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Plasma membrane inositol 1,4,5-trisphosphate receptor of lymphocytes: Selective enrichment in sialic acid and unique binding specificity

(Jurkat cells/glycosylation/inositol phosphate/calcium/lectin)

ADIL A. KHAN, JOSEPH P. STEINER, AND SOLOMON H. SNYDER*

Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205-2185

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ABSTRACT **The inositol 1,4,5-trisphosphate receptor (IP_3R) associated with plasma membranes of lymphocytes differs in terminal sugar content and binding specificity from the cerebellar receptor, which is localized to endoplasmic reticulum. Lectin column chromatography reveals that 30% of IP_3R in the thymus contains sialic acid, reflecting a plasma membrane association, in contrast to 5% of cerebellar IP_3R. IP_3R in thymus and plasma membrane fractions of Jurkat lymphocytes differs from IP_3R of Jurkat microsomes and cerebellum in inositol phosphate specificity. The plasma membrane IP_3R has lower affinity for IP_3 but higher affinity for inositol 1,3,4,5-tetrakisphosphate, which may reflect a unique regulation of calcium at the plasma membrane by inositol phosphates.**

Inositol 1,4,5-trisphosphate (IP_3), formed in response to receptor-mediated enhancement of inositolphospholipid turnover, releases Ca^{2+} from nonmitochondrial intracellular stores. IP_3 binds to a specific receptor protein, which has been purified (1), shown by reconstitution experiments to contain both an IP_3 recognition site and a Ca^{2+} channel (2), and molecularly cloned from mouse (3), rat (4), and human (5). Immunohistochemistry at the electron microscopic level in Purkinje cells of the cerebellum has revealed an association of IP_3 receptors (IP_3Rs) with subdivisions of the endoplasmic reticulum (ER) and no evidence for localization to mitochondria, the Golgi apparatus, or the plasma membrane (6–8). Electrophysiologic studies, however, have demonstrated an IP_3-mediated Ca^{2+} conductance in the plasma membrane of lymphocytes, presumably reflecting IP_3R located on the plasma membrane (9). Moreover, recent immunohistochemical studies indicate a localization of IP_3Rs to the plasma membrane of lymphocytes (A.A.K., J.P.S., M. G. Klein, M. F. Schneider, and S.H.S., unpublished work) and olfactory cilia (A. M. Cunningham, D. Ryugo, G. V. Ronnett, R. R. Reed, A. H. Sharp, and S.H.S., unpublished work).

Sugar contents of proteins vary depending on their intracellular localization. ER proteins typically contain mannose, while plasma membrane proteins contain *N*-acetylglucosamine (GlcNAc) and sialic acid, neither of which occurs in ER proteins (10). IP_3Rs appear to contain mannose, as they adhere to concanavalin A (Con A) columns, from which they are selectively eluted with methyl α-D-mannoside (1).

We report a marked enrichment of sialic acid in IP_3R from thymus. The inositol phosphate specificity and heparin sensitivity of IP_3R in thymus and Jurkat T-lymphocyte plasma membrane fractions differ from those of IP_3R in ER fractions of cerebellum and Jurkat cells.

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MATERIALS AND METHODS

Unless noted, all reagents were obtained from Sigma. Lectins were obtained from E-Y Laboratories. [^{3}H]IP_3 (17 Ci/mmol; 1 Ci = 37 GBq) and formula 963 scintillation fluid were from NEN/DuPont. Unlabeled inositol phosphates were from Calbiochem. High molecular weight standards for SDS/PAGE were from GIBCO/BRL. Adult male Sprague–Dawley rats were from Harlan–Sprague–Dawley.

