

# Inositol 1,4,5-Trisphosphate Receptors Selectively Localized to the Acrosomes of Mammalian Sperm

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**Abstract.** Calcium flux is required for the mammalian sperm acrosome reaction, an exocytotic event triggered by egg binding, which results in a dramatic rise in sperm intracellular calcium. Calcium-dependent membrane fusion results in the release of enzymes that facilitate sperm penetration through the zona pellucida during fertilization. We have characterized inositol 1,4,5-trisphosphate (IP<sub>3</sub>)-gated calcium channels and upstream components of the phosphoinositide signaling system in mammalian sperm. Peptide antibodies colocalized G $\alpha_{q/11}$  and the  $\beta$ 1 isoform of phospholipase C (PLC $\beta$ 1) to the anterior acrosomal region of mouse sperm. Western blotting using a polyclonal antibody directed against purified brain IP<sub>3</sub> receptor (IP<sub>3</sub>R) identified a specific 260 kD band in 1% Triton X-100 extracts of rat, hamster, mouse and dog sperm. In each species, IP<sub>3</sub>R immunostaining localized to the acrosome cap. Scatchard analysis of [<sup>3</sup>H]IP<sub>3</sub> binding to rat sperm sonicates revealed a curvilinear plot with high affinity ( $K_d = 26$  nM,  $B_{max} = 30$  pmol/mg) and low affinity ( $K_d = 1.6$   $\mu$ M,

$B_{max} = 550$  pmol/mg) binding sites, reflecting among the highest receptor densities in mammalian tissue. Immunoelectron microscopy confirmed the acrosomal localization in rat sperm. The IP<sub>3</sub>R fractionated with acrosomes by discontinuous sucrose gradient centrifugation and was enriched in the medium of acrosome-reacted sperm. ATP-dependent <sup>45</sup>Ca<sup>2+</sup> loading of digitonin permeabilized rat sperm was decreased by 45% in the presence of 10  $\mu$ M IP<sub>3</sub>. The IP<sub>3</sub>-mediated release of calcium was blocked by heparin. Thapsigargin, a sesquiterpene lactone inhibitor of the microsomal Ca<sup>2+</sup>-ATPase, stimulated the acrosome reaction of mouse sperm to the same extent as the Ca<sup>2+</sup> ionophore, A23187. The failure of caffeine and ryanodine to affect calcium accumulation suggested that thapsigargin acted through an IP<sub>3</sub>-sensitive store. The presence of G $\alpha_{q/11}$ , PLC $\beta$ 1 and a functional IP<sub>3</sub>R in the anterior acrosomal region of mammalian sperm, as well as thapsigargin's induction of the acrosome reaction, implicate IP<sub>3</sub>-gated calcium release in the mammalian acrosome reaction.

**T**HE acrosome is a specialized membrane-bound organelle located in the head of sperm cells that contains a rich store of hydrolytic enzymes. Binding of the sperm head to the zona pellucida (ZP)<sup>1</sup> of the egg triggers the "acrosome reaction," an exocytotic event that releases these enzymes from the acrosome to facilitate sperm penetration through the ZP, leading to sperm-egg fusion. The acrosome reaction is crucial for fertilization. Acrosomal exocytosis requires extracellular calcium, with zona binding initiating a signaling cascade that leads to calcium influx (Kopf and Gerton, 1991). The specific signal transduction mechanisms that trigger the acrosome re-

action have not been fully clarified. ZP3, a protein of the mouse ZP, initiates the signal transduction process upon sperm binding (Bleil and Wassarman, 1983). Progesterone has also been shown to induce acrosomal exocytosis (Osman et al., 1989; Blackmore et al., 1990, 1991; Roldan et al., 1994). Increases in intracellular calcium (Lee and Storey, 1989), pH (Florman et al., 1989), cAMP (Noland, T. D., D. L. Garbers, and G. S. Kopf. 1988. *Biol. Reprod.* 38:94a), IP<sub>3</sub> (Domino and Garbers, 1989; Thomas and Meizel, 1989), and diacylglycerol (DAG) (Roldan et al., 1994) have been associated with ligand-induced acrosomal exocytosis in sperm. Heterotrimeric G proteins of the G<sub>i</sub> and G<sub>z</sub> classes have been identified in mammalian sperm, while there is no evidence for the presence of G<sub>s</sub> and G<sub>o</sub> proteins (Glassner et al., 1991). Recently, the ZP has been shown to activate G<sub>i1</sub> and G<sub>i2</sub> in mouse sperm preparations (Ward et al., 1994). The direct roles and relative importance of various signaling processes in promoting calcium influx and triggering acrosomal exocytosis remain unclear.

In the present study, we demonstrate the selective local-

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1. *Abbreviations used in this paper:* DAG, diacylglycerol; BHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IP<sub>3</sub>R, inositol trisphosphate receptor; PLC $\beta$ 1, phospholipase C $\beta$ 1; ZP, zona pellucida.

ization of signaling elements related to the phosphoinositide system in mammalian sperm. The heterotrimeric G proteins  $G_q$  and  $G_{11}$  selectively activate the  $\beta$  isoforms of the phosphatidylinositol-specific PLC family, and most effectively stimulate the  $\beta_1$  isotype (Lee et al., 1992). We have localized  $G_{\alpha_{q/11}}$  and PLC $\beta_1$  to the anterior acrosomal region. We have also visualized IP $_3$ R in the acrosome cap of mammalian sperm and have enriched for IP $_3$ R protein in acrosomal subcellular fractions. We have demonstrated specific calcium release in digitonin permeabilized sperm by IP $_3$  and observed stimulation of the acrosome reaction by thapsigargin, apparently by releasing calcium from an acrosomal IP $_3$ -sensitive calcium store.

## Materials and Methods

### Materials

The elite ABC immunoperoxidase staining kit and vectashield mounting medium were purchased from Vector laboratories (Burlingame, CA), electron microscopy reagents from Polysciences (Warrington, PA), the ECL detection system from Amersham (Arlington Heights, IL), DC protein assay reagents from Biorad (Hercules, CA), precast Tris-glycine gels from Novex (San Diego, CA), [ $^3$ H]IP $_3$  and  $^{45}\text{Ca}^{2+}$  from New England Nuclear DuPont (Boston, MA), unlabeled IP $_3$  from LC laboratories (Woburn, MA), low molecular weight heparin, digitonin, phosphocreatine, and creatine phosphokinase from Sigma Chemical Co. (St. Louis, MO), M199 media from GIBCO/BRL (Gaithersburg, MD), and A23187, thapsigargin, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ), and ryanodine from Calbiochem (San Diego, CA).

**Antibodies.** Peptide antibodies to  $G_{\alpha_{q/11}}$  and PLC $\beta_1$  and the corresponding immunizing peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-IP $_3$ R polyclonal antibody used in this study has been described previously (Sharp et al., 1993). FITC-conjugated goat anti-rabbit secondary antibodies and 5-nm colloidal gold-conjugated anti-rabbit secondary antibodies were purchased from Boehringer Mannheim (Indianapolis, IN) and Amersham, respectively.

**Animal Species.** 325 g male Sprague-Dawley rats were purchased from Sasco, Inc. (Boston, MA) and CD1 retired breeder male mice from Charles River (Wilmington, MA).

[ $^3$ H]IP $_3$  binding, fractionation, and  $^{45}\text{Ca}^{2+}$  flux experiments were conducted using rat sperm in order to readily obtain sufficient sperm samples to perform the replicates required of each experiment. Acrosome reaction assays were performed using mouse sperm because this species is most widely used to study acrosomal exocytosis due to extensive characterization of the physiologic ZP3-induced mouse acrosome reaction (Bleil and Wassarman, 1980, 1983).

### Sperm Isolation and Preparation

Adult rat, mouse, hamster, and dog cauda epididymides were removed and connective tissue carefully dissected away. The caudae were immersed in PBS and under a dissecting microscope, the tubules were pierced using a 27-gauge needle. Sperm were allowed to diffuse freely from the tubules at room temperature for 5–10 min, until the media became turbid. For preparation of Triton X-100 extracts, the cauda were removed and sperm collected and centrifuged at 600 *g* for 10 min. Sperm were resuspended in PBS, centrifuged, and subsequently resuspended in buffer containing 50 mM Trizma, pH 7.4, 1 mM  $\beta$ -mercaptoethanol, 1 mM EDTA, 1% Triton X-100, and protease inhibitors (4  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, 2  $\mu\text{g}/\text{ml}$  chymotrypsin, 2  $\mu\text{g}/\text{ml}$  pepstatin). After 30 min on ice with intermittent vortexing, samples were centrifuged for 20 min in a table top microfuge at maximal speed. Supernatants were collected and protein assayed. For [ $^3$ H]IP $_3$  binding, dispersed sperm were probe sonicated (Ultrasonics W-220F) on ice at 20% with one 5-s burst. After spinning in a table top centrifuge at maximal speed for 20 min at 4°C, the pellet was resuspended by sonication in binding buffer (see below) and protein assayed. For the  $^{45}\text{Ca}^{2+}$  loading assay, sperm were dispersed and washed in PBS containing 2 mM EGTA (to remove calcium) and 1 mM  $\beta$ -mercaptoethanol. After pelleting at 600 *g*, sperm were resuspended in 20 mM Hepes (pH 7.4 with KOH) containing 1 mM  $\beta$ -mercaptoethanol followed by addition of digitonin to a final concentration of 10  $\mu\text{M}$ . Sam-

ples were incubated on ice for 10 min before addition to the  $^{45}\text{Ca}^{2+}$  loading assay.

