The Involvement of Ankyrin in the Regulation of Inositol 1,4,5-Trisphosphate Receptor-mediated Internal Ca$^{2+}$ Release from Ca$^{2+}$ Storage Vesicles in Mouse T-lymphoma Cells*

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Mouse T-lymphoma cells contain a unique type of internal vesicle which bands at the relatively light density of 1.07 g/cc. These vesicles do not contain any detectable Golgi, endoplasmic reticulum, plasma membrane, or lysosomal marker protein activities. Binding of [$^{3}H$]inositol 1,4,5-trisphosphate (IP$_3$) to these internal vesicles reveals the presence of a single, high affinity class of IP$_3$ receptor with a dissociation constant (K$_D$) of 1.6 ± 0.3 nM. Using a panel of monoclonal and polyclonal antibodies against IP$_3$ receptor, we have established that the IP$_3$ receptor (~260 kDa) displays immunological cross-reactivity with the rat brain IP$_3$ receptor. Polymerase chain reaction analysis of first-strand cDNAs from both mouse T-lymphoma cells and rat brain tissues reveals that the IP$_3$ receptor transcript in mouse T-lymphoma cells belongs to the short form (non-neuronal form) and not the long form (neuronal form) detected in rat brain tissue.

Scatchard plot analysis shows that high affinity binding occurs between ankyrin and the IP$_3$ receptor with a K$_D$ of 0.2 nM. Most importantly, the binding of ankyrin to the light density vesicles significantly inhibits IP$_3$ binding and IP$_3$-induced internal Ca$^{2+}$ release. These findings suggest that the cytoskeleton plays a pivotal role in the regulation of IP$_3$ receptor-mediated internal Ca$^{2+}$ release during lymphocyte activation.

Lymphocyte activation is initiated as a consequence of ligand-receptor binding which often generates the onset of phospholipase C activity (1-3). Hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C results in the formation of two intracellular second messengers, diacylglycerol, and inositol 1,4,5-trisphosphate (IP$_3$) (4). Diacylglycerol is an essential cofactor in activating members of the protein kinase C family of serine/threonine kinases (5). IP$_3$ is a physiological ligand known to mediate internal Ca$^{2+}$ release from intracellular Ca$^{2+}$ storage sites by binding to a specific receptor on certain intracellular membrane vesicles (4).

The IP$_3$ receptor has been identified by a number of investigators based on the specific binding of [$^{3}H$]IP$_3$ to internal vesicles in smooth muscle cells (6) and in several nonmuscle cell types such as liver (7), adrenal cortical cells (8, 9), brain cerebellum (10), and cerebellum Purkinje fibers (11). The primary intracellular storage sites for internal Ca$^{2+}$ were originally thought to be part of the endoplasmic reticulum (12-14). However, subcellular fractionation studies revealed that the distribution of Ca$^{2+}$-pumping, IP$_3$-responsive organelles does not correlate with markers for plasma membrane, endoplasmic reticulum, mitochondria, Golgi apparatus, or any other known organelles (15). Consequently, the IP$_3$-responsive vesicles appear to be a unique type which have been designated as "calciosomes" (16). Recently, an IP$_3$ receptor has been reported to exist on the plasma membrane of human T-lymphocytes (17). Since the structural and functional properties of this plasma membrane-associated IP$_3$ receptor have not been fully established, one cannot preclude the possibility that this surface IP$_3$ receptor may be structurally or functionally related to several different plasma membrane receptors for inositol 1,3,4,5-tetrakisphosphate (IP$_4$) or inositol hexakisphosphate (IP$_6$) (18, 19). Consequently, the subcellular localization of the IP$_3$ receptor still remains to be determined.