Preparation of Membranes. Tissues were obtained from adult male Sprague–Dawley rats (200–300 g) and homogenized (Brinkmann Polytron, setting 8 for 10 sec) in 50 vol of ice-cold buffer A (50 mM Tris·HCl, pH 8.3/1 mM EDTA), pelleted by centrifugation at 35,000 × *g* for 10 min and resuspended in 50 vol of buffer A with protease inhibitors (benzamidine, 360 μg/ml; leupeptin, 4 μg/ml; aprotinin, 4 μg/ml; pepstatin, 4 μg/ml). Human Jurkat T-lymphocyte plasma membranes and microsomal membranes were fractionated as described (11). In brief, Jurkat T lymphocytes (≈3 × 10^{10}) were harvested and subsequently suspended in 30 ml of 1 mM $ZnCl_2$. After 10 min at 25°C and another 10 min at 4°C, the suspension was centrifuged at 9000 × *g* for 5 min and the lysis solution was removed. The lysed cells were resuspended in 20 ml of 90 mM sodium phosphate, pH 6.5/1 mM $ZnCl_2$/5% (wt/wt) dextran/3.8% (wt/wt) polyethylene glycol on ice. This two-phase suspension was then frozen at −70°C overnight, thawed, and vigorously shaken. The suspension was centrifuged at 20,000 × *g* for 10 min at about 5°C in a Sorvall SA-600 fixed-angle rotor, and membrane material was collected at the interface of the phases. Olfactory epithelium was dissected from bovine nose into Ringer's solution with EGTA (2 mM Hepes/112 mM NaCl/3.4 mM KCl/2 mM EGTA/2.4 mM $NaHCO_3$, pH 7.4) and protease inhibitors (as above). Tissue was centrifuged at 5000 × *g* for 5 min and the supernatant was discarded. The pellet was resuspended in 30 mM Tris·HCl, pH 7.7/100 mM NaCl and agitated at 4°C for 2 min. $CaCl_2$ was added to 10 mM and the tissue was agitated for 20 min at 4°C and centrifuged at 1500 × *g* for 5 min. The supernatant was centrifuged at 15,000 × *g* for 10 min and resuspended in 30 mM Tris·HCl, pH 7.7/3 mM $MgCl_2$/0.1 mM EDTA.

IP_3 Binding. Binding of IP_3 to washed membranes was measured essentially as described (12). For cerebellar membranes, binding reactions consisted of 50 μg of washed rat cerebellar membranes, 2.0 nM [^{3}H]IP_3 (17 Ci/mmol), and buffer A to a final volume of 0.4 ml. Binding reactions for thymus and Jurkat cell membranes consisted of 400 μg and 300 μg of washed membranes, respectively, 25 nM [^{3}H]IP_3 (1.7 Ci/mmol), and buffer A to a final volume of 0.4 ml.

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_3R, IP_3 receptor; WGA, wheat germ agglutinin; LPA, *Limulus polyphemus* agglutinin; ER, endoplasmic reticulum.

*To whom reprint requests should be addressed.

Reactions were incubated to equilibrium at 4°C for 30 min and terminated by centrifugation at 10,000 × *g* for 10 min. Unbound ligand in the supernatant was removed by aspiration, and bound radioactivity in the pellets was measured by liquid scintillation spectrometry in 4 ml of formula 963 following membrane pellet solubilization with 200 μl of 1% SDS. Specific binding represented <10% of added radioactivity. Nonspecific binding was determined in the presence of 5 μM unlabeled IP_3 for cerebellar membranes, 25 μM for thymus, and 100 μM for Jurkat cell membranes.

Lectin Columns. Membrane proteins from each tissue were solubilized in 1% (vol/vol) Triton X-100. Triton X-100 concentration was then adjusted to 0.1% and the solubilized proteins from each tissue (≈2 mg/ml) were passed over the Con A-, wheat germ agglutinin (WGA)-, and *Limulus polyphemus* agglutinin (LPA)-agarose columns. For Con A columns the tissue homogenates in 50 mM Tris·HCl, pH 7.4/150 mM NaCl (Con A buffer) with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ were incubated with 1 ml of Con A-agarose for 2 hr on a rotator. The resin was then poured into a column, washed twice with 5 column volumes of Con A buffer with 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, followed by 4 hr with Con A buffer containing 2 mM EDTA and 0.5 M methyl-α-D-mannoside to elute bound proteins. WGA columns (2 ml of WGA-agarose) were treated similarly as the Con A-agarose but did not require divalent cations. The proteins bound to WGA column were specifically eluted with 0.5 M GlcNAc. For the LPA columns, tissue homogenates in 50 mM Tris·HCl, pH 8.0/150 mM NaCl (LPA buffer) with 10 mM $CaCl_2$ were incubated with 4 ml of LPA-agarose for 2 hr on a rotator. The columns were washed with 5 column volumes of LPA buffer containing 0.5 M NaCl and incubated 4 hr with LPA buffer containing 10 mM $CaCl_2$ and 0.1 M sialic acid. The lectin eluates for each tissue were resolved in 3–16% polyacrylamide gels, transferred to nitrocellulose filters, and probed with affinity-purified anti-IP_3R antibody followed by ^{125}I-labeled protein A. After exposure to film, the filters were sliced and radioactivity was determined directly in a gamma RIA counter.

