ang-II (angiotensin II)-induced down-regulation of the type I $InsP_3R$ did not cause any change in the levels of the 56 kDa polypeptide and did not promote the appearance of additional

intraluminal-protected fragments when probed with IL-3 Ab (results not shown).

DISCUSSION

The results of our study suggest that previous measurements of the half-life of the type I InsP₃R derived from pulse–chase studies reflected only the turnover rate of the small pool of newly synthesized protein and did not measure the true turnover rate of the mature protein. Mature type I InsP₃Rs in unstimulated cells appeared to be relatively stable proteins in contrast with the newly synthesized pool of receptors that were degraded much more rapidly. The InsP₃R was not unusual in this regard, since differences in the turnover rate of newly synthesized and mature proteins were commonly observed for many proteins that transit the ER during their biosynthesis. Examples include CFTR [27], erythropoeitin [28], human δ opioid receptor [29], human thyroperoxidase [30] and ApoB100 [31]. In these instances, only a small fraction (2–40%) of the newly synthesized protein escaped ER degradation that occurred predominantly by the ubiquitin/proteasome pathway (cf. [30]). For InsP₃Rs, the ubiquitin/proteasome pathway did not appear to play a major role, based on the lack of sensitivity to lactacystin in pulse–chase experiments (Figure 2). A further difference between InsP₃Rs and the aforementioned examples was that the InsP₃R was an ER resident protein and did not undergo the extensive carbohydrate processing that allows newly synthesized and mature proteins to be distinguished by differences in molecular mass. This made it difficult to estimate the fraction of newly synthesized InsP₃Rs that escape ER degradation.

Degradation of newly synthesized proteins was considered to be a reflection of the 'quality control' function of the ER, which was designed to remove misfolded or misassembled proteins (reviewed in [32]). Failure to assemble into a tetramer could be a factor contributing to the degradation of newly synthesized InsP₃R. This seems unlikely since almost all the newly synthesized proteins were found to be tetramers [7]. Conformational maturation of the newly synthesized tetramer is probably a complex process that involves interaction with ER chaperones. An alternative possibility is that failure to acquire the proper conformation contributes to the decreased stability of the newly synthesized InsP₃R. A transient association of newly synthesized InsP₃Rs with calnexin (but not calreticulin) has been observed previously [33].

A 56 kDa degradation fragment of the InsP₃R encompassing the TM domains is present in cells, even in the absence of stimulation with agonist. This fragment is larger than what would be expected from the calculated mass of just the six TM domains (35.4 kDa). This suggests that there may be additional sequences within the 56 kDa polypeptide which, based on the reactivity of Abs, probably consist of a region preceding TM-1 rather than after TM-6 (Figure 7). The C-terminus was found to be missing in the 56 kDa fragment. In addition, no intermediate fragments larger than 56 kDa were detected with the IL-3 Ab or any of the other Abs used in this study. We conclude that basal degradation of InsP₃R proceeds at both ends of the polypeptide until a limiting fragment of 56 kDa is reached. Failure to observe any larger intermediate fragments could indicate that the 56 kDa fragment was generated by a processive proteolytic mechanism. However, we cannot exclude the possibility that the concentrations of intermediate fragments were below our detection limits or that epitopes to our available Abs were destroyed by proteolysis. It seems unlikely that proteasomes contributed to the generation of the 56 kDa fragment, since lactacystin was the least effective

drug preventing accumulation of this fragment. In contrast, chloroquine, leupeptin, ALLN and ALLM were all able to reduce markedly the 56 kDa expression. As discussed further below, the different effects of NH₄Cl and chloroquine suggest that the lysosomal pathway was probably not involved. Percoll-gradient centrifugation, which separated ER and lysosomal markers, indicated that the bulk of the 56 kDa fragment remained associated with the ER fraction (results not shown). The available evidence suggests that at least the initial steps of InsP₃R degradation occur within the ER and involve a non-proteasomal pathway sensitive to inhibitors of cysteine proteases.

