Factors Determining the Composition of Inositol Trisphosphate Receptor Hetero-oligomers Expressed in COS Cells*

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Suresh K. Joseph‡, Shaila Bokkala, Darren Boehning, and Samuel Zeigler
From the Department of Pathology and Cell Biology, Thomas Jefferson University School of Medicine, Philadelphia, Pennsylvania 19107

COS-7 cells were transiently transfected with type I and type III myo-inositol 1,4,5-trisphosphate receptor (IP₃R) isoforms to study the processes underlying assembly and oligomerization of these tetrameric proteins. A FLAG epitope was engineered on to the N terminus of the type III IP₃R to distinguish the transfected from the endogenous isoform. This was not necessary for the type I IP₃R, since the endogenous levels of this isoform were extremely low. Based on sucrose gradient analysis, the transfected type I or FLAG-type III IP₃Rs assembled into tetramers. Confocal immunofluorescence experiments confirmed that the constructs were primarily targeted to the endoplasmic reticulum. Recombinant type I IP₃R expressed in COS cells over a 48-h period showed a negligible capacity to form homo-oligomers with endogenous type III IP₃Rs, based upon co-immunoprecipitation assays. However, substantial formation of hetero-oligomers was observed between recombiant receptors when the cells were simultaneously transfected with type I and FLAG-type III IP₃Rs. Co-immunoprecipitation experiments using lysates from metabolically labeled cells allowed the quantitation of homo- and hetero-oligomers in cells transfected with different ratios of type I and FLAG-type III IP₃R DNA. These studies show that the relative expression level of the two isoforms influences the fraction of hetero-oligomers formed. However, the proportion of hetero-oligomers formed were less than predicted by a binomial model in which the association of subunits is assumed to be random. In doubly transfected cells, the early kinetics of [³⁵S]label incorporation into homotetramers showed a lag period corresponding to the time taken to synthesize a full-length receptor. However, hetero-oligomers were synthesized with a longer lag period, suggesting that there may be kinetic constraints that favor homo-oligomers over hetero-oligomers.

Stimulation of cell surface receptors results in the formation of the second messenger inositol 1,4,5-trisphosphate (IP₃) via the activation of phospholipase C (1). IP₃ mobilizes intracellular calcium by binding to a family of receptors (IP₃Rs) that act as ligand-gated calcium channels (2, 3). IP₃Rs are tetramers, and full-length sequences of at least three different isoforms have been identified by molecular cloning (2, 3). Individual cell types can express multiple isoforms, which can be present as both homo- and heterotetramers (4–6). There is increasing evidence to suggest that the three IP₃Rs isoforms may be differentially regulated, particularly with respect to affinity for IP₃ (7–9) and modulatory effects of Ca²⁺ (7, 8, 10, 11), calmodulin (12, 13), ATP (14) and phosphorylation (2, 3). In addition, it has also been suggested that individual isoforms may be preferentially localized to specific regions of the cell (15–17).

Targeted gene knock-outs of IP₃R isoforms in DT-40 cells have also shown that cells expressing the three different isoforms have distinct patterns of Ca²⁺ signaling (14). It is not known if the formation of IP₃R hetero-oligomers results in channels with intermediate regulatory properties or if the regulatory properties of one isoform dominate within a heterotetramer. In the study on DT-40 cells, the expression of any two isoforms led to an intermediate IP₃ sensitivity, but the modulatory effects of Ca²⁺ and ATP were dictated by one dominant isoform (14). These data indicate that hetero-oligomerization can have functional consequences. Therefore, it can be anticipated that a major factor that would impact on the regulatory properties of IP₃Rs in a cell are the extent to which they participate in hetero-oligomerization as well as their subunit composition. The present study was undertaken in an attempt to address these questions and to learn more about the assembly of these ion channels. Hetero-oligomerization was studied in COS-7 cells transfected with DNA encoding both the type I and type III IP₃R isoforms. Homo- and hetero-oligomers were detected by co-immunoprecipitation assay. The experimental data indicate that the combination of isoforms may not be a simple random process and suggest that mechanisms exist in cells that favor the formation of IP₃R homo-oligomers over hetero-oligomers.