Other Methods. One-dimensional gel electrophoresis was as described (13), and membrane protein (14) was determined with bovine serum albumin as a standard.

RESULTS

Lectin Binding of IP_3Rs. Western blot analysis of IP_3Rs in membrane fractions of cerebellum, thymus, and olfactory cilia revealed single, discrete bands at 260 kDa staining with antiserum to IP_3R (Fig. 1). To ascertain lectin interactions of IP_3Rs from these tissues, we passed extracts over Con A-, WGA-, and LPA-agarose columns and probed the eluates with antiserum to IP_3R. Con A, WGA, and LPA bind selectively to proteins respectively enriched in mannose, GlcNAc, and sialic acid, respectively (15, 16). All three lectins bound IP_3Rs from all three tissues, but amounts varied. Con A bound quantitatively to IP_3Rs from the three tissues. By contrast, WGA interacted with 15%, 25%, and 10% of the IP_3Rs in cerebellum, olfactory cilia, and thymus, respectively. LPA bound thymus IP_3Rs most prominently, with 30% of the thymus receptors reacting, while only 10% and 5%, respectively, of cilia and cerebellar receptors bound to the LPA column.

Inositol Phosphate Specificity and Heparin Effects on IP_3 Binding in Cerebellum, Thymus, and Jurkat Cell Membranes. Saturation analysis of $[^3H]IP_3$ binding to cerebellar membranes revealed a single population of sites (K_D = 40 nM, B_{max} = 20 pmol/mg of protein) (Fig. 2*A*). By contrast rat thymus membranes displayed a curvilinear Scatchard plot with high-affinity (K_D = 45 nM, B_{max} = 0.4 pmol/mg) and low-affinity (K_D = 1.2 μM, B_{max} = 4 pmol/mg) sites (Fig. 2*B*). Differences between cerebellum and thymus could reflect receptor variations between plasma membrane and ER IP_3Rs or merely organ variation. Accordingly, we separated plasma membrane and microsomal membrane fractions from Jurkat human T-cell cultures (Fig. 2*C*). Binding of $[^3H]IP_3$ to Jurkat plasma membranes revealed a single class of low-affinity sites (K_D = 0.8–1.0 μM, B_{max} = 2.5 pmol/mg), whereas only high-affinity binding sites (K_D = 25 nM, B_{max} = 0.25 pmol/mg) were observed in the Jurkat microsomal membranes.

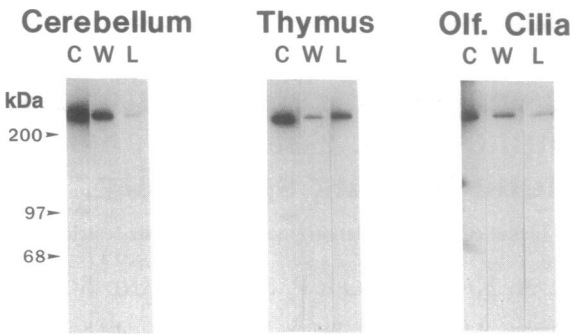

FIG. 1. Identification of glycoforms of the IP_3R. Western blot analysis of IP_3R in rat cerebellum, thymus, and olfactory cilia. For each tissue, column eluates from Con A (lanes C), WGA (lanes W), and LPA (lanes L) were analyzed. Triton X-100-solubilized proteins from each tissue were passed over the lectin-agarose columns. Lectin eluates for each tissue were resolved in 3–16% polyacrylamide gels, transferred to nitrocellulose filters, and probed with affinity-purified anti-IP_3R antibody followed by ^{125}I-protein A. After exposure to film, the filters were sliced, and the ^{125}I-protein A bound to anti-IP_3R antibody was measured in a gamma RIA counter. Data are from a representative experiment that was replicated three times.

