Molecular Biology of the Cell
Vol. 7, 949–960, June 1996

Inositol 1,4,5-Trisphosphate Receptors in Endocrine Cells: Localization and Association in Hetero- and Homotetramers

Frederick C. Nucifora, Jr.,* Alan H. Sharp,* Sharon L. Milgram,‡ and Christopher A. Ross*†§

The Johns Hopkins University School of Medicine, Laboratory of Molecular Neurobiology, Departments of *Psychiatry and †Neuroscience, Baltimore, Maryland 21205-2196; and ‡University of North Carolina-Chapel Hill, Department of Physiology, CB7545, Chapel Hill, North Carolina 27599

Submitted September 20, 1995; Accepted March 20, 1996
Monitoring Editor: Guido Guidotti

The inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular calcium channel involved in coupling cell membrane receptors to calcium signal transduction pathways within cells including endocrine cells. Several isoforms (I, II, and III) of IP_3Rs have been identified, which are encoded by separate genes, and are expressed in many tissues with differing patterns of cellular expression. We have generated specific affinity-purified polyclonal anti-peptide antibodies to each of the three isoforms. Western blot analysis of RINm5F and ATt20 cells shows high levels of endogenously expressed type I and type III IP_3R, but undetectable levels of type II. Immunofluorescence studies revealed an endoplasmic reticulum-like pattern similar to BiP, an ER marker. In contrast with previous claims, both type I and type III IP_3Rs were absent from the secretory granules of ATt20 cells. Western blots of sucrose gradients and gel filtration probed with antibodies to either type I or type III showed a molecular weight of greater than 1,000 kDa consistent with a tetrameric structure. Co-immunoprecipitation experiments indicated that most of the receptors were present as heterotetramers. Homotetramers were identified for the type III IP_3R; however, type I homotetramers were undetectable. These data suggest that molecular association of IP_3Rs into heterotetrameric forms can contribute to the complexity of the regulation of Ca^{2+} release from ER by IP_3Rs within cells.

INTRODUCTION

Calcium is a ubiquitous second messenger in neurons and other cells (Berridge and Irvine, 1989; Berridge, 1993; Simpson *et al.*, 1995). One source of calcium signaling arises from intracellular stores under the control of the second messenger inositol trisphosphate (IP_3; Ferris and Snyder, 1992; Berridge, 1993; Furuichi and Mikoshiba, 1995). Release of calcium from the endoplasmic reticulum (ER) is triggered via hormone, neurotransmitter, or growth factor stimulation of cell surface receptors, leading to G protein-linked activation of the enzyme phospholipase C and generating IP_3. IP_3 stimulates the inositol 1,4,5-trisphosphate receptor (IP_3R), resulting in calcium release. Experiments using both permeabilized cells and isolated membranes demonstrate that modulation of the IP_3R can be complex; regulation of calcium release by IP_3 may differ depending on cell type (Ehrlich and Watras, 1988; Finch *et al.*, 1991; Ferris and Snyder, 1992; Iino and Endo, 1992; Kasai *et al.*, 1993; Khodakhah and Ogden, 1993; Hajnoczky and Thomas, 1994). The IP_3R is also regulated by ATP, protein kinases, and other intracellular regulators (Ehrlich and Watras, 1988; Bezprozvanny *et al.*, 1991; Ferris *et al.*, 1991; Iino and Endo, 1992; Kasai *et al.*, 1993; Hajnoczky and Thomas, 1994).

The IP_3R has been purified from rat brain cerebellum (Supattapone *et al.*, 1988b) and localized to the ER (Mignery *et al.*, 1989; Ross *et al.*, 1989). Molecular clon-

§ Corresponding author.

ing of an IP_3R cDNA (now called type I) indicates that it encodes an ~313-kDa protein with either six or eight putative membrane spanning domains (Furuichi *et al.*, 1989; Mignery *et al.*, 1989; Mignery and Sudhof, 1990; Miyawaki *et al.*, 1990; Maeda *et al.*, 1991). The IP_3 binding domain is located at the amino-terminus; adjacent to the binding domain is a regulatory region called the coupling domain. The membrane spanning domains and the channel pore are formed by a region near the carboxyl-terminus. Biochemical studies of the purified receptor isolated from rat cerebellum suggest a tetrameric structure (Supattapone *et al.*, 1988b).

Several sources of molecular diversity exist for the IP_3R. First, multiple genes encode different IP_3Rs, termed type I, type II, and type III. The IP_3R isoforms exhibit similar molecular structures with high conservation in the binding and transmembrane domains, but somewhat less conservation in the coupling domain (Mignery *et al.*, 1990; Sudhof *et al.*, 1991; Meldolesi, 1992; Ross *et al.*, 1992; Blondel *et al.*, 1993; Maranto, 1994). The three isoforms appear to have dissimilar binding affinities for IP_3 and the expression of each isoform is tissue specific (Sudhof *et al.*, 1991; Newton *et al.*, 1994; Nakagawa *et al.*, 1991a). The type I IP_3R is expressed at very high levels in cerebellar Purkinje cells, in other neurons, and in smooth muscle. The type III receptor is expressed in intestinal epithelial cells (Maranto, 1994), and in many other cell types including endocrine cells and brain (Blondel, 1993). The type II receptor is expressed at low levels in a variety of cell types (Ross *et al.*, 1992).

A second level of diversity exists for the type I IP_3R, which has three identified regions of alternative mRNA splicing (Danoff *et al.*, 1991; Nakagawa *et al.*, 1991b; Schell *et al.*, 1993; Nucifora *et al.*, 1995). The functions of the S1 and S3 splice sites are unknown. Alternative splicing (S2) in the coupling domain (S2) may contribute to differences in regulation of the IP_3R by phosphorylation, in neurons and cells in the periphery (Danoff *et al.*, 1991; Supattapone *et al.*, 1988a).

Subcellular localization studies using antibodies to purified cerebellar receptor, or to peptides from the cloned type I receptor, indicate that IP_3Rs are present in the ER and possibly in caveolae (Ross *et al.*, 1989; Otsu *et al.*, 1990; Fujimoto *et al.*, 1992). A recent study using antibodies to peptides derived from the type III IP_3R suggests that it may be localized to secretory granules in pancreatic endocrine cells (Blondel *et al.*, 1994). However, these findings are controversial (Blondel, 1995; Meldolesi and Pozzan, 1995).

Biochemical studies of the purified cerebellar IP_3R and transfected type I IP_3R suggested that IP_3R channels associate in complexes of individual IP_3R molecules (Supattapone *et al.*, 1988b; Mignery *et al.*, 1989; Mignery and Sudof, 1990) consistent with a tetrameric structure. Cellular expression studies and monoclonal antibody studies have indicated that the monomers are associated at the COOH-terminus of the receptor (Miyawaki *et al.*, 1990; Nakade *et al.*, 1991). Cross-linking of purified IP_3Rs indicated that they associate into tetramers (Maeda *et al.*, 1991). Using CHO-K1 cells, a fibroblast-derived cell line, Monkawa *et al.* (1995) recently demonstrated that different isoforms of IP_3Rs associate as hetero-oligomers.

We have now used specific antipeptide antibodies to the type I and type III IP_3R to address the question of whether the receptors are associated as hetero- or homotetramers in endocrine cells. We have performed immunocytochemical experiments in mouse anterior pituitary corticotrope AtT-20 and rat pancreatic islet RINm5F cell lines to localize the type I and type III isoforms.