In mouse T-lymphoma cells, activation of phospholipase C by either a G$_{qo}$-like protein (3) or tyrosine kinase(s) (2) has also been shown to generate IP$_3$. Although an IP$_3$ appears to be required for inducing internal Ca$^{2+}$ release, the formation of receptor patching/capping (3, 20) and the general activation of lymphocytes (1, 2), very little is known at the present time concerning the nature of the IP$_3$ receptor and IP$_3$-inducible internal storage sites in lymphocytes. In this study, we have isolated and partially characterized a mouse T-lymphoma IP$_3$ receptor from a unique type of internal vesicle which bands at the relatively light density of 1.07 g/ml. This IP$_3$ receptor, which shares immunological cross-reactivity with the brain IP$_3$ receptor, displays both high affinity IP$_3$ binding and Ca$^{2+}$ ion channel properties. Most importantly, the interaction between mouse T-lymphoma IP$_3$ receptor and ankyrin, an important membrane-associated cytoskeletal protein, significantly inhibits IP$_3$ binding and IP$_3$-mediated internal Ca$^{2+}$ release.

MATERIALS AND METHODS

Cell Culture

The mouse T-lymphoma BW 5147 cell line (an AKR/J lymphoma line) were grown at 37°C in 5% CO$_2$/95% air using Dulbecco's...
modified Eagle's medium supplemented with 10% heat-inactivated horse serum (GIBCO), 1% penicillin, and 1% streptomycin.

**Cellular Fractionation**

The cells (suspended in 50 ml of ice-cold buffer consisting of 15 mM KCl, 1.5 mM Mg(OAc)₂, 1 mM dithiothreitol (DTT) and 10 mM HEPES (pH 7.0)) were disrupted by nitrogen cavitation in an Artisan homogenizer (Artisan Industries, Inc., Waltham, MA) held at 0°C using a pressure of 60 psi for 15 min. After disruption, 0.10 volume of 700 ml of 10% (w/v) sucrose containing 150 mM NaCl, 20 mM Tris-HEPES (pH 7.2), 0.3 mM MgCl₂, 10 mM phosphocreatine/creatine kinase (10 units/ml) (Boehringer Mannheim), 3.75 mM ruthenium red, 1 mM Mg-ATP, 0.5 mM EGTA, CaCl₂ was added to this reaction mixture for Ca²⁺ flux measurements. The amount of Ca²⁺ released from the light density vesicles was determined by a filtration method using Millipore filters (HAWP, 0.45 μm) and washing with a buffer composed of 120 mM KCl, 20 mM Tris-HEPES (pH 7.4), 150 mM NaCl, 0.05% Triton X-100 and 0.1% bovine serum albumin. The filter-associated radioactivity was analyzed by liquid scintillation counting.

**Transmission Electron Microscopy**

Lymphoma light density vesicles collected from the 15-25% sucrose interface (according to the procedures described above), were fixed with 2% glutaraldehyde in phosphate-buffered saline (pH 7.3), post-fixed with 1% OsO₄, dehydrated through a graded ethanol series, and embedded in Spurr's embedding medium. Ultrathin sections were cut on a Sorval MT2-B ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a JEOL electron microscope at 80 kV.

**Immunoblotting Techniques**

Isolated lymphoma light density vesicles (collected from 15-25% sucrose interface) and brain IP₃ receptor (obtained from rat cerebellar membranes) (10) were either directly spotted on a sheet of nitrocellulose and analyzed by a 7.5% polyacrylamide gel electrophoresis followed by transfer to nitrocellulose sheets. Subsequently, the nitrocellulose sheets were incubated with the following various immunoreagents such as monoclonal mouse anti-IP₃ receptor antibody (IPR.1; 10 μg/ml) or monoclonal anti-IP₃ receptor antibody (IPR.1; 10 ng/ml) followed by [3H]IP₃ binding. Binding was estimated in the presence of various concentrations of unlabeled IP₃ ranging from 10⁻⁶ M to 10⁻⁴ M. The binding reaction was terminated by adding 2.5 ml of cold phosphate-buffered saline (pH 7.4) and filtering through GF/B glass fiber filters that had been presoaked in phosphate-buffered saline containing 1% bovine serum albumin. The filter-associated radioactivity was analyzed by liquid scintillation counting.

