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Differences Among Type I, II, and III Inositol-1,4,5-Trisphosphate Receptors in Ligand-Binding Affinity Influence the Sensitivity of Calcium Stores to Inositol-1,4,5-Trisphosphate

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ABSTRACT

Type I, II, and III inositol-1,4,5-trisphosphate ($InsP_3$) receptors are expressed selectively in different cell lines and tissues. We examined whether type I, II, and III $InsP_3$ receptors differ in ligand-binding affinity and whether such differences influence the sensitivity of Ca^{2+} stores to $InsP_3$. Initially, SH-SY5Y human neuroblastoma cells, AR4–2J rat pancreatoma cells, and RINm5F rat insulinoma cells were studied because these cells express predominantly (>85%) type I, II, and III receptors, respectively. Immunopurification of receptors from these cell lines and measurement of $InsP_3$ binding revealed that the rank order of affinity for $InsP_3$ was type I > type II > type III (binding sites were half-maximally saturated at 1.5, 2.5, and 22.4 nM $InsP_3$, respectively). Examination of Ca^{2+} store mobilization in permeabilized cells showed that $InsP_3$ was equipotent in SH-SY5Y and AR4–2J cells but was ~5-fold less potent in RINm5F cells. In contrast, Ca^{2+} uptake and $InsP_3$-independent Ca^{2+} release were very similar in the three cell types. The binding affinity of $InsP_3$ in permeabilized SH-SY5Y, AR4–2J, and RINm5F cells correlated well with its potency as a Ca^{2+}-mobilizing agent and with binding affinity to immunopurified type I, II, and III receptors. Thus, $InsP_3$ receptor binding affinity seems to influence the potency of $InsP_3$ as a Ca^{2+}-mobilizing agent. Finally, immunopurification of type I, II, and III receptors from rat tissues revealed that the affinity differences seen in receptors purified from cultured cells are paralleled *in vivo*. In combination, the data from cell lines and rat tissues reveal that type I, II, and III receptors bind $InsP_3$ with K_d values of ~1, ~2, and ~40 nM, respectively, and that the selective expression of a particular receptor type will influence the sensitivity of cellular Ca^{2+} stores to $InsP_3$.

$InsP_3$ receptors play a crucial role in intracellular signaling as they tetramerize to form channels in endoplasmic reticulum membranes that conduct Ca^{2+} in an $InsP_3$-sensitive manner (Furuichi and Mikoshiba, 1995; Joseph, 1996). To date, the coding regions of three mammalian $InsP_3$ receptor genes have been sequenced, and in rat, their products, termed type I, II, and III receptors, are 2749, 2701, and 2670 amino acids in length, respectively (Mignery *et al*., 1990; Sudhof *et al*., 1991; Blondel *et al*., 1993; Furuichi and Mikoshiba, 1995; Joseph, 1996). Highly homologous type I, II, and III receptors also are present in other mammals (Yamada *et al*., 1994; Yamamoto-Hino *et al*., 1994).

Analyses of $InsP_3$ receptor distribution have shown that the type I receptor is expressed in many if not all tissues and in particular is in abundance in neural tissue (Furuichi and Mikoshiba, 1995; Joseph, 1996). In contrast, type II and III receptors generally are less widespread (Joseph, 1996), but they are prominent in certain tissues; for example, the type II receptor is abundant in liver (Newton *et al*., 1994; Joseph *et al*., 1995; Wojcikiewicz, 1995; De Smedt *et al*., 1997), and the type III receptor is present at high levels in pancreatic tissue (Blondel *et al*., 1993, 1994; Nathanson *et al*., 1994; Wojcikiewicz, 1995). Indeed, in certain cell types, type II or III receptors may be the predominant form, as has been shown with cells in culture (Wojcikiewicz, 1995; De Smedt *et al*., 1997). It also has become evident that type I, II, and III receptors are coexpressed within cells, as demonstrated at the level of mRNA species (Newton *et al*., 1994; Yamamoto-Hino *et al*., 1994; De Smedt *et al*., 1997) and protein (Joseph *et al*., 1995; Monkawa *et al*., 1995; Wojcikiewicz, 1995; Yule *et al*., 1997) and are free to form heterotetrameric associations (Joseph *et al*., 1995; Monkawa *et al*., 1995; Wojcikiewicz and He, 1995; Nucifora *et al*., 1996).

Characterization of the differences among type I, II, and III

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ABBREVIATIONS: $InsP_3$, inositol-1,4,5-trisphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

receptors is at a preliminary stage, and it is not yet clear what properties are conferred on a cell by the selective expression of a particular receptor type (Joseph, 1996). Perhaps the most obvious way in which $InsP_3$ receptors might differ is in their affinity for $InsP_3$. As yet, however, the affinities of native type I, II, and III receptors have not been compared directly. Nor is it known how differences among type I, II, and III receptors in binding affinity would influence the sensitivity of cellular Ca^{2+} stores to $InsP_3$.

To address these issues, we measured the binding affinity of $InsP_3$ to immunopurified type I, II, and III receptors and assessed the relationship between binding affinity and the Ca^{2+}-mobilizing potency of $InsP_3$ in cultured cells that express predominantly type I, II, or III receptors. Furthermore, we examined $InsP_3$ binding to receptors immunopurified from rat tissues to determine whether our findings *in vitro* reflect the situation *in vivo*.

Experimental Procedures

Cell culture and antisera. SH-SY5Y human neuroblastoma cells, AR4–2J rat pancreatoma cells, and RINm5F rat insulinoma cells were obtained and cultured as monolayers in dishes (15 cm in diameter) as described previously (Wojcikiewicz, 1995). Rabbit polyclonal antisera termed CT1, CT2, and CT3 were raised against the carboxyl termini of rat type I, II, and III $InsP_3$ receptors, respectively, and were affinity purified and shown to be specific as described previously (Wojcikiewicz, 1995; Wojcikiewicz *et al.*, 1994; Wojcikiewicz and He, 1995).