MATERIALS AND METHODS

Preparation of Antibodies Against Synthetic IP_3R Protein Peptides

Peptides (Figure 1) were conjugated to chicken ovalbumin, bovine thyroglobulin, and bovine serum albumin essentially as described (Harlow and Lane, 1988) using 0.1% glutaralehyde. Peptides (9–10 mg) were dissolved in 1 ml 100 mM sodium phosphate (pH 7.2) and 25 mg of carrier protein was added. An equal volume of 0.2% glutaralehyde in the same buffer was added and allowed to react for 1 h at room temperature. The reaction was stopped by the addition of 1 M glycine in 200 mM sodium phosphate buffer (pH 7.9) to a final concentration of 200 mM. The glycine was allowed to react for 1 h at room temperature and the mixture was then dialyzed extensively against phosphate-buffered saline (PBS). Rabbits were immunized using the thyroglobulin conjugates for the first two inoculations (days 0 and 14) and the bovine serum albumin conjugates for subsequent boosts (days 21 and 51 and each 1–3 mo thereafter) by Cocalico Biologicals (Reamstown, PA). Antisera were initially screened by Western blot against the ovalbumin-peptide conjugates. To purify specific antibodies, affinity columns were produced by immobilization of the ovalbumin conjugates on Affigel-15–activated agarose resin (Bio-Rad, Richmond, CA) according to the manufacturer. To reduce the concentration of nonspecific antibodies, serum (15–20 ml) was first mixed batchwise overnight at 4°C in the presence of 1 mM EDTA and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin-A, 1 μg/ml aprotinin, 0.5 μg/ml antipain, 1 μg/ml chymostatin) with an affinity resin consisting of an unrelated peptide-ovalbumin conjugate immobilized on Affigel-15. The serum was then collected and mixed batchwise with the appropriate peptide affinity matrix overnight at 4°C. The affinity matrix was poured into a column and washed sequentially with 25 ml of 0.1% Triton X-100 in 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, with 25 ml of 50 mM Tris-HCl (pH 7.4), 1 M NaCl, with 25 ml of 50 mM Tris-HCl (pH 7.4), 0.9% NaCl, and finally with 10 ml of 10 mM Tris-HCl (pH 7.4) before elution of specific antibodies with 4.5 M $MgCl_2$. Fractions (1 ml each) of the eluate containing significant absorbance at 280 nm were pooled and dialyzed extensively against 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.4), 0.9% NaCl followed by dialysis into 40% glycerol in the same buffer. Antibodies were stored at −20°C. Affinity-purified antibodies from rabbit 41 (type I IP_3R specific) were termed AP 41; those from rabbit 42 (type II IP_3R specific) were termed AP42; those from rabbit 45 (against type III IP_3R internal peptide) were termed AP45; and those from rabbit 75 (type III IP_3R C terminus specific) were termed AP75.

Cell Culture

AtT-20 and RINm5F cells were grown in DMEM-F12 containing 10% fetal clone and 10% NuSerum as described (Milgram, 1994). Cells were passaged weekly.

Membrane Preparation, SDS-PAGE, and Immunoblot Analyses

RINm5F or AtT-20 cells were homogenized in homogenization buffer (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.5 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 60 μM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). Homogenates were centrifuged at 800 × *g* for 10 min and the supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The pellets were resuspended in homogenization buffer and protein concentrations were measured using the Coomassie Plus reagent (Pierce, Rockford, IL). Proteins were subjected to electrophoresis on 3–12% gradient SDS-polyacrylamide gels (Laemmli, 1970); prestained high molecular weight standards (Life Technologies, Grand Island, NY) were run in adjacent lanes. Proteins were electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979) and Western blots were performed using affinity-purified antibodies as described previously (Sharp *et al.*, 1995). Blots were incubated overnight at 4°C with antibody AP 41 (Type I IP_3R) at a 1:450 dilution or AP75 (Type III IP_3R) at a dilution of 1:200. For peptide controls, antibodies were preincubated overnight at 4°C with the appropriate peptide at concentrations of 2–10 μg/ml. Blots were developed using Lumi Glo enhanced chemiluminescence reagent (Kirkegaard and Perry, Gaithersburg, MD).

Immunofluorescent Staining

RINm5F and AtT-20 cells were plated on poly-lysine–coated chamber slides (Nunc, Naperville, IL) and grown for 48 h in a 37°C incubator. Cells were fixed with methanol for 2 min at room temperature and washed three times in PBS. Cells were incubated in PBS containing 10% normal goat serum (Vector, Burlingame, CA) for 30 min at room temperature. Affinity-purified antibodies (AP 41, type I IP_3R [1 μg/ml] or AP 75, type III IP_3R [10 μg/ml], anti-BiP mouse monoclonal [1:500; StressGen, Victoria, BC, Canada], and rabbit anti-PC1 [1:1000; a generous gift from Richard Mains and Elizabeth Eipper]) were diluted in PBS containing 10% normal goat serum. For peptide controls, antibodies were preincubated with 2–10 μg/ml peptide overnight at 4°C before being applied to the fixed cells. Cells were incubated in primary antibody for 3 h at 37°C. After rinsing, cells were incubated for 1 h with fluorescein-conjugated secondary antisera (Jackson Immuno Research Laboratories, West Grove, PA). After three washes with PBS, cells were coverslipped using Vectashield mounting medium (Vector). Samples were examined with a Bio-Rad MRC 600 confocal microscope. The optical sections were 1 μM thick and each print image was a composite average of eight frames.

Sucrose Gradients

Membranes from RINm5F or AtT-20 cells were solubilized for 30 min on ice with 1% 3-([3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPS) in homogenization buffer and centrifuged at 100,000 × *g* for 30 min. The supernatant was applied to a 5–20% sucrose gradient containing 50 mM Tris-HCl (pH 7.4), 1% CHAPS. Gradients were centrifuged in a SW 41 rotor at 100,000 × *g* for 16 h; 500 μl fractions were collected and analyzed by Western blotting as described above.

Gel Filtration

Membranes from RINm5F were solubilized for 30 min on ice with 1% CHAPS in homogenization buffer and centrifuged at 100,000 × *g* for 30 min. The supernatant was applied to a HiPrep 16–60 Sephacryl S-300 column (Pharmacia, Piscataway, NJ) equilibrated with 50 mM Tris-HCl (pH 7.4), 0.9% CHAPS. One-milliliter fractions were collected and analyzed by Western blot as described above.

Anti-IP_3 R-linked Protein A Beads

Immunoglobulin from 5 ml of crude serum from rabbits producing antibodies against IP_3R type I (rabbit 41), IP_3R type III (rabbits 45 and 75), or control antibodies (against unrelated epitope, rabbit 81) were covalently linked to protein A-Sepharose using dimethylpimelimidate (Sigma, St. Louis, MO) as described by Husten and Eipper, 1994.

Co-immunoprecipitations

Cells were homogenized in 1% CHAPS in homogenization buffer and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was incubated with 100 μl of anti-IP_3R beads (anti-type I IP_3R or anti-type III IP_3R) for 3 h at 4°C. The beads were washed three times with 1% CHAPS in homogenization buffer and once with homogenization buffer alone. Bound proteins were eluted from the beads by boiling in Laemmli sample buffer for 5 min. The samples were electrophoresed on 5% SDS-polyacrylamide gels and transferred to nitrocellulose. IP_3R was detected by Western blot as described above.

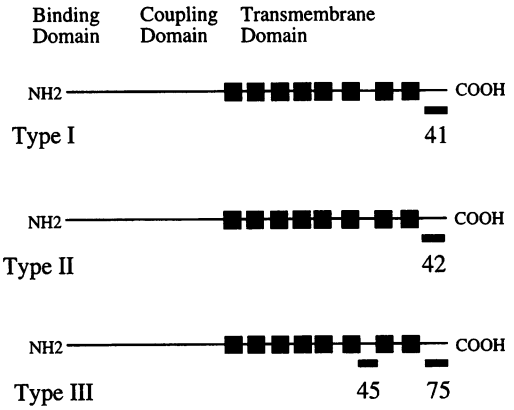
For immunodepletion experiments, the supernatant from the first antibody incubation was incubated with three sequential aliquots of the same protein A-Sepharose-antibody beads. Following each incubation, the beads were pelleted by centrifugation, the supernatant was removed, and a fresh aliquot of beads was added. The supernatant was mixed with sample buffer and samples subjected to electrophoresis on 5% SDS-polyacrylamide gels.

RESULTS

Antibody Specificity and IP_3R Isoforms in AtT-20 Cells and RINm5F Cells

To identify specific isoforms of the IP_3R, we generated antisera against peptides corresponding to unique portions of the receptor isoforms (Figure 1). The COOH-terminal regions chosen were the most divergent in amino acid sequence. In addition, for type III, an internal peptide corresponding to a unique portion of a cytoplasmic loop was chosen as indicated.