**Ankyrin Binding Assay**

Human erythrocyte ankyrin was purified by the procedure of Bennett and Stenbeck (27) and labeled with Na₂³⁻ using IODO-GEN beads. ³²P-Ankyrin (1-10 ng) was incubated with a nitrocellulose sheet coated with purified IP₃ receptor (obtained from anti-IP₃ receptor affinity column chromatography according to the procedures described above) in a binding solution containing 20 mM Tris-HEPES (pH 7.4), 150 mM NaCl, 0.05% Triton X-100 and 0.1% bovine serum albumin. The filter-associated radioactivity was analyzed by autoradiographic analysis or counted in a γ counter (for Scatchard plot analysis). Background or nonspecific binding was determined by including a large excess of unlabeled ankyrin (at least 100-fold excess) in both dot assays and Scatchard plot analysis. The results were expressed as "specific binding" in which the background level of binding was subtracted.

**Ca²⁺ Flux Measurement in Light Density Vesicles and IP₃ Receptor-containing Phospholipid Vesicles (Liposomes)**

**Ca²⁺ Flux Measurement in Light Density Vesicles--**Ca²⁺ fluxes were studied in a reaction mixture containing 120 mM KCl, 20 mM Tris-HEPES (pH 7.2), 0.3 mM MgCl₂, 10 mM phosphocreatine/creatine kinase (10 units/ml) (Boehringer Mannheim), 3.75 μM ruthenium red, 1 mM Mg-ATP, 0.5 mM EGTA. CaCl₂ was added to this solution to generate a range of free Ca²⁺ concentration between 100 and 200 nM. Subsequently, [Ca⁹⁵⁴] (5-10 μCi/ml; 20 μCi/ml; Amersham) and light density vesicles (0.5 mg/ml) were added to the reaction mixture at 30 °C for 25 min. In Ca²⁺ release experiments, IP₃ (10-100 nM) was added to these Ca⁹⁵⁴-containing vesicles. The maximal amount of Ca²⁺ release occurred 10 s after the addition of IP₃. In some cases, low density vesicles were pretreated with either ankyrin (10 μg/ml) or monoclonal anti-IP₃ receptor antibody (IPR.1; 10 μg/ml) followed by the addition of IP₃ (10-100 nM) to the reaction mixture for Ca²⁺ flux measurements. The amount of Ca²⁺ released from the light density vesicles was determined by a filtration method using Millipore filters (HAWP, 0.45 μm) and washing with a buffer consisting of 120 mM KCl and 20 mM Tris-HEPES (pH 7.2).

**Ca²⁺ Flux Measurement in IP₃ Receptor-containing Phospholipid Vesicles (Liposomes)--**Purified IP₃ receptor (obtained from the pro-
The intracellular IP3 receptor of mouse T-lymphoma cells was purified and characterized. IP3 binding was measured by a method that allows for the isolation of the IP3 receptor from the plasma membrane and the subsequent measurement of its specific activity in the presence of varying concentrations of IP3. The IP3 receptor was found to be linked to the plasma membrane and to be associated with membrane-bound ribosomes. The receptor was purified by a combination of differential centrifugation and affinity chromatography, followed by SDS-PAGE and Western blot analysis. The purified IP3 receptor was shown to bind IP3 with high specificity and affinity. The receptor was found to be localized in the light density vesicles of the lymphoma cells, and its presence was confirmed by immunohistochemical analysis. The IP3 receptor was shown to be involved in the regulation of intracellular Ca2+ levels, which is critical for the activation of many cellular functions.

In summary, the IP3 receptor of mouse T-lymphoma cells was isolated and characterized, providing new insights into the mechanisms of Ca2+ signaling in lymphocytes. The results suggest that the IP3 receptor is a critical component of the Ca2+ signaling pathway in these cells, and its modulation may have important implications for the understanding of lymphocyte function and disease.
**IPS Receptor and Ankyrin Interaction in T-lymphoma Cells**

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**FIG. 1.** Separation of various lymphoma membranes on a discontinuous sucrose gradient and enzyme marker analysis of various lymphoma membranes on a discontinuous sucrose gradient. 

**a.** Fraction A represents soluble proteins (0–15% sucrose interface); fraction B represents light density vesicle membrane (15–25% sucrose interface); fraction C represents Golgi membranes (25–35% sucrose interface); fraction D represents plasma membranes (35–40% sucrose interface); and fraction E represents lysosome membranes and other large particles (e.g. ribosomes) (40–50% sucrose interface). 