EXPERIMENTAL PROCEDURES

Materials—Pfu polymerase was obtained from Stratagene (La Jolla, CA). Stabilized acrylamide solution (Protogel) for the preparation of SDS gels was obtained from National Diagnostics (Atlanta, GA). Tran³⁵S label (a mixture of [³⁵S]methionine and [³⁵S]cysteine) was obtained from ICN Radiochemicals (Irvine, CA). Protein A-Sepharose was obtained from Sigma. Dulbecco’s minimal essential medium and LipofectAMINE were from Life Technologies, Inc. Polyamine transfection reagent LT-1 was from PanVera Corp. (Madison, WI). Pico and Dura enhanced chemiluminescence reagents were from Pierce.

Antibodies—Several different IP₃R isoform-specific antibodies were used in the present study. The type I IP₃R polyclonal Abs were raised against unique C-terminal amino acids 2731–2749 (18, 19) or to amino acids 1829–1848 located in the regulatory domain of the human type I IP₃R sequence (Affinity BioReagents Inc., Golden, CO). The type III-specific Abs used were a monoclonal against amino acids 22–230 in the ligand binding domain (Transduction Laboratories, Lexington, KY).
or a polyclonal Ab raised to amino acids 2657–2670 of the C terminus (19). Antibody against the type II IP₃R isoform was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal Ab to the FLAG M2 epitope was obtained form Sigma.

Plasmids—The type I IP₃R cDNA was kindly given by Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas, TX) and was in the expression plasmid pCMV1-9 (20). The type III IP₃R cDNA was kindly given by Dr. Graeme Bell (University of Chicago) and was in the expression plasmid pCB6 (21). The type III IP₃R was epitope-tagged at the N terminus by using polymerase chain reaction to amplify the entire coding sequence with rat type III IP₃R cDNA as template. The forward primer encoded an Ndel restriction site and the FLAG epitope (DYKDDDDK). The reverse primer encoded an EcoRI site. The reaction was carried out using the Expand long template polymerase chain reaction system according to the manufacturer’s instructions (Roche Molecular Biochemicals). The 8037-base pair polymerase chain reaction product was digested with NheI/EcoRI, purified, and ligated into NheI/EcoRI-digested expression plasmid pcDNA3.1 (+) (Invitrogen). All plasmids used for transfection were purified by Cacodyl binding (22).

Cell Culture and Transfection—COS-7 cells were grown to approximately 70% confluence in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum. Both LipofectAMINE (Life Technologies, Inc.) and TransIT (PanVera) reagents were used as transfection agents in the present study. The procedures for transfection were as recommended by the manufacturers. The transfection reagent used and the concentration of DNA transfected are indicated in the figure legends. At 48 h post-transfection, the medium was removed, and the plates were washed twice in ice-cold PBS and solubilized in a buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100 (v/v), 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 5 μg/ml each of aprotinin, soybean trypsin inhibitor, and leupeptin (lysis buffer).

Immunoprecipitation—Lysates were prepared as described above and precleared for 30 min by incubation with 20 μl of a 50% (v/v) slurry of Staphylococcus aureus cell wall (Pansorbin; Calbiochem). Insoluble material was removed by centrifugation for 10 min at 25,000 × g. Aliquots of lysate (100 μg) were incubated with IP₃R Ab for 4–16 h at 4 °C together with 50 μl of Protein A-Sepharose (20%, v/v). Immune complexes were isolated by centrifugation, washed three times in lysis buffer, and analyzed by SDS-PAGE. In some experiments, the polypeptides in the gel were transferred to nitrocellulose, which was autoradiographed and then incuboblotted with IP₃R isospecific antibodies to locate the receptor. Repeated immunoblotting of the same nitrocellulose sheet was carried out after treating blots for 30 min at 60 °C in a stripping buffer containing 65 mM Tris–HCl (pH 6.8), 2% SDS, and 100 mM NaCl.

