Muscarnic Receptor Activation Down-regulates the Type I Inositol 1,4,5-Trisphosphate Receptor by Accelerating Its Degradation*

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Stimulation of SH-SY5Y human neuroblastoma cells with carbachol, a muscarinic agonist, down-regulates the type I inositol 1,4,5-trisphosphate (InsP3) receptor by >90% with maximal and half-maximal effects after ~6 h and ~1 h, respectively. Examination of the mechanistic basis of this down-regulation revealed that carbachol increased the rate of type I InsP3 receptor degradation (radiolabeled, immunoprecipitable receptor was lost from cells with half-times of >8 h and ~1 h in the absence and presence of carbachol, respectively) and that the concentration of type I InsP3 receptor mRNA, despite a transient decrease after 3 h, did not correlate with levels of the receptor. Only those muscarinic receptor subtypes coupled to stimulation of phosphoinositide hydrolysis were capable of causing type I InsP3 receptor down-regulation.

Ca2+ mobilization was pivotal to the mechanism of receptor down-regulation, since perturbation of Ca2+ homeostasis with either EGTA or thapsigargin blocked the ability of carbachol to accelerate receptor degradation. Studies with thapsigargin also revealed that both functional InsP3-sensitive Ca2+ stores and persistent elevation of InsP3 concentration were required for down-regulation to occur.

In conclusion, phosphoinositidase C-linked muscarinic receptors down-regulate the type I InsP3 receptor by accelerating its degradation. It appears that this process is initiated by persistent discharge of intracellular Ca2+ stores via the channels formed by tetramERICally complexed type I InsP3 receptors.

Many hormones, neurotransmitters, and growth factors accelerate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) and utilize inositol 1,4,5-trisphosphate (InsP3) as an intracellular messenger (1). The primary role of InsP3 appears to be to mobilize Ca2+ from an endoplasmic reticulum-like vesicular store (1). This process is mediated by what is now known to be a family of specific membrane-located receptors (1). The gene for an InsP3 receptor (designated type I) was initially cloned and sequenced from rodent brain (2,3). This receptor is ~90% identical in human and mouse (8) and is enhanced in retinoic acid-pretreated HL-60 cells (10). Both of these regulatory effects have functional correlates, since the Ca2+ mobilizing activity of InsP3 is suppressed in muscarinic agonist-pretreated SH-SY5Y cells (11) and is enhanced in retinoic acid-pretreated HL-60 cells (12).

We now describe analyses of the possible mechanisms by which the type I InsP3 receptor is down-regulated in SH-SY5Y cells and the signals that initiate this mechanism. We have also sought to determine whether other phosphoinositidase C-linked receptors are capable of down-regulating the type I InsP3 receptor and in which cell types this process can occur.

EXPERIMENTAL PROCEDURES
Cell Culture and Pretreatment—Cell monolayers were cultured routinely in 175-cm² flasks: SH-SY5Y cells in minimal essential medium with Earle's salts supplemented with 10% newborn calf serum and antibiotics (13), A10 cells in the same medium but with 5% newborn and 5% fetal calf serum; Chinese hamster ovary (CHO) cells in minimal essential medium supplemented with 10% newborn calf serum and antibiotics, GH3 cells in Ham's F-10 with 10% fetal calf serum and antibiotics, and Swiss 3T3 and rat H9c2 cells in Hepes-buffered Dulbecco's minimal essential medium with 10% fetal calf serum and antibiotics. Rat cerebellar granule cells were prepared and cultured as described (17). Following addition of agonists or inhibitors to culture medium, cells were maintained at 37 °C for the time of pretreatment required. Cells were then removed from flasks in 155 mM NaCl, 10 mM Hepes, 0.7 mM EDTA, pH 7.4 (HBSE); SH-SY5Y, CHO, GH3, and A10 cells became detached from flasks following gentle agitation, whereas Swiss 3T3, granule, and H9c2 cells were removed mechanically. All cell types were then pelleted by centrifugation at 500 × g for 2 min, except for granule cells which were centrifuged at 1,000 × g for 3 min.