Using both AtT-20 and RINm5F cells, mouse anterior pituitary corticotrope and rat pancreatic islet cells, respectively, Western blots were performed using antibodies as shown (Figure 2). Affinity-purified antibodies generated against the type I IP_3R (AP 41) showed strong reactivity for both RINm5F cells and AtT-20 cells, with greater reactivity for the RINm5F cells. For both cell types a single band of ~260 kDa was visualized (Figure 2A). A protein of similar size was visualized in other tissues using antibodies generated against the purified cerebellar receptor (Sharp *et al.*, 1993) or using other type I IP_3R-specific antibodies (Miyawaki *et al.*, 1990; Sugiyama *et al.*, 1994). In addition, AP 41 also recognized the same ~260-kDa protein in cerebellar extracts (unpublished results). The reactivity was entirely eliminated by preabsorption with the pep-



Ab 41

GHPPHMNVNPQQPA
AA 2736-2749

Ab 42

GSNTPHENHHMPPH
AA 2688-2701

Ab 75

RRQRLGFVDVQNCMSR
AA 2655-2670

Ab 45

VSEVSVPEILEED
AA 2432-2444

Figure 1. Peptide epitopes used for generating IP_3R specific antibodies. Antibodies specific for the three isoforms of the IP_3R were generated from the C-terminus of each isoform. An additional type III antibody was produced from an internal cytoplasmic loop. The bold lines represent location of the peptide epitopes and the boxes indicate the transmembrane domains.

tide immunogen, but was unchanged when the antibody was preincubated with the type III isoform peptide immunogen (Figure 2A).

Affinity-purified antibodies to the type II (AP 42) isoform labeled a clear band at 260 kDa in Western blots prepared from primary rat glial cultures, a cell type enriched with type II IP_3R. However, type II IP_3R was not detectable in extracts prepared from RINm5F or AtT-20 cells (unpublished results); therefore the type II IP_3R isoform was not further examined.

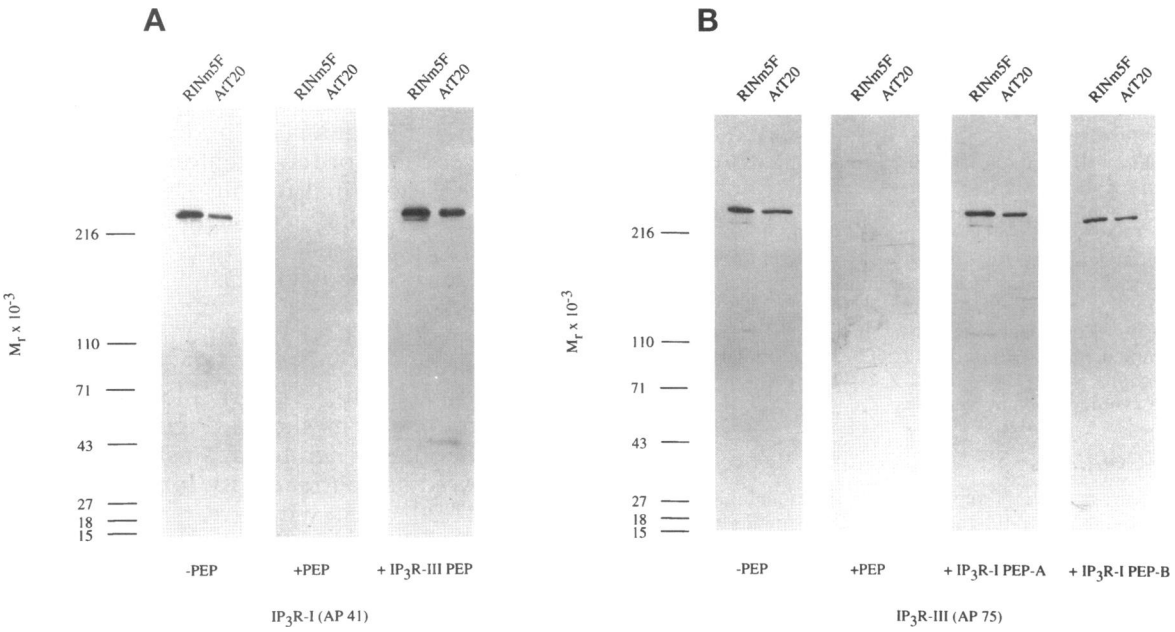


Figure 2. Western blots of RINm5F and ATt-20 cells probed with antibodies to Type I and Type III IP_3R. Membranes of RINm5F and AtT-20 cells (150 μg per lane) were subjected to electrophoresis on 3–12% SDS-polyacrylamide gradient gels. The proteins were transferred to nitrocellulose and probed with affinity-purified type-specific antibodies (A: type I IP_3R [AP 41]), (B: type III IP_3R[AP 75]). Type I immunoreactivity (A) was completely blocked by preincubation of the antibodies with the appropriate peptide immunogen (2 μg/ml; + PEP) and unaltered by preincubation with the type III peptide (2 μg/ml, + IP_3R-III). Type III IP_3R immunoreactivity was completely blocked by preincubation of the antibodies with the appropriate peptide immunogen (2 μg/ml; + PEP) and unaltered by preincubation with two different type I peptides (2 μg/ml, + IP_3R-Ia, + IP_3R-Ib). All lanes shown were from a single gel stained in a single experiment.

Western blots probed with affinity-purified rabbit anti-type III (AP75) antisera also visualized a single band at ~260 kDa, with slightly more reactivity in the RINm5F cells (Figure 2B). These results are consistent with the identification of type III IP_3R in RINm5F cells (Blondel *et al.*, 1994) as well as in the intestinal epithelium (Maranto, 1994). This reactivity was completely eliminated by preincubation with the type III peptide immunogen; by contrast, preincubation with the peptide used for the production of the type I–specific antibody did not alter the immunoreactivity (Figure 2B). Since a small portion of the type III peptide used to generate our antisera corresponds to a portion of the type I IP_3R sequence (amino acids 2657–2660, QRLG of the type III peptide versus amino acids 2730–33, QRIG of the type I sequence; three residues preceding the peptide used for Ab41), a peptide corresponding to the type I sequence including the overlapping region was produced (DDDQKQRIGLLG, with the three aspartates added to increase solubility). When AP75 was preabsorbed with this peptide there was little or no change in the intensity of the band on Western blots of RINm5F and AtT-20 cell extracts. These results demonstrate that we can specifically detect the type I and III isoforms of the IP_3R in RINm5F and AtT 20 cells, and that these cells contain relatively little of the type II isoform.

Localization of Type I and Type III IP_3R

To localize the IP_3R in AtT-20 and RINm5F cells we performed immunofluorescent staining using our type I– and type III–specific antibodies. Samples were imaged using a Bio-Rad laser scanning confocal microscope. In AtT-20 cells, type I IP_3R was localized within the cell body but not within the nucleus, con-