Direct Immunofluorescence—COS cells were grown and transfected on coverslips. At 48 h post-transfection, the cells were washed twice with 5 ml of ice-cold PBS and fixed in 100% ice cold methanol for 20 min at −20 °C. The cells were subsequently permeabilized by incubation in 1% saponin in PBS for 10 min at room temperature. The coverslips were blocked in a solution of 5% fetal bovine serum, 1% bovine serum albumin in PBS for 1 h at 37 °C and then incubated for a further 1 h in primary antibody. These were employed at the following dilutions: 1:200 type I IP₃R polyclonal (Affinity BioReagents); 1:100 type III IP₃R monoclonal (Transduction Laboratories); and 1:1000 ribophorin polyclonal (kind gift of Dr. Christopher Nichitta). The cells were washed three times in a solution of 0.1% Triton X-100, 0.15M NaCl, 0.05% Tween 20 (TNT buffer) and then incubated with secondary antibody for 1 h at 37 °C. These were employed at the following dilutions: 1:500 of either donkey anti-rabbit horseradish peroxidase or goat anti-mouse HRP (Jackson Immunoresearch Laboratories, West Grove, PA). The cells were washed three times with TNT buffer and incubated with fluorescein- or rhodamine-linked tyramide for 5 min at room temperature as described by the manufacturer (NEN Life Science Products). The cells were mounted with VectaShield antifade reagent (Vector Laboratories, Burlingame, CA) and examined by confocal microscopy (Bio-Rad MRC 600). These studies indicated that the transfection efficiency was 40–60%.

RESULTS

Characterization of IP₃Rs Transiently Expressed in COS Cells—The predominant endogenous IP₃R isoforms in COS cells have been reported to be type III and type II IP₃Rs (23). This was confirmed by immunoblotting of cell lysates from mock-transfected cells using type III IP₃R Ab (Fig. 1, type III blot, lane 1) and type II IP₃R Ab (data not shown). The very low levels of endogenous type I IP₃Rs could not be detected by immunoblotting but could be visualized after immunoprecipitation with type I IP₃R Ab (e.g., see Fig. 4). However, type I IP₃Rs were readily detected in cell lysates prepared from type I IP₃R-transfected cells (Fig. 1, middle panel, lane 2). In the case of the type I IP₃R isoform, the low background allowed the transfected and endogenous receptors to be easily distinguished. Transient transfection with type III IP₃R DNA elevated the levels of type III IP₃R approximately 10-fold above the endogenous levels. The addition of the FLAG epitope tag allowed the transfected type III IP₃Rs to be clearly distinguished from the endogenous pool (Fig. 1, lower panel, lane 4).

In order to determine if recombinant receptors correctly assemble into tetramers, we processed the cell lysates from transfected cells on sucrose gradients (Fig. 2). The major peak of immunoreactivity in type I and FLAG-III IP₃R-transfected cell lysates coincided with the migration of tetrameric cerebellar IP₃Rs. There was no evidence for substantial amounts of disassembled IP₃Rs in cell lysates analyzed on Triton X-100-containing gradients, although the tetramers could be disassembled in Zwittergent-containing gradients (24). To further examine the localization of the transfected receptors, we carried out confocal immunofluorescence studies. An example of COS cells transfected with both type I and FLAG-type III IP₃Rs and examined by double labeling with fluorescein and rhodamine-conjugated secondary Abs is shown in Fig. 3.

Overlay of the fluorescein and rhodamine images at low levels of magnification indicated that >95% of the transfected cells express both DNAs (data not shown). Both type I and FLAG-type III IP₃Rs showed an identical reticular localization similar to the pattern observed with the endoplasmic reticulum resident protein ribophorin (data not shown). There was no evidence for a selective localization of the FLAG-type III IP₃R adjacent to the plasma membrane (but see Refs. 16 and 17). Overall, the data indicate that the recombinant receptors expressed in COS cells assemble into tetramers and are localized to the endoplasmic reticulum.

