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TYPE I, II AND III INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR CO-IMMUNOPRECIPITATION AS EVIDENCE FOR THE EXISTENCE OF HETEROTETRAMERIC RECEPTOR COMPLEXES

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SUMMARY The recently described co-expression of type I, II and III inositol 1,4,5-trisphosphate ($InsP_3$) receptors in the same cell type has raised the issue of whether these proteins exist as homotetramers or heterotetramers. To address this question, $InsP_3$ receptors were immunoprecipitated with specific antibodies and then probed for co-immunoprecipitating proteins. This revealed that type I, II and III $InsP_3$ receptors co-immunoprecipitate and thus, exist in heteromeric complexes. This situation was maintained when the relative abundance of $InsP_3$ receptors was altered radically during cell differentiation. Thus, heterotetrameric $InsP_3$ receptors are likely to contribute towards signaling in cells expressing more than one receptor type.

Inositol 1,4,5-trisphosphate ($InsP_3$) receptors play a crucial role in intracellular signaling as they tetramerize in endoplasmic reticulum membranes to form channels that conduct Ca^{2+} in an $InsP_3$-sensitive manner [1,2]. To date, the coding regions of three mammalian $InsP_3$ receptor genes have been sequenced in full and their products, termed type I, II and III receptors, are >60% homologous [3-8]. Putative type IV and V receptor coding regions, which are highly homologous to type II receptor cDNA, have also been partially sequenced [9,10].

Intriguingly, recent evidence from analysis of mRNA species [10,11,12] and immunochemical studies [13,14] have shown that type I, II and III $InsP_3$ receptors can be

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Abbreviations used in text:
$InsP_3$, inositol 1,4,5-trisphosphate; CT1, CT2 and CT3, antisera against the C-termini of rat type I, II and III $InsP_3$ receptors, respectively; RA, retinoic acid.

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co-expressed in the same cell type. Thus, it has become an issue whether, in such cell types, the channels formed by $InsP_3$ receptors are homotetrameric or heterotetrameric [15]. This is of significance because type I, II and III receptors have slightly different binding affinities for $InsP_3$ [5,16] and are regulated differently by proteolysis [13] and perhaps by phosphorylation [4-6,8]; thus, homo- and heterotetramers are likely to have different characteristics. Herein, we show that type I, II and III $InsP_3$ receptors exist as stable heteromeric complexes when detergent-solubilized, suggesting that heterotetrameric $InsP_3$ receptors are present in intact cells.

EXPERIMENTAL PROCEDURES

Cell culture Monolayers of SH-SY5Y human neuroblastoma cells, AR4-2J rat pancreatoma cells and RINm5F rat insulinoma cells, and suspensions of human leukemic HL-60 cells were cultured as described [13].

Antibody production and purification To obtain antisera against individual $InsP_3$ receptors, rabbits were injected as described [13,17] with the synthetic peptides RIGLLGHPPHMNVNPQQPA, GFLGSNTPHENHHMPPH and RLGFVDVQNCMSR, which correspond to the C-termini of rat type I, II and III receptors, respectively [4-6]. Crude antisera were then affinity purified, yielding antisera CT1, CT2 and CT3, respectively, which in immunoblots are specific to their cognate proteins [13]. Due to the great similarity between human and rat receptors at their C-termini [4-8], CT1, CT2 and CT3 recognize human and rat $InsP_3$ receptors equally well [13].

Immunoprecipitation of $InsP_3$ receptors When required for experiments, cell monolayers or centrifuged HL-60 cells (500g for 10min.) were solubilized with ice-cold lysis buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 0.2mM phenylmethylsulfonyl fluoride, 1mM dithiothreitol, 10μM leupeptin, 10μM pepstatin, 0.1mM soybean trypsin inhibitor, pH 8.0) and after 30min. at 4°C, were centrifuged (38,000g for 30min. at 4°C). Protein concentration in the supernatants was then assessed [18] and was adjusted with lysis buffer to ~3mg/ml. Portions of supernatant (0.8ml) were then incubated for 1h with CT1, CT2 or CT3, and then for a further hour with Protein A Sepharose CL-4B beads (Pharmacia) as described [13]. Bound proteins were then isolated by centrifugation (16,000g for 10s), were washed 5 times with lysis buffer and finally were dissociated with 30μl x2 gel loading buffer [19] plus 5μl 8M urea. In some experiments, 0.8ml portions of supernatants from two cell types were mixed prior to addition of antisera.

