Inhibition of nuclear vesicle fusion by antibodies that block activation of inositol 1,4,5-trisphosphate receptors

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ABSTRACT Inositol 1,4,5-trisphosphate (IP₃) receptors are ligand-gated channels that release intracellular Ca²⁺ stores in response to the second messenger, IP₃. We investigated the potential role of IP₃ receptors during nuclear envelope assembly in vitro, using Xenopus egg extracts. Previous work suggested that Ca²⁺ mobilization is required for nuclear vesicle fusion and implicated IP₃ receptor activity. To test the involvement of IP₃ receptors using selective reagents, we obtained three distinct polyclonal antibodies to the type 1 IP₃ receptor. Pretreatment of membranes with two of the antibodies inhibited IP₃-stimulated Ca²⁺ release in vitro and also inhibited nuclear vesicle fusion. One inhibitory serum was directed against 420 residues within the “coupling” domain, which includes several potential regulatory sites. The other inhibitory serum was directed against 95 residues near the C terminus and identifies an inhibitory epitope(s) in this region. The antibodies had no effect on receptor affinity for IP₃. Because nuclear vesicle fusion was inhibited by antibodies that block Ca²⁺ flux, but not by control and preimmune antibodies, we concluded that the activation of IP₃ receptors is required for fusion. The signal that activates the channel during fusion is unknown.

In higher eukaryotic cells, the nuclear envelope, endoplasmic reticulum (ER), and Golgi complex are each disassembled early in mitosis (1). The end products of mitotic disassembly include small vesicles derived from the ER and nuclear envelope; these mitotically formed vesicles retain the membrane and luminal proteins of these organelles as well as their Ca²⁺ stores. During anaphase and telophase, the nuclear vesicles bind to chromosomes and fuse together to reform the nuclear envelope. We have studied the mechanism of nuclear vesicle fusion using Xenopus egg extracts (2, 3). Xenopus eggs are arrested in metaphase and contain abundant nuclear structural components (including vesicles, lamins, and nuclear pore complex subunits) in the mitotically disassembled state, stockpiled for use during early embryogenesis. We can fractionate these eggs to separate the soluble cytosolic components from the membranes. Nuclear assembly is then reconstituted in vitro by combining cytosol and membranes with an exogenous source of DNA, demembranated sperm chromatin (2). The cytosol-dependent fusion of nuclear vesicles bound to chromatin is then assayed by light microscopy.

Our previous studies (4) showed that Ca²⁺ mobilization is required for nuclear vesicle fusion and implicated the inositol 1,4,5-trisphosphate (IP₃) receptor (5, 6)—an intracellular Ca²⁺ channel—in this Ca²⁺ release event: both heparin, a nonselective inhibitor of IP₃ receptors, and the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA) inhibited fusion in an IP₃-reversible manner. To test the proposed role of IP₃ receptors during nuclear vesicle fusion, we needed reagents that selectively targeted IP₃ receptor function in Xenopus egg extracts.

The type 1 IP₃ receptor is the only isoform that has been identified in Xenopus oocytes and eggs (7); to date, no additional IP₃ receptor isoforms or other intracellular Ca²⁺ channels, such as the ryanodine receptor (8), have been reported. IP₃ receptors are located on the nuclear envelopes of Xenopus oocytes (7, 9) and other cell types (10, 11). Xenopus oocyte nuclear envelopes also contain IP₃-releasable Ca²⁺ stores (9); similarly, nuclei isolated from other cell types exhibit IP₃-stimulated Ca²⁺ release (12, 13) and ATP-dependent Ca²⁺ uptake (12). Patch- clamp experiments showed that the IP₃ receptors on Xenopus oocyte nuclei have channel properties similar to those of purified mammalian IP₃ receptors (9). For example, channel activity is gated by IP₃, modulated by cytoplasmic Ca²⁺, and can be stimulated by millimolar levels of ATP. The Xenopus nuclear channels have four conductance levels and are not desensitized by high concentrations of IP₃ (9).