The inositol phosphate specificity of IP_3Rs differs in cerebellum, thymus, and Jurkat cells. While the rank order of selectivity of IP_3 binding to the different membranes remains the same, $IP_3 > IP_4 > IP_6 > IP_1 = IP_2$, relative affinities for IP_3 and IP_4 vary markedly (Fig. 3 *A* and *B*). IP_3 inhibits binding to Jurkat plasma and thymus membranes half-maximally at 100–150 nM, while the IC_{50} for binding to Jurkat microsomal and cerebellar membranes is 30–40 nM. Conversely, IP_4 inhibits binding of $[^3H]IP_3$ to Jurkat plasma and thymus membranes half-maximally at 600–800 nM, while the IC_{50} for binding to Jurkat microsomal and cerebellar membranes is 8–10 μM. Thus, in cerebellar and Jurkat microsomes IP_3 is almost 600 times more potent than IP_4, while in thymus and Jurkat plasma membranes it is only 6 times more potent. Because IP_4 can be metabolized by a 5-phosphatase (17), we examined for possible degradation of IP_4 in Jurkat membranes under conditions identical to those used in binding assays with incubations for 30 min at 4°C. IP_4 and all its principal potential metabolites were separated by anion-exchange chromatography (18). Less than 15% degradation of IP_4 is apparent under our incubation conditions. Heparin inhibits both IP_3 binding to receptors (12) and IP_3-induced Ca^{2+} release (19). Heparin is 20–50 times more potent at the IP_3R in cerebellum than in thymus and Jurkat plasma membranes (Fig. 3).

DISCUSSION

In this study, we observe that IP_3Rs in various tissues contain sialic acid and GlcNAc, sugars that do not occur in ER proteins and are most characteristic of plasma membrane proteins (20). Evidence for an association of these sugars with IP_3Rs includes binding with LPA and WGA, lectins selective for sialic acid- and GlcNAc-containing proteins, respectively. Of the three tissues, thymus IP_3R protein appears to possess the highest sialic acid content, with 30%