### Western Blotting

Protein samples (20–50  $\mu\text{g}$ ) were subjected to electrophoresis using precast 6% or 4–12% gradient Tris-glycine gels. The separated proteins were semidry transferred to nitrocellulose and the blots blocked in 5% nonfat dry milk for 2 h, followed by overnight incubation at 4°C in antibody diluted with 3% BSA/PBS. The blots were developed using the ECL detection system according to the manufacturer's protocol. The polyclonal IP $_3$ R antibody dilution was 1:1,000 (0.5  $\mu\text{g}/\text{ml}$ ). Controls were conducted by pretreating the primary antibody with purified and reconstituted IP $_3$ R (Ferris et al., 1989; Supattapone et al., 1988) (5  $\mu\text{g}/\text{ml}$ ) overnight at 4°C.  $G_{\alpha_{q/11}}$  and PLC $\beta_1$  antibodies were diluted to 1  $\mu\text{g}/\text{ml}$ , and preabsorbed antibody was prepared by adding the immunizing peptide at 10  $\mu\text{g}/\text{ml}$  followed by overnight incubation at 4°C.

### [ $^3$ H]IP $_3$ Binding to Rat Sperm

Binding of [ $^3$ H]IP $_3$  to rat cauda sperm sonicates was measured using 12.5  $\mu\text{g}$  of protein and 2.0 nM [ $^3$ H]IP $_3$  in 0.25 ml of 25 mM Trizma, pH 8.3, containing 1 mM EDTA. Binding equilibrium was achieved after incubation for 20 min at 4°C. The equilibrated mixture was then centrifuged in a table top microfuge at maximal speed for 15 min at 4°C, the supernatant aspirated, and the pellet resuspended in 0.25 ml H $_2$ O by probe sonication. Bound radioactivity was measured by liquid scintillation in 5 ml of Formula 963. Specific binding represented <10% of added radioactivity. Binding in the presence of 25  $\mu\text{M}$  unlabeled IP $_3$  was subtracted from total binding to yield specifically bound [ $^3$ H]IP $_3$ . Competition experiments were performed with 0.001–25  $\mu\text{M}$  unlabeled IP $_3$ .

### Immunohistochemistry and Indirect Immunofluorescence

Adult Sprague-Dawley rats were perfused with PBS and 4% paraformaldehyde, and the testes and epididymides removed, postfixed and cryoprotected for frozen sectioning. Immunohistochemistry was conducted using the avidin-biotin-peroxidase system with diaminobenzidine as the chromogen, according to the manufacturer's protocol. The IP $_3$ R antibody dilution was 1:500 (1  $\mu\text{g}/\text{ml}$ ). For immunostaining of sperm, cells were harvested from the cauda epididymis as described above and dispersed in PBS. Sperm were then mixed with an equal volume of 8% freshly depolymerized paraformaldehyde and incubated on ice for 30 min. Subsequently, fixed sperm were wet-mounted on slides and allowed to air dry. The slides were then washed in PBS for 3  $\times$  5 min, 50 mM NH $_4$ Cl for 3  $\times$  15 min, PBS for 3  $\times$  5 min, permeabilized in 0.1% Triton X-100 for 15 min, rinsed in PBS for 3  $\times$  5 min, treated with 0.01% hydrogen peroxide for 10 min, and then rinsed in PBS for 3  $\times$  5 min. Slides were then blocked for 1 h at room temperature with 2% normal goat serum/1% BSA in PBS, suctioned dry, and incubated overnight at 4°C. For immunoperoxidase staining of mouse sperm with  $G_{\alpha_{q/11}}$  and PLC $\beta_1$  antibodies, slides were incubated with 1  $\mu\text{g}/\text{ml}$  antibody. Controls were conducted by preabsorbing the antibodies with 10  $\mu\text{g}/\text{ml}$  of the corresponding peptide. Slides were developed using the avidin-biotin-peroxidase system with diaminobenzidine as the chromogen. For indirect immunofluorescence staining, slides were incubated with IP $_3$ R antibody at a dilution of 1:500 (1  $\mu\text{g}/\text{ml}$ ). Controls for IP $_3$ R staining were performed using antibody preabsorbed with purified protein at 5  $\mu\text{g}/\text{ml}$ . Slides were washed in PBS for 3  $\times$  5 min, incubated for 1 h at room temperature with 30  $\mu\text{g}/\text{ml}$  FITC-conjugated goat anti-rabbit IgG secondary antibody, washed again in PBS for 3  $\times$  5 min, coverslipped with vectashield mounting medium, and examined by immunofluorescence and confocal microscopy.

### Immunoelectron Microscopy

Rat cauda sperm pellets were fixed with 2% paraformaldehyde/0.5% glutaraldehyde for 30 min on ice. Samples were centrifuged in a table top microfuge at maximal speed and washed with PBS for 3  $\times$  5 min, NH $_4$ Cl for 1  $\times$  1 h, PBS for 2  $\times$  5 min, followed by 0.1 M sodium cacodylate, pH 7.2, for 1  $\times$  10 min. Pellets were then treated with 1% OsO $_4$  in sodium cacodylate for 1 h at 4°C. After rinsing 2  $\times$  5 min in distilled water, samples were dehydrated and embedded in LR White. Polymerized blocks were sectioned on a Reichert Ultracut E microtome with a diatome low angle diamond knife. 80-nm thin sections were picked up on formvar-coated 200 mesh nickel grids. Grids were immunolabeled in droplet solutions. Sec-

tions were treated with 1% sodium-meta-periodate for 15 min followed by a 15 min rinse in TBS, 15 min in 50 mM NH<sub>4</sub>Cl, and 10 min in 1% BSA/TBS/0.05% Tween. An alternate procedure using tannic acid in place of OsO<sub>4</sub> treatment was also used (Berryman et al., 1992). Grids were treated with IP<sub>3</sub>R antibody diluted 1:250 overnight at 4°C and then washed in TBS, followed by incubation with a 1:50 dilution of 5-nm colloidal gold-conjugated anti-rabbit secondary antibody for 1 h at room temperature. The grids were then washed in TBS and distilled water, treated with 2% glutaraldehyde for 5 min, rinsed with distilled water, treated with 3% uranyl acetate for 20 min, rinsed with distilled water and blotted dry. Sections were viewed on a Zeiss TEM 10A electron microscope at 60 kV. Control grids were incubated with primary antibody pretreated with reconstituted IP<sub>3</sub>R overnight at 4°C.

### Sperm Fractionation

Rat cauda sperm were dispersed in PBS containing 2 mM EGTA and 1 mM β-mercaptoethanol, and probe sonicated at 35% for four 10-s bursts. An equal volume of 1.8 M sucrose was added and the suspension layered over a discontinuous sucrose gradient containing equal volumes of 2.05 M and 2.2 M sucrose solutions. The sample was centrifuged at 100,000 g for 1 h at 4°C. The pellet and fractions at the 2.2 M/2.05 M and 2.05 M/0.9 M interfaces were isolated, resuspended in Trizma, pH 7.4, containing 1 mM EGTA, 1 mM β-mercaptoethanol, and pelleted at 100,000 g for 45 min at 4°C. Oil immersion phase microscopy of Coomassie stained fractions (1 g Coomassie brilliant blue per liter of methanol:acetic acid:water at 5:1:5) revealed acrosome-free heads in the pellet, intact and fragmented tails at the 2.2 M/2.05 M interface and acrosomes and acrosomal ghosts at the 2.05 M/0.9 M interface. Samples were protein assayed and equal protein loads subjected to Western analysis for IP<sub>3</sub>R.

In an alternate procedure, PBS-washed sperm were pelleted at 600 g, resuspended in 0.15 M NaCl/5 mM Hepes, pH 7.3, and homogenized in a glass-Teflon homogenizer with ten strokes at low speed. The sample was layered over a discontinuous sucrose gradient containing equal volumes of 1.75 M sucrose/0.9% NaCl and 1.3 M sucrose/0.9% NaCl, and ultracentrifuged at 27,500 rpm for 2 h at 4°C. The pellet and 1.3 M/1.75 M interface were isolated, resuspended in PBS, and pelleted by ultracentrifugation at 33,000 rpm. The pelleted samples were subjected to Western analysis for IP<sub>3</sub>R.

### Acrosome Reaction Assays

For isolation of membranes released by acrosomal exocytosis, rat cauda sperm were capacitated for 1 h at 37°C in M199 media supplemented with 25 mM Hepes (pH 7.4 with KOH), 30 μg/ml sodium pyruvate and 4 mg/ml protease-free BSA. Only samples displaying more than 80% motility (as determined by cell counting) were used for further study. Sperm concentration was determined using a hemacytometer and set at 10<sup>7</sup>/ml. A 1-ml aliquot of sperm was treated with 10 μM A23187, while a control sample received vehicle (1% DMSO). After a 30-min incubation at 37°C, sperm were spun in a table top microfuge at 1,000 g. The supernatant was isolated and subsequently ultracentrifuged at 100,000 g for 1 h at 4°C. The pellets were resuspended by sonication in PBS and protein assayed. Equal protein loads were subjected to Western analysis for IP<sub>3</sub>R.

Mouse acrosome reaction assays were conducted essentially as previously described (Kinloch et al., 1991), using the supplemented M199 medium. Mouse sperm isolated from the cauda were capacitated for 1 h at 37°C. Only samples displaying vigorous motility with greater than 80% motile sperm were used for further evaluation. The sperm concentration was set at 10<sup>6</sup>/ml. 50-μl aliquots were treated with 10 μM A23187, 100 nM-10 μM thapsigargin, and vehicle (1% DMSO). After a 1-h incubation at 37°C, the samples were fixed with 50 μl of 4% paraformaldehyde on ice for 15 min. Sperm were subsequently mounted on subbed slides, air-dried, and washed with PBS 3 × 5 min, followed by Coomassie staining for 5 min. After washing with PBS 3 × 5 min, the slides were coverslipped with 30% glycerol in PBS. Experiments were done in duplicate and 200 sperm per slide were evaluated for acrosomal status by oil immersion microscopy at 100× using Nomarski optics. Control and A23187-treated sperm were additionally subjected to anti-IP<sub>3</sub>R indirect immunofluorescence in order to document the presence or absence of acrosomal IP<sub>3</sub>R in acrosome-reacted sperm.