**b.** An electron microscopic photograph of light density vesicle membranes collected from fraction C (15–25% sucrose interface) magnification $\times 80,000$. (Arrowheads in b indicate the heterogenous vesicle population.)

**c.** Immunoblot of calsequestrin (c-i) and calreticulin (c-ii) using light density vesicles (fraction B) and specific antibodies against these two proteins (sheep anti-calreticulin (c-i) and mouse anti-calsequestrin (c-ii)) (Nonimmune sheep serum and nonimmune mouse serum were used as controls. No staining was observed in these control samples (data not shown)).

**d.** $[^{3}H]$IP$_3$ binding assay; **e.** Galactosyltransferase assay (a Golgi marker); **f.** NADPH-dependent cytochrome c-reductase (an endoplasmic reticulum marker) (similar results were observed using sulphatase C assay as an independent marker of endoplasmic reticulum (data not shown)); **g.** $Na^+$/K$^+$-ATPase assay (a plasma membrane marker); **h.** $p$-N-acetyl-glucosaminidase assay (a lysosome marker). Fractions: A, 0–15% sucrose interface; B, 15–25% sucrose interface; C, 25–35% sucrose interface; D, 35–40% sucrose interface; and E, 40–50% sucrose interface. (Arrowheads in Fig. 1, c-h indicate maximal level of enrichment.)

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| Treatments                                      | Internal $[^{4}Ca^{2+}]$ release | % of maximal release |
|------------------------------------------------|---------------------------------|---------------------|
| 1. A23187                                       | 100                             | 0                   |
| 2. Buffer (no treatment)                        | 0                               | 0                   |
| 3. IP$_3$ (10–100 nM)                           | 44                              | 47                  |
| 4. Monoclonal anti-IP$_3$ receptor antibody (10 | 46                              | 46                  |
| $\mu$g/ml)                                      |                                 |                     |
| 5. Monoclonal anti-IP$_3$ receptor antibody (10 | 46                              | 46                  |
| $\mu$g/ml) + IP$_3$ (10–100 nM)                 |                                 |                     |
| 6. Nonimmune normal mouse IgG                   | 0                               | 0                   |
| (10 $\mu$g/ml)                                  |                                 |                     |

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The amount of $Ca^{2+}$ released for A23187-treated vesicle was designated as the maximal level of $Ca^{2+}$ release signal and experimental figures were compared to this maximal release. The incubation time for $[^{4}Ca^{2+}]$ release was 10 s. The data shown are the averages of triplicate determinants, which varied by less than 5% ($n=5$).

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**Effects of monoclonal anti-IP$_3$ receptor on $[^{4}Ca^{2+}]$ release from lymphoma light density vesicles**

The displacement curve reveals the presence of a single high affinity IP$_3$ receptor with a dissociation constant ($K_d$) of 1.6 ± 0.3 nM (mean ± S.D., $n=4$) (Fig. 2). The binding affinity of the light density vesicles for $[^{3}H]$IP$_3$ is comparable to those reported for internal vesicles isolated from other cell types (6).

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**Immunological Analyses of Mouse T-lymphoma IP$_3$ Receptor**—The IP$_3$ receptor in nonmuscle cells has been shown to be a homotetramer (2500 amino acid subunits, molecular mass ~260 kDa) with limited homology to the ryanodine receptor in striated muscle (38, 39). Using a polyclonal rabbit anti-IP$_3$ receptor antibody, we have determined that the T-lymphoma light density vesicles (Fig. 3, A and B) contain an IP$_3$ receptor analogous to brain IP$_3$ receptor (Fig. 3, D and E) with a molecular mass of ~260 kDa. In addition, a monoclonal mouse anti-IP$_3$ receptor antibody (IPR.1) which does not interfere IP$_3$ binding (Table II) and recognizes a short sequence at the C terminus cytoplasmic side of IP$_3$ receptor was also used. Similar immunocross-reactivity was observed using this newly developed IP$_3$ receptor antibody (Fig. 3, C and F).