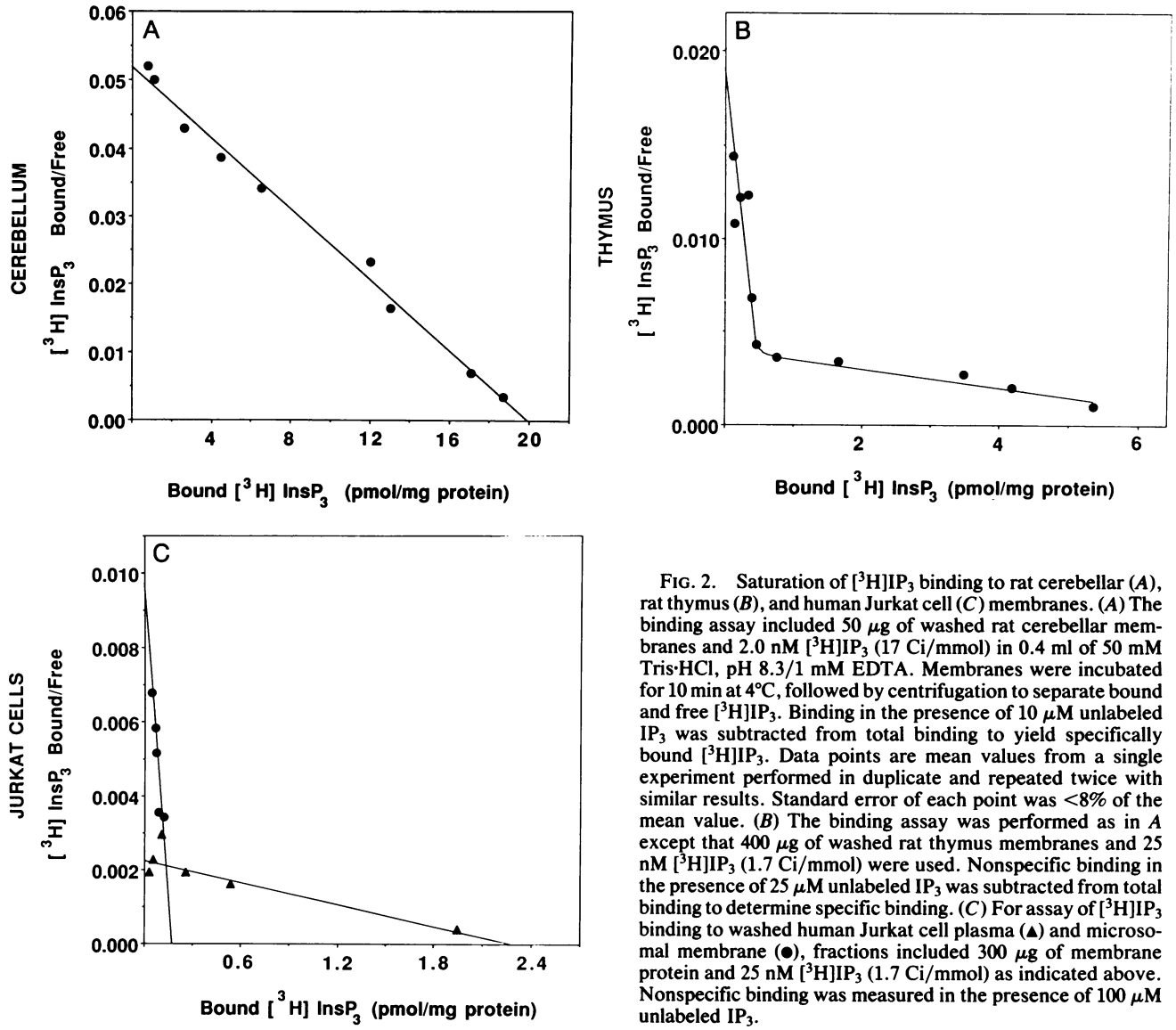

FIG. 2. Saturation of [^{3}H]IP$_3$ binding to rat cerebellar (*A*), rat thymus (*B*), and human Jurkat cell (*C*) membranes. (*A*) The binding assay included 50 μg of washed rat cerebellar membranes and 2.0 nM [^{3}H]IP$_3$ (17 Ci/mmol) in 0.4 ml of 50 mM Tris·HCl, pH 8.3/1 mM EDTA. Membranes were incubated for 10 min at 4°C, followed by centrifugation to separate bound and free [^{3}H]IP$_3$. Binding in the presence of 10 μM unlabeled IP$_3$ was subtracted from total binding to yield specifically bound [^{3}H]IP$_3$. Data points are mean values from a single experiment performed in duplicate and repeated twice with similar results. Standard error of each point was <8% of the mean value. (*B*) The binding assay was performed as in *A* except that 400 μg of washed rat thymus membranes and 25 nM [^{3}H]IP$_3$ (1.7 Ci/mmol) were used. Nonspecific binding in the presence of 25 μM unlabeled IP$_3$ was subtracted from total binding to determine specific binding. (*C*) For assay of [^{3}H]IP$_3$ binding to washed human Jurkat cell plasma (▲) and microsomal membrane (●), fractions included 300 μg of membrane protein and 25 nM [^{3}H]IP$_3$ (1.7 Ci/mmol) as indicated above. Nonspecific binding was measured in the presence of 100 μM unlabeled IP$_3$.

of its IP$_3$Rs binding to LPA, whereas in olfactory cilia and cerebellum, 10% and 5%, respectively, of the receptors bind LPA. The relative proportions of WGA binding to IP$_3$Rs in the three tissues differ from proportions with LPA. WGA binds predominantly to free GlcNAc. In sialic acid-containing proteins, such as thymus IP$_3$Rs, GlcNAc is not free but is surmounted by multiple sialic acid moieties, suggesting that the total GlcNAc content of thymus IP$_3$Rs may be substantially greater than the observed WGA-bound levels. The substantial level of sialic acid in IP$_3$Rs in the thymus, presumably reflecting thymic lymphocytes, fits with our immunohistochemical localization of IP$_3$Rs to the plasma membrane of Jurkat cells (A.A.K., J.P.S., M. G. Klein, M. F. Schneider, and S.H.S., unpublished work) and electrophysiologic evidence for IP$_3$R Ca^{2+} channels in lymphocyte plasma membranes (9). In Jurkat cells, we have separated plasma membrane and ER fractions and shown that 75–80% and 20–25% of the IP$_3$R content occurs in plasma membrane and in ER, respectively. The relatively high levels of GlcNAc and sialic acid in olfactory cilia compared with cerebellum fit with the immunohistochemical localization of IP$_3$Rs to the plasma membrane of rat olfactory cilia (A. M. Cunningham, D. Ryugo, G. V. Ronnett, R. R. Reed, A. H. Sharp, and S.H.S., unpublished work).