### <sup>45</sup>Ca<sup>2+</sup> Loading Assay

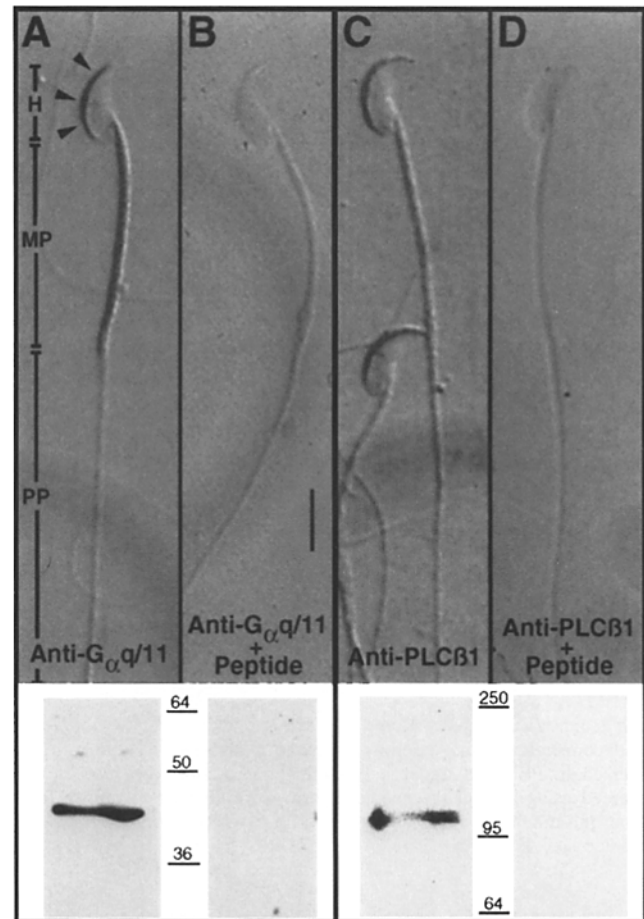
Digitonin permeabilized rat sperm at a protein concentration of 250 μg/ml were incubated for 45 min at 37°C in uptake buffer containing 20 mM

Hepes (pH 7.4 with KOH), 75 mM potassium oxalate, 5 mM sodium azide, 0.1 μM free Ca<sup>2+</sup> (as set by a Ca<sup>2+</sup>-selective electrode), 3% polyethylene glycol (average molecular weight 8,000), 10 mM phosphocreatine, 10 U/ml creatine phosphokinase, 1 mM MgCl<sub>2</sub>, 2 mM ATP, 10 mM dithiothreitol, and 120,000 cpm of <sup>45</sup>Ca<sup>2+</sup>. Drug treatments included 10 μM IP<sub>3</sub> with or without 500 μg/ml low molecular weight heparin, 10 μM A23187, 10 μM thapsigargin, 10 μM BHQ, vehicles (1% ethanol and 1% DMSO), 5 μM ryanodine, 5 mM caffeine, and a no ATP control. The 300-μl reactions were terminated by rapid filtration over 0.5% polyethyleneimine-coated glass fiber filters. Filters were washed twice with 1.5 ml of wash buffer containing 100 mM KCl, 10 mM Hepes (pH 7.4 with KOH), 5 mM MgCl<sub>2</sub>, and 1 mM EGTA at room temperature. Radioactivity was determined by liquid scintillation in 5 ml of Formula 963.

## Results

### Localization of G<sub>αq/11</sub> and PLCβ1 to the Acrosomal Region of the Mouse Sperm Head

Western analysis using peptide antibodies to G<sub>αq/11</sub> and PLCβ1 detects bands of ~42 kD and 115 kD, respectively,



**Figure 1.** Identification and localization of G<sub>αq/11</sub> and PLCβ1 in mouse sperm. Peptide antibodies to G<sub>αq/11</sub> and PLCβ1 recognize bands of ~42 kD (A) and 115 kD (C), respectively, in 1% Triton X-100 extracts of cauda epididymal mouse sperm. Immunoperoxidase staining of mouse sperm localizes G<sub>αq/11</sub> to the acrosomal region (arrowheads) of the head (H) and midpiece (MP) of the tail (A). PLCβ1 is identified in the acrosomal region and faint staining occurs in the midpiece (C). The proteins are not found in the principal piece (PP) or endpiece of the tail. Immunoreactivity is blocked when the antibodies are preabsorbed with 10 μg/ml of the corresponding peptide (B and D). Bar, 5 μm.

in 1% Triton X-100 extracts of cauda epididymal mouse sperm (Fig. 1). Immunohistochemical staining reveals a selective localization of  $G\alpha_{q/11}$  to the acrosomal cap region (*arrowheads*) and the midpiece (Fig. 1 A). No staining is evident in the principal piece or endpiece of the tail. The immunohistochemical staining and the 42-kD band on Western blot are blocked by preabsorbing the antibody with immunizing peptide (Fig. 1 B).

PLC $\beta$ 1 is also selectively localized to the anterior acrosomal region of the head with somewhat lighter staining of the midpiece and no staining of the principal piece or endpiece of the tail (Fig. 1 C). The staining pattern and the 115-kD band on Western blot are blocked by preabsorbing the antibody with immunizing peptide (Fig. 1 D).

### Localization of the IP $_3$ R to Acrosomes of Mammalian Sperm

Western blot analysis using a polyclonal antibody to purified brain IP $_3$ R, reveals a specific 260-kD band in 1% Triton X-100 extracts of rat, mouse, hamster, and dog sperm (Fig. 2). The sperm IP $_3$ R band comigrates with the cerebellar IP $_3$ R. Immunoreactivity is blocked by preincubation of the antibody with purified and reconstituted IP $_3$ R.

Rat cauda sperm possess substantial IP $_3$  binding capacity. Scatchard analysis of [ $^3$ H]IP $_3$  binding to sperm sonicates reveals a curvilinear plot containing discrete high and low affinity components with  $K_d$  values of 26 nM and 1.6  $\mu$ M, respectively (Fig. 3).  $B_{max}$  values for the high and low affinity sites are 30 and 550 pmol/mg protein, respectively, reflecting an extraordinarily high receptor density. The  $B_{max}$  value for the high affinity site in sperm is several times that reported for membranes of the rat cerebellum, the tissue generally regarded as having the highest IP $_3$ R density (Supattapone et al., 1988; Mourey et al., 1990). A curvilinear Scatchard plot is similarly observed with [ $^3$ H]IP $_3$  binding to T lymphocytes, which have high and

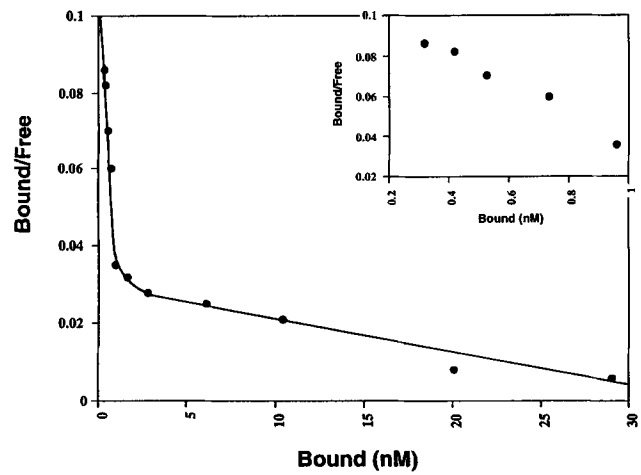


Figure 3. [ $^3$ H]IP $_3$  binding to rat sperm. Scatchard analysis of [ $^3$ H]IP $_3$  binding to rat cauda sperm sonicates produces a curvilinear plot with high affinity ( $K_d = 26$  nM,  $B_{max} = 30$  pmol/mg) and low affinity ( $K_d = 1.6$   $\mu$ M,  $B_{max} = 550$  pmol/mg) binding sites. The inset is an expansion of the 0.2–1.0 nM range of the curve which depicts the high affinity binding data. Data points are mean values from a single experiment performed in duplicate and repeated twice with similar results. The standard deviation of each point is <10% of the mean value.

low affinity sites corresponding to  $K_d$  values of 45 nM and 1.2  $\mu$ M, respectively (Khan et al., 1992). The existence of two classes of [ $^3$ H]IP $_3$ -binding sites may reflect different functional states of the IP $_3$ R or the presence of receptor subtypes with distinct binding specificities.