Using immunofluorescence staining, we have found that the IP$_3$ receptor is preferentially associated with numerous vesicular structures located in the cytoplasm (Fig. 4B). Although the resolution of light microscopy is limited, no
**FIG. 2.** Binding of $[^3H]IP_3$ to light density vesicles. Light density vesicle membranes (collected from 15–25% sucrose interface) were incubated with $[^3H]IP_3$ in the presence of various concentrations of unlabeled IP$_3$ in binding buffer as described under “Materials and Methods.”

![binding graph](image)

**TABLE II**

| Samples                          | Specific $[^3H]IP_3$ binding | CPM x $10^{3}$/mg protein |
|----------------------------------|-----------------------------|---------------------------|
| No treatment (control)           | 20.5 ± 0.8                  |
| Ankyrin-treated                  | 2.4 ± 0.2                   |
| Monoclonal anti-IP$_3$ receptor-treated | 19.8 ± 0.4                  |

Cate that no cell surface label is detected using our newly developed monoclonal anti-IP$_3$ antibody (IPR.1) (Fig. 4A). Most importantly, binding of this monoclonal anti-IP$_3$ receptor antibody to 260-kDa protein-containing low density vesicles induces a significant amount of internal Ca$^{2+}$ release analogous to IP$_3$-mediated Ca$^{2+}$ stimulation (Table I). We believe this anti-IP$_3$ receptor antibody-induced Ca$^{2+}$ release is specific, since nonimmune normal mouse IgG shows no stimulation on Ca$^{2+}$ release activity (Table I). There is no additive effect on the stimulation of internal Ca$^{2+}$ release if both a monoclonal anti-IP$_3$ receptor antibody (IPR.1) and IP$_3$ are added together to the low density vesicles (Table I). These results suggest that the epitope which this monoclonal anti-IP$_3$ antibody (IPR.1) recognizes must be very close to the proposed Ca$^{2+}$ channel region in the C terminus of the IP$_3$ receptor protein (40). Also, the binding of this antibody to IP$_3$ receptor possibly induces a conformational change of the receptor which mimics the effect of IP$_3$ binding to the receptor. Together, these findings clearly indicate that the lymphoma 260-kDa protein is an IP$_3$ receptor-like molecule (Fig. 3, C and F).

**FIG. 3.** SDS-polyacrylamide gel electrophoresis analysis of lymphoma light density vesicle membrane proteins and rat brain cerebellum membrane proteins. A, total lymphoma light density vesicle membrane proteins. B, immunoblot of lymphoma light density vesicle membrane proteins with polyclonal rabbit anti-rat brain IP$_3$ receptor antibody. (As a control, nonimmune rabbit serum was used. No staining was detected on these samples (data not shown).) C, silver staining of purified lymphoma IP$_3$ receptor obtained from monoclonal mouse anti-IP$_3$ receptor-conjugated affinity column. (As a control, nonimmune mouse serum-conjugated column was used. No protein was detected on these columns (data not shown).) D, total rat brain cerebellum membrane proteins. E, immunoblot of rat brain cerebellum membrane proteins with polyclonal rabbit anti-rat brain IP$_3$ receptor antibody. F, silver staining of purified rat brain IP$_3$ receptor obtained from monoclonal mouse anti-IP$_3$ receptor-conjugated affinity column.

Table with data

- Log(IP$_3$)(M)
Receptors in mouse T-lymphoma cells using fluorescein-conjugated monoclonal anti-IP₃ receptor. A, staining of surface exposed IP₃ receptor (note that only a background level of label was detected); B, staining of intracellular IP₃ receptor (note that a significant amount of label was detected in vesicular structures).

Fig. 4. Immunofluorescence staining of intracellular IP₃ receptors in mouse T-lymphoma cells using fluorescein-conjugated monoclonal anti-IP₃ receptor.

Purified IP₃ receptor (100 µg/ml) was incorporated into phosphatidylcholine/phosphatidylserine vesicles (liposomes) as described under "Materials and Methods." These IP₃ receptor-containing phospholipid vesicles (liposomes) were used to measure IP₃-induced Ca²⁺ release. The Ca²⁺ flux measurement was initiated by adding 2 µCi of ⁴⁵Ca²⁺ to the IP₃ receptor-containing liposomes in the presence of IP₃ (~10 nM) as described under "Materials and Methods."

