Heteroligomers of Type-I and Type-III Inositol Trisphosphate Receptors in WB Rat Liver Epithelial Cells*

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We have previously shown that a 222-kDa polypeptide co-immunoprecipitates together with the type-I myo-inositol 1,4,5-trisphosphate receptor (IP₃R) in WB rat liver epithelial cell extracts, when the immunoprecipitation is carried out with a type-I isoform specific antibody (J. Joseph, S. K. (1994) J. Biol. Chem. 269, 5673–5679). Utilizing isoform-specific antibodies raised to unique sequences within the COOH-terminal region of IP₃ receptors, we now report that the co-immunoprecipitating 222-kDa polypeptide is the type-III IP₃R isoform and that type-I IP₃R antibodies (Abs) can co-immunoprecipitate the type-I IP₃R isoform. Co-immunoprecipitation of IP₃R isoforms was not due to cross-reactivity of the antibodies for the following reasons: (a) on immunoblots the type-III antibodies did not cross-react with type-I IP₃R and vice versa; (b) inclusion of the COOH-terminal type-III peptide had no effect on the ability of type-I IP₃R Ab to co-immunoprecipitate the type-III IP₃R but blocked the ability of type-III IP₃R Ab to co-immunoprecipitate the type-I isoform; and (c) crude hepatocyte lysates contain undetectable amounts of type-I IP₃R, and immunoprecipitation with type-IIP₃RAb does not co-immunoprecipitate any other isoforms. However, type-I and type-II IP₃R isoforms were co-immunoprecipitated by their respective antibodies in hepatocyte lysates. Sucrose density gradient analysis of WB cell lysates indicated that the co-immunoprecipitating fraction is exclusively located at the density expected for tetrameric receptors, suggesting that co-immunoprecipitation was not a reflection of the nonspecific aggregation of IP₃R isoforms. Phosphorylation of either type-I or type-III immunoprecipitates by protein kinase A indicated that only the type-I IP₃R could be phosphorylated in vitro. Fractionation of WB cell membranes and immunofluorescence studies showed that the type-I and type-III isoforms have very similar sub-cellular localizations. We conclude that the WB cell contains both type-I and type-III IP₃R isoforms and that a proportion of these receptors exist as heterotetramers.

The discharge of Ca²⁺ from intracellular stores in response to the activation of cell surface receptors is mediated by the interaction of inositol 1,4,5-trisphosphate with a specific receptor that functions as a ligand-gated Ca²⁺ channel (1–3). Molecular cloning studies have revealed the presence of three types of receptors encoded by separate genes. The type-I IP₃R is particularly enriched in the cerebellum region of the brain and has a calculated molecular mass based on cDNA sequence of 313 kDa (4, 5). The purified receptor has been shown to be a homotetramer (6). The type-I receptor mRNA undergoes alternative splicing in two distinct regions of the receptor designated S1 and S2. Several studies have shown that the type-I receptor with the S2 region deleted is the predominant form in peripheral tissues (7, 8). The type-II IP₃R is 69% homologous to the type-I IP₃R and was originally described as being expressed in brain (9). Subsequent studies have since shown the presence of type-II IP₃R mRNA in several peripheral tissues (10). The type-III IP₃R has 62% homology to the type-I IP₃R and is expressed in several epithelial cells including those of the kidney, pancreas, and intestinal tract (11, 12). Only partial sequences of a type-IV (13) and type-V receptor (10) have been reported, and their distribution and properties have not been characterized.

In a previous study, we investigated the biosynthesis and turnover of IP₃R in cultured WB rat liver epithelial cells (14). We found that immunoprecipitation of ³⁵S-labeled WB cell extracts with type-I IP₃R antibody resulted in the appearance of two ³⁵S-labeled polypeptides with molecular masses of 235 and 222 kDa. Only the 235-kDa band was immunoreactive with type-I-specific IP₃R antibody. The 222-kDa band, which was present in lower amounts relative to the 235-kDa band, was not identified but was shown not to be a proteolytic clip of the type-I IP₃R. Based on the cDNA the type-III IP₃R has a calculated molecular mass of 304 kDa and would therefore be expected to run at a lower molecular mass than type-I IP₃R on SDS-PAGE. In the present study we have utilized isoform-specific IP₃R antibodies to show that the 222-kDa band indeed corresponds to the type-III IP₃R. Additional data are shown demonstrating co-localization of both isoforms in WB cells, and evidence is presented to indicate that a proportion of both isoforms exist as heterotetrameric complexes.

