The inositol 1,4,5-trisphosphate receptor (InsP3R) is activated by InsP3 binding to amino-terminal ligand binding domain (InsP3R-N). Recently we reported functional coupling of phosphatidylinositol (4,5)-bisphosphate (PIP2) to the InsP3R. Specific binding of PIP2 to InsP3R-N domain was postulated as a part of the InsP3R-PIP2 functional coupling model. Here we utilized bacterially expressed and purified InsP3R-N domain to characterize its binding specificity for InsP3, Adenophostin A (AdA) and the water-soluble PIP2 analog dioctanoyl-(4,5)PIP2 (ShPIP2). Obtained data led us to conclude that specific InsP3, AdA, and ShPIP2 binding sites are located within the InsP3R-N domain, that the extra receptor binding element responsible for enhanced binding of AdA is an integral part of the InsP3R-N domain, that ShPIP2 is able to displace InsP3 from the InsP3R-N, but InsP3 or AdA is unable to completely displace ShPIP2. These results support the InsP3R-PIP2 functional coupling model and provide novel insights into InsP3R ligand specificity.

The inositol (1,4,5)-trisphosphate receptor (InsP3R) plays a key role in the intracellular calcium (Ca2+) signaling process (1). InsP3R is a tetrameric complex (2, 3); each subunit is 2749 amino acids (predicted M.W. is 313 kDa) (reviewed in (4)). InsP3R structure consists of three distinct domains: carboxy-terminal Ca2+ channel domain, intermediate coupling domain and amino-terminal ligand-binding domain (4–6). The carboxy-terminal region is required for proper InsP3R intracellular targeting, oligomerization, and Ca2+ channel formation (7, 8). The intermediate domain, which mediates a conformational coupling between ligand-binding and Ca2+ channel opening, contains a number of modulatory sites (4). Amino-terminal ligand-binding domain (InsP3R-N) is necessary and sufficient for high-affinity InsP3 binding (5, 6, 9–12).

The InsP3R ligand binding site is highly selective for (1,4,5)InsP3. Recently two novel InsP3R agonists Adenophostins A and B have been discovered in Penicillium brevicompactum cultures (13–15). Adenophostin A (AdA) has been chemically synthesized in the laboratory (16–18). AdA is the most potent known agonist of InsP3R, approximately 10–15 fold more potent than InsP3 itself (13–16, 19–22). Structurally AdA and InsP3 are quite diverse, but there are key conserved motifs that are similar (18, 19). Since AdA displaces [3H]InsP3 from the InsP3R (13–16, 19–21) and possesses common motifs with InsP3, it is most likely that AdA interacts directly with the InsP3R ligand-binding region. However, an important aspect of AdA structure is that it possesses an adenine base and the enhanced binding seem most likely to result from an interaction of this base with a hydrophobic region of the InsP3R. Whether this region is an integral part of the InsP3R ligand binding domain or located elsewhere in the InsP3R sequence has not been addressed in the previous studies, performed with the intact InsP3R. Binding of AdA to isolated InsP3R-N domain need to be examined to answer this question.

In recent planar lipid bilayer experiments (23) we discovered that rat cerebellar InsP3R activity was increased three- to fourfold by addition of monoclonal anti-PIP2 antibody (PIP2Ab) (24) to the cytosolic side of the membrane. In complementary experiments, we demonstrated inhibition of InsP3R by exogenously added PIP2 vesicles or by the water soluble PIP2 analog D-myo-dioctanoyl-(4,5)PIP2 (ShPIP2). PIP2 vesicles and
ShPIP2 complete with [3H]InsP3 for binding to rat cer-
beellar InsP3R (23, 25). Based on these findings, we pro-
posed a novel compartmentalized Ca2+ signaling mo-
del (23), according to which a subpopulation of in-
tracellular InsP3R directly interacts with PIP2 in jux-
tapped plasma membrane leaflet. Recently a potential ro-
le for InsP3R-PIP2 complexes in activation of Imin/Icrac
signaling model and provide insights into the molecular
determinants of InsP3R-AdA interaction.