Purification of type I, II, and III receptors from cell lines. After removal of culture medium, cell monolayers were rinsed once with HBSE (155 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4) and placed on ice, and 3–6 ml of ice-cold lysis buffer (50 mM Tris-base, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μM leupeptin, 10 μM pepstatin, 0.2 μM soybean trypsin inhibitor, pH 8.0) was added to each dish. The cells were incubated for 30 min on ice and centrifuged (38,000 $\times$ *g* for 20 min at 4°). In most experiments, supernatants then were incubated at 4° with either CT1, CT2, or CT3 for 1 hr and then for an additional hour with Protein A/Sepharose CL-4B beads (10 mg/dish of cells). Immune complexes were isolated by centrifugation (500 $\times$ *g* for 2 min) and washed twice with lysis buffer. In "preclearing" experiments, RINm5F cell lysates first were incubated overnight with CT1 and Protein A beads to remove type I receptor (or vehicle and Protein A beads as a control) and then incubated with CT3 and Protein A beads to immunoprecipitate type III receptor. Finally, the washed beads were resuspended in 20 mM Tris-base and 1 mM EDTA, pH 8.0 (~0.25 ml/dish of cells).

Purification of type I, II, and III receptors from rat tissues. Cerebral cortex (~1.4 g), cerebellum (~0.25 g), liver (~3 g), and pancreas (1.4 g) from a 250-g male Sprague-Dawley rat was rinsed in phosphate-buffered saline and was then added to 40 ml of ice-cold 10 mM Tris-base, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μM leupeptin, 10 μM pepstatin, and 0.2 μM soybean trypsin inhibitor, pH 7.4. The tissues were then disrupted with 10–20 strokes of a glass-Teflon homogenizer. Homogenates were centrifuged (27,000 $\times$ *g* for 10 min at 4°), and pellets were resuspended in 12 ml of ice-cold lysis buffer. After 30 min on ice, the extract was centrifuged (27,000 $\times$ *g* for 15 min at 4°), and either CT1 (cortex and cerebellum), CT2 (liver), or CT3 (pancreas) was added to the supernatant. After 1 hr at 4°, 40 mg Protein A beads was added; 1 hr later, immune complexes were isolated by centrifugation (500 $\times$ *g* for 2 min) and two washes with lysis buffer. Finally, the washed beads were resuspended in ~1.6 ml of 20 mM Tris-base and 1 mM EDTA, pH 8.0. Samples of washed beads were electrophoresed, and gels were either silver-stained or immunoblotted as described previously (Wojcikiewicz, 1995; Wojcikiewicz and He, 1995).

$InsP_3$ binding to immunoprecipitated receptors. A previous study has shown that the Protein A bead/antibody/$InsP_3$ receptor complex remains intact during radioligand binding studies (Joseph and Samanta, 1993). Thus, washed beads (100 μl) were incubated at 4° for 30 min with [^{3}H]$InsP_3$ (specific activity, 2–5 Ci/mmol) in 35 mM Tris-base and 1.5 mM EDTA, pH 8.0 (final volume, 200 μl). Bound ligand was isolated by vacuum filtration; incubation mixtures were pipetted onto prewetted Whatman (Clifton, NJ) GF/B filters and washed twice with 4 ml of ice-cold 20 mM Tris-base and 1 mM EDTA, pH 8.0. Filters were added to vials with 0.5 ml of water and 5 ml of Ecoscint H (National Diagnostics, Atlanta, GA) and assessed for radioactivity after 48-hr extraction. Nonspecific binding was defined by including ≥10 μM nonradioactive $InsP_3$ in parallel incubations. Specific binding was analyzed with Prism (GraphPAD Software, San Diego, CA), initially fitting data to sigmoid curves of variable slope to determine concentrations that gave half-maximal saturation. The B_{max} values (the maximum number of binding sites) obtained from these analyses were then used to normalize data for presentation in Figs. 2, 3, and 7. Values of K_d (the equilibrium dissociation constant) for type I and II receptor preparations then were determined by fitting data to one-site saturation binding curves; for type III receptor preparations, specific binding was fitted to two-site saturation binding curves to define the apparent multiple sites.

$^{45}Ca^{2+}$ mobilization and $InsP_3$ binding in permeabilized cells. SH-SY5Y cells (two dishes) and AR4–2J and RINm5F cells (one dish each) were harvested in 12 ml of HBSE, centrifuged (1000 $\times$ *g* for 2 min), resuspended in 10 ml of ice-cold cytosol buffer (120 mM KCl, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 10 μM EGTA, 2 mM ATP, 20 mM HEPES, pH 7.0), centrifuged again (2000 $\times$ *g* for 2 min at 4°), and resuspended in 2 ml of cytosol buffer. Protein concentration in each cell suspension was then equalized to ~2.0 mg/ml; cells were incubated with digitonin (100 μg/ml) for 10 min at 4° and then centrifuged (2000 $\times$ *g* for 2 min at 4°). For $^{45}Ca^{2+}$-mobilization studies, pellets were resuspended in 1.4 ml of cytosol buffer and finally incubated with ~0.5 μCi of $^{45}Ca^{2+}$ for 20 min at room temperature. Portions of cell suspensions (100 μl) then were added to tubes containing 3 ml of ice-cold cytosol buffer plus stimuli. After 10–300 sec, incubations were terminated by filtration through Whatman GF/B filters, and radioactivity bound to the filters (amount of Ca^{2+} not mobilized) was assessed after the addition of 4 ml of Ecoscint H and overnight extraction. For binding studies, pellets were washed twice with 2 ml of cytosol buffer, and cell suspensions were incubated with [^{3}H]$InsP_3$ (specific activity, ~1 Ci/mmol) in a final volume of 100 μl of cytosol buffer for 15 min at 4°. The mixture then was transferred to filters and washed with 10 ml of ice-cold cytosol buffer. [^{3}H]$InsP_3$ binding was assessed and computed as described for immunoprecipitated receptors.