Fig. 5. Time course of IP₃-induced ⁴⁵Ca²⁺ flux in phospholipid vesicles (liposomes) reconstituted with the purified lymphoma IP₃ receptor (260-kDa protein). Purified IP₃ receptor was incorporated into phosphatidylcholine/phosphatidylserine vesicles (liposomes) as described under "Materials and Methods." These IP₃ receptor-containing phospholipid vesicles (liposomes) were used to measure IP₃-induced Ca²⁺ release. The Ca²⁺ flux measurement was initiated by adding 2 µCi of ⁴⁵Ca²⁺ to the IP₃ receptor-containing liposomes in the presence of IP₃ (~10 nM) as described under "Materials and Methods."

Fig. 6. The MAPPing products of the regional IP₃ receptor cDNA from mouse T-lymphoma cells and rat brain cerebellum. Lane 1, mouse T-lymphoma cells; lane 2, rat brain cerebellum; and lane 3, mouse T-lymphoma cells with GAPDH primers. (Markers for the molecular size (in base pairs) are shown on the right.)

Detection of the IP₃ Receptor Transcripts by MAPPing Techniques—Since two different IP₃ receptor transcripts derived by alternative splicing have been identified and shown to be expressed in a tissue-specific manner (neuronal versus non-neuronal tissues) (29), total RNA materials isolated from mouse T-lymphoma cells and rat brain cerebellum were analyzed for the presence of such specific transcripts by MAPPing techniques. MAPPing was developed to analyze RNAs in small numbers of cells. This technique utilizes reverse transcription of total cellular RNA to synthesize complementary DNA, followed by the polymerase chain reaction to specifically amplify DNA fragments of interest.

Previously, MAPPing techniques from various rat tissues and cell lines have revealed two distinct IP₃ receptor transcripts (29). A long form (also considered to be neuronal-specific) contains a 120-nucleotide insert between the two cAMP-dependent protein kinase phosphorylation consensus sequences, and is predominantly detected in adult brain tissues. A short form (also considered to be a non-neuronal type) lacks the insert, and is primarily found in fetal brain and peripheral tissues (29). Specific oligonucleotide primers were utilized in the PCR reactions to differentiate the long neuronal type from the short non-neuronal type of the IP₃ receptor transcripts. Our results with mouse T-lymphoma cells indicate the existence of the short form of the transcript (Fig. 6, lane 1); whereas the IP₃ receptor transcripts in the rat brain cerebellum appear to display the long form (Fig. 6, lane 2). These PCR products were further cloned using a TA cloning kit (Invitrogen Corp.) and sequenced. The nucleotide sequence data confirms that the PCR-amplified fragments represent the segment of the IP₃ receptor cDNA reported previously (29). As a control, the house keeping gene GAPDH primers were used to verify the specificity and sensitivity of MAPPing techniques (Fig. 6, lane 3). This finding suggests that only the non-neuronal form of the IP₃ receptor is synthesized in mouse T-lymphoma cells.

Interactions of IP₃ Receptors with the Cytoskeleton

Cytoskeleton proteins such as ankyrin have been shown to be involved in regulating a number of cellular activities including receptor patching and capping (41-46), cell adhesion (47-49), organelle movement, cell motility, protein secretion, and cell division (50, 51). Putney et al. (7) have reported that IP₃ receptor-containing vesicles may be attached to the plasma membrane through cytoskeletal elements such as actin. Van Bennett and co-workers (52) have also reported that...
purified lymphoma IPS receptor in the absence (i) and the presence of a 10-fold (ii) or 100-fold (iii) excess amount of unlabeled ankyrin.

![Scatchard plot analysis and dot assays of ankyrin binding to purified lymphoma IP[subscript 3] receptor.]

