

Endpoint of First Stage of Zona pellucida-Induced Acrosome Reaction in Mouse Spermatozoa Characterized by Acrosomal H^+ and Ca^{2+} Permeability: A Possible Model for the Human System

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For the capacitated mouse spermatozoon to fertilize the egg, the zona-induced acrosome reaction (ZIR) must occur and the spermatozoon must pass into the perivitelline space. The time course of the ZIR has been resolved into two stages [Lee and Storey, 1985] by use of three fluorescent probes: chlortetracycline (CTC) [Saling and Storey, 1979; Ward and Storey, 1984], 9-amino-3-chloro-7-methoxyacridine (ACMA), and 9-(N-dodecyl) aminoacridine (NDAA). The CTC assay shows three sequential fluorescent patterns on the sperm head; B-to-S and S-to-R. The B pattern has been shown to be indicative of capacitated, acrosome-intact spermatozoa. The CTC S pattern, an intermediate pattern, is that of a cell which has lost the intra- to extracellular H^+ gradient as shown by the aminoacridine pH probes. The CTC R is that of an acrosome-reacted spermatozoon. The B-to-S transition occurs concurrently with the loss of the sperm head H^+ gradient. In this study we posed the question: Is the loss of the H^+ gradient accompanied by an influx of calcium into an intracellular compartment of lower Ca^{2+} or does the influx of Ca^{2+} occur prior to the loss of the H^+ gradient? The fluorescent probe fura-2 was used to measure the cell population kinetics of Ca^{2+} influx into the sperm head by loss of fura-2, head

fluorescence. Nigericin, a K^+/H^+ exchange ionophore, was used to discharge the H^+ gradient prior to the B-to-S transition. We present evidence in this poster that the key event is Ca^{2+} influx, with loss of H^+ gradient secondary to this event.

In the mouse system it has been demonstrated that a guanine nucleotide-binding regulatory protein (Gi) is involved in the ZIR [Endo et al., 1988] by block of the ZIR by pertussis toxin (IAP). In the human system we have approached the system of sperm-egg interaction by use of solubilized zona proteins. IAP blocks the ZIR in human and has no effect on the spontaneous or ionophore-induced acrosome reaction. This implicates a Gi. These Gi proteins occupy critical roles as signal-transducing elements in coupling many ligand-receptor interactions with the generation of intracellular second messengers. These heterotrimeric plasma membrane-associated proteins are composed of distinct α -subunits, which contain a GTP-binding domain, and more highly conserved β - and γ -subunits. The α -subunits can be covalently modified by ADP-ribosylation in the presence NAD^+ by the action of a variety of bacterial toxins, including cholera toxin, pertussis toxin, and botulinum toxin. Sperm suspensions were preincubated with IAP and assayed for acrosomal status. The ability to undergo toxin-catalyzed ADP-ribosylation has been used by many investigators to identify general classes of G proteins. The ability to undergo toxin-catalyzed ADP-ribosylation has been used by many investigators to identify general classes of G proteins. We present preliminary evidence to suggest the a Gi protein is operational in the ZIR in human spermatozoa.

Materials and Methods

Sperm Capacitation and Binding to Structurally Intact Zonae pellucidae

Sperm suspensions were prepared as described by Lee and Storey [1985]. Mechanically isolated, structurally intact zonae pellucidae were prepared by forcing cumulus-free eggs through a narrow bore micropipette (i.d. = 0.75 diameter of egg with zonae). All incubations were carried out in HMB containing 4 mg/ml bovine serum albumin (BSA) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Sperm were capacitated at a concentration of $10\text{--}25 \times 10^6$ cells/ml for 90 min in the above medium containing 4 mM fura-2, AM or no fura-2, AM for NDAA experiments. After capacitation, the final sperm concentration was adjusted to 2×10^5 cells/ml by adding an aliquot of the labeled sperm suspension to a 100- μ l drop containing 100–150 mechanically isolated zonae pellucidae. The point of addition of sperm to the isolated zonae was taken to be the zero time point. Sperm were allowed to bind to the zonae for 15 min, at which time

the zonae with bound sperm were washed 3 times in HMB using a wide-bore micropipette to remove loosely associated sperm. For human experiments the culture medium HTF and modified HTF used in the handling of oocytes was purchased from Irvine Scientific. The culture medium used for incubation and washing of spermatozoa was Ham's F-10 purchased from Gibco Laboratories. The medium contained BSA at 5 mg/ml in all experiments.