Immunoblotting—The pellet from a flask of control or pretreated cells was resuspended in 10 ml of ice-cold 10 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 10 μM leupeptin, 10 μM pepstatin, pH 7.4 (homogenization buffer), was disrupted (Ultra

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‡ The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; InsP3, inositol 1,4,5-trisphosphate; CHO, Chinese hamster ovary; EST, 18S,3S-trans,epoxysoyucol-1-b-levocamido-3-methylbutane ethyl ester; t1/2, half-time of loss of radioactivity.
Turrax homogenizer, 1 × 18 a) and was centrifuged (500 × g for 10 min at 4 °C). This centrifugation step was omitted during preparation of granule cells. The supernatant was then centrifuged (38,000 × g for 10 min at 4 °C), and the pellet obtained was resuspended in homogenization buffer (3–10 mg of protein/ml), aliquoted, and frozen at −20 °C until required. Set minus preparations from both normal and insulin receptor down-regulation experiments were then subjected to electrophoresis (18), transferred to nitrocellulose, and incubated with monoclonal antibody 4C11 (at a dilution of ~1:8 of crude culture medium) or 18A10 (at a dilution of ~15,000 of purified antibody) or polyclonal antibody CT1 (at a dilution of 1:1,000 of crude serum). The nitrocellulose was then incubated with peroxidase-conjugated goat anti-rabbit or rabbit antibodies (at a dilution of ~1:500). ECL detection reagents (Amersham Corp.), and x-ray film. Immunoreactivity was quantified with a GS-670 densitometer (Bio-Rad).

Antibodies 4C11 and 18A10 were raised against the mouse type I InsP3 receptor as described (2) and have epitopes between amino acids 679 and 727, and 2736 and 2747, respectively (2, 19). Antibody 4C11 recognizes human type I and type III receptors, whereas 18A10 is type I-specific. Antibody CT1 was obtained by injecting a rabbit with a peptide corresponding to the carboxyl-terminal 19 amino acids of the rodent type I InsP3 receptor (2, 3). Antibody CT1 binds to the type I InsP3 receptor, with 3 mg of maleimide-activated keyhole limpet hemocyanin (Pierce). Initially 200 μg of antigen in complete Freund’s adjuvant was injected subcutaneously, followed 2 weeks later by subcutaneous injection of 200 μg in incomplete Freund’s adjuvant. Boosts of 100 μg of antigen were then given intravenously at 4-week intervals. Recognition in immunoblots of a ~275-kDa protein corresponding in size to the type I InsP3 receptor of rat cerebellum and SH-SY5Y cells, and the ability to immunoprecipitate a ~275-kDa protein were optimal after the first boost. Preimmune serum at the same dilution did not react with any proteins. Immunoreactivity was blocked if 0.2 mg/ml of the peptide to which the antibody was raised was included in incubations, but not by peptides from the carboxyl terminus of types I1 and I11 receptors. Interaction of 4C11, 18A10, and CT1 with the human receptor is to be expected, as the predicted amino acid sequence of the human type I InsP3 receptor is very similar to that of the mouse.

mRNA Analysis—Three flasks of control or carbachol-pretreated SH-SY5Y cells were rinsed with and harvested in 25 ml of HBSE. Following centrifugation (500 × g for 2 min), RNA was extracted using acid guanidium thiocyanate–phenol–chloroform (20). mRNA was then isolated using oligo(dT)-coupled magnetic beads (Promega). For each treatment the quality of preparation was checked visually after electrophoresis of RNA and mRNA samples on ethidium bromide–containing agarose minigel. mRNA purity and concentration were then quantitated spectrophotometrically (18); A260/A280 was always ≥ 2.0, and only minor differences in A260 of preparations from control and pretreated cells were detected. mRNA was then purified from RNA by treatment with formaldehyde, 1% agarose gels, blotted to nitrocellulose, and baked (18).

Prehybridization and hybridization in buffer containing 6 × SSPE, 2 × Denhardt’s reagent, 0.1% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA, 50% formamide and the washing of membranes were performed as described (18). The probe used, a 1.2-kilobase fragment of human type I InsP3 receptor cDNA corresponding to residues 2992–4130 of the mouse sequence, was labeled with [α-32P]dCTP using T7 DNA polymerase (Pharmacia LKB Biotechnology Inc.).

Immunoprecipitation of [35S]Methionine-labeled Receptor—Pilot experiments in which the immunoprecipitable type I InsP3 receptor of SH-SY5Y cells was labeled with [35S]Methionine either in methionine-free medium without serum or in normal SH-SYSY cell culture medium yielded similar data.

For analysis of the rate of receptor degradation, subconfluent cells in 80-cm2 flasks were labeled in 12 ml of normal culture medium with 60 μCi of [35S]Methionine for 40 h. Monolayers were then washed with 15 ml of chase medium (normal culture medium supplemented with 5 μM methionine and 20 μM Hepes, pH 7.5) and finally incubated with 10 ml of chase medium with or without carbachol. After 2–8 h of chase medium was removed, and the cells were harvested in 10 ml of HBSE and centrifuged (500 × g for 2 min).