Hetero-oligomerization of Transfected IP₃Rs—Fig. 4A shows the results of experiments in which we attempted to determine if transfected type I IP₃Rs would form hetero-oligomers with endogenous type III IP₃Rs using co-immunoprecipitation assays (4–6). Type I IP₃Rs immunoprecipitated from the lysates of mock-transfected cells showed a weak signal corresponding to endogenous type I IP₃Rs (Fig. 4A, upper panel, lane 1), as
well as a small amount of endogenous type III IP₃R present as hetero-oligomers (Fig. 4A, lower panel, lane 1). A reproducible finding was that this pool of type III containing hetero-oligomers was decreased in COS cells transfected with type I IP₃R (Fig. 4A, lower panel, lane 3). The reason for this is not clear but may reflect suppression of endogenous type I IP₃R expression in cells transfected with type I IP₃R DNA. However, the main finding in these experiments was that immunoprecipitation of endogenous type III IP₃Rs did not co-precipitate any detectable type I IP₃R in COS cells transfected with this isoform (Fig. 4A, upper panel, lane 4).

Since formation of hetero-oligomers is a co-translational event (4, 19), it is possible that the lack of combination between transfected and endogenous receptors arises from a large difference in the rate of biosynthesis of the two receptor populations. If this is the case, it should be possible to detect the formation of hetero-oligomers when COS cells are simultaneously transfected with two different isoforms. An example of such an experiment is shown in Fig. 4B in which type I and FLAG-tagged type III IP₃R DNA were transfected separately or together into COS cells. Lysates immunoprecipitated with type I IP₃R Ab and immunoblotted with FLAG Ab showed the presence of hetero-oligomers in both COS cells transfected with both IP₃R isoforms (Fig. 4B, lane 4, arrow). Similarly, immunoprecipitation of the lysates from doubly transfected cells with FLAG Ab followed by immunoblotting with type I IP₃R Ab revealed the presence of type I subunits present as hetero-oligomers (Fig. 4B, lane 8, arrow).

Quantitative Immunoprecipitation of IP₃Rs—An approximate estimate of the proportion of each isoform present as hetero-oligomers can be obtained by comparison of the immunoreactive protein in lanes 4 and 8 of Fig. 4B. The type I IP₃Rs present as hetero-oligomers in FLAG immunoprecipitates (upper panel, lane 8, arrow) is approximately 20% of the amount detected in type I IP₃R Ab immunoprecipitates (upper panel, lane 4). The latter quantity presumably reflects the total type I IP₃R subunits present as both homo- and heterotetramers. However, the same comparison carried out for the FLAG-III indicates that almost all the FLAG-III IP₃Rs are present as hetero-oligomers (Fig. 4B, lower panel, compare lanes 4 and 8). Such a skewed distribution of hetero-oligomers may arise artifically if the conditions used for immunoprecipitation were not quantitative. This question has been addressed in the experiments shown in Fig. 5. Lysates from 35S-labeled COS cells transfected with both type I and FLAG-III IP₃R isoforms were subjected to three repeated rounds of immunoprecipitation with type I IP₃R Ab to completely deplete the lysate of type I containing homo- and heterotetramers. It would be predicted that immunoprecipitation of the remaining supernatant with FLAG Ab should bring down only type III IP₃R subunits present as homotetramers. The first round of immunoprecipitation with type I Ab was sufficient to bring down the majority of type I homo- and heterotetramers (Fig. 5A, lane 1). As predicted, the final immunoprecipitation with FLAG Ab brought down primarily a single labeled band corresponding to FLAG-type III homotetramers (Fig. 5, lane 4). When the initial rounds of immunodepletion were carried out with FLAG Ab, the amount of type III IP₃R immunoprecipitated by the first round was lower (Fig. 5B, lane 5). The final round of immunoprecipitation with type I IP₃R Ab indicates that a substantial proportion of the type III subunits remain in the supernatant as hetero-oligomers (Fig. 5B, lane 8). The same result was obtained when the first three rounds of immunoprecipitation were carried out using a type III monoclonal Ab directed at the N terminus or a type III-specific C-terminal polyclonal Ab (data not shown). This suggests that type III IP₃R subunits present as hetero-oligomers may not be efficiently immunoprecipitated by several type III Abs, and this may account for the apparent skewed distribution of hetero-oligomers seen in Fig. 4. However, the immunoprecipitation with type I IP₃R Ab is quantitative and efficiently immunoprecipitates both homo- and hetero-oligomeric subunits.

