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-SYSY 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-SYSY 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-SYSY, 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 (Miglery 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 present 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).

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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 pancreataoma 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 mM leupeptin, 10 mM pepstatin, 0.2 mM soybean trypsin inhibitor, pH 8.0) was added to each dish. The cells were incubated for 30 min on ice and centrifuged (38,000 × g for 20 min at 4°C). In most experiments, supernatants then were incubated at 4°C with either CT1, CT2, or CT3 for 1 hr and then for an additional hour with Protein A/Seapharose CL-4B beads (10 mg/dish of cells). Immunoprecipitates were isolated by centrifugation (500 × 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 control) 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 were rinsed in phosphate-buffered saline and then were added to 40 ml of ice-cold 10 mM Tris-base, 1 mM EDTA, 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 × g for 10 min at 4°C), and pellets were resuspended in 12 ml of ice-cold lysis buffer. After 30 min on ice, the extract was centrifuged (27,000 × g for 15 min at 4°C), and either CT1 (cortex and cerebellum), CT2 (liver), or CT3 (pancreas) was added to the supernatant. After 1 hr at 4°C, 40 mg Protein A beads was added; 1 hr later, immune complexes were isolated by centrifugation (500 × g for 2 min) and two washes with lysis buffer. Finally, the washed beads were resuspended in 1.4 ml of cytosol buffer 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°C 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 Ecoscent 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 assessed by washing (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 × g for 2 min), resuspended in 10 ml of ice-cold cytosol buffer (120 mM KCl, 2 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 10 μM EGTA, 2 mM ATP, 20 mM HEPES, pH 7.0), centrifuged again (2000 × g for 2 min at 4°C), 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°C and then centrifuged (2000 × g for 2 min at 4°C). 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 stimulator. 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 Ecoscent 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°C. 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/Seapharose CL-4B was from Pharmacia (Piscataway, NJ). $[^{3}H]$InsP$_3$ (21 Ci/mmol) and $^{45}$CaCl$_2$ (~800 Ci/mmol) 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 InsP₃ 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 InsP₃ 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 InsP₃, respectively (the values for type II and III receptors being significantly greater than that for type I receptor, \( p < 0.01 \)) and that each receptor type bound approximately equivalent amounts of InsP₃ 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 (\( p < 0.01 \)), 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 (\( p < 0.01 \)). Thus, type II and III receptors seem to bind InsP₃ with ~2-fold and ~30-fold lower affinity than the type I receptor, respectively.

**Binding of InsP₃ 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 CTI, only type III receptor was recovered (Fig. 3A, lane 2), indicating that type I/III 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 (\( p < 0.01 \)). Thus, type II and III receptors seem to bind InsP₃ with ~2-fold and ~30-fold lower affinity than the type I receptor, respectively.

![Fig. 1. Composition of InsP₃ receptors immunoprecipitated from cell lines.](image1)

![Fig. 2. Binding of InsP₃ to receptors immunoprecipitated from cell lines.](image2)
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 \(\text{InoP}_3\), respectively, and Hill slopes of \(-0.81\) and \(-0.97\), respectively). These data and the fact that precleared 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 \(\text{InoP}_3\) and that binding to type 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 \((\sim 47\) nM, Fig. 3B) is identical to the \(K_d\) value of the low affinity binding site in heterotetramer-containing type III receptor preparations \((\sim 48\) nM, Fig. 2).

**InsP\(_3\) binding and \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) stores to \(\text{InsP}_3\). Thus, we measured \(\text{InsP}_3\) binding and \(\text{Ca}^{2+}\) mobilization in digitonin-permeabilized SH-SY5Y, AR4–2J, and RINm5F cells under identical conditions. Fig. 4 shows that \(\text{InsP}_3\) 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 \(\text{InsP}_3\) as a \(\text{Ca}^{2+}\)-mobilizing agent also varied considerably, whereas maximal \(\text{Ca}^{2+}\) release was similar for the three cell types (Fig. 5A). For SH-SY5Y, AR4–2J, and RINm5F cells, \(EC_{50}\) values were \(22 \pm 2, 20 \pm 2,\) and \(94 \pm 6\) nM \(\text{InsP}_3\), respectively, and Hill slopes were \(1.9 \pm 0.1, 1.8 \pm 0.1,\) and \(2.0 \pm 0.1\), respectively (eight independent experiments; Fig. 5A). Thus, because \(\text{InsP}_3\) has similar \(\text{Ca}^{2+}\)-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 \(\text{Ca}^{2+}\) release kinetics could influence \(EC_{50}\); however, the rates of \(\text{InsP}_3\)-induced \(\text{Ca}^{2+}\) efflux were essentially identical for each cell type (Fig. 5B). Second, the extent to which \(\text{Ca}^{2+}\) stores are loaded can influence \(EC_{50}\) (Parys et al., 1993); however, the extent of \(\text{Ca}^{2+}\) uptake was very similar for each cell type (Fig. 5, legend). Third, as-yet-undefined factors might suppress \(\text{Ca}^{2+}\) release from RINm5F cells; however,
ionomycin, which acts independently of InsP₃ receptors, released Ca²⁺ 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 ⁴⁵Ca²⁺ release in 5-min incubations at 25° were very similar for the three cell types (EC₅₀ = 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 InsP₃-independent Ca²⁺-handling properties of SH-SY5Y, AR4–2J, and RINm5F cells are very similar and thus the relatively high EC₅₀ value of InsP₃ in permeabilized RINm5F cells is a reflection of the predominance of low affinity type III receptors in this cell type.

**Binding to InsP₃ 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 InsP₃ 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 immunobotted 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 immunoprecipitation indicates that heterotrameric InsP₃ receptors are expressed in liver and pancreas.

Initial analysis (Fig. 7A) showed that InsP₃ 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 ≤ 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 ≤ 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 Kᵥ 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 Kᵥ value was defined by one-site analysis. The Kᵥ 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 InsP₃ receptors bind InsP₃ with different affinities and that these differences seem to influence the potency of InsP₃ as a Ca²⁺-mobilizing agent. This provides an indication of how selective expression of a particular InsP₃ receptor type...
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 $\sim 1$, $\sim 2$, and $\sim 40$ 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 C6H10T1/2 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$^{2+}$-handling 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 immunochromchemical 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 $\beta$ 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
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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Fig. 7. Binding of InsP3, 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 [3H]InsP3 for 20 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 InsP3 receptor preparations.