Electrophoresis and immunoblotting Samples from immunoprecipitations or HL-60 cell microsomes, prepared as described [13], were subjected to denaturing electrophoresis in 4% polyacrylamide gels and were either silver-stained or transferred to nitrocellulose [19]. Nitrocellulose was then incubated with CT1, CT2 or CT3, peroxidase-conjugated anti-rabbit IgG secondary antibody, enhanced chemiluminescence detection reagents (Amersham Corp.) and X-ray film [19]. Immunoreactivity was quantitated by densitometric scanning. In some experiments, samples were run in 7% gels and were probed with an antibody that recognizes endoplasmic reticulum Ca^{2+}-ATPase [20] (kindly provided by Dr D H MacLennan, University of Toronto).

Miscellany Secondary antibody, molecular mass markers, urea, dithiothreitol, retinoic acid, protease inhibitors, silver staining reagents and Triton X-100 were obtained from Sigma; reagents and equipment for electrophoresis were from BioRad. All data presented are mean ± SEM or representative of ≥ 3 independent experiments.

RESULTS AND DISCUSSION

To establish whether or not $InsP_3$ receptors exist in heteromeric complexes, we examined their ability to co-immunoprecipitate after solubilization with 1% Triton X-100.

We initially focussed on AR4-2J cells, as they co-express type I, II and III receptors (in the relative proportions 12, 86 and 2%) and for comparison examined SH-SY5Y and RINm5F cells, which express, respectively, 99% type I receptor and 96% type III receptor [13].

The proteins immunoprecipitated with the type I, II and III receptor-specific antisera CT1, CT2 and CT3 are depicted in Fig 1a. As expected from the predominance of type I receptor immunoreactivity in SH-SY5Y cells [13], only CT1 (lane 2) specifically immunoprecipitated detectable amounts of protein from this cell type (presumably the type I receptor, M_r ~279,000) and no co-immunoprecipitating proteins of similar M_r were

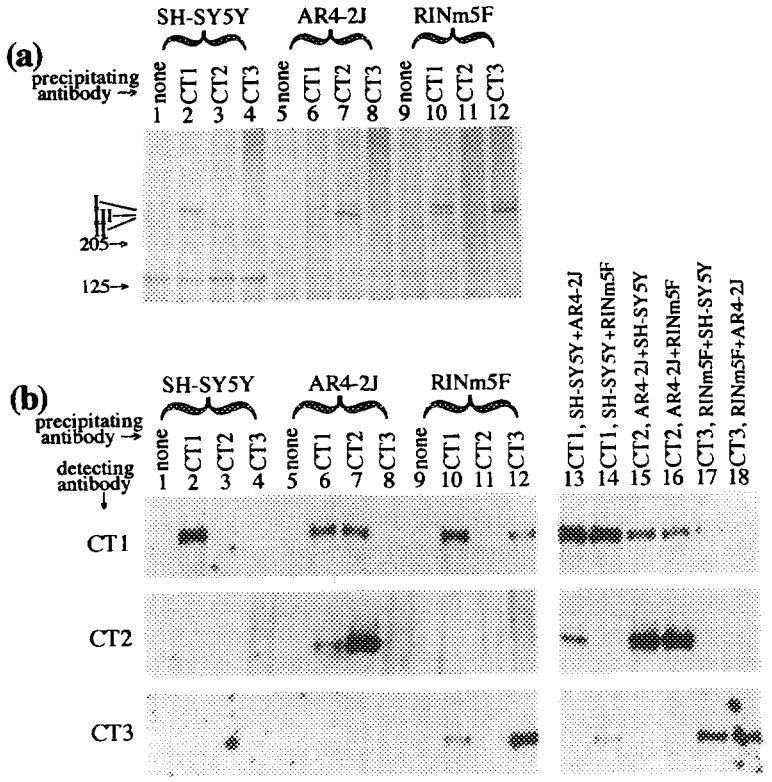

Figure 1. Immunoprecipitation of $InsP_3$ receptors from cell extracts.

Triton X-100 cell extracts were incubated with or without antisera. Immunoprecipitated proteins were then subjected to electrophoresis and were either silver-stained or transferred to nitrocellulose. (a) Silver-stained gel of proteins immunoprecipitated from SH-SY5Y (lanes 1-4), AR4-2J (lanes 5-8) and RINm5F cells (lanes 9-12) with CT1 (lanes 2, 6 and 10), CT2 (lanes 3, 7 and 11) or CT3 (lanes 4, 8 and 12). The positions to which immunoprecipitated type I, II and III receptors and the markers myosin (M_r = 205,000) and ß-galactosidase (M_r = 125,000) migrated are indicated. M_r values for type I, II and III receptors were 279±4, 257±4 and 263±5 x 10^3, respectively. (b) Nitrocellulose-bound proteins immunoprecipitated from SH-SY5Y, AR4-2J or RINm5F cell extracts (lanes 1-12) or from mixed extracts (lanes 13-18) were probed with either CT1, CT2 or CT3. The images shown correspond to the M_r 2-4 x 10^5 region of gels.