The inability of the IL-3 or TM-1 Ab to immunoprecipitate the 56 kDa fragment from cell lysates precluded the use of metabolic labelling studies to establish a precursor–product relationship between the full-length and 56 kDa fragments. However, when the formation of 56 kDa fragment was blocked by treatment with drugs, there was no measurable increase in the levels of full-length InsP₃R. This result suggests that the basal turnover of the InsP₃R was slow and that only a small fraction of InsP₃Rs was converted into a 56 kDa fragment under our experimental conditions. The 56 kDa fragment was not observed in cerebellum microsome membranes, indicating differences in the extent of accumulation of the 56 kDa polypeptide or the presence of alternative pathways of InsP₃R disposal in some cell types.

Analysis of WB cell lysates on gel filtration columns indicated that the 56 kDa fragment is monomeric. Previous studies have established that the TM domains in the InsP₃R were the primary structural determinants for the formation of tetramers [34–36]. When [³⁵S] methionine pulse-labelled WB cells were analysed on gel filtration columns, there was no evidence that free InsP₃R monomers accumulated to any extent during the biosynthesis of this protein [7]. This was consistent with the idea that oligomerization mediated by the TM domains was a co-translational process [35,36]. We therefore propose that free InsP₃R monomers were present only during InsP₃R degradation and that the 56 kDa fragment was derived from the cleavage of an InsP₃R monomer pool. The mechanism by which InsP₃R tetramers were dissociated into monomers remains to be determined. It should be noted that an assembly determinant was present in the C-terminal tail of the InsP₃R [37]. Potentially, cleavage of this portion of a tetrameric receptor could itself promote dissociation into monomers. Previous studies have shown that a mutant construct expressing the distal portion of the InsP₃R, containing the TM-5 and TM-6 domains and the C-terminal tail, encoded an ion channel that is constitutively gated in the open state [38]. The removal of the C-terminal tail in the 56 kDa fragment may have the functionally beneficial effect of allowing degradation to proceed via a pathway that avoided the generation of Ca²⁺ channels that were constitutively active.

A C-terminal fragment of 16 kDa was observed to accumulate in cells treated with NH₄Cl, but not in cells treated with chloroquine. We also observed that chloroquine was more effective than NH₄Cl in decreasing the levels of 56 kDa fragment (Figure 3B). Since both drugs were known to alkalinize the lysosomal compartment, the results suggest that their effects were not the simple result of pH changes in the lysosomal compartment. For example, it had been reported that chloroquine modulated gene expression and inhibited InsP₃ binding to the InsP₃R by mechanisms that were independent of pH changes [39,40]. Differences in the behaviour of NH₄Cl and chloroquine were also observed previously in the proteolytic processing of the amyloid precursor protein [41]. The 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein measurements made in the present study indicated that only NH₄Cl alkalinized the cytosolic pH. The NH₄Cl-induced increase in cytosolic pH could inhibit the normal trafficking
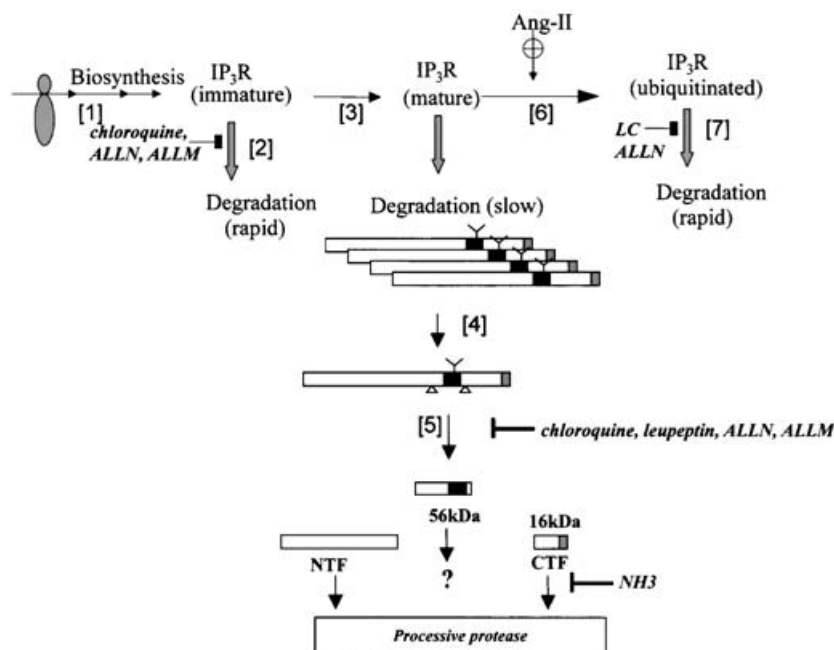
Figure 8   Model of InsP_3R degradation