Cell suspensions were used when the effects of EGTA and thapsigargin on receptor degradation were examined. In these experiments subconfluent cells in 175-cm2 flasks were labeled in 20 ml of normal culture medium with 125 μCi of [35S]Methionine for 40 h, monolayers were washed with 10 ml of chase medium, harvested in 10 ml of HBSE, and centrifuged at 500 × g for 2 min. The pellet was then washed once in chase medium and finally resuspended in 2 ml of chase medium/flask of cells. After division into 1-ml aliquots, the cell suspensions were incubated for 6 h with stimuli or inhibitors at 37 °C with occasional shaking and finally were centrifuged (500 × g for 1 min).

For each experiment and suspension experiment were then resuspended in 0.9 ml of ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 μg/ml aprotinin, 10 μM peptatin and leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, pH 8.0) and transferred to 1.5-ml microcentrifuge tubes. After incubation on ice for 30 min, lysates were centrifuged (13,000 × g for 8 min at 4 °C), and the supernatants were transferred to tubes containing 100 μl of 20% non-fat dried milk, 0.25% Tween 20, and 12 μl of crude CT1 antisera. After 40 min on ice, 300 μl of protein A-Sepharose slurry (Pharmacia) was added, and after a further 30 min at 4 °C, immune complexes were purified by washing the Sepharose beads five times with 1 ml of lysis buffer. After the addition of 30 μl of 2 × gel loading buffer (18) and 5 μl of 8 μl aera, bound proteins were eluted by heating the beads for 3 min at 100 °C. Proteins were then subjected to electrophoresis, and gels were fixed with 10% acetic acid, 50% methanol, impregnated with Amplify (Amersham), dried, and autoradiographed.

Phosphorylation—Cells from 175-cm2 flasks were harvested in HBSE, centrifuged (500 × g for 2 min), washed twice in phosphate-free Krebs-Hepes buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 10 mM glucose, 25 mM NaHCO3, 10 mM Hepes, pH 7.4), and finally resuspended in the same buffer (2 ml/flask of cells) containing 0.2 mM/ml [32P]P. Cells were then aliquoted into 1-ml portions and incubated for 1 h at 37 °C. After the addition of stimuli and further incubation at 37 °C, cells were centrifuged (500 × g for 1 min) and resuspended in 0.9 ml of ice-cold lysis buffer supplemented with the phosphatase inhibitor p-nitrophenyl phosphate (7.6 mM). The type I InsP3 receptor was then isolated as for [35S]Methionine-labeled cells, except that 18A10 (10 μl of crude culture medium) was the immunoprecipitating antibody.

Measurement of InsP3 Mass—Cells were subcultured into multiwell dishes (1.8 cm2/well) and after 24 h of normal culture medium. Incubation was terminated by removal of media and the addition of 150 μl of 0.5 M trichloroacetic acid and 40 μl of 10 mM EDTA. Cells were then transferred to 1.5-ml microcentrifuge tubes, 200 μl of 1:1 freon/ octylamine was added, and following centrifugation (13,000 × g for 5 min), samples were assayed for InsP3 mass as described (21).

Measurement of Ca2+ Mobilization—InsP3-induced Ca2+ mobilization from electroperelemassized cells was assessed as described (14).

Miscellany—Conjugated goat anti-rabbit and rabbit antibodies, prestained molecular mass markers, receptor agonists, dithiothreitol, protease and phosphatase inhibitors, thapsigargin, 1-methionine and (2S,3S)-trans-epoxysuccinyl-7-leucylamido-3-methylbut~e~llepoly~e~ster (E64) were from Bachem. RNA size markers were from Promega. 1-[35S]Methionine (1,000 Ci/mmol), [32P] (10 Ci/mml), and [α-32P]dCTP (3,000 Ci/mmoll were from Amersham. Protein was assayed with Coomassie Blue using bovine serum albumin as a standard (22). Unless stated otherwise, data shown are representative of at least two independent experiments, and percentage changes quoted or plotted graphically are the means of values from those experiments.

RESULTS

Detection of Type I InsP3 Receptor Down-regulation—Antibody 4C11 recognizes a protein in rat cerebellum preparations with an apparent molecular mass ~ of 275 kDa (Fig. 1, lane 1), which corresponds to the 313-kDa product of the rodent type I InsP3 receptor gene (2, 3). This antibody also recognizes a protein with the same mobility in SH-SY5Y cells which corresponds in size to the human type I InsP3 receptor (Fig. 1, lane 2). Pretreatment of SH-SY5Y cells in culture with carbachol for 6 h reduced the intensity of this ~275 kDa band by 92% (Fig. 1, lane 3). This finding is in close agreement with our previous study on SH-SY5Y cells using antibody 18A10 (15), in which carbachol induced an ~90% fall in type I InsP3 receptor immunoreactivity by 6 h (half-maximal effect at ~1 h) which was maintained for up to 24 h. As 4C11 and 18A10 recognize amino-terminal and carboxyl-terminal regions of the type I InsP3 receptor, respectively, these data show that the cellular concentration of the entire type I InsP3 receptor polypeptide is reduced.