MATERIALS AND METHODS

Antibodies—The isoform-specific antibodies used in these studies were raised against the unique COOH-terminal sequences of IP₃ receptors. The type-I Ab was a polyclonal anti-peptide antibody raised to amino acids 2731–2749 of the rat type-I IP₃R and has previously been characterized (15, 16). Two different type-III Abs were used in the present studies. The anti GST-H3CT polyclonal antibody was raised against a glutathione S-transferase fusion protein made with the 27 COOH-terminal amino acids of the human type-III IP₃R. This antibody

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‡ The abbreviations used are: IP₃R, myo-inositol 1,4,5-trisphosphate receptor; IP₃, myo-inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; Ab, antibody; PAGE, polyacrylamide gel electrophoresis.
was affinity purified using an Affi-Gel fusion protein column (12). The second type-III antibody was a monoclonal anti-peptide antibody raised to amino acids 2657–2671 of the human type-III IP3R (17). This and additional monomodal antibodies to the type-I and type-II IP3R isoforms were a kind gift of Dr. Mamoru Hasegawa (Kyowa Hakko Kogyo Ltd., Tokyo, JAPAN). The reactivity of these monoclonal antibodies in hematopoietic cells has been detailed previously (17). Antibodies to the rough ER marker protein ribophorin-I and to the nuclear marker protein lamin-B were generously provided by Dr. Christopher Nichitta (Duke University, Durham, NC) and Dr. Gerd Maul (Wistar Institute, Philadelphia, PA), respectively. Antibody to the plasma membrane marker Na+/K+-ATPase (α-subunit) was purchased from Upstate Biotechnology, Inc. (NY). All polyclonal antibodies used to block the polypeptides in the gel were transferred to nitrocellulose that was then autoradiographed and then immunoblotted with type-III antibody to locate the receptor. Immunoreactive bands were detected with an enhanced chemiluminescence assay (Amersham Corp.). When necessary, immunoblots were stripped and then incubated for a further 10 min in phosphate-buffered saline containing 0.1% saponin. Nonspecific binding sites were blocked by 0.1% saponin in ice-cold phosphate-buffered saline solution. The polypeptides were transferred to nitrocellulose, which was then autoradiographed (Auto-rad, A). The same nitrocellulose sheet was then sequentially immunoblotted with type-III Ab (B), stripped, immunoblotted with type-I Ab (C), stripped, and immunoblotted with both Abs (D).

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**Fig. 1.** Co-immunoprecipitation of type-III IP3R with type-I IP3R in 35S-labeled WB extracts. Cells were labeled with Trans35S-label and immunoprecipitated with either type-I Ab (lane 1) or type-III Ab (lane 2) as described under "Materials and Methods." The immunoprecipitates were run out on a 5% SDS gel together with an unlabelled sample of WB cell extract (lane 3). The polypeptides were transferred to nitrocellulose, which was then autoradiographed (Auto-rad, A). The same nitrocellulose sheet was then sequentially immunoblotted with type-III Ab (B), stripped, immunoblotted with type-I Ab (C), stripped, and immunoblotted with both Abs (D).
conclusion relies on the antibodies being entirely specific for their respective antigens. The recognition properties of the type-III polyclonal antibody (GST-H3CT Ab; Ref. 12) is shown in Fig. 2A. Detergent extracts of cerebellum microsomes, WB cells and hepatocytes were immunoblotted against type-I and type-III antisera. As expected, the type-I receptor that is enriched in cerebellum produces a strong signal when probed against type-I Ab (Fig. 2A, upper panel). However, the cerebellar type-I IP₃R shows no cross-reactivity to the type-III Ab (Fig. 2A, lower panel). Sufficient amounts of type-I and type-III receptor are present in 50 μg of WB cell extract proteins to enable a clear signal to be obtained from immunoblotting without the necessity of immunoprecipitating the extracts. This is not the case for hepatocytes where a weak signal for type-I is seen only when 200 μg of extract protein is loaded into a gel lane. The amount of type-III receptor in hepatocyte extracts was below the detection limit. This is in agreement with a recent study using reverse transcriptase-polymerase chain reaction that found only 3% of the total IP₃R mRNA in rat liver encoded the type-III isoform (10). The co-immunoprecipitation of IP₃R isoforms seen in Fig. 1 could be duplicated using monodonal antibodies to type-I and type-III IP₃R (data not shown). The immunoblot in Fig. 2B (lane 2) shows that the type-I monodonal Ab also selectively recognizes only the lower 222-kDa 35S-labeled band in the type-I monodonal Ab immunoprecipitates.