Human sperm ejaculates were obtained from healthy, known fertile, adult donors via masturbation after 36–48 h abstinence. The ejaculates were allowed to liquify at room temperature for 15–30 min. All ejaculates had initial parameters of at least 65% motility, 60% motility, 60% normal morphology, and cell counts of $35 \times 10^6/\text{ml}$ or more. After the initial ejaculate had liquified, it was mixed with 3 volumes F-10 containing 5 mg/ml BSA. The sperm was washed by centrifugation in 12-ml conical tubes at 600 g for 10 min. The supernatant was removed and the sperm pellet gently overlaid with 2 ml Ham's F-10 containing 5 mg/ml BSA. The centrifuged tube was inclined at 45° and the motile sperm allowed to swim up out of the pellet for 30 min. This technique is a modification of the method of Overstreet et al. [1976]. The sperm sample obtained by this method showed motility percentages over 90% consistently. In experiments, IAP was added to the sperm suspension to a final concentration of 1 mg/ml. Sperm suspensions were incubated for 3 h to capacitate prior to addition of A23187 or solubilized human zona proteins.

Preparation of Mechanically Isolated, Structurally Intact Human Zonae pellucidae

Oocytes were obtained from IVF patients whom had at least one oocyte fertilized per treatment cycle to exclude possible zona abnormalities. All zona were removed from oocytes which were previously inseminated and did not fertilize within 72 h postretrieval. Zona were removed from oocytes judged as mature, immature, or postmature at the time of oocyte retrieval. All oocytes were cultured in HTF medium supplemented with 5 mg/ml ultrapure BSA. Each oocyte was removed from the culture dish using a drawn micropipet and placed into 100 μl of Hepes-buffered (10 mM) HTF, (MHTF) at pH 7.4 in a lux 50-well culture dish. The oocyte was transferred through three 100- μl drops to remove residual cumulus and serum albumin. The oocyte was transferred to a fourth drop of MHTF, pH 7.4, containing 1 mg/ml EDTA and 10 mg/ml Lima bean trypsin inhibitor. The oocyte was transferred to a fourth drop of the above medium and aspirated into a pipet 0.75 diameters of the egg. This facilitates rupture of the cytoplasm and extrusion of the intact zona pellucida. The intact zona is then transferred through four washes of the above medium. The intact zona is then placed into 100 μl of 1% Triton X-100 buffered with 10 mM ADA, 1 M NaCl and 1 mM benzamidine in DH_2O for 1 min. The intact zona are then transferred through four washes of 1 mM benzamidine in DH_2O and twice through DH_2O . The zona was then placed in a 1-ml nunc vial containing 50 μl of glycerol supplemented with 20 mg/ml ultrapure BSA and stored at 0°C until used. Induction of the acrosome reaction was initiated after a 3-hour incubation period in medium containing 5 mg/ml ultrapure BSA. Two methods were used to induce acrosome reactions: treatment with A23187 [Wolf et al., 1985; Byrd and Wolf, 1986], or incubation with acid-solubilized human zona pellucida. Human zona were removed from oocytes that were not fertilized in vitro and stored as described previously. 30–50 zonae were pooled into one tube and washed by centrifugation $3 \times$ with culture medium. The supernatant was removed and acidified (pH 4.0) Ham's F-10 without BSA was added to bring the zonae