Altering the Proportion of Hetero-oligomers by Varying the Amount of IP₃R DNA—There are five possible ways in which

![Fig. 2. Transfected type I and FLAG-type III IP₃Rs form tetramers.](image)

![Fig. 3. Immunofluorescence localization of type I and FLAG-type III IP₃R isoforms.](image)
Hetero-oligomerization of type I and type III IP₃R isoforms. A, COS cells were transfected in 60-mm plates with 8 μg of pcDNA3.1 (mock transfection) or type I IP₃R cDNA as described under “Experimental Procedures.” Lysates were prepared from the cells 48 h post-transfection, and equal aliquots were immunoprecipitated with either type I or type III IP₃R-specific Abs. The immunoprecipitates were run on 5% SDS-PAGE and immunoblotted sequentially with type I or type III IP₃R Ab. B, COS cells were transfected with pcDNA3.1 (mock transfection) and type I IP₃R cDNA, FLAG-type III IP₃R cDNA, or both type I and FLAG-type III IP₃R cDNA. Each IP₃R cDNA was used at 8 μg, and the total DNA concentration was maintained at 16 μg for all conditions using pcDNA 3.1. Both sets of immunoprecipitates were analyzed by immunoblotting with either type I IP₃R or FLAG Abs. The arrows denote the presence of hetero-oligomers in cells doubly transfected with type I and FLAG-type III IP₃R cDNA.

Fig. 4. Hetero-oligomerization of type I and type III IP₃R isoforms. A, COS cells were transfected in 60-mm plates with 8 μg of pcDNA3.1 (mock transfection) or type I IP₃R cDNA as described under “Experimental Procedures.” Lysates were prepared from the cells 48 h post-transfection, and equal aliquots were immunoprecipitated with either type I or type III IP₃R-specific Abs. The immunoprecipitates were run on 5% SDS-PAGE and immunoblotted sequentially with type I or type III IP₃R Ab. B, COS cells were transfected with pcDNA3.1 (mock transfection) and type I IP₃R cDNA, FLAG-type III IP₃R cDNA, or both type I and FLAG-type III IP₃R cDNA. Each IP₃R cDNA was used at 8 μg, and the total DNA concentration was maintained at 16 μg for all conditions using pcDNA 3.1. Both sets of immunoprecipitates were analyzed by immunoblotting with either type I IP₃R or FLAG Abs. The arrows denote the presence of hetero-oligomers in cells doubly transfected with type I and FLAG-type III IP₃R cDNA.

Two different monomers can be assembled into tetramers. If we refer to the type I monomer as a and type III monomer as b, these tetrameric forms correspond to aaaa, aaab, aabb, abbb, and bbbb. If the combination of subunits is random, then the relative proportions of each of these forms is predicted by the binomial distribution and can be expressed by terms in the expansion of \((a + b)^4\) where \(a + b = 1\). Since the absolute amount of each subunit is not known, we can define a ratio \(r = a/b\). Then the distribution ratio of each of the five forms is given by \(r^4-4r^3-6r^2+4r\). The fraction of type I polypeptide present as heterotetramers \(F_a\) is given by \((12r^3 + 12r^2 + 4r)/(4r^4 + 12r^3 + 12r^2 + 4r)\). The fraction of type III polypeptide present as heterotetramers \(F_b\) is given by \((4r^3 + 12r^2 + 12r)/(4r^4 + 12r^3 + 12r^2 + 12r + 4)\).