A further criteria of specificity in antibody recognition is that immunoprecipitation of the target antigen should be suppressed by inclusion of the peptide epitope used in immunization. In the case of type-I IP₃R antibody, we have previously shown that the CT-1 peptide blocks the immunoprecipitation of both 35S-labeled bands from WB extracts (14). In Fig. 3 unlabeled WB extracts were immunoprecipitated with polyclonal type-I or type-III antibody in the presence or absence of CT-3 peptide. The immunoprecipitates were immunoblotted with either type-I (Fig. 3A) or type-III-specific antibodies (Fig. 3B). The results show that the CT-3 peptide does not influence the ability of type-I antibody to immunoprecipitate the type-I IP₃R or to co-immunoprecipitate the type-III IP₃R. However, the CT-3 peptide markedly inhibits the ability of the type-III antibody to immunoprecipitate the type-III IP₃R and co-immunoprecipitate the type-I IP₃R isoform. This selectivity of peptide inhibition suggests that co-immunoprecipitation of receptors is not due to a lack of specificity in the recognition properties of the IP₃R antibodies.

In Vitro Phosphorylation of IP₃R Immunoprecipitates—In our previous study we showed that the 222-kDa polypeptide co-immunoprecipitating with the type-I IP₃R was not a substrate for protein kinase A. If the 222-kDa polypeptide is the type-III IP₃R, it would be predicted that the type-III IP₃R would also not be a substrate for protein kinase A in vitro. It should be noted that the primary sequence of the type-III IP₃R does contain five consensus protein kinase A phosphorylation sites (12). Fig. 4 shows the result of protein kinase A phosphorylation of type-I and type-III immunoprecipitates prepared from WB cell extracts. As observed previously (14), the WB type-I IP₃R is readily phosphorylated by protein kinase A (Fig. 4, lane 1). The type-III immunoprecipitates were phosphorylated to a lesser extent and only a single phosphorylated band was observed (Fig. 4, lane 2). By immunoblotting of this lane (Fig. 4, lanes 3 and 4), it could be demonstrated that the single phosphorylated band corresponded to the type-I IP₃R and that the type-III IP₃R itself was not an protein kinase A substrate. The data do not exclude the possibility that the type-III isoform may be fully phosphorylated under these conditions.

Subcellular Localization of IP₃R Isoforms in WB Cells—We have used subcellular fractionation and immunocytochemistry as two different approaches to compare the distribution of IP₃R isoforms in WB cells. Fig. 5 shows the distribution of type-I and type-III IP₃R in WB membranes fractionated on a sucrose density gradient. Both isoforms had a similar distribution profile with peak immunoreactivity being located toward the bottom of the gradient. The distribution profile of IP₃R did not overlap exactly with any of the three membrane markers examined. In particular, only a minor proportion of IP₃R isoforms were found in the fractions containing ribophorin-I, a marker of the rough ER. A greater degree of overlap was obtained with those fractions containing nuclear and plasma membrane markers. The similar localization of both receptor isoforms was also observed when the peak fractions of IP₃R immunoreactivity were pooled and fractionated further on a 15% Percoll gradient (data not shown). A similar localization of both iso-
forms was also observed in immunofluorescence studies of WB cells (Fig. 6). Immunofluorescence of type-I and type-III Ab in WB cells shows that these isoforms are excluded from the nucleus and are widely distributed throughout the cell in both diffuse and punctate structures (Fig. 6, A and B). With both isoforms the intensity of staining was highest in the perinuclear region. In agreement with the fractionation experiments (Fig. 5), the pattern of labeling with IP₃R Abs was not identical to the pattern of labeling seen with anti-ribophorin Ab, which would be expected to stain rough ER membranes (Fig. 6C). The fluorescence signal was inhibited when type-III (Fig. 6D) or type-I Ab (data not shown) was preblocked with their respective antigenic peptides.

Heterotetrameric Formation or Nonspecific Aggregation of Receptor Complexes—Nonspecific association of the two homotetrameric isoforms would be expected to generate large multimeric complexes with molecular masses that greatly exceed 10⁶ daltons. However, complexes that are heterotetramers of two isoforms would still have a molecular mass of approximately 10⁶ daltons. To identify the molecular mass of the complexes, we subjected WB cell lysates to sucrose gradient centrifugation (Fig. 7A). Sufficient lysate protein was loaded to enable the profile of both IP₃R isoforms in the gradient to be determined by immunoblotting alone. These results showed that both isoforms had a similar distribution in the gradient. The predominant peak of immunoreactive protein was located at the migration position of tetrameric cerebellar type-I IP₃R (Fig. 7B). In addition, a minor peak was seen at the position corresponding to IP₃R monomer, and immunoreactive protein was also observed in intermediate fractions. When the fractions were immunoprecipitated with type-I Ab and then immunoblotted with type-I Ab, the overall profile of type-I IP₃R was identical to that observed by direct immunoblotting of the fractions. This is the anticipated result if the antibody shows no preference for any particular oligomeric structure. Immunoblotting of the type-I immunoprecipitates with type-III anti-body indicated that the co-immunoprecipitating type-III IP₃R was restricted to just three fractions that coincide with the expected position of tetrameric receptor (Fig. 7A). The same fractions also contained the type-I IP₃R that was co-immunoprecipitated by type-III Ab (data not shown). There was no evidence from these experiments for the presence of large multimeric heterocomplexes migrating at the bottom of the gradient. We conclude that a fraction of type-I and type-III IP₃R isoforms in WB cells exist as heterotetramers.