MATERIALS AND METHODS

Expression and Purification of Recombinant
InsP3R-N-His Protein

GST-InsP3R-N-His protein was expressed in 2xYT media for 18 h at room temperature in protease-
deficient BL21 E. coli strain by 0.7 mM IPTG induction
(at OD600 = 0.6). Cells grown in 1 liter culture were cooled on ice, collected by 10 min centrifugation at
5,000 rpm (Beckman JA-10) and resuspended in 50 ml of imidazole lysis buffer (50 mM imidazole pH 6.8, 100
mM NaCl, 10 mM EDTA, 1 mM DTT) with addition of
imidazole lysis buffer (50 mM imidazole pH 6.8, 100
mM NaCl, 1 mM EDTA, 1 mM DTT) and precipitated with 12.5%
washed 3 times with the low salt buffer (20 mM
imidazole pH 6.8, 1 M NaCl) and pelleted at 2,500 rpm for 3 min in the microcentrifuge, the supernatant was collected in the
fresh tube, mixed with 0.5 ml of His.Bind beads and incubated in the His binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 NaCl) for 30 min at end-
over-end shaker. The His.Bind beads were pelleted by centrifugation, washed with the His binding buffer and the InsP3R-N-His protein was eluted from the His.Bind
beads in 10 ml of the elution buffer (20 mM Tris-HCl, pH 7.9, 500 mM imidazole, 0.5 NaCl). Obtained sample
was dialyzed overnight in the cold room against 2 liters of PBS (14,000 MWCO, Spectrum) and stored at 4°C for prior to binding assays.

[3H]InsP3 Binding Assay

Specific [3H]InsP3 binding was performed with minor
modifications of the procedure described previously
(23). Briefly, 10–20 µg of purified InsP3R-N-His protein was incubated on ice with 10 nM [3H]InsP3 in the
binding buffer (50 mM Tris-HCl, pH 8.3, 1 mM EDTA,
1 mM DTT, 100 mM NaCl) and precipitated with 12.5% PEG and 1.2 mg/ml γ-globulin at 14,000 × g. Precip-
itates were quickly washed with the binding buffer,
dissolved in Soluene and their [3H] content was deter-
mined by liquid scintillation counting (Beckman
LS6500). Non-specific counts, determined in the pres-
ence of 25 µM non-labeled InsP3, were subtracted from the total to yield specific binding. Various amounts of hydrophilic ShPIP2 (dissolved as 1 mM stock in the
binding buffer), InsP3 (1 mM stock in water) and AdA
(100 µM stock in water) were added to the binding
buffer in [3H]InsP3 competition experiments as indicated.

[125]I]ShPIP2 Binding Assay

[125]I]ShPIP2 was generated essentially as described in
(27). Briefly, 0.25 mg (300 nmol) of ShPIP2 amino-
derivative (NH2-ShPIP2, MW 821) (28) was dissolved
in 0.1 M sodium-borate buffer. Labeled phospholipids were separated from the glycine
conjugates and hydrolysis products by gel filtration on
Sephadex G-10 (Pharmacia), collected in 0.6 ml frac-
tions and analyzed by γ-counting. Fractions containing
[125]I]ShPIP2 (M.W. 1191) were pulled together to yield
3 ml of 100 µM stock in 0.1 M sodium-borate buffer. The binding experiments were performed as described above for [3H]InsP3 using 5 µM [125]I]ShPIP2 concentration. Following PEG/γ-globulin precipitation and brief wash, bound [125]I]ShPIP2 was quantified by γ-counting
(Packard Cobra). Non-specific counts, determined in the
presence of 100 µM non-labeled ShPIP2, were sub-
tracted from the total to yield specific [125]I]ShPIP2
binding.
Materials

Rat InsP$_3$R-I cDNA was a kind gift of Thomas C. Südhof (UTSW Medical Center) (29). Adenophostin A (AdA) was synthesized as described (18). The synthesis of D-my-o-dioctanoyl-(4,5)PIP$_2$ (ShPIP$_2$) and NH$_2$-ShPIP$_2$ was previously described (28). Sodium salt form of AdA, ShPIP$_2$ and NH$_2$-ShPIP$_2$ was used. ShPIP$_2$ was >95% pure based on NMR and TLC analysis. Stearoyl-arachidonyl-(4,5)PIP$_2$ (SA-PIP$_2$) was from Boehringer Mannheim. Glutathione-agarose beads and thrombin from Pharmacia. His.Bind beads and T7.Tag monoclonal antibody are from Novagen. (1,4,5)InsP$_3$ from LC Laboratories. [$^3$H]InsP$_3$ from Amersham. [$^{125}$I]-Bolton-Hunter reagent from ARC. Soluene from Packard Inst. Western Max detection kit (Amresco) was used for Western blotting. Other chemicals were from Sigma or Intermountain Scientific Corp.