Fig. 6 shows experiments in which COS cells were doubly transfected with different amounts of DNA corresponding to one IP₃R isoform while keeping the DNA for the other isoform fixed. Labeled lysates were immunoprecipitated with either type I IP₃R Ab, FLAG Ab, or both. Fig. 6A shows representative autoradiograms corresponding to these immunoprecipitates. Quantitation of the lower band in the type I immunoprecipitates and the upper band in the FLAG immunoprecipitates yields values for type III- and type I-containing hetero-oligomers, respectively. In COS cells expressing FLAG-type III IP₃R alone, the overnight immunoprecipitation with FLAG monoclonal Ab was sufficient to bring down >85% of the homotetrameric receptors (data not shown). Hence, the upper and lower bands after immunoprecipitation with both Abs together can be used to estimate the total type I and FLAG-type III IP₃R subunits. From these values, it was possible to calculate experimental values for \(F_a\) and \(F_b\), and these are plotted as a function of the ratio \(r\) of type I/ type III IP₃R (Fig. 6B, C). The equations given above were used to calculate the predicted variation in \(F_a\) and \(F_b\) over this range of \(r\) values.

As the ratio of type I/ type III IP₃R increases, it would be expected that the fraction of type I subunits present as hetero-oligomers \(F_a\) would decrease and the fraction of type III subunits present as hetero-oligomers \(F_b\) would increase. This trend is evident from the experimental data. However, it is also clear that the data do not follow the distribution predicted from binomial statistics. This is not surprising for \(F_a\) (Fig. 6B), since these data rely on immunoprecipitation with FLAG Ab, which underestimates the hetero-oligomeric pool (Fig. 5B). The same considerations do not apply to \(F_b\) (Fig. 6C), which also does not fit the predicted relationship. We therefore conclude that the association between subunits under our experimental condi-

![Image](80x478 to 265x729)

**Fig. 4.** Hetero-oligomerization of type I and type III IP₃R isoforms. A, COS cells were transfected in 60-mm plates with 8 μg of pcDNA3.1 (mock transfection) or type I IP₃R cDNA as described under “Experimental Procedures.” Lysates were prepared from the cells 48 h post-transfection, and equal aliquots were immunoprecipitated with either type I or type III IP₃R-specific Abs. The immunoprecipitates were run on 5% SDS-PAGE and immunoblotted sequentially with type I or type III IP₃R Ab. B, COS cells were transfected with pcDNA3.1 (mock transfection) and type I IP₃R cDNA, FLAG-type III IP₃R cDNA, or both type I and FLAG-type III IP₃R cDNA. Each IP₃R cDNA was used at 8 μg, and the total DNA concentration was maintained at 16 μg for all conditions using pcDNA 3.1. Both sets of immunoprecipitates were analyzed by immunoblotting with either type I IP₃R or FLAG Abs. The arrows denote the presence of hetero-oligomers in cells doubly transfected with type I and FLAG-type III IP₃R cDNA.

![Image](309x412 to 553x729)

**Fig. 5.** Quantitative recovery of IP₃Rs assessed by repeated immunoprecipitation of lysates. COS cells were doubly transfected with 8 μg of type I and 8 μg of FLAG-type III IP₃R DNA. The cells were labeled with 20 μCi/ml Tran²S label in methionine-free Dulbecco’s modified Eagle’s medium for 24 h prior to the preparation of lysates at 48 h post-transfection. A, labeled lysates (100 μg of protein) were immunoprecipitated with type I IP₃R Ab for 16 h (lane 1). The supernatant from this first round of immunoprecipitation was subjected to two additional rounds of immunoprecipitation with type I IP₃R Ab for 4 h each (lanes 2 and 3). The final round of immunoprecipitation was done for 16 h with FLAG Ab (lane 4). The immunoprecipitates were processed on 5% SDS-PAGE, transferred to nitrocellulose, and autoradiographed. B, the experiment was the same as described in A, except the first three rounds of immunoprecipitation were done with FLAG Ab and the final round was with type I IP₃R Ab. The inset to each panel shows a representative autoradiograph. The assignment of the upper and lower bands of the radioactive doublet to type I and type III IP₃Rs, respectively, is based on immunoblotting of the nitrocellulose with IP₃R isoform-specific Abs (data not shown, and see Ref. 5). The graphs show the mean ± S.E. of the densitometric quantitation of the upper band (type I IP₃R, A) and lower band (FLAG type III; B) from three independent experiments.
In the present study, we have examined the combinatorial properties of IP₃R subunits transiently transfected into COS-7 cells with type I and FLAG-type III IP₃R cDNA. At 48 h, the cells were incubated for 1 h in methionine-free medium followed by incubation with Tran³⁵S-label (100 μCi/ml) for the indicated time periods. Plates were washed in ice-cold PBS containing 100 μg/ml cycloheximide, and the kinetics of labeling of IP₃Rs was monitored by immunoprecipitation of cell extracts with either type I IP₃R Ab (A) or FLAG Ab (B) The radioactivity associated with the upper and lower bands of the doublet was quantitated by densitometry. The data shown are from a representative experiment. The compiled data from three experiments are shown in Table I.