Miscellany. Peroxidase-conjugated antibodies, molecular mass markers, dithiothreitol, protease inhibitors, ATP, $InsP_3$, and digitonin were obtained from Sigma Chemical (St. Louis, MO). Protein A/Sepharose CL-4B was from Pharmacia (Piscataway, NJ). [^{3}H]$InsP_3$ (21 Ci/mmol) and $^{45}CaCl_2$ (~800 Ci/mol) were from New England Nuclear Research Products (Boston, MA). Deviations from mean values are given as mean ± standard error. Statistical significance was determined by unpaired Student's *t* test.

Results

Binding to $InsP_3$ receptors immunoprecipitated from cell lines. Previous analysis of a range of cultured cell lines established the $InsP_3$ receptor content of SH-SY5Y cells to be ≥99% type I; that of AR4–2J cells to be 12% type I, 86% type II, and 2% type III; and that of RINm5F cells to be 4% type I and 96% type III (Wojcikiewicz, 1995). Thus, for the current study, SH-SY5Y, AR4–2J, and RINm5F cells were

used as sources of type I, II, and III receptors, respectively. Immunopurification of receptors from these cell lines with the specific antisera CT1, CT2, and CT3 (Fig. 1) shows that the receptors migrate at slightly different rates and that a protein of the same size as the SH-SY5Y cell type I receptor (*lane 1*) coimmunoprecipitates with type II receptor from AR4–2J cells (*lane 2*) and type III receptor from RINm5F cells (*lane 3*). Previous immunoblotting studies have defined this as type I receptor (Wojcikiewicz, 1995) and indicate that some of the $InsP_3$ receptor complexes purified from AR4–2J and RINm5F cells are heterotetrameric. It is estimated that the type II and III receptor preparations contain ~10% and ~5% type I receptor, respectively (Wojcikiewicz, 1995; Fig. 1).

The characteristics of $InsP_3$ binding to the type I, II, and III receptor preparations were defined (Fig. 2). Initial analysis (Fig. 2A) showed that half-maximal saturation of binding sites occurred at 1.5, 2.5, and 22.4 nM $InsP_3$, respectively

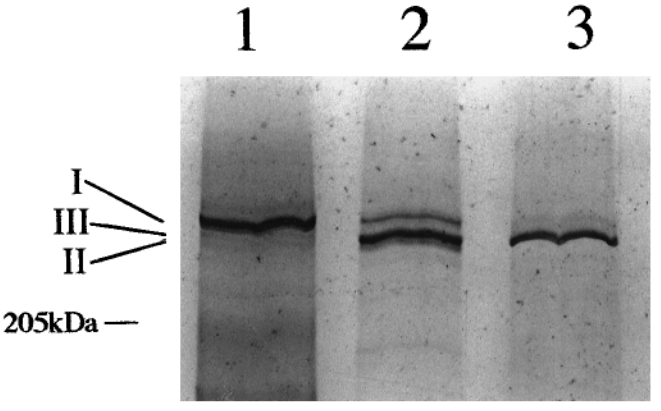

Fig. 1. Composition of $InsP_3$ receptors immunoprecipitated from cell lines. $InsP_3$ receptors were immunoprecipitated from SH-SY5Y (*lane 1*), AR4–2J (*lane 2*), and RINm5F (*lane 3*) cell lysates with CT1, CT2, and CT3, respectively, and were electrophoresed in 4% gels and silver stained. Shown is the ~150–400-kDa region of a gel (representative of five independent experiments). The positions to which myosin mass marker (205 kDa) and type I, II, and III receptors migrate, ~270, ~255, and ~260 kDa, respectively (Wojcikiewicz, 1995), are indicated.

(the values for type II and III receptors being significantly greater than that for type I receptor, $p \leq 0.01$) and that each receptor type bound approximately equivalent amounts of $InsP_3$ when saturated (see Fig. 2 legend). Although Hill slopes were ~1, indicating that a single affinity predominated in each preparation, that for the type III receptor preparation (0.86 ± 0.03) was significantly <1 ($p \leq 0.01$), indicating that multiple sites might be present (see Fig. 2 legend). Scatchard analysis (Fig. 2B) confirmed that sites with different affinities were present in the type III receptor preparation. Although it is clear from Fig. 1 that the type II receptor preparation also contains type I receptor, this is not reflected in the Hill slope being <1 or in curvilinear Scatchard plots, presumably because the affinities of type I and II receptors are so similar. Thus, one-site analysis was used to define K_d values for type I and II receptor preparations and two-site analysis was used for the type III receptor preparation. K_d values obtained were 1.4 ± 0.2 nM for type I receptor, 2.4 ± 0.2 nM for type II receptor, and 1.1 ± 0.5 and 47.8 ± 7.1 nM for the type III receptor preparation, with the latter site representing 87 ± 2% of total (at least four independent experiments). Both the type II receptor and the predominating low affinity site in the type III receptor preparation (presumably the type III receptor) were of lower affinity than the type I receptor ($p \leq 0.01$). Thus, type II and III receptors seem to bind $InsP_3$ with ~2-fold and ~30-fold lower affinity than the type I receptor, respectively.

