Degradation of Inositol 1,4,5-Trisphosphate Receptors during Cell Stimulation Is a Specific Process Mediated by Cysteine Protease Activity*

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Inositol 1,4,5-trisphosphate (InsP$_3$) receptors are down-regulated in response to chronic activation of certain cell surface receptors because their degradation is accelerated. Studies on the nature of the down-regulatory process and the protease(s) responsible for receptor degradation are described here. InsP$_3$ receptor down-regulation was not accompanied by parallel changes in the concentrations of several other relevant proteins (endoplasmic reticulum Ca$^{2+}$-ATPase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and protein kinases $\alpha$ and $\epsilon$). Thus, the down-regulatory process selectively targets InsP$_3$ receptors for degradation. Furthermore, down-regulation was unaffected by brefeldin A and NH$_4$Cl, indicating that InsP$_3$ receptor degradation occurs without removal of receptors from the endoplasmic reticulum and independently of functional lysosomes. Analysis of InsP$_3$ receptor immunofluorescence confirmed that the receptors are not redistributed prior to or during down-regulation. Finally, of a range of protease inhibitors tested, only N-acetyl-Leu-Leu-norleucinal blocked down-regulation. Thus, cysteine protease activity accounts for InsP$_3$ receptor degradation and analysis of proteolysis in permeabilized cells indicates that this activity is calpain. Thus, InsP$_3$ receptor down-regulation appears to result from the highly selective calpain-mediated degradation of InsP$_3$ receptors. Calpain activity may be stimulated by high concentrations of Ca$^{2+}$ that are thought to be found in the vicinity of activated InsP$_3$ receptors.

Phosphoinositidase C (PC$^1$)-mediated phosphatidylinositol 4,5-bisphosphate hydrolysis leads to the formation of two intracellular signaling molecules, inositol 1,4,5-trisphosphate (InsP$_3$) and 1,2-diacylglycerol (1, 2). These molecules then bind to and activate, respectively, InsP$_3$ receptors and members of the protein kinase C (PKC) family (1, 2). InsP$_3$ receptors play a crucial role in intracellular signaling as they form channels that conduct Ca$^{2+}$ in an InsP$_3$-sensitive manner (1, 3-5). Of the InsP$_3$ receptors fully sequenced (types I, II, and III), the type I receptor is the most widely expressed and appears to be ubiquitous in animal tissues (4, 5). The primary intracellular location of the type I receptor is the endoplasmic reticulum (ER) and its primary function appears to be to regulate Ca$^{2+}$ release from this organelle (3-5).

The importance of the type I InsP$_3$ receptor is reflected by the extent to which it is regulated; Ca$^{2+}$, ATP, phosphorylation, and cytoskeletal elements can all regulate receptor function (4-6). Furthermore, receptor concentration can be altered, since activation of certain PC-linked receptors reduces cellular type I InsP$_3$ receptor levels with half-maximal effects at -1 h (7-10). This down-regulation has been observed in a number of cell types stimulated with a variety of agonists; for example, in SH-SY5Y human neuroblastoma cells stimulated with carbachol, a muscarinic agonist (7, 8), and in AR4–2J rat pancreaticoma cells stimulated with cholecystokinin (9). As InsP$_3$ receptor down-regulation suppresses Ca$^{2+}$ mobilization (7), the down-regulatory process may serve to limit Ca$^{2+}$-mediated effects during chronic cell stimulation.