concentration to 10 zonae/ μl . The zonae were incubated at 37 °C for a period of 1 h in order to dissociate the zona proteins. The final solution at pH 7.4 containing solubilized zonae pellucidae was added immediately to an appropriate volume of capacitated sperm suspension to achieve a final concentration of 2 zonae/ μl [Cross et al., 1986]. In the treatment with A23187, stock A23187 was added to an appropriate volume of capacitated spermatozoa to achieve a final concentration of 10 μM which equals 0.1% DMF (v/v). DMF alone was added to a second aliquot to 0.1% (v/v). Incubation was continued at 37 °C for an additional 2 h after the addition of either A23187 or zonae proteins. At the end of the incubation with either ionophore or solubilized zonae (5 h total), 5- μl aliquots were removed from each sample for the CTC assay. The remainder of the samples were prepared for the FITC-PSA lectin assay.

Fluorescence Assays

Fura-2, AM was dissolved as a 10-mM stock and aliquoted into 10-ml vials in DMF and kept in a light-shielded container at 4 °C at all times. NDAA was dissolved at 10 mM in DMF and kept under similar conditions. In a third set of experiments the ionophore nigericin was added to the final wash drop containing zonae with bound labeled sperm (fura-2,AM or NDAA) to a concentration of 2.5 mg/ml. The binding of sperm to the isolated zonae pellucidae was scored by phase-contrast microscopy ($\times 400$), with a Nikon Optiphot microscope equipped with interchangeable phase-contrast and epifluorescence optics. The bound sperm were then examined by epifluorescence microscopy ($\times 400$) for fura-2,AM or NDAA fluorescence. Mn^{2+} was added to final concentration of 0.25 mM in fura-2,AM experiments. NDAA solution was added to a final concentration of 10 mM in those experiments. Control zonae had an equal volume of DMF (NDAA carrier) added. In no experiment did the DMF concentration exceed 0.1%. After 5 min incubation, 10 zonae with bound sperm (fura-2,AM or NDAA labeled) were placed on a slide and covered with a coverslip. The remaining zonae with bound sperm were placed in the incubator for later observations. At the time of assay, 10 washed zonae with bound labeled sperm were placed on a slide immediately after which a coverslip was attached. These slides were placed on a warmed (37 °C) stage and viewed at 30-min intervals with phase-contrast and epifluorescence optics. Fluorescence loss was observed at the time intervals postbinding. At the time of assay, 9.9. μl of HMB containing 10 washed isolated zonae with bound fura-2,AM labeled sperm or NDAA were added to a warmed slide. Fluorescence excitation was at 405 nm with a half band width of 30 nm, using the excitation unit of the spectrum analyzer and the Optiphot 'V' filter cassette with excitation transmission between 395 and 425 nm with peak at 405 nm for NDAA and fura-2,AM. The emission was filtered through the DM 445 dichroic mirror of the filter cassette and a 470K guard filter. The 405 nm excitation of the fura-2,AM corresponds to the Ca^{2+} free form of the probe [Gryniewicz et al., 1985]. Controls for fura-2,AM experiments contained an equal amount of DMF (fura-2,AM carrier).