Binding of $InsP_3$ to type III receptor homotetramers. To establish that binding to the type III receptor preparation was not due to some kind of interaction with the trace amounts of type I receptor present therein (Fig. 1, *lane 3*), we sought to purify type III receptor homotetramers by preclearing RINm5F cell lysates of type I/III receptor heterotetramers before immunoprecipitation with CT3. Such an approach was used by Nucifora *et al.* (1996). Immunoblots of a control type III receptor preparation (Fig. 3A, *lane 1*) confirm that both type III and I receptors are present therein. In contrast, when lysates were precleared with CT1, only type III receptor was recovered (Fig. 3A, *lane 2*), indicating that type I/III

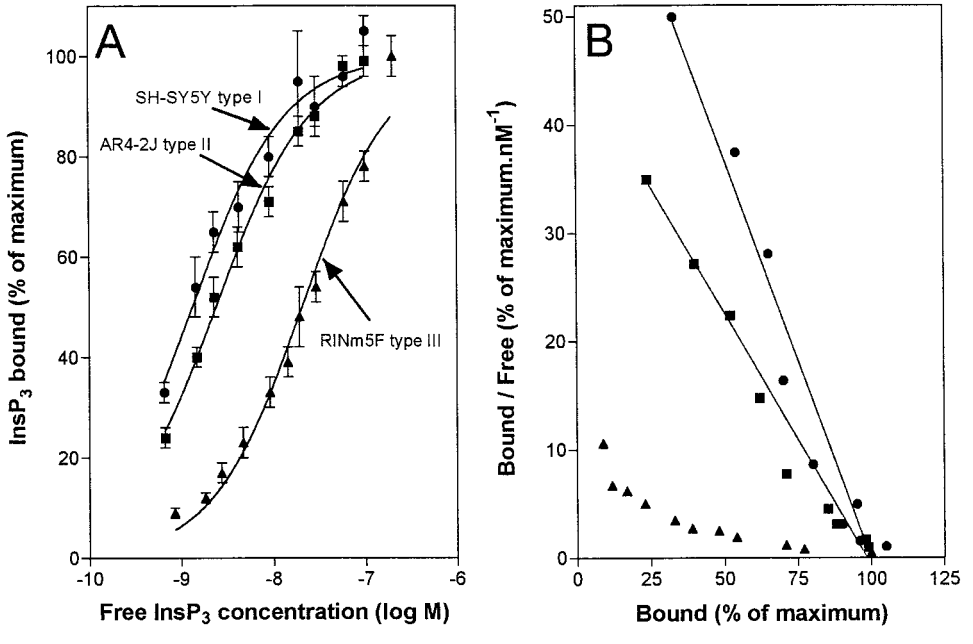

Fig. 2. Binding of $InsP_3$ to receptors immunoprecipitated from cell lines. Approximately equivalent amounts of type I (●), type II (■), and type III (▲) $InsP_3$ receptor preparations were incubated with 1–200 nM [^{3}H]$InsP_3$ for 30 min at 4°. A, Specific binding to the receptor preparations fitted to sigmoid curves of variable slope. Data shown are mean ± standard error of at least four independent determinations in which half-maximal saturation occurred at 1.5 ± 0.2, 2.5 ± 0.2, and 22.4 ± 2.7 nM, respectively, and Hill slopes were 0.94 ± 0.15, 0.88 ± 0.07, and 0.86 ± 0.03, respectively. B_{max} values were 800 ± 200, 1300 ± 400, and 1300 ± 300 cpm, respectively. B, Scatchard plots of the specific binding data (mean from at least four independent experiments) with lines of best fit for the type I and II receptor preparations. The type III receptor data were not related linearly, indicating that multiple binding sites are present.

receptor heterotetramers were removed and that this preparation consists exclusively of type III receptor homotetramers. Fig. 3B shows that binding to control or type I receptor precleared (homotetrameric) type III receptor preparations was similar but subtly different (half-maximally saturation at ~34 and ~47 nM $InsP_3$, respectively, and Hill slopes of ~0.81 and ~0.97, respectively). These data and the fact that preclearing reduced maximal binding by only ~10% are consistent with removal of trace amounts of a high affinity binding site (the type I receptor) from the type III receptor preparation. Thus, it is clear that RINm5F cell type III receptors do bind $InsP_3$ and that binding to type I/III receptor heterotetramers does not compromise the conclusions from Fig. 1 regarding type III receptor binding affinity. Finally, it is noteworthy that half-maximal saturation of homotetrameric type III receptors (~47 nM, Fig. 3B) is identical to the K_d value of the low affinity binding site in heterotetramer-containing type III receptor preparations (~48 nM, Fig. 2).

$InsP_3$ binding and Ca^{2+} mobilization in permeabilized SH-SY5Y, AR4–2J, and RINm5F cells. We next sought to determine whether the type I, II, and III receptor affinity differences also were seen with membrane-associated receptors and, if so, whether they influenced the sensitivity of Ca^{2+} stores to $InsP_3$. Thus, we measured $InsP_3$ binding and Ca^{2+} mobilization in digitonin-permeabilized SH-SY5Y, AR4–2J, and RINm5F cells under identical conditions. Fig. 4 shows that $InsP_3$ binding affinity differs in the three cell types (half-maximal saturation at 25, 31, and 260 nM, respectively) and that although absolute values of affinity were ~1 order of magnitude higher than that seen with immunoprecipitated type I, II, and III receptors (most likely because of differences in buffer composition used in Figs. 4 and 2), the relative differences between the cell types parallel those among type I, II, and III receptors. This indicates that immunoprecipitated receptors remain in native form despite being solubilized and bound by antibody and validates the conclusions from Fig. 2.