To test the role of IP₃ receptors in nuclear vesicle fusion, we used antibodies directed against different structural domains of the receptor. The channel is a tetramer of 307-kDa subunits, and each subunit has an IP₃-binding site near its N terminus (8). The large middle region, designated the coupling domain, contains consensus sites for phosphorylation and ATP binding and may regulate receptor activity (7). The coupling domain links the N-terminal IP₃-binding site to transmembrane domains near the C terminus, which form the channel; Ca²⁺ flux through the channel is mediated by the cytoplasmic C-terminal region (5, 6). In this report, we used antibodies to the coupling domain and the cytoplasmic C terminus of the mouse type 1 IP₃ receptor, which is 90% identical to the type 1 receptor in Xenopus (7). Our approach was to pretreat the Xenopus egg membrane fraction with sera to allow antibody binding to IP₃ receptors, wash the membranes, and then assay their ability to bind IP₃, undergo IP₃-stimulated Ca²⁺ flux, and form nuclear envelopes.

MATERIALS AND METHODS

Antisera Production. The M and 3′β₁ sera as defined in Fig. 1 were raised in rabbits immunized with the appropriate GST-fusion proteins electroeluted from SDS/PAGE gel slices (D.D.L., unpublished data). Antigen for the 3′β₂ serum was prepared by eliciting the soluble GST-fusion protein from glutathione-Sepharose beads (K.M.C.S., unpublished data).

Membrane and Cytosol Fractions from Xenopus Eggs. Egg extracts were prepared and fractionated as described (16). In brief, the eggs were lysed by a 10,000 g centrifugation, and the crude cytoplasm was centrifuged at 200,000 g to fractionate the cytosol and membranes. The membrane fraction consists of mitochondria and the mitotic forms of all membranous organelles. The cytosol fraction is arrested in interphase by adding cycloheximide and is also supplemented with

Abbreviations: IP₃, inositol 1,4,5-trisphosphate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid; GST, glutathione S-transferase.†To whom reprint requests should be addressed.

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an ATP-regenerating system. This interphase cytosol promotes nuclear envelope assembly when reconstituted with nuclear vesicles and chromatin.

**Pretreatment of Membranes with Antibodies.** Membranes (10 µl at 100 mg/ml) were pretreated for 1 hr (or 30 min) at 23°C with an equal volume of immune or preimmune serum. All reagents used in pretreatments were incubated with Calcium Sponge S (Molecular Probes) to remove contaminating divalent cations. Unbound antibodies were removed by dilution into 100 µl of membrane wash buffer (250 mM sucrose/50 mM KCl/2.5 mM MgCl2/50 mM Hepes, pH 8.0/1 mM dithiothreitol/0.5 mM ATP/1 µg of aprotinin per ml/1 µg of leupeptin per ml) and centrifugation (100,000 × g for 15 min at 4°C; Beckman TL-100).

Longer pretreatment time (1 hour versus 30 min) was responsible for the decrease in the extent of Ca²⁺ release from vesicles treated with M preimmune serum, relative to 3'β₁ preimmune serum (Fig. 2). Serum pretreatment appeared to decrease the total amount of IP₃-releasable Ca²⁺, but the rate of Ca²⁺ release was similar in all preimmune and buffer

**Fig. 1.** (A) Diagram of the mouse type 1 IP₃ receptor (2749 amino acids) (6, 14). Thick vertical lines indicate the six transmembrane spans (15); the N- and C-terminal domains are cytoplasmic. The bars labeled M (amino acids 1379–1798) and 3'β₁ (amino acids 2604–2698) indicate regions fused to glutathione S-transferase (GST) and used to generate polyclonal antisera. Antisera 3'β₁ and 3'β₂ were generated by using independent preparations of GST-fusion protein. (B and C) Immune sera specifically immunoprecipitate IP₃ receptors from Xenopus egg membranes. Gels of the immunoprecipitates are visualized by silver stain (B), and by Western blotting with the 3'β₂ antiserum (C). Stars indicate the IP₃ receptor.