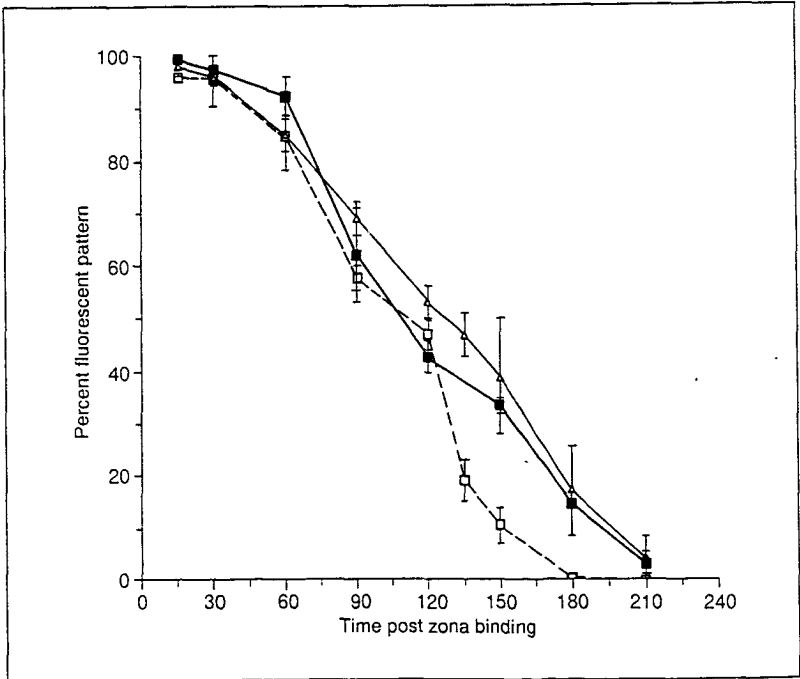
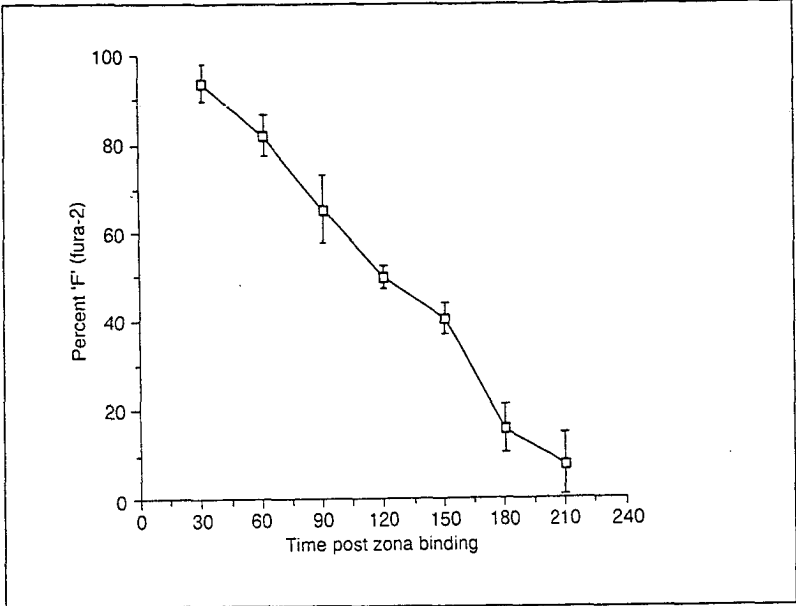
The CTC stock solution was prepared as previously described [Ward and Storey, 1984; Lee and Storey, 1985, 1989]. At the appropriate times during incubations (see Discussion), 5 μl of sperm suspension was placed on a warmed (37 °C) slide and an equal volume of CTC stock solution added, followed within 30 s by 0.05 μl 12.5% glutaraldehyde in 1 M Tris buffer (pH 7.8). The slides were kept in a humidified, light-shielded container until scoring. The sperm were examined at 400 \times for CTC fluorescence patterns with an Olympus BHZ fluorescent microscope equipped with phase-contrast and

epifluorescent optics. A minimum of 200 spermatozoa were scored for each determination for each time point of individual experiments.

The fluorescent FITC-PSA lectin was prepared as previously described by Cross et al. [1986]. At the appropriate times postincubation the spermatozoa were centrifuged at 600 g for 10 min and the supernatant removed. 50–200 μ l of methanol was added to the pellet and resuspended. The methanol spermatozoa suspension was allowed to sit at 4 °C overnight. 20-ml aliquots were placed onto cleaned slide and allowed to air dry. 20 ml of FITC-PSA lectin at a concentration of 100 mg/ml was added to each slide and allowed to incubate in a humidified, light-shielded container for 10 min. Unbound FITC-PSA was rinsed away by dipping each slide into a beaker of deionized water for approximately 15 s. Water was drained from the slide and the remainder wiped away from the center where the labeled sperm are located. A drop of mounting medium is added to the slide and a cover glass attached. A minimum of 200 cells were scored for each determination of the percentage of FITC-PSA lectin binding spermatozoa in each time point for each experiment.