To date, investigation of the mechanism of InsP$_3$ receptor down-regulation has shown that it results from accelerated receptor degradation (10). This acceleration is not mediated by PKC and appears to require persistent receptor activation by InsP$_3$ (7, 8). It also requires InsP$_3$ receptor-mediated Ca$^{2+}$-flux, since thapsigargin, which discharges ER Ca$^{2+}$ stores independently of InsP$_3$ receptors (1, 3), blocks carbachol-induced InsP$_3$ receptor down-regulation in SH-SY5Y cells (10). An additional link with Ca$^{2+}$ comes from a study showing that a Ca$^{2+}$-independent cytosolic cysteine protease activity, perhaps calpain (11), can cleave purified type I InsP$_3$ receptor (12). As yet, however, it is not known whether the InsP$_3$ receptor degradation seen in intact cells (7-10) reflects a general increase in proteolysis or is specific, whether InsP$_3$ receptors are relocated prior to their degradation, and which activity is responsible for InsP$_3$ receptor degradation. Here we report on the specificity and site of InsP$_3$ receptor degradation in intact cells and provide evidence that a cysteine protease activity, most likely calpain, is responsible for InsP$_3$ receptor down-regulation.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Pretreatment—SH-SY5Y cells, generously provided by either Dr. J. L. Beidler (Sloan-Kettering Cancer Center, New York) or Dr. S. K. Fisher (University of Michigan), and AR4–2J cells (obtained from ATCC) were cultured routinely as described (9). When appropriate, stimuli or inhibitors were added directly to culture medium and cells were maintained at 37°C for the time of pretreatment required. Antibodies—Specific type I receptor antisera was produced from a peptide corresponding to the C-terminal 19 amino acids of rat and human type I InsP$_3$ receptor and was then affinity purified (9, 10). The other antisera used were: anti-rabbit cardiac sarcoplasmic reticulum Ca$^{2+}$-ATPase (crude rabbit serum C4, generously provided by Dr. D. H. MacLennan, University of Toronto (13), anti-rat PKC$\alpha$ and $\epsilon$ (mouse monoclonal antibodies, obtained from Transduction Laboratories), and

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$^1$ The abbreviations used are: PC, phosphoinositidase C; InsP$_3$, inositol 1,4,5-trisphosphate; PKC, protein kinase C; ER, endoplasmic reticulum; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ALLN, N-acetyl-Leu-Leu-norleucinal.
anti-rat 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, crude rabbit serum, generously provided by Dr. P. A. Edwards, University of California, Los Angeles).

Analysis of InsP$_3$ Receptor Down-regulation—Control or pretreated cell monolayers were harvested in 155 mM NaCl, 10 mM Hepes, 1 mM EDTA, pH 7.4, and were collected by centrifugation at 400 × g for 2 min. Permeabilized pellets were then resuspended in ice-cold homogenization buffer (10 mM Tris, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiotreitol, 10 μM leupeptin, 10 μM pepstatin, 0.2 mM soybean trypsin inhibitor, pH 7.4) and were disrupted with a Polytron homogenizer (1 × 10 s). Homogenates were then centrifuged (38,000 × g for 10 min at 4 °C) and microsomal preparations obtained were resuspended in homogenization buffer and frozen until required. For analysis of type I InsP$_3$ receptor, Ca$^{2+}$-ATPase, and PKC isoforms, microsomal preparations were denatured at 100 °C for 5 min in SDS gel loading buffer (14); for HMG-CoA reductase analysis, samples were incubated at 20 °C for 45 min in SDS gel loading buffer (14) plus 6 M urea. Samples (10 μg of protein/ lane) and prestained molecular mass markers were then subjected to electrophoresis in polyacrylamide gels, were transferred to nitrocellulose, incubated with antisera, peroxidase-conjugated secondary antibodies, ECL detection reagents (Amersham), and x-ray film (14).

Analysis of InsP$_3$ Receptor Degradation in Permeabilized Cells—For permeabilization, pellets of harvested SH-SY5Y cells were resuspended in 120 mM KCl, 1 mM EDTA, 20 mM Tris, pH 7.4 (Buffer A). The suspension was then treated with digitonin (75 μg/ml) for 10 min at 20 °C and was centrifuged (1000 × g for 2 min). The pellet of permeabilized cells was then washed and finally resuspended in ice-cold Buffer A. To prepare cytosol, pellets of harvested SH-SY5Y cells were resuspended in ice-cold 1 mM EGTA, 1 mM dithiotreitol, 20 mM Tris, pH 7.4, and were disrupted with 25 strokes of a Dounce tissue grinder. The homogenate was then centrifuged at 40,000 × g for 10 min at 4 °C and the supernatant was used as a cytosol preparation.