2 T. Furuichi and K. Mikoshiba, unpublished data.

3 T. Furuichi and K. Mikoshiba, submitted for publication.
Type I InsP₃ Receptor Down-regulation

Analysis of Type I InsP₃ Receptor mRNA Concentration—The effects of muscarinic stimulation on type I InsP₃ receptor mRNA concentration were examined. Fig. 2A shows that a probe derived from human type I InsP₃ receptor cDNA hybridized with a ~10-kilobase mRNA species from control cells (lane 1) and that the concentration of this species was the same after treatment with carbachol for 20 h (lane 2). The concentration of this species, which is the appropriate size for the precursor of InsP₃ receptor polypeptides, was, however, reduced by shorter incubations with carbachol, decreasing sharply after 1 h to a nadir of 38 ± 15% of control levels at 3 h (Fig. 2B). Despite this decrease, these data reveal no correlation between mRNA levels and the kinetics of changes in type I InsP₃ receptor concentration (15).

Analysis of Receptor Degradation—We next examined the possibility that degradation of the type I InsP₃ receptor was accelerated by carbachol. SH-SY5Y cells were labeled for 40 h with [³⁵S]methionine and the rate at which radioactivity was lost from immunoprecipitable type I InsP₃ receptor during subsequent incubations in medium containing 5 nm nonradioactive methionine was examined. Fig. 3A shows that the half-time of loss of radioactivity (t₁/₂) in unstimulated cells was > 8 h, whereas in the presence of carbachol, t₁/₂ was < 2 h. These data indicate that an increase in the rate of type I InsP₃ receptor degradation accounts for its down-regulation. This effect was specific, as carbachol did not alter the overall [³⁵S] content of the array of proteins detected after electrophoresis of crude cell lysates.

Effects of Perturbation of Ca²⁺ Homeostasis on Receptor Down-regulation and Degradation—As Ca²⁺ mobilization is pivotal to the intracellular signaling initiated by phosphoinositide C-linked muscarinic receptors (23, 24), the effects of perturbing Ca²⁺ homeostasis were examined. We have previously shown that reducing extracellular Ca²⁺ concentration to ~200 nm with EGTA blocks the ability of carbachol to cause type I InsP₃ receptor down-regulation (15). Fig. 3B shows that this results from a reversal of the ability of carbachol to accelerate receptor degradation; carbachol accelerates degradation in cells suspended in normal medium (lanes 1 and 2) but not in cells incubated with EGTA (lanes 3 and 4).

The effects of thapsigargin were also examined. By inhibiting endoplasmic reticulum ATPases (25), thapsigargin releases intracellular Ca²⁺ stores including those normally mobilized by InsP₃ (24–26) and in some cells, including SH-SY5Y cells, stimulates Ca²⁺ entry via "capacitative refilling" (1, 26). These effects lead to persistent increases in cytosolic Ca²⁺ concentra-

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4 R. J. H. Wojcikiewicz and S. R. Nahorski, unpublished data.
5 D. G. Lambert and S. R. Nahorski, unpublished data.
Receptor Down-regulation in Other Cell Types—To characterize further type I InsP₃ receptor down-regulation, the effects of a range of agonists at phosphoinositidase C-linked receptors were examined in a variety of cell types. In SH-SY5Y cells, bradykinin as well as muscarinic receptors are coupled to phosphoinositidase C activation, although bradykinin produces a much smaller and more transient response than carbachol, and after incubation with 20 μM bradykinin for 12 h, the intracellular InsP₃ concentration was no different from control values.⁶ Fig. 6A (lanes 2–6) shows that although carbachol caused down-regulation with a half-maximal effect at ~0.1 μM, bradykinin was ineffective (lane 7). Similar effects of carbachol were seen in rat cerebellar granule cells (Fig. 6B), in which carbachol also persistently enhances InsP₃ formation (17).

As SH-SY5Y and granule cells express multiple muscarinic receptors subtypes (17, 31) we sought to determine which ones initiate down-regulation. Of the five subtypes known, m₁, m₃, and m₅ are coupled preferentially to activation of phosphoinositidase C, and m₂ and m₄ to inhibition of adenylate cyclase and activation of K⁺ channels (23). Thus, we examined the effects of carbachol in CHO cells transfected with human m₁, m₂, and m₃ receptor cDNA (23). In these cells, phosphoinositidase C-linked receptors were immunoprecipitated using antibody CT1 and isolated in thapsigargin (lanes 3–6). The graph depicts the loss of radioactivity from the type I InsP₃ receptor expressed as a percentage of control values (lane 1).