Results

The Ca^{2+} probe fura-2 gives fluorescence over the entire mouse sperm cell after preincubation with fura-2 to 'load' the dye into the cell. But a more regionalized fluorescence over the anterior portion of the sperm head is seen in the presence of 0.25 mM Mn^{2+} to act as quencher for extracellular fura-2. The fluorescence over the anterior head represents fura-2 in the Ca^{2+} free form, trapped in an intracellular space inaccessible to divalent ions, e.g. the fluorescence quenching divalent cation Mn^{2+} [Gryniewicz et al., 1985]. This is evidently the acrosome itself or acrosome plus periacrosomal space. The head fluorescence is lost after time in sperm bound to isolated, structurally intact mouse zonae pellucidae (fig. 1). In the absence of added Mn^{2+} , fluorescence over the entire cell remains unchanged with time under these conditions. The kinetics of the loss of fura-2 fluorescence from the acrosomal region gives a characteristic 30-min lag period previously demonstrated with the fluorescent probes CTC, ACMA, and NDAA (fig. 2). The time course kinetics of loss of fura-2 fluorescence is not disturbed by the addition of the ionophore nigericin, which abolishes the head fluorescence of the pH probes ACMA and NDAA (fig. 3), in zona bound mouse spermatozoa. The ionophore nigericin acts by equilibrating the H^+ gradient across membranes by K^+/H^+ exchange. Loss of ACMA and NDAA fluorescence indicates loss of this gradient between the intracellular space over the anterior head and the suspending medium [Lee and Storey, 1985, 1989]. Fura-2 enables us to separate the loss of the H^+ gradient from



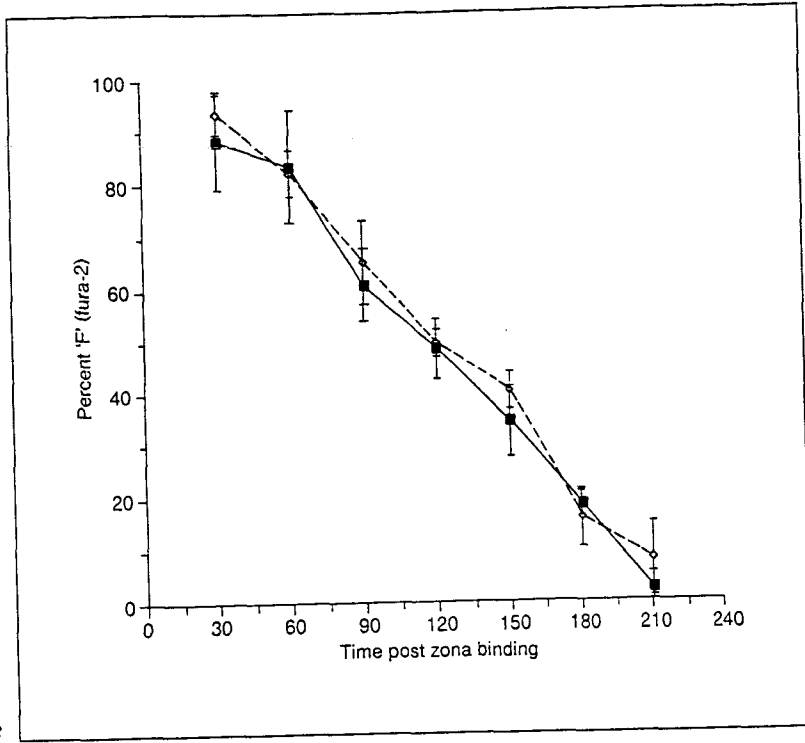


Fig. 1. Time course of the percentage of capacitated mouse sperm bound to isolated zona pellucida showing pattern 'F' (fura-2) head fluorescence. Note the characteristic 30-min 'lag' time prior to the onset of loss of fura-2 fluorescence loss. Each point represents the mean \pm SD of 5 replicate experiments with a minimum of 10 zona pellucida scored per experiment per point.