**Fig. 2.** M and 3'β₁ antisera inhibit IP₃-stimulated Ca²⁺ release from membranes. Graphs show the time course of IP₃-stimulated Ca²⁺ release from membranes pretreated with M (A), 3'β₁ (B), or 3'β₂ (C) immune ("imm") or preimmune ("pre") serum. Stars indicate the time of IP₃ injection. The fura-2 fluorescence ratio plotted is directly proportional to the extract free Ca²⁺ concentration. Each pretreatment was performed two (3'β₂) or four (M and 3'β₁) times with at least two different extracts and yielded consistent results; typical time courses are shown.
controls. This effect of serum on the total Ca\(^{2+}\) released was nonspecific; pretreatment with sera, but also with other reagents, damages membranes, and this damage increases with the length of pretreatment and the number of washing steps.

**Efficiency of Antibody Binding to Intact Vesicles.** Membranes were pretreated with the M, 3′β1, or 3′β2 immune sera as described above and then incubated in cytosol for 1 hr at 23°C. The membranes were pelleted [10 min at 14,000 rpm (12,000 \( \times \) g) in an Eppendorf Microfuge at 4°C], and the antibodies that remained bound were quantified as described (17) by incubating membranes with an excess of alkaline phosphatase-conjugated secondary antibodies, developing the enzyme label with the soluble substrate p-nitrophenyl phosphate (Sigma), and reading the absorbance at 405 nm. Based on the absorbance readings, the relative amounts of M, 3′β1, and 3′β2 antibodies bound to membranes were 0.98, 1.0, and 0.89, respectively.

**Ca\(^{2+}\) Flux Measurements.** Serum-pretreated membranes were resuspended in 100 \( \mu \)l of cytosol containing 50 \( \mu \)M fura-2-dextran (Molecular Probes). Samples (2 \( \mu \)l) were immersed in silicon oil and injected with IP\(_3\) (\( \sim 20 \) nl of a 1 mM stock solution) by using a Picospritzer II apparatus (General Valve, Fairfield, NJ). The basal free Ca\(^{2+}\) concentration in the extract (1.0 on the y axis of Fig. 2) is generally 350–450 nM (data not shown; see ref. 4), which is very similar to that in the intact egg (\( \sim 400 \) mM). The 350 nm/380 nm ratio imaging of fura-2 fluorescence was done as described (4) except that free Ca\(^{2+}\) concentrations were not calculated. The relative change in the fura-2 fluorescence ratio is proportional to the free Ca\(^{2+}\) concentration and is the most direct way to express this data. Fluorescence ratios were normalized to facilitate the comparison of Ca\(^{2+}\) release between different samples: the ratio at each time point was divided by the average fluorescence ratio of the sample before IP\(_3\) addition.

**Nuclear Assembly Reactions and Fusion Assays.** Sperm chromatin free from nuclear membrane was prepared as described (16) and placed with pretreated membranes and cytosol at 25°C to initiate nuclear assembly. Nuclear envelope formation and growth were assayed by phase-contrast microscopy, and nuclear envelope surface areas were calculated as described (4). The effects of all three sera on vesicle fusion and nuclear envelope assembly were reproducible in different batches of extracts. However, the extracts themselves varied in robustness. The extract used in Fig. 3C was quite robust and grew nuclei to an unusually large size; the extract used in Fig. 3A and B was more typical.

**Measurement of IP\(_3\) Binding Affinity.** IP\(_3\) binding assays were done as described (18) with several modifications. Membranes were washed in PHEDS buffer (20 mM Hepes, pH 7.5/10% sucrose/1 mM EGTA/1 mM dithiothreitol/1 mg of apotinin per ml/1 mg leupeptin per ml), pelleted at 200,000 \( \times \) g for 15 min at 4°C, and pretreated with antibodies as described above. "Buffer" indicates membranes that were pretreated with HECK buffer (25 mM Hepes, pH 7.5/5 mM EGTA/0.1 M KCl). After pretreatment, membranes were diluted with HECK buffer to a final protein concentration of 0.4 mg/ml and incubated with 2 nM [\(^{3}H\)]IP\(_3\) (21 Ci/mmol; NEN; 1 Ci = 37 GBq) for 15 min at 4°C. Aliquots (495 \( \mu \)l) were placed in tubes containing 5 \( \mu \)l of nonradioactive IP\(_3\) to give a range of unlabeled IP\(_3\) concentrations from 0 to 200 nM. The blank tube contained 1.4 \( \mu \)l of unlabeled IP\(_3\) to measure nonspecific binding. The samples were incubated for 15 min at 4°C to allow displacement of bound [\(^{3}H\)]IP\(_3\). Membranes were pelleted in an Eppendorf Microfuge at 14,000 rpm for 15 min at 4°C and solubilized in 1% SDS. [\(^{3}H\)]IP\(_3\) displacement was measured by counting both the membrane-bound (pellet) and free (supernatant) label from each tube and subtracting the nonspecific binding (<10% of total). To determine the apparent dissociation constant (K\(_d\)), the displacement curves from each of three or more independent binding assays were fit with a logistic function by using the program SIGMAPLOT (Jandel, San Rafael, CA), and the results were averaged.