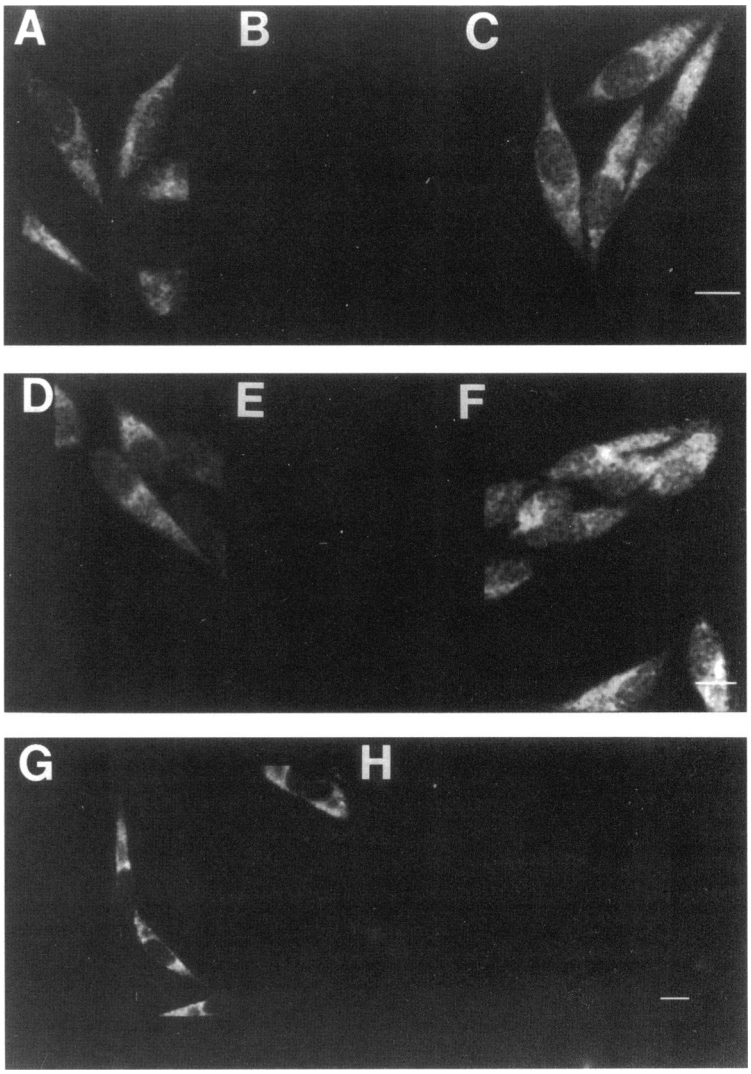


Figure 3. Immunocytochemistry of type I and type III IP_3R in AtT-20 cells with confocal imaging. AtT-20 cells were labeled with 1 μg/ml affinity-purified anti-type I IP_3R (A). Immunoreactivity was absent when preincubated with the peptide immunogen (2 μg/ml; B) or unaltered when type I antibodies were preabsorbed with 2 μg/ml of the type III immunogen (C). AtT-20 cells were labeled with 10 μg/ml of affinity-purified anti-type III IP_3R antibodies (D). Immunoreactivity was absent when preincubated with the peptide immunogen (10 μg/ml; E) or unaltered when type III antibodies were preabsorbed with 10 μg/ml of the type I immunogen (F). AtT-20 cells were labeled with a 1:500 dilution of anti-BiP (G) and minus primary antibody (H). Cells were imaged using a confocal microscope. Bar, 10 μm.

sistent with the previously demonstrated ER localization pattern (Figure 3A). Staining was completely blocked by preincubation with the type I peptide and unchanged by preabsorption with the type III peptide (Figure 3, B and C). The type III IP_3R displayed a similar localization (Figure 3D). Again, labeling was completely eliminated by preincubation with the peptide immunogen but unaltered by preincubation with the type I peptide (Figure 3, E and F). Therefore, the similar distributions of type I and type III IP_3Rs are not a result of cross-reactivity of the anti-peptide antisera. The steady state distribution of the type I and type III IP_3Rs was very similar to the distribution of the resident ER protein, BiP (Figure 3G) (Huovila *et al.*, 1992). Similar results were obtained when RINm5F cells were stained with the isoform-specific IP_3R antibodies (Figure 4). The type I and type III receptors were distributed in a similar localization pattern and this pattern was similar to the staining of RINm5F cells with the BiP antisera. The pattern of labeling for isoforms of IP_3Rs was more punctate than that of BiP, suggesting that the IP_3Rs might be present on a subcompartment of the ER. However, it is possible that the difference was due to different intensity of staining or of background fluorescence.

Previous experiments suggested that the IP_3R might be localized within secretory granules in endocrine cells (Blondel *et al.*, 1994, 1995). To determine whether type I or type III IP_3R was localized in secretory granules, we used AtT-20 cells, because these cells extend clear processes, whereas RINm5F cells stay compact. We compared the distribution of type I and type III IP_3R to the distribution of the endogenous secretory granule protein, prohormone convertase 1 (PC1). PC1 is involved in the proteolytic processing of Proopiomelanocortin, which occurs in secretory granules;

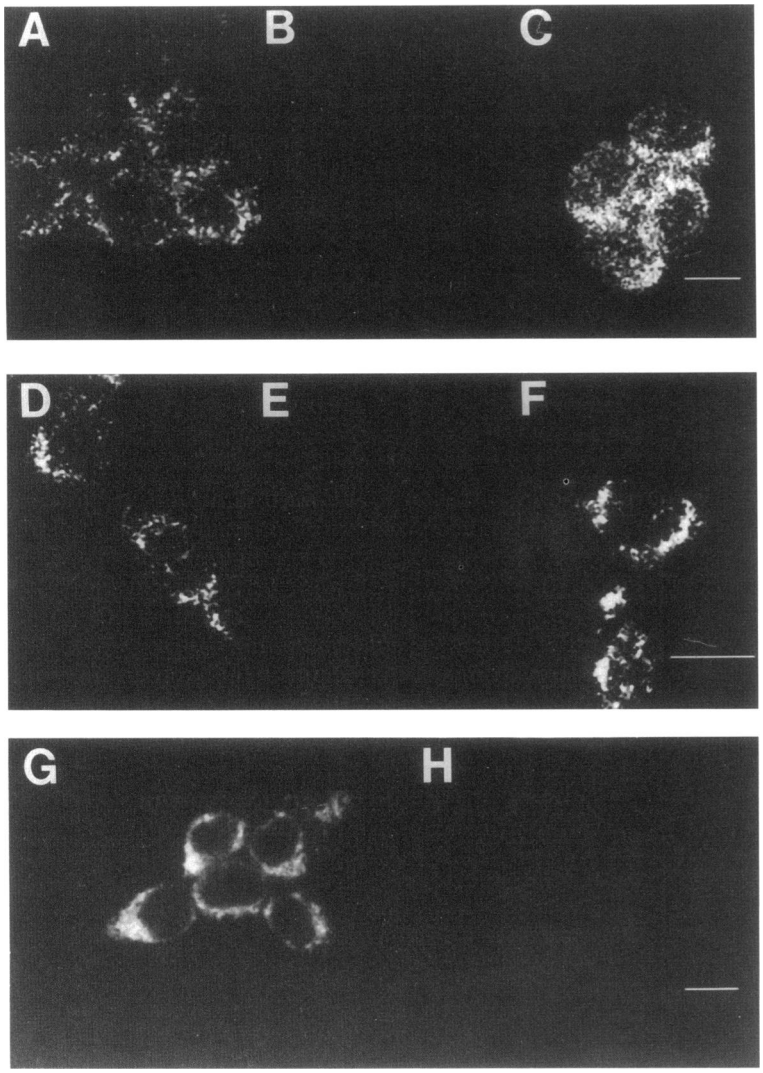


Figure 4. Immunocytochemistry of type I and type III IP_3R in RINm5F cells with confocal imaging. RINm5F cells were labeled with 1 μg/ml affinity-purified anti-type I IP_3R (A). Immunoreactivity was absent when preincubated with the peptide immunogen (2 μg/ml; B) or unaltered when type I antibodies were preabsorbed with 2 μg/ml of the type III immunogen (C). RINm5F cells were stained with 10 μg/ml of affinity-purified anti-type III IP_3R antibodies (D). Immunoreactivity was absent when preincubated with the peptide immunogen (10 μg/ml; E) or unaltered when type III antibodies were preabsorbed with 10 μg/ml of the type I immunogen (F). RINm5F cells were labeled with a 1:500 dilution of anti-BiP (G) and minus primary antibody (H). Bar, 10 μm.

therefore, PC1 serves as an excellent marker for this organelle (Zhou and Mains, 1994). Immunofluorescent staining of IP_3R or PC1 was combined with visualization of the cell profiles using phase contrast microscopy (Figure 5). The distribution of type I- and type III IP_3Rs was restricted to the cell body. The proteins were not detectable in the peripheral processes of AtT-20 cells, a region known to contain a dense accumulation of secretory granules. A region of less intense labeling was seen adjacent to the nucleus in each cell in a pattern resembling the Golgi apparatus (Figure 5, A and B) (Milgram *et al.*, 1993). This distribution was essentially identical to that obtained when cells were stained with BiP antibody (Figure 5C). By contrast PC1, an endogenous secretory granule protein, showed a very different pattern of localization. PC1 accumulated in the distal processes of AtT-20 cells (Figure 5D); this area corresponds to the region of low to undetectable reactivity for both the type I and type III IP_3Rs.