Fig. 2. Time course of the percentage of capacitated mouse sperm bound to isolated zona pellucida showing pattern 'B' (CTC), 'A' (ACMA), or 'N' (NDAA). Open squares denote scoring of sperm bound using the CTC fluorescence pattern 'B'. Closed squares denote scoring of sperm bound using the ACMA fluorescent assay having the fluorescent pattern 'A'. Open triangles denote scoring of bound sperm using the NDAA fluorescent assay having the fluorescent pattern 'N'. Each point represents the mean \pm SEM of 20 replicate experiments, with a minimum of 10 zona pellucida scored per experiment per point.

Fig. 3. Time course of the percentage of capacitated mouse sperm bound to isolated zona pellucida showing pattern 'F' (fura-2) head fluorescence \pm nigericin. Open diamonds denote control spermatozoa loaded with fura-2. Closed boxes represent sperm scored in the presence of the ionophore nigericin. Note the characteristic 30-min 'lag' time prior to the start of loss of fura-2 fluorescence. Each point represents the mean \pm SD of 5 replicate experiments, with a minimum of 10 zona pellucida scored per experiment per point.

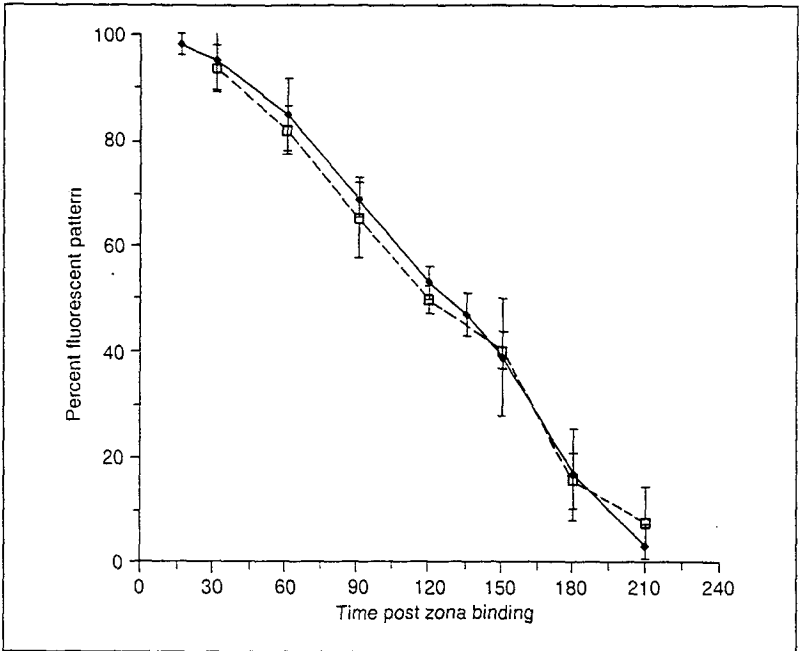


Fig. 4. Time course of the percentage of capacitated mouse sperm bound to isolated zona pellucida showing pattern 'F' (fura-2), and pattern 'N' head fluorescence. Open boxes denote spermatozoa scored after loading with the Ca^{2+} probe fura-2. Closed diamonds denote spermatozoa scored using the fluorescent pH probe NDAA. Each point represents the mean \pm SD of 5 replicate experiments with a minimum of 10 zona pellucida scored per experiment.

the influx of Ca^{2+} in the early stages of the ZIR in mouse spermatozoa. The implication is that loss of the H^+ gradient as it occurs during the ZIR is a consequence of Ca^{2+} influx, not a precursor to it (fig. 4).