**Immunoprecipitations, Gels, and Immunoblotting.** For each immunoprecipitation, 200 \( \mu \)g of membranes were solubilized in 400 \( \mu \)l of radioimmuno precipitation assay (RIPA) buffer (10 mM Tris/0.1% SDS/1% deoxycholate/1% Nonidet P-40/0.15 M NaCl/1 mM EDTA/10 \( \mu \)g of aprotinin per ml/10 \( \mu \)g of leupeptin per ml, pH 7.6) and precleared with protein A beads. Immune or preimmune serum (4 \( \mu \)l) was added, and the samples were incubated for 5 hr at 23°C. Antibodies were isolated on protein A-conjugated beads and eluted with SDS reducing sample buffer at 100°C for 5 min. Two sets of immunoprecipitates were subjected to SDS/7% PAGE: one set was silver-stained as described (19), and the other was transferred to nitrocellulose and incubated with 3′β2 antiserum at 1:1000 dilution for 12 hr at 4°C. Bound antibodies were detected by sequential exposure to alkaline phosphatase-conjugated secondary antibodies (Bio-Rad) and phosphatase substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). On the silver-stained gel, faint bands that probably correspond to IgGs and dimers are visible: one between 105 and 216 kDa and the other between 70 and 105 kDa.

**RESULTS**

Three polyclonal antisera were raised against two distinct regions of the mouse type 1 IP\(_3\) receptor (Fig. 1A): the coupling domain (antisera M; 420 residues) and the cytoplasmic C terminus (antisera 3′β1 and 3′β2; 95 residues). The immune, but not the preimmune, sera specifically recognized the *Xenopus* IP\(_3\) receptor. All three antisera immunoprecipitated from the *Xenopus* egg membrane fraction a protein of \~250 kDa (Fig. 1B and C) consistent with the mobility of IP\(_3\) receptors on SDS/PAGE (7, 8, 18) and recognized the same protein on Western blots (Fig. 1C).

To determine if the antibodies affected IP\(_3\) receptor function, we measured IP\(_3\)-stimulated Ca\(^{2+}\) release in vitro. Isolated membranes were preincubated with either immune or preimmune serum, washed to remove unbound antibodies, and resuspended in cytosol containing 50 \( \mu \)M fura-2-dextran. A 2-\( \mu \)l droplet of this mixture was suspended in silicon oil on a microscope coverslip, and cytosolic free Ca\(^{2+}\) levels were measured by ratio-imaging before and after injecting exogenous IP\(_3\) into the droplet (final IP\(_3\) concentration, 10 \( \mu \)M). Pretreatment with the M or 3′β1 antisera significantly inhibited Ca\(^{2+}\) release from luminal stores relative to preimmune controls (Fig. 2A and B). The initial rate of Ca\(^{2+}\) release was decreased by a factor of 9 by M antisera or 6 by 3′β1 antisera, and each decreased the peak free Ca\(^{2+}\) concentration by a factor of 3. Pretreatment with the 3′β2 antisera had no reproducible effect on Ca\(^{2+}\) flux (Fig. 2C). We ascribe the different effects of the 3′β1 and 3′β2 antisera to recognition of inhibitory and noninhibitory epitopes, respectively, within the 3′β region.