Inositol phosphate specificity and affinity for heparin differ markedly in plasma membrane compared with microsomal preparations of Jurkat cells. The similar pattern of binding in thymus and Jurkat cell plasma membranes indicates that they both reflect plasma membrane IP$_3$R, while similarities of cerebellar and Jurkat microsomal preparations presumably relate to ER IP$_3$R. The differing inositol phosphate specificities of the two forms of IP$_3$R may reflect functional distinctions. Thus, the lower affinity of the plasma membrane receptor for IP$_3$ may relate to the presumed higher concentration of IP$_3$ at plasma membrane, where it is synthesized, than at the ER. The higher affinity of plasma membrane IP$_3$R for IP$_4$ may relate to the putative role of IP$_4$ in regulating Ca^{2+} entry at the plasma membrane (21). Whether differences between plasma membrane and ER IP$_3$Rs reflect different glycosylation patterns or distinct amino acid sequences is unclear. Subtypes of IP$_3$R protein derive both from alternative splicing (22, 23) and from distinct genes (24, 25).

Why are IP$_3$Rs localized in distinct subcellular compartments of various tissues? IP$_3$ releases limited intracellular stores of Ca^{2+} from ER in response to rapid signaling from neurotransmitters and hormones acting at plasma membrane receptors, as is most apparent in neurons. In lymphocytes, inositolphospholipid turnover mediates the major changes in cellular proliferation that follow antigenic stimulation, requiring prolonged increases in intracellular Ca^{2+} derived from extracellular sources (26). The enhanced Ca^{2+} influx secondary to receptor triggering in lymphocytes represents a com-