The potency of $InsP_3$ as a Ca^{2+}-mobilizing agent also varied considerably, whereas maximal Ca^{2+} release was similar for the three cell types (Fig. 5A). For SH-SY5Y, AR4–2J, and RINm5F cells, EC_{50} values were 22 ± 2, 20 ± 2, and 94 ± 6 nM $InsP_3$, respectively, and Hill slopes were 1.9 ± 0.1, 1.8 ± 0.1, and 2.0 ± 0.1, respectively (eight independent experiments; Fig. 5A). Thus, because $InsP_3$ has similar Ca^{2+}-releasing potency in SH-SY5Y and AR4–2J cells but is much less potent in RINm5F cells, EC_{50} values and binding affinity do correlate (compare Figs. 5A and 4).

To validate the argument that binding affinity influences potency, other factors that could cause the differences seen in Fig. 5A were examined and discounted. First, variation in Ca^{2+} release kinetics could influence EC_{50}; however, the rates of $InsP_3$-induced Ca^{2+} efflux were essentially identical for each cell type (Fig. 5B). Second, the extent to which Ca^{2+} stores are loaded can influence EC_{50} (Parys *et al.*, 1993); however, the extent of Ca^{2+} uptake was very similar for each cell type (Fig. 5, legend). Third, as-yet-undefined factors might suppress Ca^{2+} release from RINm5F cells; however,

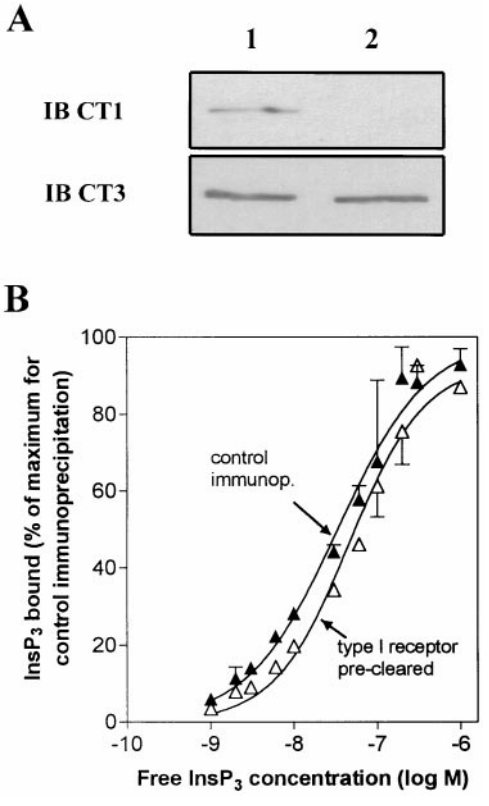

Fig. 3. Binding of $InsP_3$ to type III receptor homotetramers. $InsP_3$ receptors were immunoprecipitated with CT3 from RINm5F cell lysates that had been pretreated with either vehicle (*control immunop.*) or CT1 (*type I receptor pre-cleared*). The immunoprecipitates then were either electrophoresed in 4% gels and immunoblotted or incubated with 1–1000 nM [^{3}H]$InsP_3$ for 30 min at 4°. A, Immunoblots with CT1 (*IB CT1*) or CT3 (*IB CT3*) of control (*lane 1*) or type I receptor precleared (*lane 2*) type III receptor preparations (representative of two independent determinations). B, Specific binding to control (▲) and type I receptor precleared (△) type III receptor preparations fitted to sigmoid curves of variable slope. Data shown are mean ± range of two independent determinations in which half-maximal saturation occurred at 34 ± 6 and 47 ± 1 nM, respectively, and Hill slopes were 0.81 ± 0.07 and 0.97 ± 0.05, respectively. Maximum binding to the type I receptor precleared preparation was 93 ± 1% of that to the control preparation.

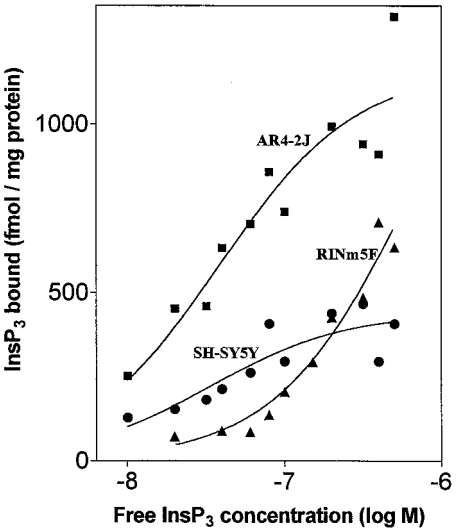

Fig. 4. Binding of $InsP_3$ to permeabilized cell lines. Digitonin-permeabilized SH-SY5Y (●), AR4–2J (■), and RINm5F (▲) cells were incubated with 10–500 nM [^{3}H]$InsP_3$ for 15 min at 4°. Data shown are specific binding from one experiment representative of three independent experiments with similar results in which half-maximal saturation occurred at 25 ± 3, 31 ± 5,and 260 ± 30 nM, respectively, and B_{max} values were 570 ± 140, 1440 ± 70, and 960 ± 40 fmol/mg of protein, respectively (mean ± standard error; three independent experiments).

ionomycin, which acts independently of $InsP_3$ receptors, released Ca^{2+} to a similar extent (Fig. 5A) and with similar kinetics (Fig. 5B) in SH-SY5Y, AR4–2J, and RINm5F cells. Furthermore, the characteristics of thapsigargin-induced $^{45}Ca^{2+}$ release in 5-min incubations at 25° were very similar for the three cell types (EC_{50} = 0.7 ± 0.1, 0.6 ± 0.1, and 0.9 ± 0.1 μM, respectively; five independent experiments). Finally, RINm5F cells could be resistant to or adversely affected by digitonin. However, data essentially identical to those shown in Fig. 5A were obtained when cells were permeabilized electrically (R. J. H. Wojcikiewicz, and S. G. Luo, unpublished data).