Immunoperoxidase staining of the rat testis and epididymis demonstrates a selective localization of IP $_3$ R in maturing sperm cells. In rat testis, the developing acrosomes of round spermatids stain for IP $_3$ R (Fig. 4 A). These cells undergo morphologic differentiation and are ultimately re-

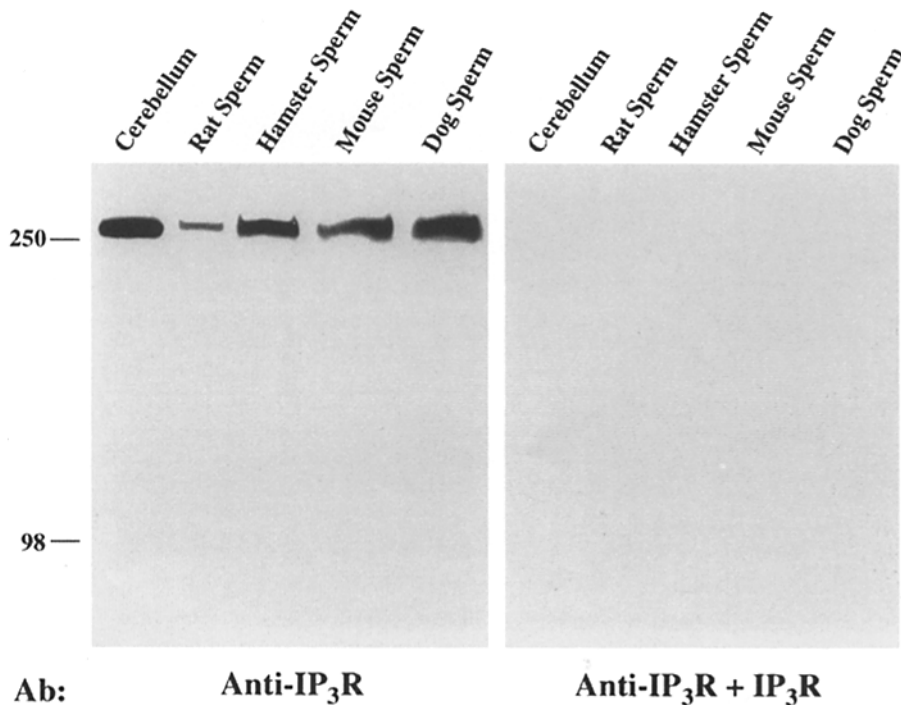
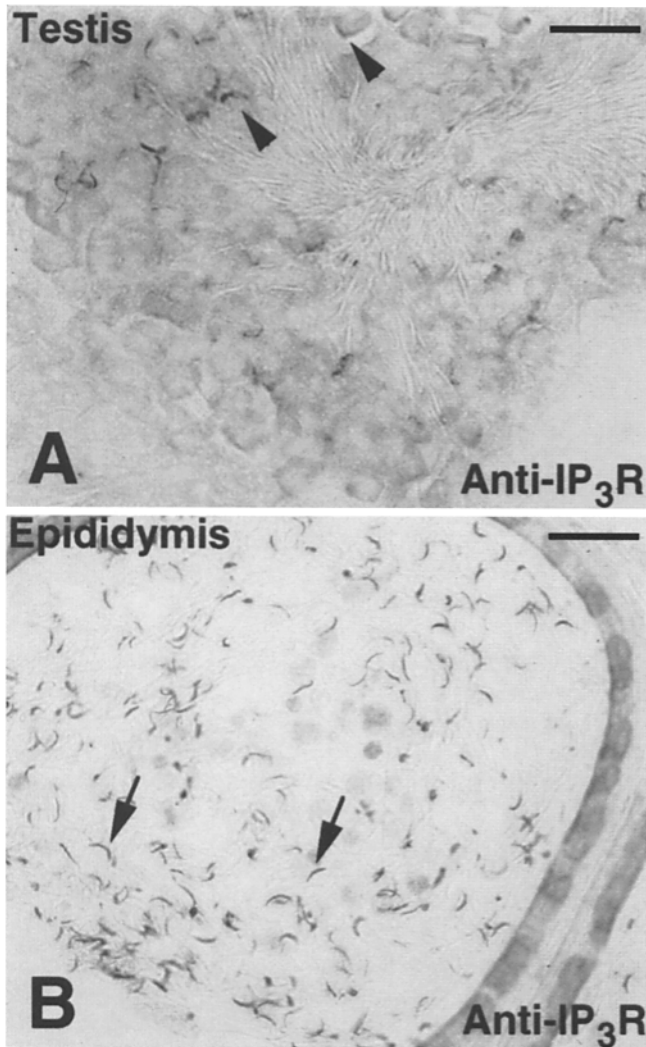
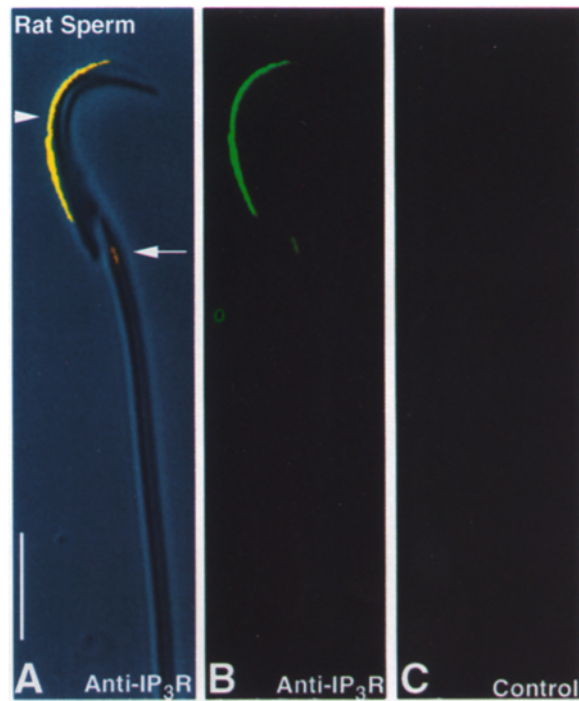


Figure 2. Identification of IP $_3$ R in mammalian sperm by Western blotting. IP $_3$ R antibody (1:1,000, 0.5  $\mu$ g/ml) detects a specific band of ~260 kD in 1% Triton X-100 extracts of rat, hamster, mouse, and dog sperm. The sperm bands comigrate with IP $_3$ R protein detected in the cerebellum. Preincubation of the antibody with purified and reconstituted brain IP $_3$ R blocks the immunoreactivity.



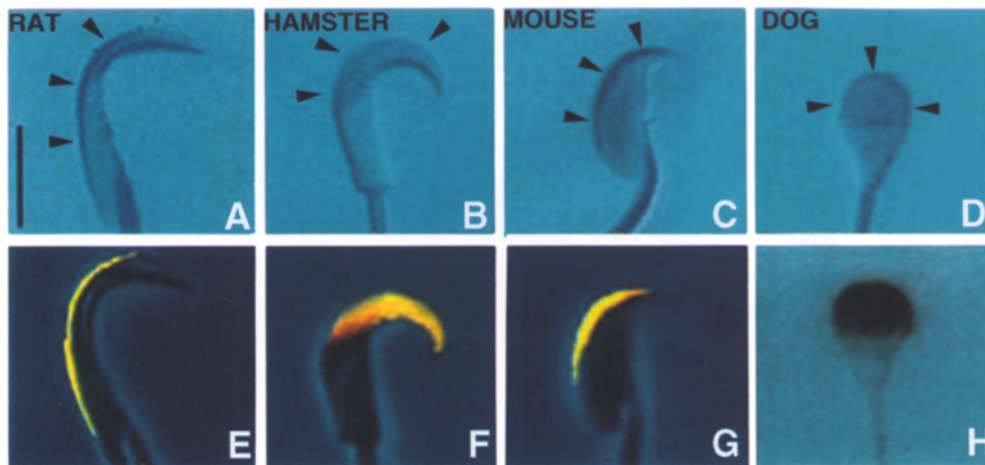
**Figure 4.** Immunohistochemical detection of IP<sub>3</sub>R in rat testis and epididymis. (A) Immunohistochemical staining of the adult testis with IP<sub>3</sub>R antibody highlights the crescent shape of the developing acrosome (arrowheads) in spermatids. Bar, 30  $\mu$ m. (B) The acrosomes (arrows) of epididymal sperm stain positive for IP<sub>3</sub>R. Bar, 40  $\mu$ m.



**Figure 5.** Indirect immunofluorescence staining of rat sperm with IP<sub>3</sub>R antibody. (A) Indirect immunofluorescence confocal microscopy of positively stained rat sperm overlaid on the corresponding phase image reveals strong immunoreactivity of the acrosome cap (arrowhead). A focus of staining also occurs in the proximal midpiece (arrow). The fluorescent staining pattern (B) is completely blocked when the antibody is preabsorbed with pure IP<sub>3</sub>R (C). Bar, 5  $\mu$ m.

leased into the lumen of the seminiferous tubules. Sperm acquire the potential for motility and egg binding as they traverse the epididymis. Even at low magnification, a cross section through an epididymal tubule reveals intense immunoperoxidase staining of the acrosomal crescent of maturing sperm (Fig. 4 B).

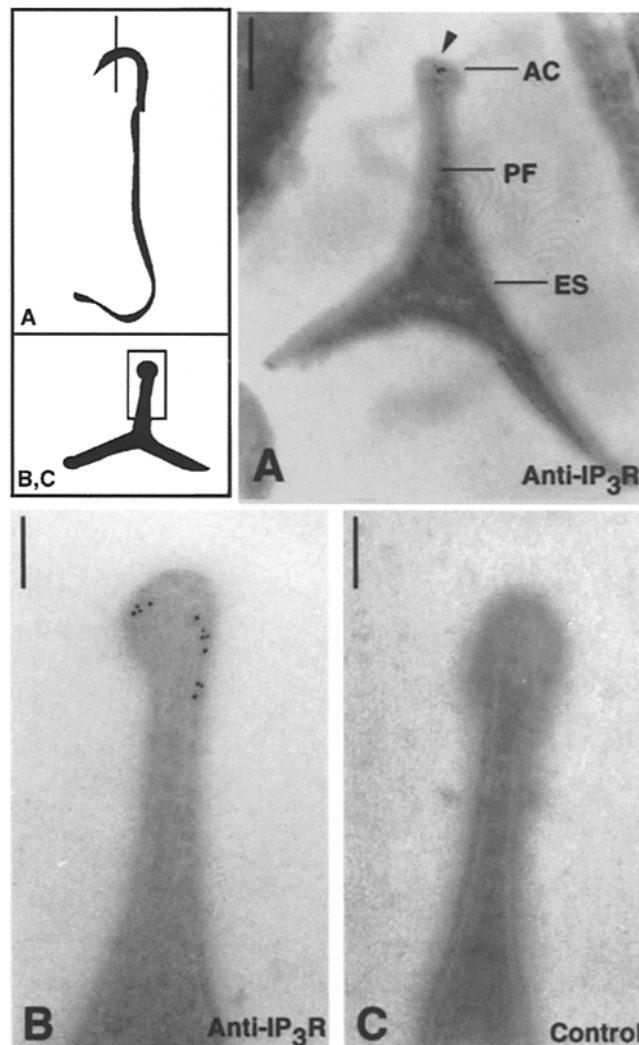
Indirect immunofluorescence confocal microscopy reveals high densities of discretely localized IP<sub>3</sub>R in the acrosome cap of rat sperm (Fig. 5, A and B). A focus of staining is also present at the proximal midpiece of rat sperm (Fig. 5, A and B). An IP<sub>3</sub>-gated calcium store at the



**Figure 6.** Colocalization of IP<sub>3</sub>R with the acrosomes of mammalian sperm. The distinct shapes of the acrosomes of rat (A), hamster (B), mouse (C), and dog (D) sperm are visualized by Coomassie staining (arrowheads). Using IP<sub>3</sub>R antibody, indirect immunofluorescence confocal microscopy of rat (E), hamster (F), and mouse (G) sperm and immunoperoxidase staining of dog sperm (H) demonstrates the acrosomal localization. Bar, 4.2  $\mu$ m.

proximal midpiece may participate in regulating motility in response to signals received by putative odorant receptors recently identified on the rat sperm midpiece (Walensky et al., 1995). IP<sub>3</sub>R staining is completely blocked by preabsorption with pure IP<sub>3</sub>R protein (Fig. 5 C).