seen. Note that the bands at $M_r \leq 240,000$ in lanes 2-4 were also isolated when antibody was omitted (lane 1) and are, therefore, proteins unrelated to $InsP_3$ receptors. Similar analysis of AR4-2J cells revealed that both CT1 and CT2 immunoprecipitated readily detectable amounts of protein (lanes 6 and 7), again, consistent with the relative abundance of $InsP_3$ receptors in this cell type [13]. Significantly though, a doublet was clearly evident in lane 7; the larger of the two proteins co-migrating with the SH-SY5Y cell type I receptor, and the smaller protein (presumably the type II receptor) with M_r ~257,000. Thus, CT2 can immunoprecipitate the type I receptor when type I and II receptors are co-expressed. Similarly, both CT1 and CT3 clearly immunoprecipitated two proteins from RINm5F cells (lanes 10 and 12), the smaller protein (presumably the type III receptor) migrating with M_r ~263,000. Size differences between the receptors are to be expected as type II and III receptors are composed of fewer amino acids than type I receptor [3-8] and the receptors may be glycosylated differently [21].

To unequivocally identity the co-immunoprecipitating proteins, samples were probed with CT1, CT2 and CT3 (Fig 1b, lanes 1-12). This revealed that CT2 does indeed immunoprecipitate both type I and II receptors from AR4-2J cells (lane 7). Further, CT1 can now be seen to do the same (lane 6), and the RINm5F cell proteins immunoprecipitated by CT1 and CT3 are indeed type I and III receptors (lanes 10 and 12). That co-immunoprecipitation of type II and III receptors was not clearly evident (lanes 7, 8, 11 and 12) reflects the paucity of type III receptors in AR4-2J cells and type II receptor in RINm5F cells, since in COS-1 cells, which express similar amounts of type II and III receptors [13], co-immunoprecipitation of these proteins was readily detectable (data not shown).

To establish that co-immunoprecipitation reflects the existence of stable $InsP_3$ receptor heteromers and not an artefact, the following control experiments were performed. Firstly, to test for the possibility that $InsP_3$ homotetramers dissociate in lysis buffer and then artefactually reassociate into heteromeric complexes, cell extracts were mixed and then immunoprecipitated (Fig1b, lanes 13-18). Large increases in co-immunoprecipitation would be seen if dissociation/reassociation was occurring; for example, CT1 would immunoprecipitate much more type II receptor from SH-SY5Y plus AR4-2J cells, than from AR4-2J cells alone. However, this did not occur (compare the CT2 panel of Fig 1b, lanes 13 and 6). Similarly, CT1 immunoprecipitated equivalent amounts of type III receptor from SH-SY5Y plus RINm5F cells and RINm5F cells alone (compare the CT3 panel of Fig 1b, lanes 14 and 10). Analogous results were obtained when CT2 and CT3 (Fig 1b, lanes 15-18) were analyzed. Secondly, immunoprecipitates were probed with an antibody against endoplasmic reticulum Ca^{2+}-ATPase [20]. This tests for the possibility that large complexes containing a mixture of proteins, and possibly homotetramers of different $InsP_3$ receptors, are extracted and then precipitated.

However, Ca^{2+}-ATPase, which was abundant in cell extracts was not detected in immunoprecipitates (data not shown). Finally, results identical to that seen in Fig 1a were obtained when 1% sodium cholate or 1% CHAPS were used to solubilize cells (data not shown). Thus, $InsP_3$ receptor co-immunoprecipitation reflects the presence in cell extracts of stably associated heteromeric receptor complexes. As $InsP_3$ receptors retain their tetrameric nature after Triton X-100 solubilization [22], these data suggest that $InsP_3$ receptors are heterotetrameric in intact cells.

We also sought to determine whether heteromer formation was maintained when the relative abundance of $InsP_3$ receptors was altered radically. We thus studied HL-60 cells, which respond to the differentiating agent retinoic acid (RA) with changes in $InsP_3$ receptor mRNA levels [12]. As expected, RA altered $InsP_3$ receptor expression (Fig 2), increasing type I and II receptor concentration 1.7±0.1 fold and 18.3±1.4 fold after 4d. Receptor relative abundance was also changed; type II receptor comprised 13±3% of total in control cells and 60±2% of total after exposure to RA for 4d. Type III receptor immunoreactivity was not detected.