RESULTS

The InsP$_3$R-N region (M1-F580, MW 62 kDa) of rat InsP$_3$R-I cDNA (29) was amplified by PCR and ligated into pGEX-KG vector with the addition of carboxy-terminal 6-His-tag to yield pGEX-InsP$_3$R-N-His construct (Fig. 1A). The construct obtained was transformed into protease-deficient BL21 E. coli strain. InsP$_3$R-N-His protein was expressed by IPTG induc-
tion and purified by batch-affinity purification on glutathione beads and His.Bind beads as described in Methods. Final sample and the samples from intermediate stages of purification were separated by SDS-gel electrophoresis and analyzed by Coomassie staining (Fig. 1B) and Western blotting with monoclonal T7.Tag (anti-His tag) antibody (Fig. 1C). Described procedure typically yielded 0.5–1 mg of at least 95% pure (Fig. 1B, lane 6) InsP3R-N-His protein from 1 liter of BL21 culture.

It has been previously reported that InsP3R-N region is sufficient to form a specific InsP3 binding site (5, 6, 9–12). Here we utilized recombinant InsP3R-N to characterize interactions of InsP3R ligand binding domain with a novel superpotent InsP3R agonist Adenophostin A (AdA) (13–16, 19–22) and the InsP3R antagonist water-soluble PIP2 analog D-myo-dioctanoyl-(4,5)PIP2 (ShPIP2) (23). The structures of InsP3, AdA and ShPIP2 are compared on Fig. 2. Similar to previous reports, we detected specific binding of [3H]InsP3 to InsP3R-N domain (Fig. 3, open squares). We further demonstrated that AdA binds to InsP3R-N with a 10–15 fold higher affinity than InsP3 itself (Fig. 3, filled circles), similar to conclusions reached in binding experiments with cerebellar microsomes. Thus, the high potency of AdA to induce Ca2+ release from InsP3-sensitive stores (13–16, 19–21) and to activate InsP3R in bilayers (22) indeed results from the increased affinity for the InsP3R ligand-binding site.

Inhibition of [3H]InsP3 binding to rat cerebellar microsomes by PIP2 vesicles and ShPIP2 has been previously demonstrated (23, 25). Does PIP2 compete with InsP3 for the InsP3R ligand-binding site or exerts negative effects on InsP3 binding via long-range allosteric interactions? To resolve this question, we tested an ability of ShPIP2 to compete with [3H]InsP3 for binding to InsP3R-N. In quantitative agreement with the earlier results (23), micromolar concentrations of ShPIP2 displaced [3H]InsP3 from the InsP3R-N (Fig. 3, open circles). The fit to the data (Fig. 3, smooth curve) yielded an IC50 equal to 11 μM ShPIP2. Similar results were obtained when stearoyl-arachidonyl-(4,5)PIP2 vesicles were used in competition experiments instead of ShPIP2 (data not shown).

PIP2 can displace InsP3 from the InsP3R ligand-binding site (Fig. 3). Does InsP3R-N specifically bind ShPIP2? To answer this question we took advantage of NH2-ShPIP2 derivative (28). Following the procedure from (27), we generated [125I]ShPIP2 as described in Methods. The reagent thus generated was utilized in binding experiments with recombinant InsP3R-N. The reagent thus generated was utilized in binding experiments with recombinant InsP3R-N. PIP2 in micromolar concentrations was able to displace [125I]ShPIP2 from the InsP3R-N (Fig. 4, open circles), supporting a specific InsP3R-N association with

![Chemical structure of InsP3 and AdA](image)

**FIG. 4.** ShPIP2 binding to InsP3R-N. [125I]ShPIP2 (above) at 5 μM concentration was used as specific ligand for InsP3R-N. Competitive displacement experiments were performed with InsP3 (open squares, n = 3), AdA (filled circles, n = 2) and ShPIP2 (open circles, n = 2) at concentrations as indicated. Specific [125I]ShPIP2 binding at each concentration of a competitor was normalized to binding obtained in the absence of competitor in the same series of experiments and pooled together. The data are presented as means ± SEM.
ShPIP2. A fit to the data (Fig. 4, smooth curve) yielded an IC50 equal to 0.83 μM ShPIP2. Binding of [125I]-ShPIP2 was not affected by the His tag at InsP3R-N carboxy-terminal, as we obtained similar results with the GST-InsP3R-N protein which lacked His tag (data not shown). Interestingly, InsP3 and AdA were only partially effective in displacing [125I]-ShPIP2 from the InsP3R-N binding site (Fig. 4, open squares and filled circles), which argues against a simple single-site competition model.