IP<sub>3</sub>R is also localized to acrosomes in mouse, hamster, and dog sperm (Fig. 6, E–H). In each species, the shape of the anterior acrosome is distinct and can be visualized by Coomassie staining (Fig. 6, A–D). IP<sub>3</sub>R localization conforms to the shape of the acrosome in each case. In contrast to the rat, no IP<sub>3</sub>R staining is detected in the proximal midpiece of hamster, mouse, or dog sperm.



**Figure 7.** Localization of IP<sub>3</sub>R to the acrosome by immunoelectron microscopy. A transverse section through the rat sperm head adjacent to the tip (*schematic: A*) produces a profile (*schematic: B,C*) containing the acrosome cap (AC), the perforatorium (PF), and the equatorial segment (ES). The plasma membrane, which encircles the profile, is lost in this section. (A) Using IP<sub>3</sub>R antibody and a 5-nm gold-conjugated secondary antibody, immunolabeling is detected on the anterior acrosome (*arrow*). Bar, 200 nm. (B) A higher power view of the upper half of the transverse profile (boxed in *schematic: B, C*) shows that IP<sub>3</sub>R labeling follows the contour of the outer acrosomal membrane. Bar, 100 nm. (C) When the antibody is preincubated with purified and reconstituted IP<sub>3</sub>R, the acrosomal immunoreactivity is blocked. Bar, 100 nm.

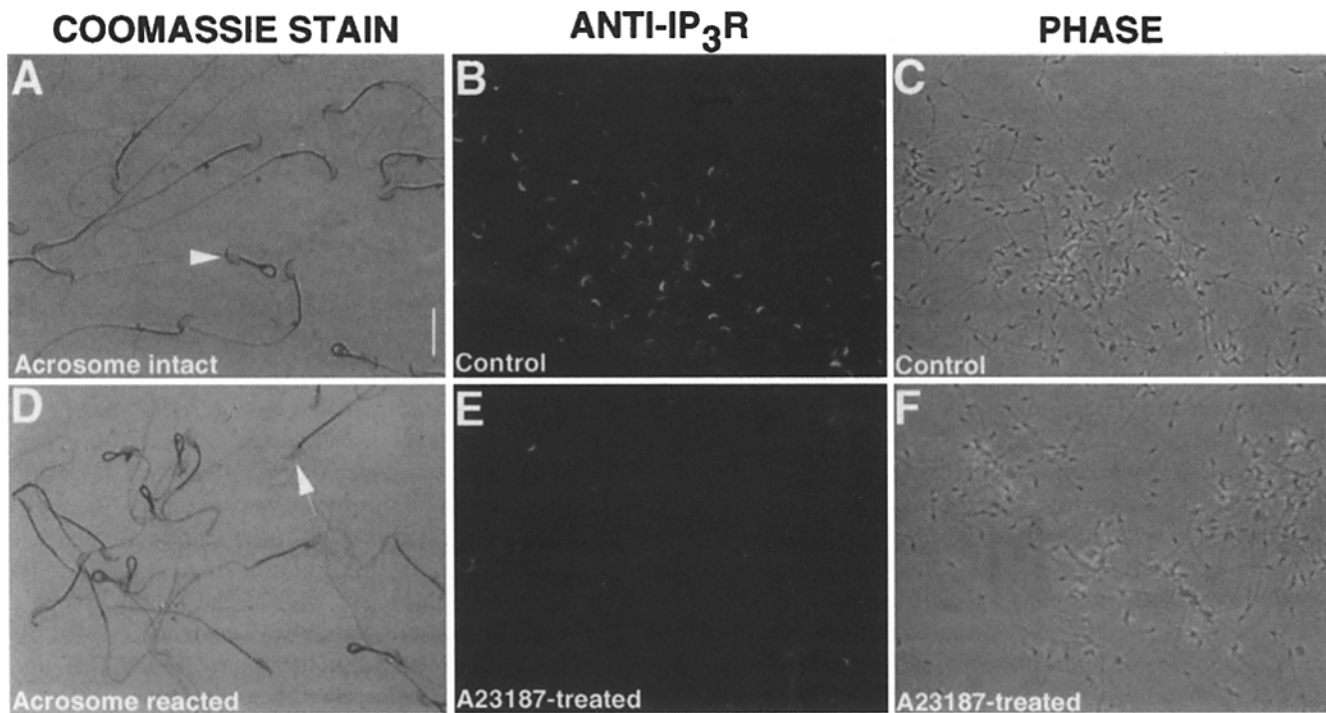
At the electron microscopy level, we detect specific immunogold labeling of the acrosome (Fig. 7 A). The distribution of the gold particles conforms to the localization of the outer acrosomal membrane (Fig. 7 B). There is no labeling of the perforatorium or the equatorial segment (Fig. 7 A). The nuclear envelope and the plasma membrane (in sections where it remained intact) similarly exhibit no immunolabeling (data not shown). Preincubation of IP<sub>3</sub>R antibody with pure IP<sub>3</sub>R protein abolishes acrosomal labeling.

The acrosome is bound by two discrete membranes, the outer and inner acrosomal membranes. During the acrosome reaction, the outer acrosomal membrane and the overlying plasma membrane fuse and vesiculate, resulting in release of the acrosomal contents and loss of the overlying membranes. The inner acrosomal membrane is subsequently exposed on the sperm head surface. To confirm the association of IP<sub>3</sub>R with the outer acrosomal membrane, we conducted indirect immunofluorescence staining of acrosome intact and A23187 acrosome-reacted mouse sperm. Acrosomal IP<sub>3</sub>R staining of intact sperm is absent in acrosome-reacted sperm (Fig. 8).

To further examine the association of IP<sub>3</sub>R with the acrosome and acrosomal membrane, we performed two sperm fractionation procedures (Purvis et al., 1982; Zahler and Doak, 1975). Rat sperm were probe sonicated, layered over a discontinuous sucrose gradient, and three fractions were isolated, Coomassie stained and visualized by phase microscopy. The 2.05 M/0.9 M interface contains acrosomes and acrosomal ghosts (Fig. 9, a, A), the 2.2 M/2.05 M interface contains intact and fragmented tails, and the pellet is enriched in acrosome-free heads (Fig. 9, a, B). Western blot analysis of the acrosome and acrosome-free head fractions reveals IP<sub>3</sub>R protein in the acrosome fraction (Fig. 9, a, C). In a second procedure, rat sperm were homogenized using a glass-Teflon homogenizer, layered over a discontinuous sucrose gradient, and the 1.3 M/1.75 M interface containing acrosomal membranes isolated and analyzed by Western blotting. IP<sub>3</sub>R protein is also enriched in this acrosomal membrane fraction (data not shown). In a final experiment to link IP<sub>3</sub>R to the acrosome, we capacitated rat sperm and induced the acrosome reaction with 10 μM A23187. After centrifugation at 1,000 g, the medium containing membranes released during acrosomal exocytosis was centrifuged at 100,000 g for 1 h at 4°C. The 100,000 g pellet of sperm treated with A23187 is enriched with IP<sub>3</sub>R protein compared to the corresponding fraction of untreated capacitated sperm (Fig. 9 b). Because dying cells and a percentage of capacitated cells can auto-acrosome react in a calcium-containing media, the presence of some IP<sub>3</sub>R protein is expected in the control fraction.

#### <sup>45</sup>Ca<sup>2+</sup> Loading of Digitonin Permeabilized Rat Sperm

We used digitonin permeabilized rat sperm loaded with <sup>45</sup>Ca<sup>2+</sup> to examine IP<sub>3</sub>-mediated calcium release from intracellular stores. Sodium azide was included in the reaction buffer at a concentration known to inhibit mitochondrial <sup>45</sup>Ca<sup>2+</sup> accumulation (Arab et al., 1990; Verma et al., 1992). <sup>45</sup>Ca<sup>2+</sup> accumulated in the presence of ATP, which activates Ca<sup>2+</sup>-ATPase loading of intracellular stores (Verma et al., 1990). No calcium accumulation occurs in the absence of ATP or in the presence of thapsigargin or



**Figure 8.** Acrosomal anti-IP<sub>3</sub>R immunofluorescence is lost in acrosome-reacted sperm. Capacitated mouse sperm were treated with 10  $\mu$ M A23187 to induce acrosomal exocytosis. Control sperm treated with 1% DMSO are acrosome intact by Coomassie staining (A) and demonstrate the characteristic acrosomal anti-IP<sub>3</sub>R immunofluorescence (B). A23187-treated sperm undergo the acrosome reaction and lose the outer acrosomal membrane, the overlying plasma membrane, and acrosomal contents. A23187-treated sperm no longer exhibit Coomassie staining (D) or anti-IP<sub>3</sub>R staining (E) of the acrosome. Panels C and F are the corresponding phase images of B and E, respectively. Bars: (A and D) 14  $\mu$ m; (B, C, E, and F) 28  $\mu$ m.