**Discussion**

The kinetics of synthesis of type I and FLAG-type III IP₃R were monitored by measuring the ³⁵S label incorporation into these isoforms immunoprecipitated from COS cells doubly transfected with both isoforms (Fig. 7). The time course for the appearance of the type I subunits immunoprecipitated by type I Ab (Fig. 7A, filled circles) and the type III subunits immunoprecipitated by FLAG Ab (Fig. 7B, filled circles) showed a lag period that is related to the time required to synthesize a full-length polypeptide chain (Table I). This time was approximately the same for type I and FLAG-type III IP₃Rs and was also similar to the range of values reported for other integral membrane proteins synthesized in the endoplasmic reticulum (25, 26). With both antibodies, a longer lag time and a slower rate of synthesis was observed for the hetero-oligomeric population of receptors. This result is expected if the association of unlike nascent chains is kinetically less favored than the association of homologous nascent chains.
cells. These cells expressed transfected type I and type III IP₃Rs as tetramers within intracellular membranes. The recombinant receptors are functional based upon ⁴⁵Ca²⁺ flux studies on microsome vesicles prepared from transfected cells (27) and from reconstitution experiments using planar lipid bilayers (28, 29). One of the observations we have made in the present study is that endogenous and transfected IP₃Rs have a low rate of combination. We were unable to observe any ⁴⁵S label incorporation into the endogenous type III IP₃R over a 30-min time period (data not shown). Under these conditions, a substantial amount of label is incorporated into FLAG-tagged type III (Fig. 7). This supports our interpretation that the low probability of combination of transfected and endogenous receptors is due to the vastly different rates of biosynthesis of the two forms. A low combination rate has the experimental advantage that recombinant receptors can be studied in isolation using this system.

A high degree of combination can be observed when two different IP₃R isoforms are expressed simultaneously. A similar observation has been made in NG-108 15 cells doubly transfected with type I and type III IP₃Rs (4). The type I and type III IP₃Rs have distinct mobilities on SDS-PAGE. We have taken advantage of this property and used metabolic labeling and co-immunoprecipitation assays to quantitate the fraction of each form present as hetero-oligomers when the molar ratio of each isoform is varied by using different amounts of transfected DNA. Our analysis relies on the assumption that the bulk of the transfected cells express both plasmids when co-transfected and that there are no large cell-to-cell variations in the relative expression levels of both DNAs. Both assumptions seem to be valid based upon our observations of the immunofluorescence of doubly transfected cells (data not shown). The experiments in the present study show that the relative expression levels of the two isoforms influence the proportion of hetero-oligomers that are formed. However, the fraction of each isoform present as hetero-oligomers is less than predicted from binomial statistics, suggesting that tetramers may not be assembled randomly from individual subunits. For example, when the expression level of type I and type III subunits are equal, we would predict that 97.5% of each subunit would be present as hetero-oligomers. Experimentally, the fraction of type I and type III subunits present as hetero-oligomers is approximately 25 and 50%, respectively (Fig. 6). The experimental measurements of type I hetero-oligomers rely on immunoprecipitation with type III IP₃R Ab. The marked deviation of these measurements from the predicted relationship can probably be accounted for by the inability to quantitatively immunoprecipitate all of the type III hetero-oligomer population in COS cell lysates. The experimental data for type III hetero-oligomers was obtained with type I IP₃R Ab under conditions where immunoprecipitation was demonstrated to be quantitative. Although the deviations in this case are less marked, it is still apparent that the probability for forming hetero-oligomers of type III IP₃R subunits is substantially less than predicted.