In the human system we have used solubilized zona proteins to demonstrate the ZIR. This reaction is inhibited by preincubation during capacitation of the spermatozoa with IAP which ADP ribosylates the α -subunit of the G_i protein heterodimer. The spontaneous and ionophore-induced acrosome reactions are not affected by IAP (fig. 5). The inhibition by IAP of the ZIR implicates a G_i protein is involved in the modulating of this reaction. The loss of acrosomal status was monitored using the CTC and PSA-FITC fluorescent labeling probes. Both fluorescent probes give similar results in monitoring human spermatozoa acrosomal status.

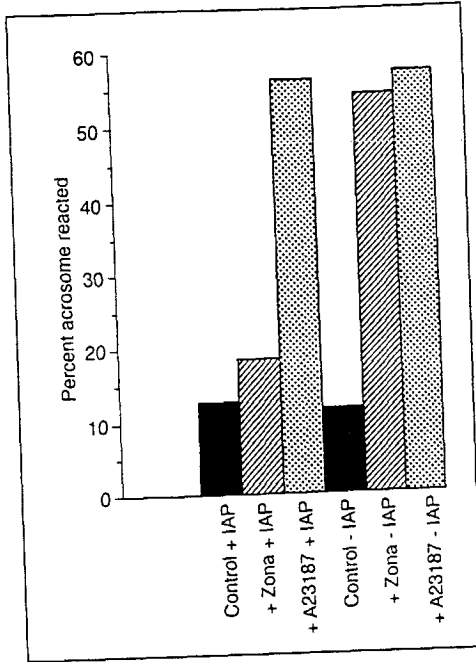


Fig. 5. Percentage of human spermatozoa acrosome reacted scored via FITC-PSA lectin \pm 1 mg/ml IAP + 2 zona/ml or 10 mM A23187. Dark bars represent control sperm incubated 3 h \pm IAP and incubated an additional 2 h. Striped bars represent sperm incubated 3 h \pm IAP 2 zona/ml for an additional 2 h. Note the lack of acrosome reaction in the IAP-treated sperm. Mottled bars represent sperm preincubated 3 h \pm IAP + 10 mM A23187 for an additional 2 h.

Discussion

In this study we have addressed the question of intracellular Ca^{2+} localization in the mouse sperm by observation of single cells bound to zonae. Localization was achieved by utilizing the fluorescent quenching property of Mn^{2+} to eliminate extracellular fluorescence. The fluorescence observed in the presence of Mn^{2+} must be from fura-2 in an intracellular compartment not accessible to extracellular Mn^{2+} and, by extension, not accessible to extracellular Ca^{2+} . From its location on the sperm head, we infer that the fluorescence is emanating from the acrosome and or acrosomal space overlying the acrosome. This fluorescence has the same regional location as that of monoclonal antibodies.

It should be noted that the 4 μM fura-2,AM used during the loading procedure was immediately diluted to 0.08 μM upon adding the sperm to the drops of media containing the zona and this dilution was followed by three washes with fresh media to insure reduction of free fura-2,AM.

The onset of membrane permeability to Ca^{2+} is synchronous with the onset of membrane permeability to H^+ , as indicated by loss of pattern N (fig. 2). The most probable explanation is that interaction of zona protein, in the case of the mouse ZP3, with the plasma membrane occurs through a receptor-mediated pathway which leads to a generalized permeability of both the plasma membrane and outer acrosomal membrane. The influx of Ca^{2+} into, and H^+ gradient loss from the acrosomal compartment appear to be parallel reactions. In mouse and possibly human sperm, the ZIR has the elements of a ligand receptor-mediated process, involving such participants in an intracellular signal transduction-mediated process as G_i proteins, protein kinase C, phospholipase C, and a possible receptor for the zona ligand that triggers the reaction. Precisely how these elements interact is as yet unclear, but the results of this study and some preliminary work carried out in our laboratory with the human system indicate that the endpoint of the process is free access of extracellular cations through the plasma and outer acrosomal membranes, allowing their entry into the acrosomal compartment.

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