BHQ, both of which specifically inhibit the Ca<sup>2+</sup>-ATPase of nonmitochondrial intracellular membranes (Thastrup et al., 1990; Llopis et al., 1991). The calcium ionophore, A23187, also prevents calcium loading. 10  $\mu$ M IP<sub>3</sub> decreases <sup>45</sup>Ca<sup>2+</sup> accumulation in sperm by 45% (Fig. 10), which is similar to the effect observed in cerebellar microsomes (Verma et al., 1992). Actions of IP<sub>3</sub> on sperm calcium are blocked by heparin, an IP<sub>3</sub>R antagonist. In contrast, caffeine and ryanodine, which selectively release calcium from the calcium-induced-calcium-release (CICR) pool (Ehrlich et al., 1994), display no effect on <sup>45</sup>Ca<sup>2+</sup> loading. The inability of IP<sub>3</sub> to release all of the accumulated calcium is consistent with data from the cerebellum, where IP<sub>3</sub> and caffeine in combination can release no more than 65% of the accumulated calcium (Verma et al., 1992).

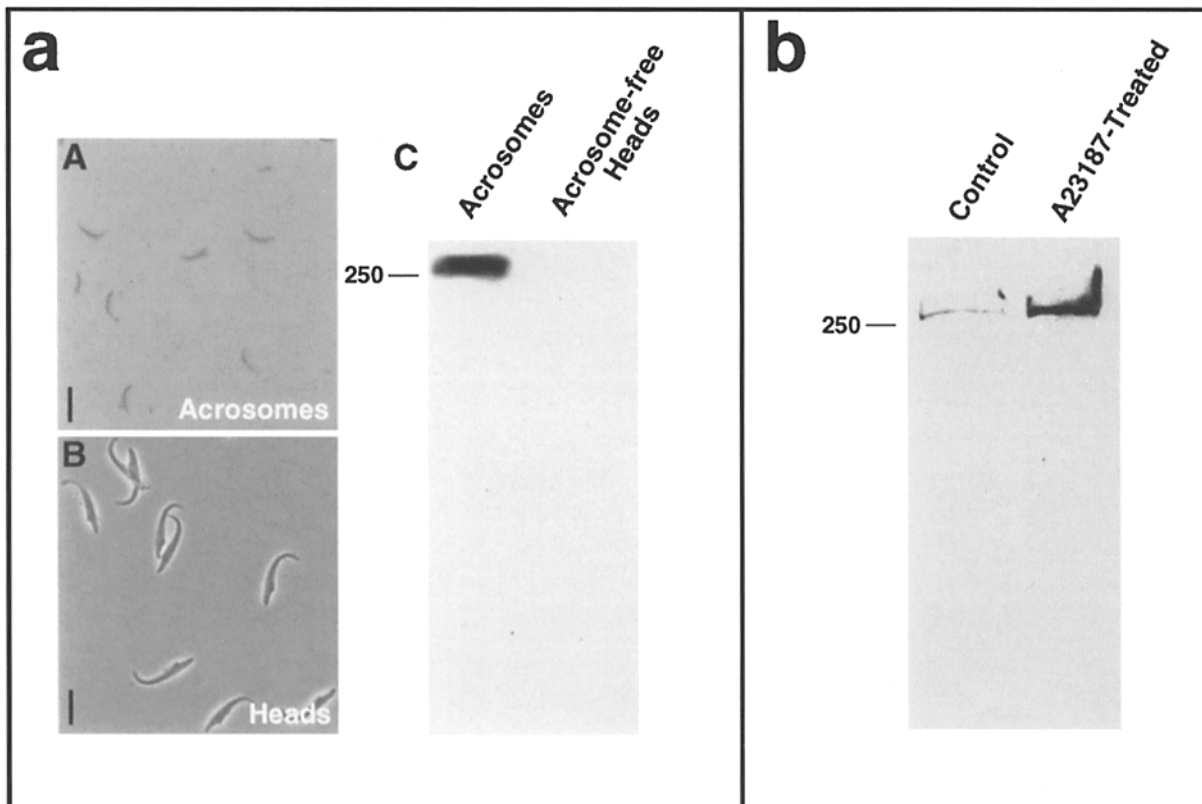
#### **Induction of the Acrosome Reaction by Thapsigargin**

Thapsigargin selectively inhibits the Ca<sup>2+</sup>-ATPase of nonmitochondrial intracellular membranes and does not affect the plasma membrane pump (Lytton et al., 1991). The failure of caffeine or ryanodine to influence calcium accumulation suggests that thapsigargin releases calcium from IP<sub>3</sub>-sensitive stores. To determine whether an IP<sub>3</sub>-gated calcium store participates in acrosomal exocytosis, we examined the influence of thapsigargin on the acrosome reaction of mouse sperm. Thapsigargin triggers acrosomal

exocytosis with a maximal effect equivalent to the maximal response elicited by the calcium ionophore, A23187 (Fig. 11). The potency of thapsigargin in stimulating the acrosome reaction (EC<sub>50</sub> = 0.5  $\mu$ M) resembles its potency in elevating the intracellular calcium levels of pancreatic acinar cells (Metz et al., 1992). Thapsigargin also induces the acrosome reaction of human sperm (Meizel and Turner, 1993).

#### **Discussion**

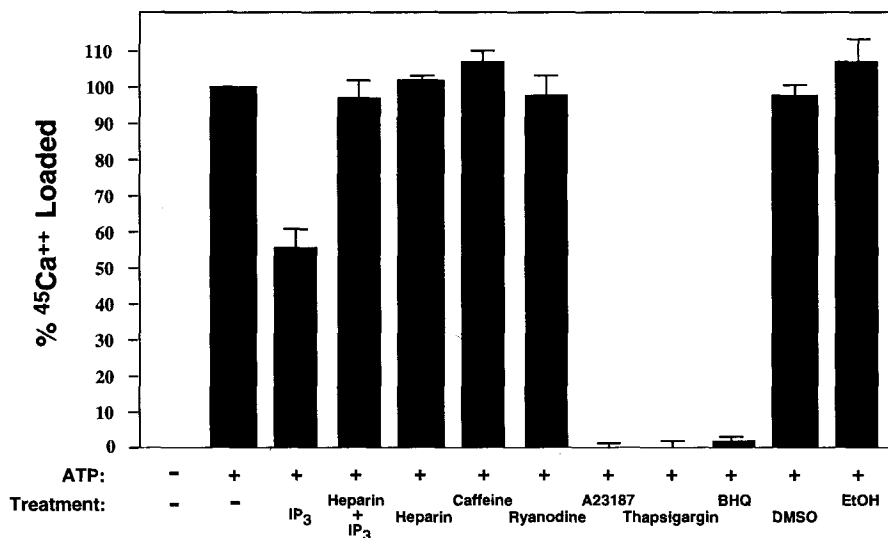
The main findings of this study are the selective localization and functional association of IP<sub>3</sub>R with the acrosomes of mammalian sperm. The level of IP<sub>3</sub> binding in sperm is among the highest observed in mammalian tissues. This is particularly striking since the IP<sub>3</sub>R is selectively localized to the acrosomal membrane, which constitutes only a small fraction of sperm membranes. Acrosome-reacted sperm lose their acrosomal IP<sub>3</sub>R immunostaining, and the medium in which the reaction takes place is correspondingly enriched with IP<sub>3</sub>R protein compared to control samples. These findings are consistent with the release of the outer acrosomal membrane and associated IP<sub>3</sub>R from the sperm head into the medium during acrosomal exocytosis. Sperm IP<sub>3</sub>R are functional, as evidenced by the IP<sub>3</sub>-mediated activation of calcium channels in a reverse <sup>45</sup>Ca<sup>2+</sup> flux assay using digitonin permeabilized sperm. Recently, type 1 and type 3 ryanodine receptors have been identified in



**Figure 9.** (a) IP<sub>3</sub>R is associated with sperm acrosomes. Fractionating rat sperm sonicates over a discontinuous sucrose gradient produces a layer enriched in acrosomes and acrosomal ghosts at the 2.05 M/0.9 M interface (A) and a pellet containing acrosome-free heads (B). Western blotting of equal protein loads demonstrates IP<sub>3</sub>R in the acrosome-enriched fraction but not in the acrosome-free head fraction (C). (b) Capacitated rat sperm (10<sup>7</sup>/ml) were treated with 10 μM A23187 to induce the acrosome reaction, and control samples received vehicle (1% DMSO). Sperm were pelleted at 1,000 g and the supernatant containing membranes lost during acrosomal exocytosis was centrifuged at 100,000 g. The pellets were resuspended and subjected to Western analysis using anti-IP<sub>3</sub>R antibody. The sample derived from acrosome-reacted sperm is enriched with IP<sub>3</sub>R protein compared to untreated control samples. Bars: (A) 12 μm; (B) 6 μm.