The hypothetical scheme depicts the pathways of degradation of newly synthesized InsP_3R, mature InsP_3R and InsP_3R degradation occurring in the presence of agonists. The various steps depicted are: biosynthesis of newly synthesized InsP_3R [1]; degradation of immature, newly synthesized InsP_3Rs [2]; conformational maturation of the receptor involving interaction with chaperones [3]; dissociation of mature InsP_3R tetramers to monomers [4]; and cleavage of mature InsP_3R monomer on either side of the TM domains (filled box) to release the 56 kDa fragment [5]. The N-terminal fragment (NTF) or the C-terminal fragment (CTF) did not accumulate in cells. When cells were pretreated with NH$_4$Cl, a 16 kDa CTF was observed. In the presence of Ang-II, the InsP_3R was ubiquitinated [6] and rapidly degraded by the proteasome [7]. See the text for additional description.

and/or proteolytic processing of the C-terminal portion of the InsP_3R leading to the accumulation of the 16 kDa peptide. Alternatively, NH$_4$Cl could activate an alkaline protease cleavage of the C-terminal tail that normally did not occur under physiological conditions. The available experimental results did not allow us to distinguish between these possibilities.

Figure 8 displays a model of InsP_3R degradation that incorporates both data and hypothesis regarding the individual steps that may be involved. We have found that the pathway of newly synthesized and mature receptor degradation had markedly different kinetics and they were depicted as separate pathways. However, their sensitivities to protease inhibitors were similar. It is therefore possible that these pathways are the same and that the different kinetics arose from the utilization of different substrates (i.e. newly synthesized versus mature InsP_3Rs). In the model, basal degradation of mature InsP_3Rs was proposed to involve the disassembly of the tetrameric receptor and the formation of a deglycosylated 56 kDa fragment. The 56 kDa fragment was depicted as a product of proteolytic cleavage of the monomeric full-length InsP_3R on either side of the TM domains. These initial cleavages were inhibited by chloroquine, leupeptin, ALLN or ALLM. The N- and C-terminal fragments that were formed did not accumulate in cells, but were rapidly hydrolysed by a processive protease that was lactacystin-insensitive, or by proteases that formed fragments that were too small to detect by the methods employed in the present study. Proteolysis of the C-terminal fragment was inhibited by NH$_3$, and led to the accumulation of a 16 kDa fragment. The final fate of the 56 kDa TM domain fragment is unclear. When Ang-II was added to induce proteasomal degradation of InsP_3Rs, there was no evidence for the accumulation of 56 kDa or any other protected fragments. This indicated that the entire molecule, including the TM domains,

was degraded processively by the proteasomal pathway, probably by a mechanism that involved dislocation of the protein into the cytosol. Further characterization of the pathway of InsP_3R degradation will be focused on studying the disassembly of InsP_3R tetramers and the identification of the proteolytic enzymes and compartments involved in the degradation pathway.

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