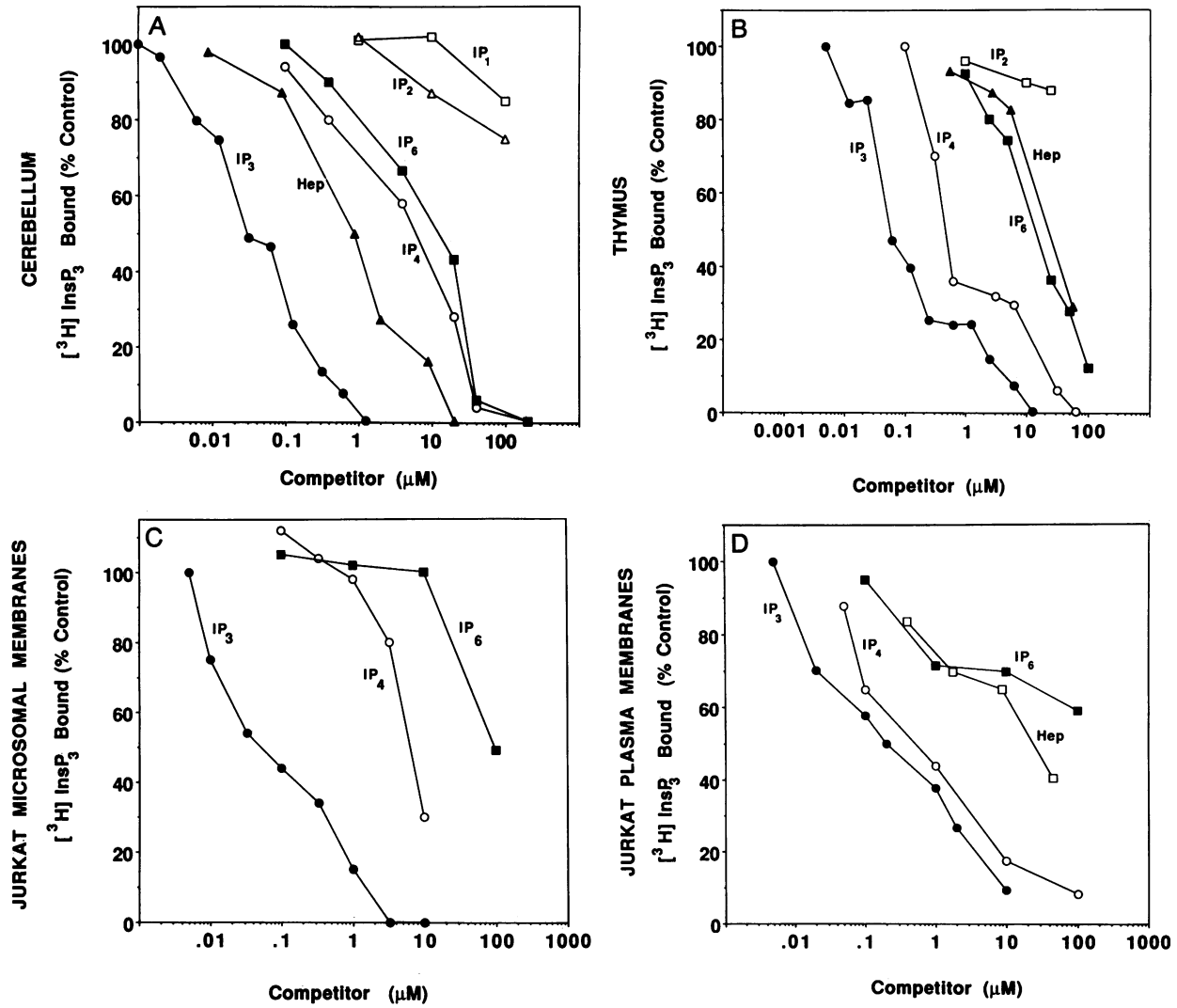

FIG. 3. Specificity of [^{3}H]IP$_3$ binding to cerebellar membranes (*A*), thymus membranes (*B*), Jurkat microsomal membranes (*C*), and Jurkat plasma membranes (*D*). Binding of 2.0 nM [^{3}H]IP$_3$ (17 Ci/mmol) to 50 μg of washed rat cerebellar membranes was assayed as described in Fig. 2*A* in the presence of various concentrations of inositol mono-, bis-, tris-, tetrakis-, or hexakisphosphate or heparin (Hep, M_r = 5000). Binding of 25 nM [^{3}H]IP$_3$ (1.7 Ci/mmol) to 300 μg of thymus membranes and 200 μg of Jurkat microsomal and plasma membrane fractions was as described in Fig. 2 *B* and *C*, respectively. Data points are mean values from a single experiment performed in duplicate and repeated twice with similar results. Standard error of each point was <8% of the mean value.

mon signaling pathway for lymphocyte receptors, whose importance is demonstrated by the abrogation of interleukin 2 mRNA accumulation when Ca^{2+} entry is blocked by the Ca^{2+} chelator EGTA (27). Patch-clamp studies have demonstrated the absence of voltage-gated Ca^{2+} channels in T-cell membranes (28). A plasma membrane IP$_3$R in lymphocytes could be ideally suited to mediate the influx of large amounts of Ca^{2+} in response to antigenic challenge.

In olfactory cilia, plasma membrane IP$_3$R may fulfill several roles. The cilia possess very little internal volume, which is filled with microtubules and possesses negligible ER (29). Signal transduction in olfactory cilia involves odorant binding to presumed receptors on the plasma membrane followed by increases in inositolphospholipid turnover and adenylyl cyclase activity (30–32). Our laboratory has obtained evidence that inositolphospholipid turnover may precede adenylyl cyclase activation. Thus, activation of adenylyl cyclase by odorants in olfactory neuronal cultures requires substantial levels of intracellular Ca^{2+}, which can only be obtained following IP$_3$-induced Ca^{2+} release (32). Moreover, odorants enhance inositolphospholipid turnover prior to stimulating an increase in adenylyl cyclase, and IP$_3$ injections into these cells increase adenylyl cyclase activity (32). Thus, within the plasma membrane of olfactory cilia, odorant activation of receptors triggers production of IP$_3$ to release Ca^{2+}, which then activates adenylyl cyclase. These events would require a plasma membrane IP$_3$R to utilize external Ca^{2+} closely adjacent to the plasma membrane rather than drawing on internal sources.

In the brain, previous immunohistochemical studies have revealed IP$_3$R in Purkinje cells of the cerebellum in subdivisions of the ER, with no evidence for plasma membrane IP$_3$R (6–8). Besides neuronal cells, IP$_3$R occurs in nerve terminals throughout the brain (33, 34) and retina (35). A major function of Ca^{2+} in nerve terminals is to modulate neurotransmitter release, which involves Ca^{2+} entry from outside the cell. Conceivably, IP$_3$R in plasma membrane of nerve terminals plays a role in this process.

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