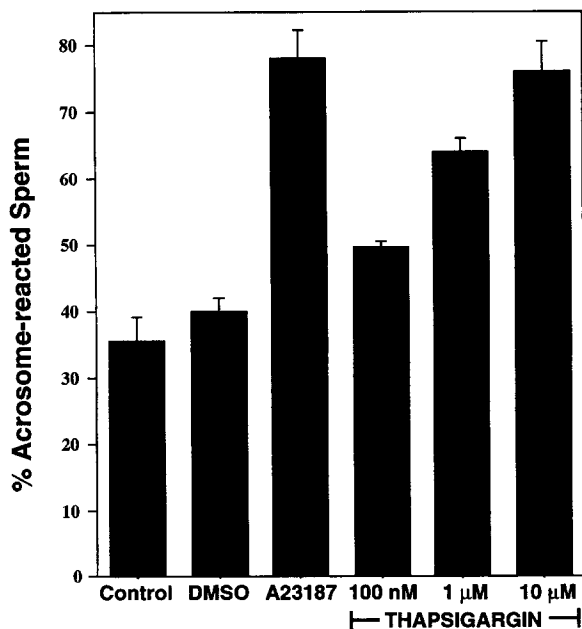
murine testis and in purified populations of spermatocytes and spermatids (Giannini et al., 1995). Although the presence or absence of ryanodine receptors in mature sperm awaits further study, we were unable to detect an effect of

caffeine or ryanodine on sperm <sup>45</sup>Ca<sup>2+</sup> accumulation, suggesting that the phosphoinositide system is the major mechanism for intracellular calcium release in sperm. Accordingly, the finding that thapsigargin, which acts selec-



**Figure 10.** <sup>45</sup>Ca<sup>2+</sup> loading of digitonin permeabilized sperm. Digitonin permeabilized rat sperm accumulate <sup>45</sup>Ca<sup>2+</sup> in an ATP-dependent manner. In the absence of ATP or in the presence of the intracellular Ca<sup>2+</sup>-ATPase inhibitors thapsigargin (10 μM) and BHQ (10 μM), no loading is observed. 10 μM IP<sub>3</sub> decreases <sup>45</sup>Ca<sup>2+</sup> accumulation by 45%. The IP<sub>3</sub> effect is reversed by heparin (500 μg/ml). In contrast, caffeine (5 mM) and ryanodine (5 μM) have no effect on <sup>45</sup>Ca<sup>2+</sup> accumulation. The drug vehicles ethanol (1%) and DMSO (1%) similarly have no effect on <sup>45</sup>Ca<sup>2+</sup> accumulation. Specific loading represents ≤10% of added radioactivity. Experiments were performed in duplicate and repeated at least three times. Bars represent standard deviations of the mean.





**Figure 11.** Induction of the acrosome reaction by thapsigargin. Thapsigargin triggers the acrosome reaction of capacitated mouse sperm in a dose-dependent manner from 100 nM to 10  $\mu$ M. The maximal thapsigargin effect is equivalent to that elicited by the calcium ionophore, A23187 (10  $\mu$ M). Experiments were performed in duplicate and repeated twice. For each reaction sample, sperm were fixed, Coomassie stained, and 200 sperm per slide evaluated for acrosomal status under oil immersion microscopy at 100 $\times$  using Nomarski optics. Bars represent standard deviations of the mean.

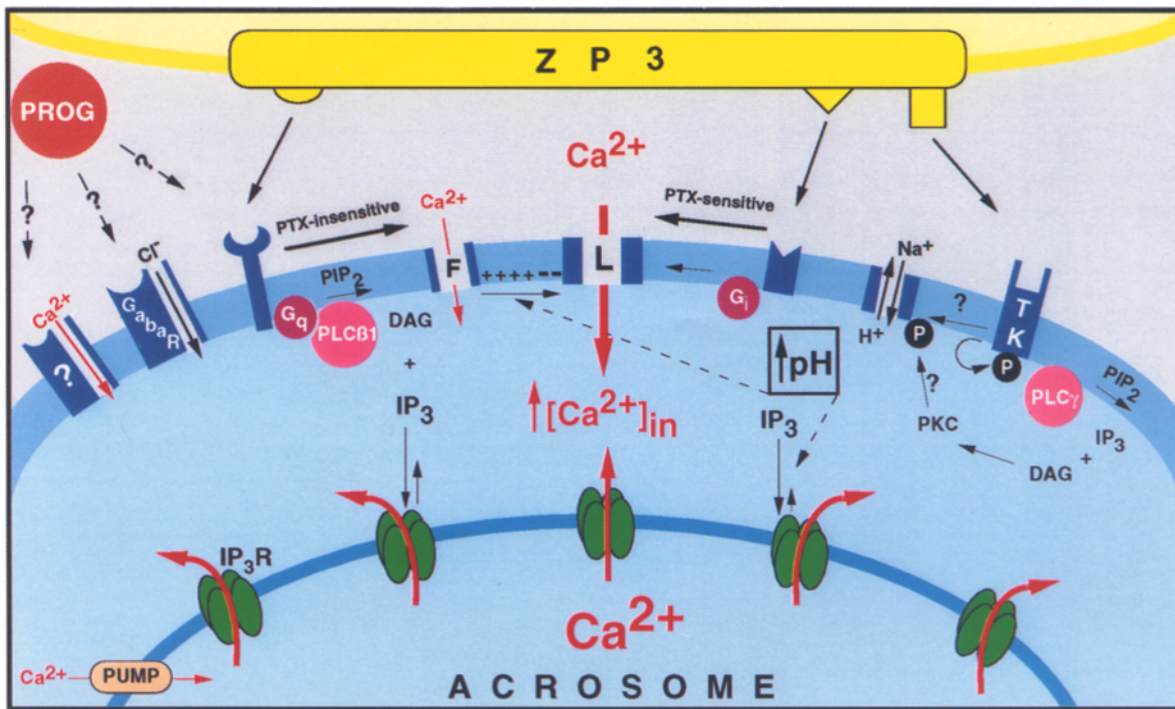
tively upon the intracellular calcium release system, triggers acrosomal exocytosis strongly implicates  $IP_3$ -mediated calcium release in promoting the acrosome reaction.

The acrosomal vesicle is similar to somatic secretory granules in that it packages proteins at an acidic pH and is exocytosed in response to specific ligand stimulation. The localization of  $IP_3$ R to the acrosome suggests that this organelle additionally functions as a calcium store. Whereas the endoplasmic reticulum is the most widely recognized subcellular localization of  $IP_3$ Rs,  $IP_3$ -gated calcium channels have been identified in the plasma membrane (Khan et al., 1992), nuclear membrane (Malviya et al., 1990), and nerve terminals (Peng et al., 1991). The finding that  $IP_3$  directly triggers calcium release from bovine adrenal medullary secretory vesicles, indicates that  $IP_3$ Rs are a component of chromaffin granules (Yoo et al., 1990). A recent study localized  $IP_3$ R subtype 3 to the insulin and somatostatin secretory granules of pancreatic  $\beta$ -cells and  $\delta$ -cells, respectively (Blondel et al., 1994). These vesicles have been shown to release calcium upon cell stimulation (Abrahamsson et al., 1981). Calcium loading of secretory granules is a well known phenomenon, and may play a functional role in stimulus-secretion (Nicaise et al., 1992). High capacity calcium-binding proteins typically present in the core of secretory granules participate in calcium sequestration. Using video enhanced fluorescence microscopy of fura-2 loaded mouse sperm, (Herrick, S. B., and R. A. Cardullo. 1994. *Mol. Biol. Cell.* 5:346a) recently lo-

calized intracellular calcium to the acrosomal crescent. Interestingly, an endoplasmic reticulum protein, calreticulin, has been identified as a component of the rat sperm acrosome (Nakamura et al., 1993). Calreticulin possesses high and low affinity calcium-binding sites and may participate in acrosomal calcium sequestration. The identification of  $IP_3$ R in  $\beta$ - and  $\delta$ -secretory granules has generated the hypothesis that secretagogue-induced  $IP_3$  production triggers calcium release from the granules that directly regulates exocytosis (Blondel et al., 1994).  $IP_3$ -induced calcium mobilization has been directly shown to activate nuclear vesicle fusion in vitro (Sullivan et al., 1993).  $IP_3$ -gated calcium release from the acrosome may likewise play an important role in promoting membrane fusion events and resultant acrosomal exocytosis.

What is the signal transduction mechanism leading to acrosomal exocytosis? The specific ligands known to trigger the acrosome reaction include ZP3 (Bleil and Wassarman, 1983), a glycoprotein of the mouse ZP, and progesterone (Osman et al., 1989; Blackmore et al., 1990, 1991; Roldan et al., 1994), a steroid present in the cumulus oophorus which surrounds the egg. Several sperm ZP3-binding proteins are candidate ZP3 receptors (Leyton et al., 1989, 1992; Miller et al., 1992; Cheng et al., 1994). Indeed, ZP3 may have multivalent interactions with sperm surface proteins (Kopf and Gerton, 1991). A cell surface receptor has been hypothesized to transduce the progesterone signal (Blackmore et al., 1991; Mendoza and Tesarik, 1993). Progesterone may act through a sperm GABA<sub>A</sub>-like receptor (Wistrom and Meizel, 1993; Roldan et al., 1994) and perhaps additionally through direct activation of an unidentified calcium channel (Roldan et al., 1994). Although multiple signal transduction components have been identified and hypothesized to play important roles in acrosomal exocytosis, the specific nature of the ligand-receptor interaction, the relevant signaling pathways activated, and the sequence of transduction events remain unclear.