Co-immunoprecipitation of Receptor Isoforms in Hepatocyte Lysates—In order to determine if co-immunoprecipitation of isoforms is a unique property of WB cell lysates or to type-I and -III isoforms, we carried out experiments with hepatocyte lysates. Hepatocytes are reported to contain predominantly type-I and type-II mRNA (10), and this has been verified at the protein level by immunoblotting.² Fig. 8A shows a type-I IP₃R Ab immunoblot of type-I, -II, and -III immunoprecipitates. It is clear that the type-II antibody can co-immunoprecipitate the type-I IP₃R (Fig. 8A, lane 2). Similarly, type-I antibody co-immunoprecipitates some type-II IP₃R (Fig. 8B, lane 1). In contrast to WB cells, the hepatocytes contain little type-III IP₃R, and under the conditions of the experiment, the type-III antibody does not bring down detectable amounts of the type-III isoform (data not shown). The type-III antibody also does not co-immunoprecipitate any type-I or type-II IP₃R (lanes 3 in Fig. 8, A and B). This attests to the specificity of the type-III antibody. As a further test of specificity, the COOH-terminal peptides of the types I and II IP₃R were included during the immunoprecipitation protocol. The results show that the CT-2 peptide blocks the ability of the type-II antibody to bring down its target antigen (Fig. 8B, lane 7) and the co-immunoprecipitating type-I IP₃R (Fig. 8A, lane 7). The CT-1 peptide did not interfere with the activity of type-II IP₃R Ab (Fig. 8A, lane 6). However, the CT-1 peptide blocked the ability of the type-I antibody to bring down its target antigen (Fig. 8A, lane 4) and the co-immunoprecipitating type-II IP₃R (Fig. 8B, lane 4). The CT-2 peptide did not interfere with the activity of type-I IP₃R Ab (Fig. 8A, lane 5). These data show that co-immunoprecipitation of IP₃Rs extends to other combinations of isoforms and to other cell types besides WB cells.

DISCUSSION

The results of the present study establish that the lower component of the ³⁵S-labeled doublet of polypeptides immunoprecipitated by the type-I IP₃R Ab from WB cell lysates is the type-III IP₃R isoform. Although the presence of multiple IP₃R isoforms in specific cell types has been inferred from mRNA analysis (10, 22, 23), the quantitation of relative levels of immunoreactive protein and the subcellular localization of multiple isoforms has been analyzed in very few cell types. In PC12 cells, an IP₃R antibody that does not discriminate between isoforms has been shown to detect a doublet of polypeptides of which only the upper band is the type-I IP₃R (22). The Jurkat T-cell has been shown to contain all three isoforms, mast cells contain both type-I and type-II, and only the type-II isofrom can be detected in macrophages (17). WB rat liver epithelial cells contain high levels of both type-I and type-III IP₃R isoforms with no detectable type-III isoform (data not shown). Isolated hepatocytes are different in that they have a much lower total expression of receptors and that they contain the type-I and type-II IP₃R as the predominant isoforms. A quantitation of the relative abundance of the three isoforms in several cell lines and tissues has recently been published (24). In a megakaryocytic cell line, fluorescence confocal microscopy

²C. Lin, G. Hajnoczky, S. K., Joseph, T. G. Schneider, and A. P. Thomas, manuscript in preparation.
was used to show that the type-I and type-II receptors are localized differently (17). This is clearly different from our results in WB cells where no gross differences in localization of type-I and type-III isoforms were noted from subcellular fractionation or immunofluorescence measurements. Both approaches also showed that the localization of the IP$_3$R isoforms was distinct from the distribution of ribophorin-I, a rough ER marker. This supports the conclusion that in some cell types the IP$_3$R may be localized in a subcompartment of the ER (25–27).