DISCUSSION

We used bacterially expressed and purified to >95% homogeneity recombinant InsP3R ligand-binding domain (InsP3R-N) to characterize its binding specificity for InsP3, AdA and ShPIP2. The results obtained lead us to conclude that (i) specific InsP3, AdA and ShPIP2 binding sites are located within the InsP3R-N domain, (ii) the extra receptor binding element responsible for enhanced binding of AdA is an integral part of the InsP3R-N domain, (iii) ShPIP2 is able to displace InsP3 from the InsP3R-N, but InsP3 or AdA unable to completely displace ShPIP2.

In the context of InsP3R-PIP2 functional coupling model we previously proposed specific association of PIP2 with the InsP3R ligand-binding domain (23), and the data reported here support this hypothesis. The stretch of 15 amino acids is spliced out from the SI site within type I InsP3R-N domain (Fig. 1A) in a tissue- and developmentally-specific manner (30). Both SI splice variants display similar InsP3 binding properties (9), and the significance of splicing at the SI site for InsP3R physiological function is unknown. Here we used SI – splice variant of rat type I InsP3R-N (29), the predominant isoform in the cerebellum. Notably, 6 out of 15 amino acids in the SI splice insert (VDPDF-EFECLEFOPS) are negatively charged (underlined), and the SI + splice isoform of type I InsP3R-N may not bind PIP2, as effectively as SI – isoform due to electrostatic repulsion between negatively charged PIP2, and the cluster of negative charges within SI splice insert. We are in the process of testing this hypothesis experimentally by using methodology outlined in the present paper.

Although ShPIP2 is able to completely displace InsP3 from the InsP3R-N (Fig. 3), InsP3 or AdA cannot completely displace ShPIP2 (Fig. 4). These data argue against simple one-site competition model. One possibility is that the InsP3R-binding site is a part of the PIP2-binding site within the InsP3R-N sequence. Structural determinants involved in InsP3 binding to InsP3R-N have been extensively characterized by site-directed mutagenesis (31) and we are taking similar approach to map out determinants responsible for PIP2 binding. Another related possibility is that the InsP3R-N domain adopts different conformations in InsP3-bound and ShPIP2-bound states. According to this idea, induced-fit conformational changes in InsP3R-N upon ShPIP2 binding lead to dramatic reduction in affinity for InsP3. The fact that in our experiments ShPIP2 was 10-fold more potent in displacing [125I]-ShPIP2 (Fig. 4, IC50= 0.83 μM) than [3H]InsP3 (Fig. 3, IC50 = 11 μM) from InsP3R-N may also relate to induced-fit conformational changes in the InsP3R-N structure. Different conformations of InsP3R ligand-binding domain in InsP3- and PIP2-occupied states constitute a key element of the InsP3R-PIP2 coupling model (23), and the availability of sufficient quantities of purified recombinant InsP3R-N (Fig. 1, lane 6) should allow us to test this hypothesis by biochemical techniques. Because AdA activate (13–16, 19–22) and ShPIP2 inhibit (23) InsP3R in functional experiments, we predict that InsP3- and AdA-bound conformations of InsP3R-N will be similar to each other and different from the ShPIP2-bound InsP3R-N conformation.

ACKNOWLEDGMENTS

We are grateful to T. C. Sudhof for the gift of rat InsP3R-I cDNA. I.B. is thankful to S. Bezprozvannaya for tremendous support and encouragement of his work. L.G. is on leave from the Institute of Cytology Russian Academy of Sciences. Work was supported by AHA (I.B.), Robert A. Welch Foundation (I.B. and J.R.F.), NIH (NS38082, I.B., and GM3278, J.R.F.) and the Welcome Trust (045491, B.V.L.P.)

REFERENCES