Type I and Type III IP_3Rs Form Tetramers in AtT-20 and RINm5F Cells

Previous experiments suggested that the type I IP_3R exists in a tetrameric structure; however, little is known regarding the type III isoform. To determine the molecular weight of the endogenous type III isoform, we used gel filtration analysis. Gel filtration experiments using RINm5F cells and analyzed by Western blot using the type III–specific antibody demonstrated a sharp peak with all of the reactivity in Figure 6, lanes 36–38, corresponding to a molecular weight of greater than 1000 kDa.

We also used sucrose gradients to corroborate these results. Identical results were obtained for both RINm5F and AtT-20 cells. For both type I and type III IP_3Rs, the fraction containing the band of greatest intensity corresponded to a molecular weight above 1,000 kDa (fraction four). No significant reactivity for either isoform was seen corresponding to the molecular weight of the monomer or dimer (260 kDa or 510

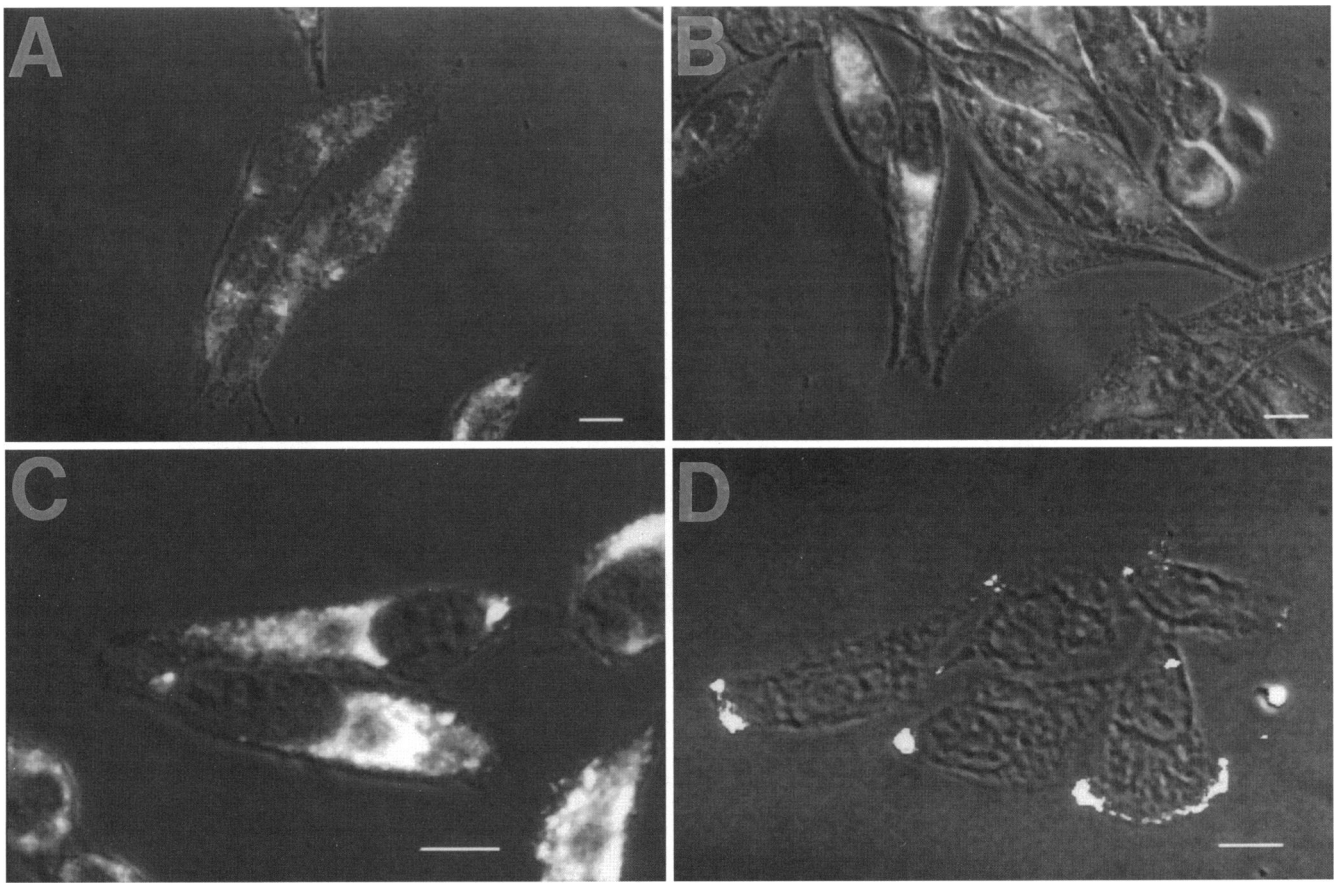


Figure 5. Immunocytochemistry of type I and type III IP_3R in AtT-20 cells by confocal combined with a phase contrast image. AtT-20 cells labled with affinity-purified anti-type I IP_3R (A), anti-type III IP_3R (B), and anti-BiP antibodies (C) as described. Cells were labeled with a 1:1000 dilution of PC1 (D), a protein expressed in secretory granules of antibody against these cells. Bar, 10 um.

kDa) in either gel filtration or sucrose gradient experiments. Therefore we conclude that both the type 1 and type III IP_3Rs exist in a tetrameric structure in endocrine cells.

Heterotetrameric Complexes of Type I and Type III IP_3R Isoforms

To determine whether this native molecular complex contained only one isoform of the receptor or could contain mixtures of the two isoforms, we performed co-immunoprecipitation studies (Figure 7, A and B). Homogenates were incubated with control antisera or isoform-specific antisera, and the immunoprecipitated proteins were analyzed on Western blots. In RINm5F cells, control antibody beads or protein A-Sepharose beads did not precipitate IP_3R proteins (Figure 7, A and B); however, type I- or type III-specific antibody beads precipitated the appropriate IP_3R isoform, detected on Western blots following immunoprecipitation.

When immunoprecipitating with the type III antiserum (rabbit 75) and detecting with the type I antibody (AP 41) on Western blots, a clear band of type I IP_3R was consistently visualized (although the intensity of the band varied somewhat from experiment to experiment) (Figure 7A). To confirm these results, a different type III IP_3R antipeptide antiserum was used in co-immunoprecipitation experiments; our unpublished results indicate that this antiserum (rabbit 45) also immunoprecipitated type III as well as type I IP_3R protein. In addition, samples immunoprecipitated using the type I antibody (AP 41), displayed immunoreactivity toward type III IP_3Rs (Figure 7B). Similar results were obtained in AtT-20 cells. Therefore we conclude that in pancreatic islet and anterior pituitary corticotrope cell lines, the IP_3Rs exist as heterotetramers.

Homotetrameric Complexes Are also Formed by the Type III IP_3R

We next sought to determine whether homotetramers of IP_3R also exist. Homogenates of RINm5F cells were depleted of the IP_3R type I by incubating cell extracts with excess type I–specific protein A beads until no type I protein was present in the supernatant (compare homogenate to depleted supernatant, Figure 8, A and B). However, a band was detectable with the type III antibody in this immunodepleted supernatant (Figure 8C), suggesting the existence of type III IP_3R homotetramers. Experiments were also performed to determine if type I homotetramers exist. RINm5F cell homogenates were depleted of type III IP_3R, and Western blots of the depleted supernatant were analyzed using type I– and type III–specific antisera. Although both type III and type I receptors were detectable in the homogenate (Figure 9, A and C), neither type I nor type III IP_3R was detected in the depleted supernatant (Figure 9, B and D). Based on these data we conclude that the type III IP_3R is present as a homotetrameric structure, while homotetramers of the type I isoform are not detectable.