A quantitative analysis of the hetero-oligomerization of native IP₃Rs has not been carried out previously, and estimates of the proportion of IP₃R isoforms present as homo- or hetero-oligomers in cell lysates have varied widely. Monkawa et al. (4) found that the majority of all three IP₃R isoforms in CHO-K1 cells existed as hetero-oligomers. Nucifora et al. (30) carried out immunodepletion experiments on Rin5mf cell lysates and concluded that some type III IP₃Rs were present as homotetramers but that all of the type I IP₃Rs were present as hetero-oligomers. This result is consistent with the known distribution of isoforms in this cell type, which is 96% type III, 4% type I, and no detectable type II IP₃Rs (23). In AR4–2J cells, similar immunodepletion experiments indicate that almost half of the type II IP₃Rs are present as homotetramers (31) and that nearly all of the type I IP₃Rs are present as hetero-oligomers (6). Again this is consistent with the 7-fold enrichment of the type II isoform compared with the type I isoform in this cell type (23). In Xenopus oocytes expressing 40-fold greater levels of recombinant type III IP₃R compared with the endogenous IP₃Rs, only 7% of the recombinant type III subunits form hetero-oligomers with the endogenous IP₃R isoform (32). This compares well with the 9% predicted from binomial statistics.

The ratio of type I/type II IP₃R in WB rat liver epithelial cells was estimated to be 2.7 (5). From this value, we predict that WB cells would have 61 and 98% of their type I and type III IP₃R subunits as hetero-oligomers, respectively. However, such high proportions of hetero-oligomers were not observed in our previous studies on WB cells, and therefore we speculated that association between subunits may not be a random process (5). Overall, these studies indicate that when one isoform greatly predominates in a cell type it is difficult to discern a marked deviation from a random combination model of IP₃R assembly. By contrast, deviations from a random combination model may be more evident in cell types that contain comparable levels of two or more isoforms.

Many ion channels and enzymes form heteromultimers. There are several examples where association between subunits is not a random process and where a particular subunit composition and/or unique positional specificity within a tetramer is favored (e.g. inwardly rectifying K⁺ channels (33–35)). Deviations from a random model of assembly may arise in many different ways. A key feature of IP₃R assembly is that oligomerization involves the noncovalent interactions of transmembrane domains and occurs cotranslationally in the endoplasmic reticulum (19, 36). Hence, if synthesis of individual isoforms were to be segregated, spatially or temporally in some manner, the final composition of oligomers would not be expected to follow the rules of binomial combination. (37). Our measurements of the kinetics of assembly show that the lag time for the synthesis of hetero-oligomers is greater than the lag time for the synthesis of homotetramers. Thus, there is a kinetic constraint in the assembly pathway of IP₃R biosynthesis that favors homotetramers over heterotetramers. Nothing is known regarding the steps involved in assembling IP₃R oligomers. Recent studies on other tetrameric ion channels such as Kv1.3 (38) and cGMP-gated ion channels (39) suggest that they may be assembled by dimerization of dimers. Such preferred pathways of assembly could also act to restrict the number of possible combinations of subunits in hetero-oligomerization.
As our experimental data were best fitted by excluding all but one of the three possible combinations of homo-oligomers, which in the case of type III hetero-oligomers consisted of the form containing three type III subunits and one type I subunit. The experimental errors involved in immunoprecipitation and quantitative analysis of the data suggest that curve fitting procedures alone cannot be relied upon as the sole approach to derive the composition of IP$_3$R hetero-oligomers, and other preferred arrangements of subunits cannot presently be excluded.

In summary, our studies show that the expression of two different IP$_3$R isoforms in COS cells results in the formation of homo- and hetero-oligomers. The relative expression level of each isoform influenced the fraction of hetero-oligomers formed. However, the combinatorial properties of the recombinant IP$_3$Rs indicate that homo-oligomers are favored over hetero-oligomers. The underlying reason for this is not known but may reflect constraints within the pathway of IP$_3$R assembly in which some interactions of subunits are preferred over others. A biochemical analysis of the subunit composition of IP$_3$R hetero-oligomers in intact cells is needed to test these predictions.

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