SH-SY5Y cells in multwell dishes were incubated in normal culture medium in the absence or presence of 1 mM carbachol with either vehicle (0.1% dimethyl sulfoxide, lanes 1 and 2) or 2 μM thapsigargin (lanes 3 and 4) in the absence or presence (+) of 1 mM carbachol. The position of the type I InsP₃ receptor is indicated by the arrow.

Table I

| Condition                              | InsP₃ pmol/mg protein |
|----------------------------------------|----------------------|
| Carbachol                              | 15.9 ± 1.7           |
| Carbachol with thapsigargin            | 35.3 ± 4.0*          |
| Carbachol with thapsigargin + carbachol| 22.4 ± 3.0           |
| Carbachol with thapsigargin            | 38.7 ± 2.2*          |

*p < 0.001 comparing InsP₃ concentration in the absence or presence of carbachol.

⁶ G. B. Willars and S. R. Nahorski, manuscript in preparation.
Type I InsP₃ Receptor Down-regulation

Fig. 5. Phosphorylation of the type I InsP₃ receptor in intact SH-SY5Y cells. SH-SY5Y cells in suspension were labeled with ²²P, for 1 h at 37 °C and then incubated further at 37 °C with either vehicle (lane 1), 1 mM carbachol (lane 2), 10 µM forskolin (lane 3), or 10 µM forskolin for 10 min (lane 4). The type I InsP₃ receptor was then immunoprecipitated with antibody 18A10 and isolated in a 6% polyacrylamide gel. The position of the type I InsP₃ receptor is indicated with an arrow.

Fig. 6. Analysis of type I InsP₃ receptor immunoreactivity in a range of cell types. Samples (30 µg of protein/lane) from control or pretreated cells were analyzed as in Fig. 1 except that antibody CT1 was used. Panel A, SH-SY5Y cells were incubated for 18 h with either vehicle (lane 2), 0.1 µM carbachol (lane 3), 1 µM carbachol (lane 4), 10 µM carbachol (lane 5), 1 mM carbachol (lane 6), or 20 µM bradykinin (lane 7). Rat cerebellum (2 µg) was run in lane 1. Panel B, rat cerebellar granule cells were incubated for 18 h with either vehicle (lane 1) or 1 mM carbachol (lane 2). Panel C, CHO-m1 cells (lanes 1 and 2), CHO-m2 cells (lanes 3 and 4) and CHO-m3 cells (lanes 5 and 6) were incubated for 18 h with either vehicle (lanes 1, 3, and 5) or 1 mM carbachol (lanes 2, 4, and 6). Panel D, GH3 cells were incubated for 18 h with either vehicle (lane 1) or 2 µM thyrotropin-releasing hormone (lane 2). Panel E, A10 cells were incubated for 18 h with either vehicle (lane 1), 2.5 µM angiotensin II (lane 2), 100 nM endothelin I (lane 3), or 1 µM phenylephrine (lane 4). Panel F, Swiss 3T3 cells were preincubated for 18 h in serum-free medium containing 1% bovine serum albumin and were then incubated in the same medium for 6 h with either vehicle (lane 1), 10% fetal calf serum (lane 2), 500 nM bombesin (lane 3), or 100 nM endothelin I (lane 4). Panel G, H9c2 cells were incubated for 18 h with either vehicle (lane 1) or 1 µM phenylephrine (lane 2).

dase C-linked receptor activation elevates InsP₃ concentration for hour-long periods (32), whereas m2 receptors do not stimulate InsP₃ formation.⁴ Fig. 6C shows that carbachol causes type I InsP₃ receptor down-regulation in those cells expressing m1 and m3 receptors, but not in those expressing m2 receptors. Fig. 7 provides the functional correlate of this finding, since InsP₃-induced °Ca²⁺ mobilization was suppressed only in those cells expressing m1 and m3 receptors. Thus, it can be concluded that only the phosphoinositidase C-linked muscarinic receptors are capable of causing InsP₃ receptor down-regulation.

Fig. 7. Effects of pretreatment with carbachol on InsP₃-induced Ca²⁺ mobilization in permeabilized CHO cells expressing m1 (panel A), m2 (panel B), and m3 (panel C) receptors. Control cells (○) or cells that had been pretreated with 1 mM carbachol for 20 h (●) were harvested, permeabilized electrically, and loaded with °Ca²⁺. Ca²⁺ mobilized in response to a range of InsP₃ concentrations was then assessed after incubations for 2 min at 20 °C. Data shown are the means of three independent experiments. Concentrations of InsP₃ causing half-maximal stimulation (mean ± S.E., n = 3) in control and carbachol-treated CHO-m1 cells were 0.10 ± 0.01 and 0.27 ± 0.01 µM, respectively, and in CHO-m3 cells were 0.14 ± 0.02 and 0.28 ± 0.05 µM, respectively.