DISCUSSION

In these studies we have used antisera specific for type I and type III IP_3R isoforms to localize the endogenous receptors to the ER in endocrine cell lines. Our results are consistent with previous localization studies of the type I isoform in the brain and types I and III in gastrointestinal epithelial cells, and in COS-7 cells

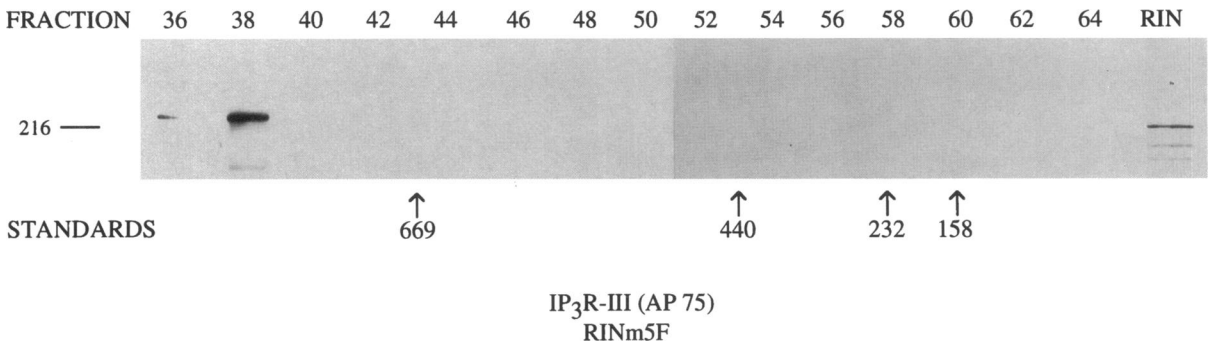


Figure 6. Gel filtration of type III IP_3R in RINm5F cells. CHAPS soluble extracts of RINm5F cells were layered over a HiPrep 16–60 Sephacryl S-300 column. One-milliliter fractions were collected and type III IP_3R was detected by Western blot. The position of standards run on the same column were determined by Coomassie blue staining of SDS-polyacrylamide gels of each fraction. The standards used, their native molecular weight and peak fraction on the gradients were aldolase (158 kDa), fraction 60; catalase (232 kDa), fraction 58; ferritin (440 kDa), fraction 53; and thyroglobulin (669 kDa), fraction 43. Peak reactivity was seen in fraction 38 for type III IP_3R, corresponding to a molecular mass greater than 1,000 kDa. No reactivity was seen in fractions corresponding to molecular weight of dimers or monomers (520 or 260).

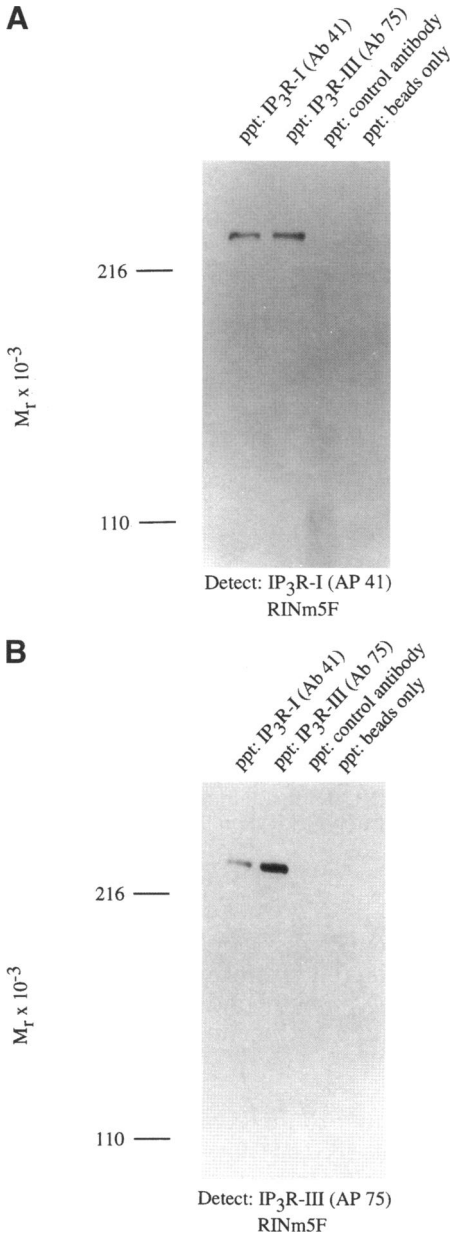


Figure 7. Immunoprecipitation of type I and type III IP_3R in RINm5F cells. IP_3R were immunoprecipitated with anti-type I and anti-type III antibodies, subjected to SDS-PAGE, and transferred to nitrocellulose. When Western blots were analyzed with anti-type I IP_3R (A), a band of approximately 260 kDa was obtained. No band was seen for either a control antibody or protein A-Sepharose beads alone. Analagous results were obtained when Western blots were analyzed with anti-type III IP_3R (B).

transfected with a partial cDNA encoding the type III receptor isoform.

Previous studies have suggested a localization of the type III receptor to secretory granules in rat pancreatic islet β cells (Blondel *et al.*, 1994, 1995). More recently the same group suggested that the type III receptor is localized in secretory granules in RINm5F cells (Blondel, 1995), although the interpretation of these studies is controversial (Blondel, 1995; Meldolesi and Pozzan, 1995). By contrast, our studies do not support the hypothesis of localization of type III IP_3Rs to secretory granules. Both RINm5F cells and AtT-20 cells, labeled using different antibodies specific for the type III receptor, clearly show an ER distribution pattern with low density in the Golgi region, consistent with the type III IP_3R being an ER resident protein. Furthermore, in AtT-20 cells that extend clear distal processes containing secretory granules, the distribution of the type III IP_3R did not overlap with the distribution of the secretory granule-specific protein PC1 (Figure 5) (Zhou and Mains, 1994). The reason for the difference between our results and the results of Blondel *et al.* (1994) is uncertain.

Previous studies have shown that different isoforms of IP_3R can coexist within the same cell (Sugiyama *et al.*, 1994). Such co-expression could involve tetramers of each isoform separately (conceivably in separate sub populations of ER) or could involve hetero-oligomers of different isoforms associating together. Our studies clearly show that in two different cell lines (RINm5F and AtT20), type I and type III isoforms associate into hetero-oligomers (Figure 7). Based on our gel filtration and sucrose gradient experiments (Figure 6), we propose that both type I and type III IP_3Rs most likely form heterotetramers. Because the immunoprecipitation is not quantitative, we cannot address the stoichiometry of this association and it is quite possible that the stoichiometry of IP_3R oligomerization may differ among different cell types. The existence of heterotetramers is consistent with our immunofluorescent localization of type I and type III isoforms in the same cellular compartments in AtT-20 cells and RINm5F cells. Our results parallel closely the results of Monkawa *et al.* (1995) in a recent independent study of IP_3R isoform hetero-oligomerization in CHO-K1 cells.

In addition, our results demonstrate that a fraction of type III receptors exists in a homotetrameric form (Figure 8). Our results suggest that this fraction is likely to be a small portion of the total, on the order of 10%, although this is only a semi-quantitative estimate. It is possible that a very small fraction of the type I receptor also exists in homotetrameric form, but was undetectable in our system. Our methods do not allow us to determine whether the homotetramers and heterotetramers are present on similar or different subcompartments of the ER. This would require double label experiments using high resolution electron microscopy. It is also possible that type III homotetramers are selectively targeted to the cell membrane. Physiological experiments using Jurkat cells indicate that calcium influx stimulated by depletion of ER stores is not mediated by type I IP_3Rs (Jayaraman *et al.*,

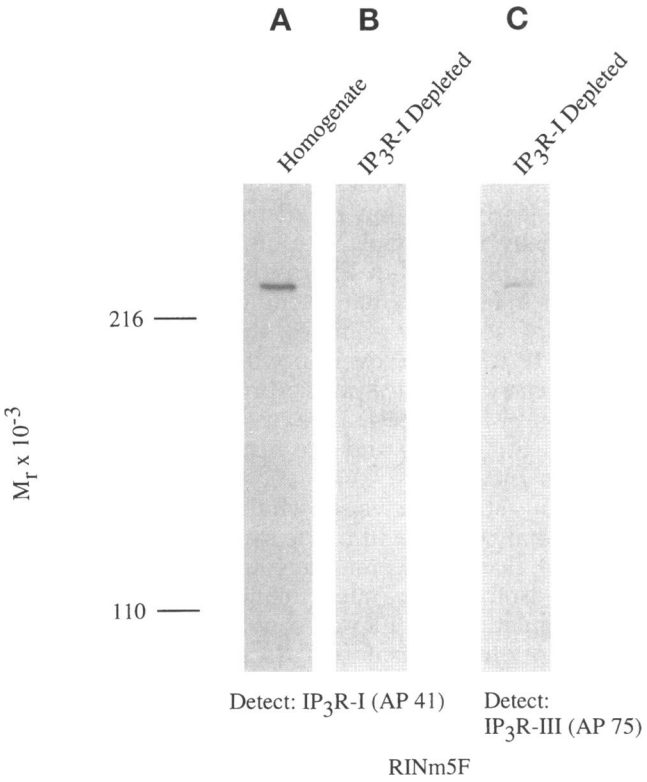


Figure 8. Immunodepletion of type I from supernatant demonstrates type III homotetramers. Western blots of type I IP_3R in the homogenate of RINm5F cells (A) and supernatant immunodepleted using anti-type I antibody beads (B). (C) Type III IP_3Rs remaining in the type-I depleted supernatant.