Analysis of control HL-60 cells with CT1 and CT2 showed again that type I and II receptors co-immunoprecipitate (Fig 3a-c, lanes 2 and 3). Consistent with the modest induction of type I receptor by RA (Fig 2), this agent had little effect on the amount of type I receptor immunoprecipitated by CT1 (Fig 3a and b, compare lane 2 with 5). In contrast, the amount of type II receptor immunoprecipitated by CT1 was significantly raised by RA (Fig3a and c, compare lane 2 with 5), indicating that newly synthesized

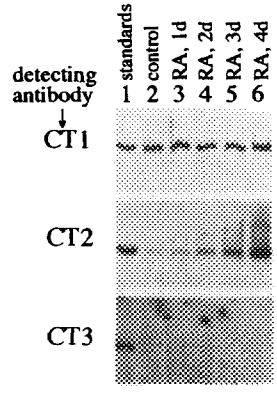

Figure 2. Effects of retinoic acid on $InsP_3$ receptor levels in HL-60 cells.
HL-60 cells were cultured with 2μM retinoic acid (RA) for 1-4 days or without RA (control). Cell microsomes (lanes 2-6; 10μg protein/lane) and standards (lane 1; 2ng type I, II or III $InsP_3$ receptor immunoprecipitated from SH-SY5Y, AR4-2J or RINm5F cells) were then subjected to electrophoresis, were transferred to nitrocellulose and were probed with CT1, CT2 or CT3 at dilutions that produced the same band intensity from each standard. Thus, HL-60 cell immunoreactivity reflects $InsP_3$ receptor concentration.

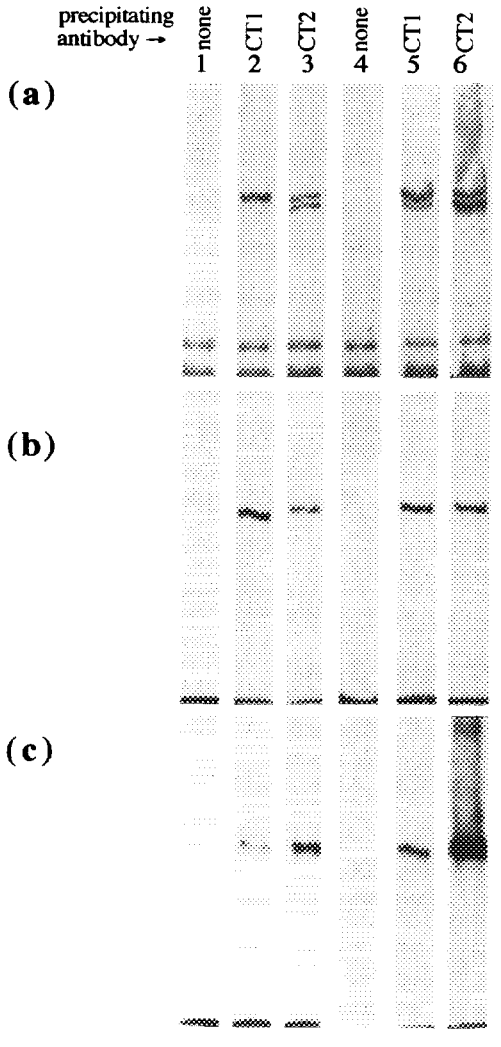

Figure 3. Effects of changing $InsP_3$ receptor relative abundance on co-immunoprecipitation.

Extracts of control HL-60 cells (lanes 1-3) or of cells pretreated with 2μM RA for 4d (lanes 4-6) were incubated without antibody or with CT1 or CT2 as indicated. Immunoprecipitated proteins were then electrophoresed and either (a) silver-stained or transferred to nitrocellulose and probed with (b) CT1 or (c) CT2. The positions to which M_r markers and type I and II receptors migrated are indicated.

type II receptor associates with type I receptor. Similarly, the amount of type I receptor immunoprecipitated by CT2 was increased markedly by RA (Fig 3a and b, compare lane 3 with 6). Thus, the dramatic induction of type II receptor by RA increases the abundance of heteromers.

In total, the data presented show that type I, II and III receptors can exist as heteromers, implying that the $InsP_3$-sensitive Ca^{2+} channels of cells in which $InsP_3$

receptors are co-expressed are heterotetrameric. Support for this view comes from a study in which mutant $InsP_3$ receptors were expressed in NIH 3T3 cells [23]. At present, the functional and regulatory features of such channels are completely unknown. However, as $InsP_3$ binds with slightly different affinities to type I, II and III receptors [5,16] it can be predicted that channels with a range of sensitivities to $InsP_3$ would be formed in cells co-expressing multiple receptors. Such a range may explain certain poorly understood aspects of Ca^{2+} store mobilization; for example, the smoothly graded Ca^{2+} mobilization seen in response to increasing hormone concentration [15,24].

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