Type I InsP₃ receptor immunoreactivity was also detected in GH₃ rat pituitary cells (Fig. 6D), in A10 rat smooth muscle cells (Fig. 6E), in Swiss mouse 3T3 fibroblasts (Fig. 6F), and in rat H9c2 cardiac muscle cells (Fig. 6G). However, none of the agonists tested altered the level of immunoreactivity. This may be because the agonists used did not stimulate phosphoinositide hydrolysis persistently. For example, thyrotropin-releasing hormone produces a robust increase in phosphoinositide hydrolysis and InsP₃ formation within seconds of its addition to GH₂ cells (33), but when measured after 12 h of incubation, InsP₃ mass in cells stimulated with 2 µM thyrotropin-releasing hormone was the same as that in control cells (25.2 ± 0.8 and 26.1 ± 0.7 pmol/mg protein, respectively; mean ± S.E. of sextuplicate incubations).

DISCUSSION

The main conclusion to be drawn from the data presented is that the type I InsP₃ receptor of SH-SY5Y cells is down-regu-
lated during muscarinic stimulation because it is degraded more rapidly than in resting cells. The $t_{1/2}$ of the receptor in carbachol-stimulated cells was $\approx 1$ h, whereas in resting cells the $t_{1/2}$ was $>8$ h. This latter value immediately rules out a reduction in the rate of receptor synthesis as the sole cause of down-regulation, even if type I InsP$_3$ receptor production was blocked totally upon addition of carbachol, receptor concentration would only fall with a half-time of $>8$ h. Rather, the increase in the rate of receptor degradation is alone sufficient to reduce rapidly the cellular concentration of the type I InsP$_3$ receptor. Indeed, $t_{1/2}$ in carbachol-stimulated cells concurs well with the time at which down-regulation of the type I InsP$_3$ receptor is half-maximal (15).

Despite the evidence against a role for regulation of receptor synthesis as the cause of down-regulation, type I InsP$_3$ receptor mRNA levels did fall after 3 h of stimulation but then returned to basal levels. Such complex changes during muscarinic stimulation are common to other mRNA species, for example, m2 and m3 muscarinic receptor mRNA in cerebellar granule (34) and SH-SY5Y (35) cells, and suggest that reduced type I InsP$_3$ receptor synthesis may contribute toward down-regulation at certain times. However, verification of this suggestion will require that the rates of mRNA translation are measured directly.

The studies with CHO cells revealed that only phosphoinositidase C-linked muscarinic receptor subtypes caused down-regulation. Although stimulation of these receptors initiates a cascade of intracellular signaling (23), the primary response to receptor activation appears to be confined to the breakdown of PIP$_2$ and the formation of the second messengers InsP$_3$ and 1,2-diacylglycerol. InsP$_3$ releases intracellular Ca$^{2+}$ stores via InsP$_3$ receptors and also seems to be involved in causing entry of Ca$^{2+}$ into the cell, either directly by interacting with plasma membrane InsP$_3$ receptors (1) or indirectly via capacitative refilling of discharged intracellular stores (1, 26). In contrast, 1,2-diacylglycerol activates protein kinases C (36). Although protein kinase C activation does not initiate type I InsP$_3$ receptor down-regulation (15), the data presented here indicate that both InsP$_3$ and Ca$^{2+}$ stores have roles to play in this process. Initial evidence for this came from the finding that acceleration of receptor degradation was blocked by chelation of extracellular Ca$^{2+}$ with EGTA. This manipulation inhibits Ca$^{2+}$ influx and also eventually depletes intracellular Ca$^{2+}$ stores. However, as Ca$^{2+}$ derived from both stores and entry acts as a feedback activator of phosphoinositide hydrolysis during muscarinic stimulation of SH-SY5Y cells (24), depletion of extracellular Ca$^{2+}$ not only suppresses Ca$^{2+}$ mobilization but also markedly inhibits PIP$_2$ hydrolysis. Thus, treatment with EGTA does not discriminate between Ca$^{2+}$ mobilization or InsP$_3$ as the signal that initiates acceleration of receptor degradation.