**FIG. 7.** Scatchard plot analysis and dot assays of ankyrin binding to purified lymphoma IP[subscript 3] receptor. [superscript 125]I-Ankyrin (1–10 ng) was incubated with a nitrocellulose sheet coated with purified IP[subscript 3] receptor (obtained from monoclonal mouse anti-IP[subscript 3] receptor (IPR.l) affinity column chromatography) according to the procedures described under "Materials and Methods." The results were expressed as "specific binding" in which the background level of binding was subtracted. A, Scatchard plot analysis of ankyrin binding to purified lymphoma IP[subscript 3] receptor. B, dot assays of [superscript 125]I-Ankyrin (5 ng) binding to purified lymphoma IP[subscript 3] receptor in the absence (i) and the presence of a 10-fold (ii) or 100-fold (iii) excess amount of unlabeled ankyrin.

**Fig. 8.** Effects of ankyrin on [superscript 45]Ca[superscript 2+] release in lymphoma light density vesicles. In these experiments, the amount of Ca[superscript 2+] released for IP[subscript 3]-treated vesicle was designated as the maximal level (or 100%) of Ca[superscript 2+] release signal and experimental figures were compared to this maximal release. The incubation time for [superscript 45]Ca[superscript 2+] flux was approximately 20 s. A, IP[subscript 3] (100 nm) alone; B, ankyrin (10 [mu]g/ml) + IP[subscript 3] (100 nm); C, monoclonal anti-IP[subscript 3] receptor antibody (10 [mu]g/ml); D, ankyrin (10 [mu]g/ml) + monoclonal anti-IP[subscript 3] receptor antibody (10 [mu]g/ml).

A complex consisting of IP[subscript 3] receptor, ankyrin, and GP180 can be isolated from brain tissue. In this study, we have used a newly developed in vitro assay which involves the use of nitrocellulose papers coated with purified lymphoma IP[subscript 3] receptor (obtained from anti-IP[subscript 3] antibody (IPR.l) affinity column chromatography as shown in Fig. 3C) to determine IP[subscript 3] receptor's binding to [superscript 125]I-ankyrin. Our data clearly indicate that the lymphoma IP[subscript 3] receptor binds to ankyrin (Fig. 7).

To further establish the specificity and affinity of IP[subscript 3] receptor's binding to ankyrin, we have incubated [superscript 125]I-ankyrin with IP[subscript 3] receptor-coated nitrocellulose sheets in the absence (Fig. 7B (i)) and presence of various concentrations of unlabeled ankyrin (Fig. 7B (ii) and (iii)). Scatchard plot analysis reveals the presence of a single high affinity class of ankyrin binding sites on IP[subscript 3] receptor with a dissociation constant (Kd) of 0.2 nm (Fig. 7A).

Finally and most importantly, we have found that the binding of ankyrin to the IP[subscript 3] receptor in light density vesicles significantly inhibits IP[subscript 3] binding (Table II) and IP[subscript 3]-stimulated internal Ca[superscript 2+] release (Fig. 8, A and B). This ankyrin-mediated inhibitory effect on IP[subscript 3] function in these vesicles (Fig. 8, A and B). Alternatively, ankyrin binding may cause some conformational changes at the regulatory domain(s) of IP[subscript 3] receptor resulting in a decrease in IP[subscript 3] binding as well as a reduction in the potency of IP[subscript 3] in releasing Ca[superscript 2+] as shown previously by protein kinase A-mediated phosphorylation in cerebellar membranes (53). Currently, we are using in vitro mutagenesis and deletion mutational techniques to define further the ankyrin-binding domain(s) on mouse T-lymphoma IP[subscript 3] receptor.

Ankyrin is well known to bind a number of plasma membrane-associated proteins including band 3 (54); two other members of the anion exchange gene family (55, 56), Na+/K+-ATPase (57–59), amiloride-sensitive Na+ channel (60), the voltage-dependent Na+ channel (61, 62) and GP85(CD44) (43–48). The fact that ankyrin also binds to intracellular proteins such as the IP[subscript 3] receptor on light density vesicles suggests that ankyrin may be involved in multiple functions during cellular regulation. In lymphocytes, for example, ankyrin may play a pivotal role in linking surface adhesion molecules (e.g. GP85(CD44)) and intracellular Ca[superscript 2+] storage organelle membrane proteins (e.g. IP[subscript 3] receptor). This ankyrin-based linkage between plasma membrane proteins and organelle Ca[superscript 2+] storage channel molecules may be critically important for receptor-mediated signal transduction during lymphocyte activation.

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