Taken together, these data indicate that the $InsP_3$-independent Ca^{2+}-handling properties of SH-SY5Y, AR4–2J, and RINm5F cells are very similar and thus the relatively high EC_{50} value of $InsP_3$ in permeabilized RINm5F cells is a reflection of the predominance of low affinity type III receptors in this cell type.

Binding to $InsP_3$ receptors immunoprecipitated from rat tissues. To confirm that the differences in binding affinity seen in cultured cells are representative of the situation *in vivo*, we immunopurified $InsP_3$ receptors from rat tissues, using cerebellum and cerebral cortex as sources of type I receptor, liver as a source of type II receptor, and pancreas as a source of type III receptor. The type I receptor preparations were essentially homogeneous with no "contamination" from the faster migrating type II or III receptors (Fig. 6, *lanes 1 and 2*). In contrast, it is estimated from the silver-stained and immunoblotted gels that the type II receptor preparation contains ~25% type I receptor (*lane 3*) and that the type III receptor preparation contains ~20% type II receptor and ~5% type I receptor (*lane 4*) [the migration position of the pancreatic type III receptor in the silver-stained gel (Fig. 6, *lane 4*) is anomalously low due to distortion of the gel during photography; the body of data from these experiments showed that migration of the pancreatic type III receptor was between that of type I and type II receptors]. Such coimmunoprecipitation indicates that heterotetrameric $InsP_3$ receptors are expressed in liver and pancreas.

Initial analysis (Fig. 7A) showed that $InsP_3$ binding to the preparations from cortex, cerebellum, liver, and pancreas was half-maximal at 0.7, 1.2, 1.7, and 20.9 nM, respectively, with the values for liver and pancreas preparations being significantly greater ($p \leq 0.01$) than that for cortex (see Fig. 7 legend). Furthermore, although the curves for cortex and cerebellum had Hill slopes of ~1, those for liver (0.78 ± 0.05) and pancreas (0.57 ± 0.08) were significantly <1 ($p \leq 0.01$), indicating that multiple binding sites were present in these preparations (see Fig. 7 legend). This also is illustrated by Scatchard analysis; the data points for liver and particularly pancreas are not related linearly (Fig. 7B). Thus, one-site analysis was used to define K_d values for binding to cortex and cerebellum preparations and two-site analysis was used to define the sites present in liver and pancreas preparations. However, two-site analysis of binding to the liver preparation did not yield consistent data, presumably because of the closeness of the affinities of type I and II receptors; thus, the liver preparation K_d value was defined by one-site analysis. The K_d values obtained were 0.7 ± 0.1 nM for cortex, 1.2 ± 0.2 nM for cerebellum, 1.8 ± 0.2 nM for liver (see preceding explanation), and 0.5 ± 0.1 and 43 ± 1 nM for pancreas, with the latter site representing 75 ± 5% of the total (at least four independent experiments).

Discussion

The major findings presented herein are that type I, II, and III $InsP_3$ receptors bind $InsP_3$ with different affinities and that these differences seem to influence the potency of $InsP_3$ as a Ca^{2+}-mobilizing agent. This provides an indication of how selective expression of a particular $InsP_3$ receptor type