Permeabilized cells and cytosol were then co-incubated without or with Ca$^{2+}$ and protease inhibitors in 45 mM KCl, 0.375 mM EDTA, 0.375 mM EGTA, 0.375 mM dithiotreitol, 15 mM Tris, pH 7.4 (final volume, 40 μl). After 25 min at 37 °C, reactions were quenched with SDS gel loading buffer (14) and samples were probed for type I receptor immunoreactivity.

Immunofluorescence Microscopy—SH-SY5Y cells, grown on coverslips, were rinsed with Dulbecco’s phosphate-buffered saline supplemented with Ca$^{2+}$-Mg$^{2+}$ and then fixed in the same buffer plus 3.6% paraformaldehyde (Acros Organics). All subsequent steps were performed in Ca$^{2+}$-Mg$^{2+}$-free phosphate-buffered saline; cells were washed once for 2 min, permeabilized with 0.2% Triton X-100 for 10 min, washed 3 times (10 min/wash), incubated with type I InsP$_3$ receptor-specific antibody plus 10% fetal calf serum for 1 h, washed 3 times, incubated with rhodamine-labeled donkey anti-rabbit IgG (Jackson) plus 10% fetal calf serum for 1 h and finally washed 3 times. To define nonspecific fluorescence, cells were incubated with antibody preadsorbed with peptide antigen (10 μg/ml). Coverslips were then rinsed with water, mounted in 90% glycerol, 0.1% p-phenylenediamine and immobilized with nail polish. Photomicrographs were generated from 10 s exposures using a Nikon Microphot-FXA microscope equipped for analysis of rhodamine fluorescence.

Miscellaneous—Peroxidase-conjugated antibodies, molecular mass markers, dithiotreitol, cholecystokinin (Tyr-(SO$_3$H)-fragment 26–33 amide), carbachol, protease inhibitors, brefeldin A, cholesterol, 25-hydroxycholesterol, Ca$^{2+}$-ATPase (buffer A), and protease inhibitors in 45 mM KCl, 0.375 mM EDTA, 0.375 mM EGTA, 0.375 mM dithiotreitol, 15 mM Tris, pH 7.4 (final volume, 40 μl). After 25 min at 37 °C, reactions were quenched with SDS gel loading buffer (14) and samples were probed for type I receptor immunoreactivity.

Analysis of the specificity of proteolysis during cell activation—First, SH-SY5Y cells were incubated without stimulus (lane 1) or with 1 mM carbachol for 1 (lane 2), 2 (lane 3), or 4 h (lane 4). Cells were then harvested and probed with antisera against type I InsP$_3$ receptor or HMG-CoA reductase. Bands corresponding to these proteins were detected at ~260 and 97 kDa, respectively. b, AR4–2 cells were incubated in serum-free culture medium for 18 h and were then incubated for 6 h without stimulus (lanes 1 and 2), with 0.5 μM cholecystokinin (lanes 3 and 4), or with 15 μg/ml cholesterol plus 1.5 μg/ml 25-hydroxycholesterol (lanes 5 and 6). Cells were then harvested and probed with antisera against type I InsP$_3$ receptor or HMG-CoA reductase. Bands corresponding to these proteins were detected at ~260 and 97 kDa, respectively. Data from duplicate culture dishes are shown as the reduction in HMG-CoA reductase immunoreactivity was relatively minor.