More revealing are the results obtained with thapsigargin. Without significantly raising InsP$_3$ levels, thapsigargin persistently discharges intracellular Ca$^{2+}$ stores, including those normally released by InsP$_3$ (24, 26), and also, because of capacitative refilling, causes Ca$^{2+}$ entry (26). However, thapsigargin alone did not accelerate the degradation of or cause down-regulation of the type I InsP$_3$ receptor. These data together with the observation that that persistent depolarization of SH-SY5Y cells with K$^+$ does not cause down-regulation (15) show that accelerated degradation is not simply a consequence of chronic release of intracellular Ca$^{2+}$ stores, Ca$^{2+}$ entry, or elevation of cytosolic Ca$^{2+}$ concentration. Most significantly, thapsigargin blocked the ability of carbachol to cause down-regulation without inhibiting its ability to elevate InsP$_3$ concentration. Thus, persistent activation of PIP$_2$ hydrolysis alone is not sufficient to cause down-regulation. Furthermore, as the InsP$_3$ formed in the presence of thapsigargin will be unable to enhance the passage of Ca$^{2+}$ through the channels formed by tetrameric InsP$_3$ receptors, because the gradient driving this movement will have been discharged, it appears that functional intracellular Ca$^{2+}$ stores are required for down-regulation to occur. This linkage of Ca$^{2+}$ stores with the mechanism of type I InsP$_3$ receptor down-regulation is given credence by the observation that half-maximal inhibition of type I InsP$_3$ receptor immunoreactivity was seen at $\approx 0.1$ \mu mol carbachol. This value is very close to that which gives half-maximal Ca$^{2+}$ mobilization during prolonged incubations of SH-SY5Y cell monolayers with carbachol (0.3 \mu mol).6 Thus, we propose that the signal that initiates acceleration of type I InsP$_3$ receptor degradation is the efflux of Ca$^{2+}$ from intracellular stores via the InsP$_3$ receptor itself. Perhaps the conformational changes that appear to accompany type I InsP$_3$ receptor activation (5) and the flux of Ca$^{2+}$ (12) expose the type I InsP$_3$ receptor to a degradative pathway.

Regarding the nature of this pathway, it has been shown recently that calpain cleaves the type I InsP$_3$ receptor into fragments of $\approx 130$ and $\approx 95$ kDa (27). However, cleavage by calpain was not responsible for the down-regulation seen in SH-SY5Y cells, as EST did not block this process, and immunoreactive fragments did not appear as type I InsP$_3$ receptor levels declined. Neither does phosphorylation of the type I InsP$_3$ receptor seem to play a role in its down-regulation. Although the receptor is clearly a phosphoprotein in resting SH-SY5Y cells, the extent of its phosphorylation was unaltered by carbachol. It is noteworthy that forskolin also had no effect on the extent of phosphorylation. Thus, at least in SH-SY5Y cells, activation of the two major signaling pathways used by hormone and neurotransmitter receptors does not regulate type I InsP$_3$ receptor phosphorylation. This contrasts with data obtained from cell-free systems (29).

Investigation of whether or not down-regulation occurred in other cell types revealed that muscarinic receptor stimulation was effective in reducing immunoreactivity in CHO cells expressing phosphoinositidase C-linked muscarinic receptors and also in cerebellar granule cells. The latter finding is particularly significant as it shows that down-regulation is not just a feature of cell lines, but can also occur in primary cultures. In each of these cell types, muscarinic receptor activation stimulates PIP$_2$ hydrolysis persistently (14, 17, 32), and it is likely that other receptors that share this characteristic (37, 38) will be those with the capacity to cause InsP$_3$ receptor down-regulation. It will also be interesting to ascertain how persistent phosphoinositidase C activation relates to the observation that GTP-binding proteins that couple receptors to phosphoinositidase C in CHO-m1 cells are down-regulated during chronic stimulation with carbachol (39). In contrast to muscarinic receptors, however, many phosphoinositidase C-linked receptors are subject to rapid and severe desensitization (37, 38). The transience of the responses generated by such receptors may explain why, for example, bradykinin did not cause type I InsP$_3$ receptor down-regulation in SH-SY5Y cells and why in the other cell types examined, none of the agonists tested was effective.

As yet we have only examined type I InsP$_3$ receptor down-regulation. This is the predominant type in the central nervous system (40) and in SH-SY5Y cells appears to mediate the majority of Ca$^{2+}$ store mobilization. This can be concluded because down-regulation of the type I InsP$_3$ receptor by $\approx 90\%$ suppressed markedly InsP$_3$ action; maximal Ca$^{2+}$ mobilization was reduced by half, and the potency of InsP$_3$ was reduced 3-fold (14). Similarly, in CHO cells, type I InsP$_3$ receptor down-regulation reduced the maximal effect of InsP$_3$ by one-third and its potency 2--3-fold. Nevertheless, significant amounts of types II and III receptor are expressed in brain (40) and in other tissues.
and cell lines (8, 9), and it will be fascinating to see whether these too are down-regulated.

In conclusion, this study has shown that persistent muscarinic receptor stimulation causes severe down-regulation of the type I InsP3 receptor in several cell types. Acceleration of type I InsP3 receptor degradation provides the mechanistic basis for this regulation, and sustained InsP3 generation and Ca2+ release from intracellular stores appear to be essential in the initiation of this process.