The present study demonstrates that antibodies specific to one IP$_3$R isoform can co-immunoprecipitate additional isoforms. The same finding was also noted by Wojcikiewicz in a recent study (24). Co-immunoprecipitation of different voltage-gated K$^+$ channels (28, 29) has been used as evidence for the formation of heteromultimeric complexes in vivo. However, the validity of these conclusions relies on the absolute specificity of the antibodies that are used in this experimental approach. The isoform specificity of the IP$_3$R anti-peptide antibodies used in the present studies are based on the following observations:

(a) The antibodies are raised against the nonconserved COOH-terminal regions and do not cross-react with multiple isoforms on immunoblots. Thus, the type-III polyclonal antibody does not react with the type-I IP$_3$R (from cerebellum or WB cells), and the type-I polyclonal does not recognize the type-III IP$_3$R.

(b) The ability of an isoform-specific antibody to immunopre-
co-immunoprecipitation occurs as a result of the association of body but not by any other peptides. These results imply that is blocked by the COOH-terminal cognate peptide of that anti-cipitate that isoform and co-immunoprecipitate other isoforms and not as a result of cross-reactivity. (c) The type-III IP₃R is absent from hepatocyte lysates. Immunopre-cipitation with the type-III antibody does not bring down any type-I or type-II IP₃R, as would be expected if the observed antibody interactions are specific. (d) Finally, the experimental ob-servations on co-immunoprecipitation and subcellular local-ization have been validated with two different antibodies for each isoform (data not shown).

We have interpreted our results to indicate that a pool of IP₃ receptors in WB cells exists as heterotetramers. The conclusion that the heterocomplexes are heterotetramers rather than large aggregates of receptor is based on our demonstration that the co-immunoprecipitating fractions of receptor have a migra-tion position on sucrose density gradients that is consistent with that expected of a heterotetramer. Such experiments also revealed that freshly prepared WB cell lysates contained low amounts of disassembled receptors, including a pool of free monomers. The same observation was made in our previous studies when the lysates were fractionated by gel filtration (14). It is therefore possible that the heterotetramers asse-mbled after lysis of the cells and do not occur in vivo. However, the proportion of ⁴³S-labeled type-I and type-III receptor im-munoprecipitated by type-I antibody was not altered by lysing the cells in radioligand assay buffer (which contained 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100) or by pretreating the intact cells for 1 h with 100 μg/ml of the cleavable cross-linking agent diithiobis(succinimidylpropionate) (data not shown). Because the amount of co-immunoprecipitating receptor cannot be changed by these experimental manipulations, we conclude that heteroligomerization of IP₃R pre-exists in the native WB cell membranes.

A random association of two different monomers can generate five possible combinations of tetramers. The proportion of homotetrameric and heterotetrameric receptor would be de-pendent on the relative amounts of the type-I and type-III isoforms. The ratio of type-I and type-III isoforms in WB cells cannot be easily estimated by comparing immunoblots because of the difficulties of standardizing and comparing the reactiv-ities of two different antibodies. An alternative is to quantitate the ⁴³S-labeled type-I and type-III isoforms immunoprecipi-tated by their respective antibodies from the lysate of ⁴³S-labeled cells (cf. Fig. 1A). A type-I/type-III ratio of 2.7 ± 0.2 (n = 3) is obtained by this method. Assuming an entirely random association, the relative proportions of the five tetramer forms can be calculated from the expansion of the polynomial (a + b)^4 = 1, where a and b are the relative concen-trations of the two different monomers (30). Taking a as the...
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The formation of heteromultimeric members of the IP3 receptor family is restricted to a localized pool of receptors. The inability to phosphorylate the type-III IP3R by protein kinase A also points to important differences in the regulation of IP3R isoforms. Heteroligomerization of isoforms may differ by an order of magnitude (22). A mathematical model has been used to describe the kinetics of Ca2+ release from rat basophilic leukemia cells (30). Heteroligomeric IP3R ion channels would be expected to have a wide range of sensitivity to IP3, and potentially could have different channel gating characteristics. The inability to phosphorylate the type-III IP3R by protein kinase A also points to important differences in the regulation of IP3R isoforms. Heteroligomerization of isoforms may thus provide an additional mechanism that generates considerable diversity in the regulation of the IP3R Ca2+ current channel.

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Note Added in Proof—After submission of this paper, evidence for heterotetramer formation of IP3R subunits was reported in several cell types (Monkawa, T., Miyawaki, A., Sugiyama, T., Yoneshima, H., Yamamoto-Hino, M., Furuchi, T., Saruta, T., Hasegawa, M., and Mikoshiba, K. (1995) J. Biol. Chem. 270, 14700–14704).

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