To determine if the antibodies remained bound to membranes at equivalent levels after pretreatment, we pretreated membranes with M, 3′β1, or 3′β2 immune sera, incubated the membranes in cytosol for 1 hr, and then quantitated antibody binding. The relative amounts of M, 3′β1, and 3′β2 antibodies bound to membranes were 0.98, 1.0, and 0.89, respectively. This result showed that similar amounts of all three immune antibodies continued to bind membrane IP\(_3\) receptors after a 1-hr exposure to cytosol.

We controlled for nonspecific serum effects by saturating the antigen-binding sites of the immune M and 3′β1 sera with the corresponding fusion proteins prior to membrane pretreatment. IP\(_3\)-stimulated Ca\(^{2+}\) release occurred normally in membranes incubated with the preadsorbed sera, compared with mock-pretreated controls (data not shown). This showed that
the inhibition of IP3-stimulated Ca\(^{2+}\) flux by the M and 3'\(\beta_1\) immune sera was due to specific immune recognition of the IP3 receptor.

It was formally possible that the immune sera behaved as agonists to stimulate channel opening, which might discharge the luminal Ca\(^{2+}\) stores during pretreatment. To test this possibility, untreated vesicles were mixed with cytosol containing fura-2-dextran and placed in silicon oil on a coverslip. We then measured free Ca\(^{2+}\) concentrations before and after injecting either M or 3'\(\beta_1\) immune serum into the droplet (final antibody concentration, 0.1 mg per ml). No Ca\(^{2+}\) release was detected over a 3-min time course (data not shown). In separate experiments, we showed that the antibodies did not affect IP3 binding to the receptor (Table 1). Based on these controls and the experiments in Fig. 2, we concluded that the M and 3'\(\beta_1\) antibodies bind to IP3 receptors and specifically inhibit channel opening in response to IP3 binding.

The above experiments showed that all three immune antibodies bind to Xenopus IP3 receptors, but only the M and 3'\(\beta_1\) antibodies inhibit channel opening in response to exogenous IP3. We therefore used these antibodies to test the hypothesis that IP3 receptor activation is required for nuclear vesicle fusion. Membranes were pretreated as before with immune or preimmune serum, washed, and resuspended in cytosol plus sperm chromatin free of nuclear membrane. These nuclear assembly reactions were monitored over time by light microscopy to assay vesicle fusion and nuclear envelope growth. Vesicle fusion was inhibited significantly in samples pretreated with M or 3'\(\beta_1\) antiserum, relative to preimmune controls (Fig. 3A and B). In contrast, pretreatment with 3'\(\beta_2\) antiserum had no detectable effect on fusion or nuclear envelope growth (Fig. 3C). The noninhibitory 3'\(\beta_2\) antibodies provided a further control: they showed that antibody binding per se to this C-terminal region does not sterically inhibit fusion. Consistent with this, Fab fragments made from either M or 3'\(\beta_1\) antiserum also inhibited nuclear vesicle fusion, albeit less efficiently than intact antibodies (data not shown; we attribute the difference to dilution of the Fab fragments). Because fusion was inhibited by the M and 3'\(\beta_1\) antibodies, which block Ca\(^{2+}\) flux, but not by 3'\(\beta_2\) antibodies, we concluded that IP3 receptor activation is required for vesicle fusion during nuclear envelope assembly.

**DISCUSSION**

We characterized two domain-specific antibodies as selective antagonists of type 1 IP3 receptors. We found that the M and 3'\(\beta_1\) antibodies specifically inhibited channel opening in *vitro* in response to IP3 binding. When vesicles were pretreated with antibodies that inhibit channel activation, nuclear vesicle fusion was strongly inhibited. These results demonstrate a clear correlation between channel activation and vesicle fusion during nuclear envelope assembly.

Our findings are unrelated to previous studies in which nuclear envelope breakdown was used to monitor the onset of mitosis during early embryogenesis. For example, in sea urchin embryos IP3 stimulates early entry into mitosis; thus, all mitotic events, including nuclear envelope breakdown, occur prematurely (20). However, there is no evidence that IP3 signaling is directly involved in the mechanism of nuclear envelope disassembly. In *Xenopus* egg extracts that contain the activated mitotic cdc2 kinase, mitotic nuclear breakdown occurs normally when heparin is used to block IP3 receptor activation and when free Ca\(^{2+}\) is buffered by BAPTA or EGTA (K.M.C.S. and K.L.W., unpublished data).