Results

Specificity of InsP$_3$ Receptor Degradation—To gain insight into the down-regulatory process, other relevant proteins were quantified. Initially examined were ER Ca$^{2+}$-ATPase, which like the type I InsP$_3$ receptor is an ER membrane protein involved in Ca$^{2+}$ homeostasis (3, 13), and PKC isoforms α and ε, which like InsP$_3$ receptors can be down-regulated during cell stimulation because of accelerated proteolysis (16–19). Fig. 1a shows that while type I InsP$_3$ receptor immunoreactivity in SH-SY5Y cells was dramatically reduced by exposure to carbachol (half-maximal effect at ~1 h), Ca$^{2+}$-ATPase and PKCα and ε immunoreactivity remained unchanged for up to 4 h. Thus, InsP$_3$ receptor down-regulation does not reflect a general in-
cells were preincubated with a range of protease inhibitors and were then stimulated with carbachol for 2 h (Fig. 4). The agents tested were the cysteine protease inhibitor N-acetyl-Leu-Leu-norleucinal (ALLN, also known as calpain inhibitor I), the metalloprotease inhibitors phosphoramidon and N-carbobenzoxy-Gly-Phe (CBZ-Gly-Phe), the serine protease inhibitor 4-amidinophenylmethanesulfonyl fluoride (APMSF), the aspartic protease inhibitor pepstatin, the serine/cysteine protease inhibitor leupeptin, and 3,4-dichloroisocoumarin, and the serine/cysteine protease inhibitor chloroisocoumarin (34), and leupeptin (35) can to an extent enter cells and inhibit proteolysis. Fig. 4 shows that the down-regulation in digitonin-permeabilized cells only when cytosol was present (compare lanes 3 and 6) and that calpain inhibitor peptide inhibited this proteolysis with half-maximal effect at 0.1–1 μM (37). Fig. 5 shows that Ca2+ was effective in stimulating InsP3 receptor degradation in digitonin-permeabilized cells and that calpain activity is responsible for InsP3 receptor degradation under these conditions. As ALLN also inhibited InsP3 receptor proteolysis (lanes 7–9) with similar potency to that seen in intact cells (Fig. 4), it is likely that the inhibitory effect of ALLN in intact cells is also due to inhibition of calpain activity. It remains a possibility, however, that a cysteine protease activity other than calpain degrades the InsP3 receptor in intact cells, since ALLN is also known to inhibit other cysteine proteases (38).

**DISCUSSION**

The finding that InsP3 receptors are degraded specifically in stimulated intact cells (Fig. 1) immediately places constraints on possible mechanisms that might account for InsP3 receptor down-regulation, since activation of a protease with freedom to degrade a broad range of substrates is clearly untenable. Moreover, as neither brefeldin A nor NH4Cl inhibited down-regulation and the type I InsP3 receptor was not redistributed during cell stimulation (Figs. 2 and 3), it appears that InsP3 receptors are degraded while occupying their normal intracellular sites (i.e. the membranes of intracellular Ca2+ stores). These findings, together with evidence that InsP3 binding initiates receptor degradation (7, 8), restricts the plausible mechanisms that could account for down-regulation to two possibilities; either (i) a protease is stimulated specifically in the vicinity of activated InsP3 receptors, or (ii) a conformational change in InsP3 receptors during InsP3 binding makes them susceptible to proteolysis. Support for the first possibility comes from the findings that Ca2+ mobilization via InsP3 receptors is required for acceller-
Fig. 5. InsP₃ receptor degradation in permeabilized cells. SH-SYSY cells were permeabilized with digitonin and were incubated either without cytosol (lanes 1–3) or with cytosol (lanes 4–13) for 25 min at 37 °C (except for lanes 1 and 4 which were quenched prior to incubation). Ca²⁺ (1.5 mM) was included in the incubations shown in lanes 3 and 6–13 (free Ca²⁺ concentration ~750 μM) together with ALLN (lanes 7–9) or calpain inhibitor peptide (CIP) (lanes 11–13). Samples were then probed for type I InsP₃ receptor immunoreactivity.

As yet, however, the factors which govern that these proteins should be targets for calpain(s) are unknown. Perhaps the factor that unifies InsP₃ receptors, PKCe and PC-β3 is their localization to membranes; the latter two proteins become membrane-bound upon cell stimulation (19, 44). Membranes may provide either a lipid co-factor that facilitates calpain activation (39) or, because of the presence of Ca²⁺ channels, may be the only sites at which Ca²⁺ concentration is sufficiently high to raise calpain activity.

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