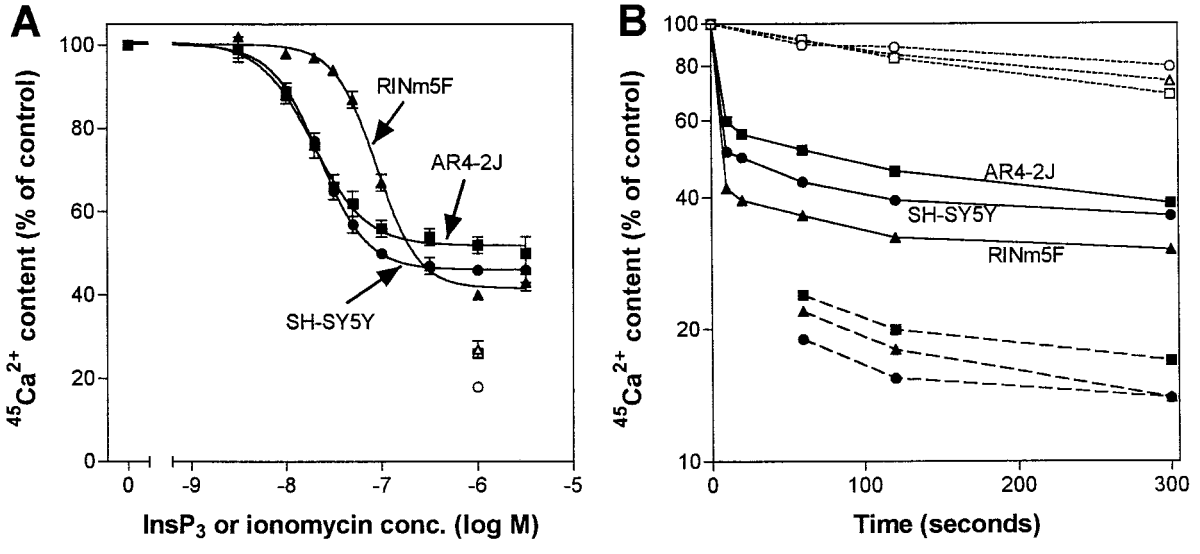

Fig. 5. Ca^{2+} mobilization from SH-SY5Y, AR4–2J. and RINm5F cells. Digitonin-permeabilized $^{45}Ca^{2+}$-loaded SH-SY5Y (○, ●), AR4–2J (□, ■), and RINm5F (△, ▲) cells were added to tubes containing $InsP_3$ or ionomycin, and the amount of $^{45}Ca^{2+}$ remaining sequestered after incubations ($^{45}Ca^{2+}$ content) was used as an index of Ca^{2+} mobilization. A, Ca^{2+} mobilization after 120-sec incubation with 0.003–3 μM $InsP_3$ (*solid symbols*) or 1 μM ionomycin (*open symbols*) (mean ± standard error from at least three independent experiments). Ca^{2+} content was calculated as a percentage of that remaining sequestered after incubation for 120 sec in the absence of $InsP_3$ (33 ± 1, 31 ± 2 and 33 ± 3 × 10^3 cpm, respectively). B, Time course of Ca^{2+} mobilization during incubation without stimulus (*open symbols*, *dotted line*), with $InsP_3$ (*closed symbols*, *solid line*; 0.3 μM for SH-SY5Y and AR4–2J cells and 1 μM for RINm5F cells), or with 1 μM ionomycin (*closed symbols*, *broken line*) (mean from at least two independent experiments).

will influence cell signaling. Importantly, this is the first study in which type I, II, and III receptors have been analyzed under identical experimental conditions and thus provides an accurate assessment of their differences.

Our findings from cell lines and rat tissues that type I, II, and III receptors bind $InsP_3$ with K_d values of ~1, ~2, and ~40 nM, respectively, are broadly in agreement with previous studies, the most significant of which also concluded that the type III receptor has relatively low affinity at physiological or subphysiological Ca^{2+} concentrations (Newton *et al*., 1994; Yoneshima *et al*., 1997). In some of these previous studies (Sudhof *et al*., 1991; Newton *et al*., 1994), $InsP_3$ interaction with the ligand binding domains of type I, II, and III receptors was assessed, either the soluble ligand-binding domains of type I and II receptors (yielding K_d values of 90 and 27 nM, respectively) or the ligand-binding domains of type I and III receptors fused to glutathione-*S*-transferase (yielding K_d values of 6 and 67 nM, respectively). These results led to the conclusion that the rank order of affinity was type II > type I > type III (Sudhof *et al*., 1991; Newton *et al*., 1994). Although we agree that the type III receptor has by far the lowest affinity, our conclusion is that the affinity order is type I > type II > type III. The reason for the discrepancy may be that we measured binding to whole $InsP_3$ receptors in tetrameric association rather than to monomeric receptor fragments; monomeric receptor fragments may bind $InsP_3$ differently to native receptors.

Is there a basis for the type III receptor being so different from types I and II receptors ? Over their entire sequences, type I and II receptors are 69% identical and type I and III receptors are 62% identical; in the ligand-binding domain (the amino-terminal 576 amino acids), the similarity is 77% and 73%, respectively (Mignery *et al*., 1990; Sudhof *et al*., 1991; Blondel *et al*., 1993; Newton *et al*., 1994; Joseph, 1996). Thus, gross sequence differences do not explain the variation in K_d value. Furthermore, all of the 10 basic residues within the ligand-binding domain of the type I receptor that seem to be involved in $InsP_3$ binding are conserved in type II and III receptors (Yoshikawa *et al*., 1996). Thus, at the present, the low affinity of the type III receptor cannot be explained.

We also conclude that the affinity differences among type I, II, and III receptors contribute to setting the EC_{50} value of $InsP_3$ as a Ca^{2+}-mobilizing agent. Thus, the difference in K_d value between type I and III receptors (~40-fold) dictates that the $InsP_3$ receptors in permeabilized SH-SY5Y and RINm5F cells are half-maximally saturated at 25 and 260 nM, respectively (a difference of ~10-fold), and thus that the potency of $InsP_3$ will be greater in SH-SY5Y cells than in RINm5F cells (EC_{50} values differ by ~5-fold). A similar difference has been measured between A7r5 and $C_3H10T_{1/2}$ cells, which express predominantly type I and type III receptors, respectively (De Smedt *et al*., 1997). However, our data also show that the relationship between half-maximal saturation and EC_{50} value is not directly proportional. Indeed, although the binding affinity of type I receptor preparations and SH-SY5Y cells was slightly higher than that of type II receptor preparations and AR4–2J cells, the EC_{50} values for $InsP_3$ in SH-SY5Y and AR4–2J cells were identical. Thus, a factor or factors other than binding affinity also must influence EC_{50}; this does not seem to be variation among the cells in Ca^{2+} store characteristics because the $InsP_3$-independent Ca^{2+}-handling properties of the three cell types essentially were identical. Rather, $InsP_3$ receptor density may be the factor because it is known from transfection studies that increasing the $InsP_3$ receptor density increases the potency of $InsP_3$ (Miyawaki *et al*., 1990). Because AR4–2J and RINm5F cells have two to three times as many $InsP_3$-binding sites as SH-SY5Y cells, a finding that agrees with previous immunochemical studies (Wojcikiewicz, 1995), one would expect EC_{50} values in AR4–2J and RINm5F cells to be closer to the SH-SY5Y cell value than that predicted from binding affinity differences alone.

The overall significance of these findings is that the Ca^{2+} stores of cells that express predominantly type II or type III receptors will be less sensitive to cell surface receptor-generated $InsP_3$ than would cells expressing predominantly type I receptors. For example, hepatocytes seem to express predominantly the type II receptor (Joseph *et al*., 1995; Wojcikiewicz, 1995; De Smedt *et al*., 1997) and thus would be expected to have slightly lower sensitivity to $InsP_3$ than would cells (e.g., neuronal cells) that express predominantly the type I receptor (Furuichi and Mikoshiba, 1995; Joseph, 1996). More significant reductions in sensitivity to $InsP_3$ are to be expected in cells that express the type III receptor predominantly, such as pancreatic islet β cells (Blondel *et al*., 1993, 1994). Remembering that variation in receptor density may compensate for or exacerbate these differences, it will be fascinating to test this hypothesis directly by comparing Ca^{2+} release from these cell types under identical experimental conditions.