One of our inhibitory sera, 3'\(\beta_1\), is directed against 95 residues within the cytoplasmic C-terminal region. A monoclinal antibody (mAb 18A10) to a C-terminal epitope has been shown to inhibit receptor activation *in vivo* and *in vitro* (21). However, the 18A10 epitope (amino acids 2736–2747; ref. 22) is distinct from the 3'\(\beta_1\) polypeptide (amino acids 2604–2698). Thus, the 3'\(\beta_1\) antiserum defines a new inhibitory epitope(s) within the C-terminal domain. The inhibitory epitopes recognized by the 3'\(\beta_1\) and M sera have not yet been identified.

The affinity of *Xenopus* egg membranes for IP3, ~11 nM, was comparable to that reported for *Xenopus* oocyte microsomes, 5 nM (18), and our antiserum had no effect on the affinity of receptors for IP3. In contrast, mAb 18A10 increases receptor affinity for IP3 about 2-fold (22). The additional effect of mAb 18A10 on IP3 binding suggests that this antibody inhibits channel activity by a different mechanism than the 3'\(\beta_1\) antibodies.

![Fig. 3](image-url)  
**Fig. 3.** M and 3'\(\beta_1\) antisera inhibit nuclear vesicle fusion. Membranes were preincubated with M (A), 3'\(\beta_1\) (B), or 3'\(\beta_2\) (C) antiserum ("imm") or the corresponding preimmune serum ("pre"). Each graph plots the change in average nuclear envelope surface area in units of 10\(^{-3}\) μm\(^2\). The error bars represent one standard error of the mean. A star indicates the time point by which nuclear vesicle fusion had formed intact nuclear envelopes around 100% of the chromatin. Nuclear envelope formation did not occur to completion in the 3'\(\beta_1\) immune sample (B) and occurred simultaneously in the 3'\(\beta_2\) immune and preimmune samples (C). Serum pretreatment had no detectable effect on vesicle binding to chromatin or chromatin structure. This experiment was done three times for 3'\(\beta_2\) and more than five times each for M and 3'\(\beta_1\); typical time courses are shown.
There are now three lines of evidence that IP$_3$ receptors mediate vesicle fusion during nuclear envelope assembly: fusion is inhibited by BAPTA (which suppresses mobilized Ca$^{2+}$ and also competes with IP$_3$ for binding to IP$_3$ receptors; ref. 23), fusion is inhibited by heparin (a potent but nonselective antagonist of IP$_3$ receptors), and fusion is inhibited by two antibodies that selectively block IP$_3$ receptor activation. Our experiments did not address the location of the IP$_3$ receptors involved in nuclear vesicle fusion. We cannot exclude the possibility that the fusion of chromatin-bound vesicles is triggered by activated IP$_3$ receptors on other vesicles, but this is unlikely given that free Ca$^{2+}$ ions are rapidly cleared from the cytoplasm after mobilization (24). We hypothesize that the function of IP$_3$ receptors in nuclear vesicle fusion is analogous to that of voltage-gated Ca$^{2+}$ channels in synaptic vesicle fusion (25, 26). However, we do not understand the signaling mechanism that triggers channel opening during fusion, nor do we know how, or if, this signal is coupled to cell cycle progression (specifically, exit from metaphase). The mass of IP$_3$ increases at the time of first cleavage in Xenopus embryos (27), which coincides roughly with the time of nuclear envelope assembly. It is therefore possible that the receptors are activated or sensitized to IP$_3$ generated elsewhere during anaphase and telophase; however, for this model to be feasible, these nonlocal sources of IP$_3$ must be operative in our extracts, which are stably arrested in interphase. We favor the notion that IP$_3$ receptors are activated by a signal generated locally, at the site of fusion (28). This signal could involve local IP$_3$ production, sensitization of the receptor (for example, via phosphorylation), or mechanical coupling of IP$_3$ receptors to components of the fusion complex.

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