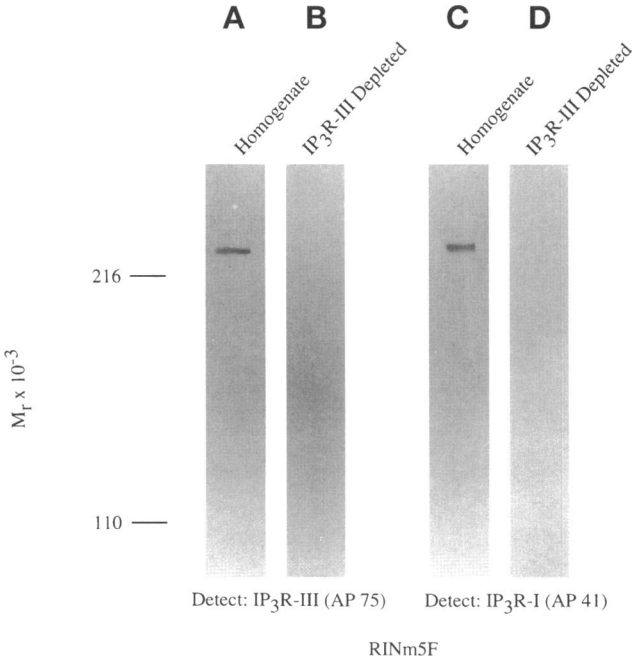


Figure 9. Immunodepletion of type III from supernatant demonstrates nondetectability of type I homotetramers. Western blots of type III IP_3R in the homogenate of RINm5F cells (A) and supernatant immunodepleted using anti-type III antibody beads (B). Western blot of type I IP_3Rs in RINm5F homogenate (C) and lack of immunoreactivity of type I IP_3R in the type III IP_3R-depleted supernatant (D).

1995). Other studies (Khan *et al.*, 1992a,b) have suggested that IP_3R's are present on the cell membranes of T-lymphocytes and can gate calcium upon stimulation.

Previous studies have shown that functional heterogeneity of IP_3R-mediated calcium release in neurons and other cells can be regulated through different mechanisms (Ferris *et al.*, 1990, 1991; Nakade *et al.*, 1991; Khodakhah and Ogden, 1993; Simpson *et al.*, 1995). There may be multiple pathways of second messenger regulation that may differ among cell types. Some of this heterogeneity could arise from the presence of different IP_3R isoforms with different affinities for IP_3. Our results suggest an additional level of complexity, since a single isoform can exist in heterotetramers as well as in homotetramers, possibly producing functionally different calcium release channels.

Similarly, ligand-gated ion channels including the acetylcholine and glycine receptors often exist as hetero-oligomers of subunits encoded by related genes (Halvorsen and Berg, 1990; Vernallis *et al.*, 1993; Becker, 1995). The constituents of these hetero-oligomers are believed to be developmentally regulated with consequent changes in function (Takahashi *et al.*, 1992). The different isoforms of the IP_3R may also undergo developmental and cell-type–specific regulation, contributing further to molecular heterogeneity of IP_3Rs.

Note added in proof: Ravazzola *et al.*, (1996) have suggested that AB3 (but not AP45) can cross react with insulin, explaining in part the differences between our results and those of Blondel *et al.* (1994).

ACKNOWLEDGMENTS

This work was supported by the National Institute of Mental Health under grant MH-43040. We thank Drs. Richard Mains and Betty Eipper for providing antisera to PC1, AtT-20 cells, and their helpful discussions; Linda Hester for providing the RINm5F cells; Mike Delannoy for his expertise with the confocal imaging; Debbie Pollard for expert word processing; and Cindy Geppi for help in preparing the manuscript.

REFERENCES

Becker, C.-M. (1995). Glycine receptors: molecular heterogeneity and implications for disease. Neuroscientist *1*, 130–141.

Berridge, M.J. (1993). Inositol triphosphate and calcium signaling. Nature *361*, 315–325.

Berridge, M.J., and Irvine, R.F. (1989). Inositol phosphate and cell signaling. Nature *341*, 197–205.

Bezprozvanny, I., Watras, J., and Ehrlich, B.E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751–754.

Blondel, O. (1995). IP_3 receptors and secretory granules. Trends Neurosci. *18*, 340–341.

Blondel, O., Bell, G.I., and Seino, S. (1995). Inositol 1,4,5-trisphosphate receptors, secretory granules and secretion in endocrine and neuroendocrine cells. Trends Neurosci *18*, 157–161.

Blondel, O., Moody, M.M., DePaoli, A.M., Sharp, A.H., Ross, C.A., Swift, H., and Bell, G.I. (1994). Localization of inositol trisphosphate receptor subtype 3 to insulin and somatostatin secretory granules and regulation of expression in islet and insulinoma cells. Proc. Natl. Acad. Sci. USA *1*, 7777–7781.

Blondel, O., Takeda, J., Janssen, H., Susumu, S., and Bell, G.I. (1993). Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP_3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract and other tissues. J. Biol Chem. *15*, 11356–11363.

Danoff, S.K., Ferris, C.D., Donath, C., Fischer, G.A., Munemitsu, S., Ullrich, A., Snyder, S.H., and Ross, C.A. (1991). Inositol 1,4,5-trisphosphate receptors: distinct neuronal and nonneuronal forms derived by alternative splicing differ in phosphorylation. Proc. Natl. Acad. Sci. USA *88*, 2951–2955.

Ehrlich, B.E., and Watras, J. (1988). Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. Nature *366*, 583–586.

Ferris, C.D., Huganir, R.L., Bredt, D.S., Cameron, A.M., and Snyder, S.H. (1991). Inositol trisphosphate receptor: phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. Proc. Natl. Acad. Sci. USA *88*, 2232–2235.

Ferris, C.D., Huganir, R.L., and Snyder, S.H. (1990). Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides. Proc. Natl. Acad. Sci. USA *87*, 2147–2151.

Ferris, C.D., and Snyder, S.H. (1992). Inositol phosphate receptors and calcium disposition in the brain. J. Neurosci *12*, 1567–1574.

Finch, E.A., Turner, T.J., and Goldin, S.M. (1991). Calcium as a coagonist of the inositol 1,4,5-trisphosphate receptor-induced calcium release. Science *252*, 443-446.

Fujimoto, T., Shinji, N., Miyawaki, A., Mikoshiba, K., and Ogawa, K. (1992). Localization of inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae. J. Cell Biol. *119*, 1507–1513.

Furuichi, T., and Mikoshiba, K. (1995). Inositol 1,4,5-trisphosphate receptor Ca^{2+} signaling in the brain. J. Neurosci. *64*, 953–960.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989). Primary structure and function expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature *342*, 32–38.

Hajnoczky, G., and Thomas, A.P. (1994). The inositol trisphosphate calcium channel is inactivated by inositol trisphosphate. Nature *370*, 474–477.

Halvorsen, S.W., and Berg, D.K. (1990). Subunit composition of nicotinic acetylcholine receptors from chick ciliary ganglia. J. Neurosci. *10*, 1711–1718.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Huovila, A.-P., Eder, A.M., and Fuller, S.D. (1992). Hepatitis B surface antigen assembles in a post-ER pre-Golgi compartment. J. Cell Biol. *118*, 1305–1320.