Finally, given that the sequences of type I, II, and III

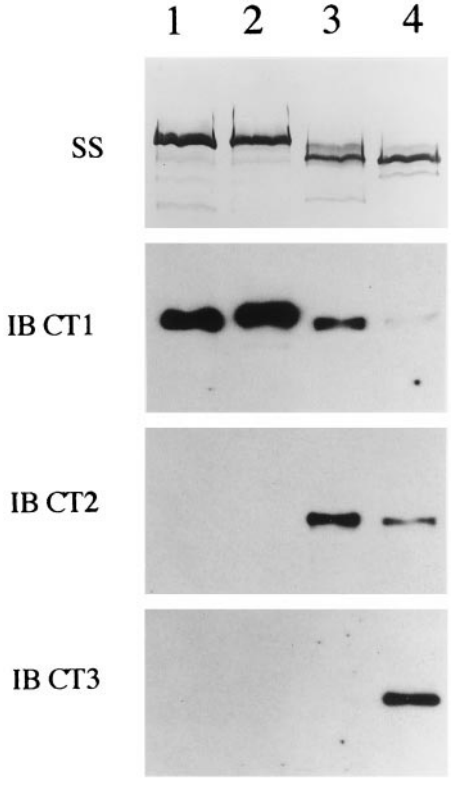

Fig. 6. Composition of $InsP_3$ receptors immunoprecipitated from rat tissues. $InsP_3$ receptors were immunoprecipitated from cerebellum (*lane 1*) and cerebral cortex (*lane 2*) with CT1, from liver with CT2 (*lane 3*), and from pancreas with CT3 (*lane 4*) and were electrophoresed in 4% gels and either silver-stained (*SS*) or immunoblotted (*IB*) with CT1, CT2, or CT3. Data are representative of four independent experiments.

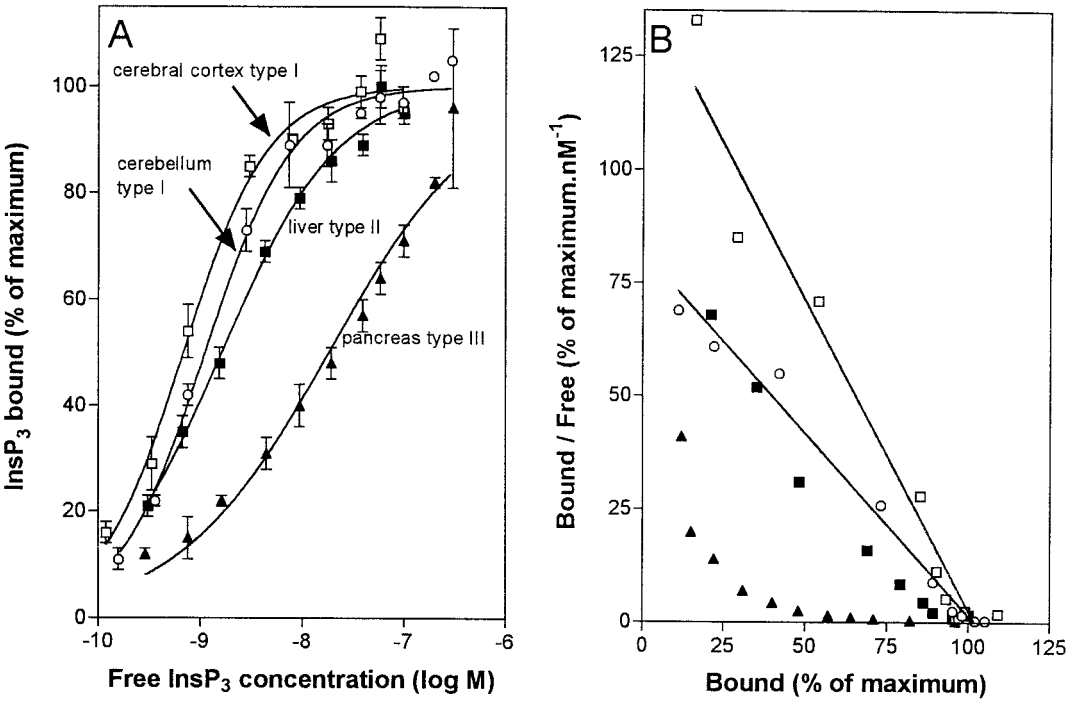

Fig. 7. Binding of $InsP_3$ to receptors immunoprecipitated from rat tissues. Type I receptors purified from cerebral cortex (□) or cerebellum (○), type II receptors purified from liver (■), and type III receptors purified from pancreas (▲) were incubated with 1–300 nM [^{3}H]$InsP_3$ for 30 min at 4°. A, Specific binding to the receptor preparations fitted with sigmoid curves of variable slope. Data are mean ± standard error of four independent determinations in which half-maximal saturation occurred at 0.7 ± 0.1, 1.2 ± 0.2, 1.7 ± 0.2, and 20.9 ± 3.6 nM, respectively, and Hill slopes were 1.10 ± 0.16, 1.13 ± 0.05, 0.78 ± 0.05 and 0.57 ± 0.08, respectively. B, Scatchard plots of the specific binding data (mean from four independent experiments), with lines of best fit for cortex and cerebellum type I $InsP_3$ receptor preparations.

receptors are divergent throughout their entire lengths, it is to be expected that they will differ in ways other than in ligand binding affinity, such as in their ability to be phosphorylated. Our future studies are aimed at defining these differences.

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