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REFERENCES

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Furuchi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989) Nature 342, 32–38
3. Mignery, G. A., Newton, C. L., Archer, B. T., III, and Sudhof, T. C. (1990) J. Biol. Chem. 265, 12678–12685
4. Miyawaki, A., Furuchi, T., Ryu, Y., Yoshikawa, S., Nakagawa, T., Sai toh, T., and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4911–4915
5. Mignery, G. A., and Sudhof, T. C. (1990) EMBO J. 9, 3883–3888
6. Chadwick, C. C., Sai toh, T., and Fleischer, S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2132–2136
7. Nakagawa, T., Okano, H., Furuchi, T., Aruga, J., and Mikoshiba, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6244–6248
8. Sudhof, T. C., Newton, C. L., Archer, B. T., III, Us hikaryov, Y. A., and Mignery, G. A. (1991) EMBO J. 10, 3199–3206
9. Bressollet, O., Takeda, J., Jansen, H., Seino, S., and Bell, G. I. (1993) J. Biol. Chem. 268, 11356–11363
10. Missiaen, L., De Smedt, H., Droogmans, G., and Casteels, R. (1992) Nature 357, 599–602
11. Bepprovann, I., Watras, J., and Ehrlich, B. E. (1991) Nature 351, 751–754
12. Watras, J., Bepprovann, I., and Ehrlich, B. E. (1991) J. Neurosci. 11, 3239–3245
13. Lambert, D. G., and Nahorski, S. R. (1990) Biochim. Biophys. Acta 1029, 244–256
14. Wójcikiewicz, R. J. H., and Nahorski, S. R. (1991) J. Neurochem. 55, 555–562
15. Wójcikiewicz, R. J. H., Nakada, S., Mikoshiba, K., and Nahorski, S. R. (1992) J. Neurochem. 59, 383–388
16. Bradford, P. G., Waeg, X., Jin, Y., and Hui, P. (1992) J. Biol. Chem. 267, 20953–20964
17. Whitlam, E. M., Challiss, R. A. J., and Nahorski, S. R. (1991) Eur. J. Pharmacol. 206, 181–189
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Nakade, S., Maeda, N., and Mikoshiba, K. (1991) Biochem. J. 277, 125–131
20. Chomczynski, P., and Sacci, N. (1987) Anal. Biochem. 162, 156–159
21. Challiss, R. A. J., and Nahorski, S. R. (1990) Methods Neurosci. 18, 224–244
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
23. Hulman, E. C., Birdsell, N. J. M., and Buckley, N. J. (1990) Annu. Rev. Pharmacol. Therapeutics 30, 683–673
24. Wójcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1994) J. Neurochem., in press
25. Thastrup, O., Cullen, P. J., Drohak, B. K., Hanley, M. R., and Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470
26. Putney, J. W., Jr. (1990) Cell Calcium 11, 611–624
27. Magnusson, A., Haug, I. S., Walass, I., and Ostvold, A. C. (1993) FEBS Lett. 323, 229–232
28. McGowan, E. B., Becker, E., and Dettwiler, T. C. (1989) Biochem. Biophys. Res. Commun. 158, 432–435
29. Ferris, C. D., and Snyder, S. H. (1992) J. Neurosci. 12, 1567–1574
30. Lambert, D. G., and Nahorski, S. R. (1990) Biochem. Pharmacol. 40, 2291–2295
31. Wall, S. J., Yasuda, R. P., Li, M., and Wolfe, B. B. (1991) Mol. Pharmacol. 40, 783–789
32. Tobin, A. B., Lambert, D. G., and Nahorski, S. R. (1992) Mol. Pharmacol. 42, 1042–1048
33. Gerashengorm, M. C. (1986) Annu. Rev. Physiol. 48, 515–526
34. Fukumori, F., Hough, C., and Chuang, D.-M. (1991) J. Neurochem. 56, 716–719
35. Steel, M. C., and Buckley, N. J. (1993) Mol. Pharmacol. 43, 694–701
36. Nishiura, Y. (1992) Science 258, 607–614
37. Wójcikiewicz, R. J. H., Tobin, A. B., and Nahorski, S. R. (1993) Trends Pharmacol. Sci. 14, 279–285
38. Menetti, F. S., Takemura, H., Oliver, K. G., and Putney, J. W., Jr. (1991) Mol. Pharmacol. 40, 727–733
39. Mitchell, F. M., Buckley, N. J., and Milligan, G. (1993) Biochem. J. 298, 495–499
40. Furuchi, T., Simon-Chazottes, D., Fujino, I., Yamada, N., Hasegawa, M., Miyawaki, A., Yoshikawa, S., Gueret, J.-L., and Mikoshiba, K. (1993) Receptors Channels 1, 11–14