Husten, E.J., and Eipper, B.A. (1994). Purification and characterization of Pam-1, an integral membrane protein involved in peptide processing. Arch. Biochem. Biophys. *312*, 487–492.

Iino, M., and Endo, M. (1992). Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. Nature *360*, 76–78.

Jayaraman, T., Ondriasova, E., Ondrias, K., Harnick, D.J., and Marks, A. (1995). The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling. Proc. Natl. Acad. Sci. USA *92*, 6007–6011.

Kasai, H., Yue, X.L., and Miyashita, Y. (1993). Subcellular distribution of Ca^{2+} release channels underlying Ca^{2+} waves and oscillations in exocrine pancreas. Cell *74*, 669–677.

Khan, A.A., Steiner, J.P., Klein, M., Schneider, M., and Synder, S.H. (1992a). IP_3 receptor: localization to plasma membrane of T cell and cocapping with the T cell receptor. Science *257*, 815–818.

Khan, A.A., Steiner, J.P., and Snyder, S.H. (1992b). Plasma membrane inositol 1,4,5-trisphosphate receptor of lymphocytes: selective enrichment in sialic acid and unique binding specificity. Proc. Natl. Acad. Sci. USA *89*, 2849–2853.

Khodakhah, K., and Ogden, D. (1993). Functional heterogeneity of calcium release by inositol trisphosphate in single Purkinje neurons, cultured cerebellar astrocytes, and peripheral tissues. Proc. Natl. Acad. Sci. USA *90*, 4976–4980.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Maeda, N., Takashi, K., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991). Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. J. Biol. Chem. *266*, 1109–1116.

Maranto, A.R. (1994). Primary structure, ligand binding, and localization of the human type 3 inositol 1,4,5-trisphosphate receptor expressed in intestinal epithelium. J. Biol. Chem. *269*, 1222–1230.

Meldolesi, J. (1992). Multifarious IP_3 receptors. Curr. Biol. *2*, 393–394.

Meldolesi, J., and Pozzan, T. (1995). IP_3 receptors and secretory granules. Trends Neurosci. *18*, 340–341.

Mignery, G.A., Newton, C.L., Archer, B.T.I., and Sudhof, T.C. (1990). Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. *265*, 12679–12685.

Mignery, G.A., and Sudhof, T.C. (1990). The ligand binding site and transduction mechanism in the inositol-1,4,5-trisphosphate receptor. EMBO J. *9*, 3893–3898.

Mignery, G.A., Sudhof, T.C., Takei, K., and De Camilli, P. (1989). Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature *342*, 192–195.

Milgram, S.L., Eipper, B.A., and Mains, R.E. (1994). Differential trafficking of soluble and integral membrane secretory granule-associated proteins. J. Cell Biol. *124*, 33–41.

Milgram, S.L., Mains, R.E., and Eipper, B.A. (1993). COOH-terminal signals mediate the trafficking of a peptide processing enzyme in endocrine cells. J. Cell Biol. *121*, 23–36.

Miyawaki, A., Furuichi, T., Maeda, N., and Mikoshiba, K. (1990). Expressed cerebellar-type inositol 1,4,5-trisphosphate receptor, P_{400}, has calcium release activity in a fibroblast L cell line. Neuron *5*, 11–18.

Monkawa, T., Miyawaki, A., Sugiyama, T., Yoneshima, H., Yamamoto-Hino, M., Furuichi, T., Sauta, T., Hasegawa, M., and

Mikoshiba, K. (1995). Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor subunits. J. Biol. Chem. *270*, 14700–14704.

Nakade, S., Maeda, N., and Mikoshiba, K. (1991). Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analyzed using region-specific monoclonal antibodies. J. Biol. Chem. *277*, 125–131.

Nakagawa, T., Olano, H., Furuichi, T., Aruga, J., and Mikoshiba, K. (1991a). The subtypes of the mouse inositol 1,4,5-trisphosphate receptor are expressed in a tissue-specific and developmentally specific manner. Proc. Natl. Acad. Sci. USA *88*, 6244–6248.

Nakagawa, T., Shiota, C., Okano, H., and Mikoshiba, K. (1991b). Differential localization of alternatively spliced transcripts encoding inositol 1,4,5-trisphosphate receptors in mouse cerebellum and hippocampus: in situ hybridization study. J. Neurochem. *57*, 1807–1810.

Newton, C.L., Mignery, G.A., and Sudhof, T.C. (1994). Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate ($InsP_3$) receptors with distinct affinities for $InsP_3$. J. Biol Chem. *269*, 28613–28619.

Nucifora, F.C., Jr., Li, S.-H., Danoff, S., Ullrich, A., and Ross, C.A. Inositol (1,4,5) trisphosphate receptor type 1: a third alternatively spliced variant. Mol. Brain Res. (*in press*).

Otsu, H., Yamamoto, A., Maeda, N., Mikoshiba, K., and Tashiro, Y. (1990). Immunogold localization of inositol 1,4,5-trisphosphate ($InsP_3$) receptor in mouse cerebellar Purkinje cells using three monoclonal antibodies. Cell Struct. Funct. *15*, 163–173.

Ross, C.A., Danoff, S.K., Schell, M.J., and Snyder, S.H. (1992). Three additional inositol 1,4,5-trisphophate receptors: molecular cloning and differential localization in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA *89*, 4265–4269.

Ross, C.A., Meldolesi, J., Milner, T.A., Satoh, T., Supattapone, S., and Snyder, S.H. (1989). Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons. Nature *339*, 468–470.

Schell, M.J., Danoff, S.K., and Ross, C.A. (1993). Inositol (1,4,5)-trisphosphate receptor: characterization of neuron-specific alternative splicing in rat brain and peripheral tissues. Mol. Brain Res. *17*, 212–216.

Sharp, A.H., Dawson, T.M., Ross, C.A., Fotuhi, M., Mourey, R.J., and Snyder, S.H. (1993). Inositol 1,4,5-trisphosphate receptors: immunohistochemical localization to discrete areas of rat central nervous system. Neurosci. *53*, 927–942.

Sharp, A.H., *et al.* (1995). Widespread expression of Huntington's disease gene (IT15) protein product. Neuron *14*, 1065–1074.

Simpson, P.B., Challiss, R.A.J., and Nahorski, S.R. (1995). Neuronal Ca^{2+} stores: activation and function. Trends Neurosci. *18*, 299–306.

Sudhof, T.C., Newton, C.L., Archer, B.T., 3rd, Ushkaryov, Y.A., and Mignery, G.A. (1991). Structure of a novel $InsP_3$ receptor. EMBO J. *10*, 3199–3206.

Sugiyama, T., Furuya, A., Monkawa, T., Yamamoto-Hino, M., Satoh, S., Ohmori, K., Miyawaki, A., Hanai, N., Mikoshiba, K., and Haegawa, M. (1994). Monoclonal antibodies distinctively recognizing the subtypes of inositol 1,4,5-trisphosphate receptor: application to the studies on inflammatory cells. FEBS Lett. *354*, 149–154.

Supattapone, S., Danoff, S.K., Theibert, A., Joseph, S.K., Steiner, J., and Snyder, S.H. (1988a). Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. Proc. Natl. Acad. Sci. USA *85*, 8747–8750.

Supattapone, S., Worley, P.F., Baraban, J.M., and Synder, S.H. (1988b). Solubilization, purification, and characterization of an inositol trisphosphate receptor. J. Biol. Chem. *263*, 1530–1534.

Takahashi, T., Momiyama, A., Hirai, K., Hishinuma, F., and Akagi, H. (1992). Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. Neuron *9*, 1155–1161.

Towbin, H., Staehlin, T., and Gorden, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Vernallis, A.B., Conroy, W.G., and Berg, D.K. (1993). Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron *10*, 451–464.

Zhou, A., and Mains, R.E. (1994). Endoproteolytic processing of proopiomelanocortin and prohormone convertases 1 and 2 in neuroendocrine cells overexpressing prohormone convertases 1 or 2. J. Biol. Chem. *269*, 17440–17447.