Like many ligand-activated secretory events, the physiologic acrosome reaction is calcium-dependent and has been long known to require extracellular calcium (Yanagimachi and Usui, 1974). In a wide range of somatic cell types, ligand-mediated generation of  $IP_3$  followed by calcium release from  $IP_3$ -gated internal stores promotes extracellular calcium influx across the plasma membrane (Berridge and Irvine, 1989). This phenomenon of capacitative calcium entry is well documented but the mechanism is incompletely understood (von Tscharner et al., 1986; Putney, 1990; Bird et al., 1991; Schilling et al., 1992). In many secretory cells, the initial focal release of calcium from  $IP_3$ -gated intracellular stores has a priming effect on exocytosis (Marty, 1991; Thorn, 1993; Blondel et al., 1994). In chromaffin cells and basophils, the release of intracellular calcium alone by ligand-activated generation of  $IP_3$  or by treatment with intracellular calcium release agents such as thapsigargin, is insufficient to promote secretion in the absence of extracellular calcium (Cheek and Barry, 1993; MacGlashan and Botana, 1993). The requirement of extracellular calcium to induce acrosomal exocytosis likewise suggests that  $IP_3$ -gated acrosomal calcium release may be insufficient to trigger acrosomal exocytosis in the absence of external calcium. However, under physiological condi-



**Figure 12.** Model of ligand-induced signaling events in the mammalian acrosome reaction leading to increased intracellular calcium in the sperm head. ZP3 (*above*) may have multivalent interactions with the sperm plasma membrane (*below*) and thereby trigger multiple signaling pathways. A pertussis-insensitive pathway may involve those signal transduction components identified in this study. Receptor activation of  $G_q$  stimulates PLC $\beta$ 1 activity resulting in phosphatidylinositol 4,5-bisphosphate hydrolysis and the generation of IP $_3$  and DAG. Binding of IP $_3$  to IP $_3$ Rs localized to the outer acrosomal membrane would trigger release of acrosomal calcium. Subsequent capacitative calcium entry through focal, voltage-insensitive channels (*F*) would further elevate intracellular calcium, trigger membrane depolarization, and activate L-like calcium channels (*L*). A pertussis-sensitive pathway involves ZP3 activation of  $G_i$  which may directly activate L-like calcium channels, resulting in extracellular calcium influx. ZP3 binding to p95, a putative receptor tyrosine kinase (*TK*), may result in receptor phosphorylation and PLC $\gamma$  stimulation. Activation of a Na $^+$ -H $^+$  exchanger would cause a Na $^+$  influx, H $^+$  efflux, a rise in intracellular pH, membrane depolarization, and resultant calcium influx through L-like channels. The increase in pH would enhance IP $_3$ -gated calcium release. Progesterone (*PROG*) can trigger calcium influx and initiate the acrosome reaction in a pertussis-insensitive manner. Progesterone may operate through a receptor-operated calcium channel, a GABA $_A$ -like receptor (*GabaR*), or a  $G_q$ -coupled receptor. The ligand-induced elevations of sperm intracellular calcium levels are required for membrane fusion events and resultant acrosomal exocytosis.

tions, IP $_3$ -gated calcium release may be an important mechanism for activating the extracellular calcium influx required for the acrosome reaction.

Roldan and colleagues (1994) have demonstrated that progesterone and ZP activate sperm phosphatidylinositol 4,5-bisphosphate (PIP $_2$ ) hydrolysis, which produces both IP $_3$  and DAG. The generation of DAG is believed to be important in activating phospholipase A $_2$ , which generates fusogenic free fatty acids and lysophosphatides (Roldan and Harrison, 1993). Roldan and colleagues (1994) measure increases in whole cell DAG mass in response to ZP and progesterone, and indicate that the ligand-induced second messenger elevation is dependent upon extracellular calcium. Chelation of extracellular calcium, however, can deplete intracellular calcium and thereby preclude PLC activation. In addition, measuring whole cell DAG mass may be insufficient to detect ligand-induced generation of DAG in the absence of extracellular calcium due to the focal nature of second messenger elevations typical of polarized secretory processes (Marty, 1991; Thorn, 1993). Ultimately, it will be important to determine if the extracellular calcium-dependent increases in whole cell DAG mass correlate with acrosomal exocytosis. Our  $^{45}\text{Ca}^{2+}$  ac-

cumulation data indicate that sperm can load calcium in an ATP-dependent manner into an IP $_3$ -gated store and that thapsigargin can completely prevent this accumulation. The association of ATPase activity with the outer acrosomal membrane (Gordon et al., 1978; Gordon, 1973), coupled with the finding that thapsigargin can stimulate acrosomal exocytosis in human (Meizel and Turner, 1993) and mouse sperm, suggests that calcium release from the acrosome participates in triggering acrosomal exocytosis. Thus, the finding that early events of the acrosome reaction require micromolar Ca $^{2+}$  levels and later events require millimolar levels (Roldan and Harrison, 1993), is consistent with initial ligand-induced generation of IP $_3$  and acrosomal calcium release, followed by extracellular calcium influx.

What agonist-induced calcium responses have been observed in mammalian sperm? Florman et al. (1992) (Florman, 1994) have characterized ZP-induced calcium channel activity in bovine sperm. Florman has identified two agonist-dependent calcium entry mechanisms. The first is a poorly selective, QNB-sensitive, and voltage-insensitive cation channel that is responsible for transient and focal calcium elevations in the sperm head. A transient, focal in-

tracellular calcium elevation was similarly observed in mouse sperm heads in response to ZP (Storey et al., 1992). The second activity has several pharmacological characteristics of L-type voltage-sensitive calcium channels and is responsible for sustained calcium elevations throughout the entire sperm cell. This secondary massive influx of calcium is believed to be essential for acrosomal exocytosis. ZP3-activation of the first cation channel may produce membrane depolarization resulting in activation of sperm "L-like" channels. Whereas the focal calcium conductance is pertussis-insensitive, the L-like channel activity is pertussis-sensitive. ZP-induced acrosomal exocytosis is inhibited by QNB presumably by blocking focal channels. In the presence of pertussis toxin, the ZP-induced acrosome reaction is also inhibited, despite activation of focal channels. These findings suggest that sperm L-like channels are regulated by dual voltage-sensitive and voltage-insensitive mechanisms (Florman, 1994).

The inhibition of ZP3-induced acrosomal exocytosis with pertussis toxin (Endo et al., 1987), indicates that G proteins participate in regulating this secretory event. In mouse sperm preparations,  $G_{i1}$  and  $G_{i2}$  are directly activated by ZP3 in a pertussis-sensitive manner (Ward et al., 1994). The mechanism by which ZP3 activates L-like calcium channels and the manner in which pertussis-sensitive G proteins are involved in mediating this process is unknown. Another mechanism for triggering membrane depolarization, L-like channel activation, and calcium influx has been proposed by Fraser et al. (1993a,b) (Fraser, 1994) and involves early activation of a  $Na^+-H^+$  exchanger. Fraser et al. (1993a) have demonstrated that the  $Na^+$ -ionophore monensin can trigger acrosomal exocytosis. Activation of  $Na^+$  entry and  $H^+$  extrusion would promote alkalinization, membrane depolarization, and subsequent activation of sperm L-like calcium channels. This sequence of events is supported by the ability of nifedipine to block the monensin-induced acrosome reaction (Fraser, 1993b). The mechanism by which the sperm  $Na^+-H^+$  exchanger is activated in response to ligand stimulation is not known. In somatic cells, agonist-induced activation of  $Na^+-H^+$  exchangers has been shown to occur through phosphorylation by protein kinase A, protein kinase C, and tyrosine kinase activities (Manganel and Turner, 1989, 1991; Borgese et al., 1992; Grinstein and Rothstein, 1986; Grinstein et al., 1989). A 95-kD candidate sperm receptor for ZP3 has tyrosine kinase activity (Leyton et al., 1992; Saling, 1991) and could potentially activate a sperm  $Na^+-H^+$ -exchanger (Fraser, 1994).

In this study, we have identified components of the phosphoinositide signaling pathway, including  $G_{\alpha_{q11}}$ , PLC $\beta$ 1, and an  $IP_3$ -gated calcium store in the acrosomal region of mammalian sperm. Recently,  $G_q$  has independently been identified in the acrosomal region and midpiece of mouse sperm by P. E. Visconti and G. S. Kopf (personal communication). These pertussis-insensitive signaling components may promote acrosomal exocytosis by mobilizing intracellular calcium. In insulin secreting  $\beta$ -TC3 cells, the muscarinic agonist carbachol stimulates the phosphoinositide pathway resulting in intracellular calcium release and the potentiation of glucose induced insulin secretion (Baffy et al., 1993). Cellular injection of anti- $G_q$  antibodies inhibits the carbachol effect. Interestingly, a muscarinic antagonist, QNB, has been shown to attenuate ZP-induced focal, transient  $Ca^{2+}$  elevations in the mouse sperm head (Storey et

al., 1992) and inhibits focal channel activity in bovine sperm (Florman, 1994). Thus, ZP3 may initially activate a Gq-coupled receptor resulting in  $IP_3$ -production, acrosomal calcium release and subsequent focal capacitative calcium entry through voltage-insensitive channels in the sperm head plasma membrane. This focal elevation of intracellular calcium may participate in L-like channel activation by triggering membrane depolarization as proposed by Florman (1994). ZP3 binding to a  $G_i$ -linked receptor may directly activate L-like calcium channels, producing a global influx of extracellular calcium. Phosphorylation activation of a  $Na^+-H^+$  exchanger by PKC or a receptor tyrosine kinase may additionally promote L-like channel activation by increasing  $Na^+$ , extruding  $H^+$ , increasing pH, and causing membrane depolarization. It is noteworthy that the increase in pH during the acrosome reaction would potentiate calcium release from the acrosome, since binding of  $IP_3$  to  $IP_3$ Rs and subsequent calcium release through  $IP_3$ Rs are both favored by alkaline pH (Brass and Joseph, 1985; Worley et al., 1987). A model of ligand-induced signaling events leading to increased sperm intracellular calcium is presented in Fig. 12.

There are multiple signal transduction components in the acrosomal region of mammalian sperm poised to execute a single exocytotic event in response to the appropriate ligand. ZP3 may activate several signaling pathways through multivalent interactions with the sperm plasma membrane. The sequence of signaling events and the manner in which they are integrated to produce exocytosis is not definitively known. However, there appears to be alternate routes to exocytosis. Whereas ZP3 triggers the acrosome reaction in a pertussis-sensitive fashion, progesterone has been shown to initiate the acrosome reaction in a pertussis-insensitive manner (Tesarik et al., 1993). Multiple physiologic inducers or inhibitors may participate in the regulation of acrosomal exocytosis. Identification and characterization of the specific sperm receptors that trigger signaling pathways in response to physiologic ligands, will contribute enormously to our future understanding of this critical exocytotic event. It is clear, however, that elevated intracellular calcium is central to induction of the acrosome reaction. Whereas  $IP_3$ -gated calcium release is well known to be essential for egg activation (Miyazaki et al., 1992; Xu et al., 1994), our data suggests that calcium flux through sperm  $IP_3$ Rs may likewise play a pivotal role in the fertilization process.

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