Molecular Human Reproduction Vol.8, No.1 pp. 37–47, 2002

An anti-actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm

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We report an inhibitory effect of an anti-actin monoclonal antibody (mAb) on the human zona pellucida (ZP)-induced acrosome reaction (AR). Motile sperm were incubated with native human ZP for 2 h in medium containing either the anti-actin mAb, an irrelevant control mAb or cytochalasins B or D (40 μmol/l). Sperm bound to the ZP were recovered and the AR was determined by fluorescein-labelled *Pisum Sativum* agglutinin. Anti-mouse immunoglobulin G (mIgG) Dynabeads, immunofluorescence and immunogold were used to detect the location of the anti-actin mAb in sperm. The anti-actin mAb significantly inhibited the ZP-induced AR (equivalent to cytochalasins), the ionophore A23187-induced AR and hyperactivation of sperm in medium. After incubation with anti-actin mAb, anti-mIgG beads bound to the head of >50% of sperm recovered after binding to the ZP and 10% of sperm remaining in the medium. The proportion of sperm that bound anti-mIgG beads after recovery from binding to the ZP in the presence of the anti-actin mAb was significantly correlated with the ZP-induced AR in the absence of the antibody. Immunofluorescence and immunogold demonstrated entry of the anti-actin mAb into sperm. This study suggests that the sperm plasma membrane becomes permeable to the anti-actin mAb during capacitation and initiation of the AR.

Key words: actin/actin monoclonal antibody/cytochalasin/human acrosome reaction/sperm

Introduction

The human acrosome and the acrosome reaction (AR) are very important for sperm–oocyte interaction during fertilization. Human sperm without an acrosome, for example round-headed acrosomeless sperm, do not bind to and penetrate the zona pellucida (ZP) or fuse with the oolemma of ZP-free oocytes *in vitro* (von Bernhardi *et al*., 1990; Bourne *et al*., 1995). It is believed that acrosome-intact sperm bind to the ZP and the AR occurs on the surface of the ZP, triggered by ZP glycoproteins such as ZP3 (Bleil and Wassarman, 1988; Tesarik, 1989; Yanagimachi, 1994; Wassarman, 1999). There is a close relationship between the ZP-induced AR and sperm-ZP penetration *in vitro* (Liu and Baker, 1996a,b). In conventional IVF, defects of sperm–ZP interaction and sperm-ZP penetration are the main sperm dependent causes of failure of fertilization (Liu and Baker, 2000). Inhibition of acrosin activity with trypsin inhibitors completely blocks sperm-ZP penetration and reduces the AR of sperm bound to the ZP (Liu and Baker, 1993; Llanos *et al*., 1993). The importance of the ZP-induced AR for sperm-ZP penetration during the human fertilization process is supported by our discovery of patients with disordered ZP-induced AR, which is a natural cause of failure of sperm-ZP penetration and failure of fertilization *in vitro*, despite normal semen analysis and normal sperm–ZP binding (Liu and Baker, 1994).

There are many studies in the literature on the spontaneous and stimulated AR using chemical and biological inducers such as calcium ionophore A23187, human follicular fluid and progesterone (Tesarik, 1985; Aitken *et al*., 1993; Brucker and Lipford, 1995). Because of the limited availability of human ZP material, there are fewer studies on the mechanism of the AR induced with native human ZP (Cross *et al*., 1988; Coddington *et al*., 1990; Hoshi *et al*., 1993; Bielfeld *et al*., 1994; Morales *et al*., 1994; Liu and Baker, 1996a,b). We have found that there is no correlation between calcium ionophore A23187-induced AR and human ZP-induced AR *in vitro* (Liu and Baker, 1996a,b). In human IVF, only a proportion of sperm are capable of binding to the ZP. Sperm bound to the

ZP are selected due to better morphology and an intact acrosome (Liu and Baker, 1992; Garrett *et al*., 1997). The ZP-induced AR is limited to those sperm that are capable of binding to the ZP and therefore ZP should be used as the physiological inducer to study the mechanism of the human AR.

The ZP-induced AR is a complex process and the molecular mechanisms are not fully understood. Although calcium influx plays a central role in the AR, many other factors are involved, including several signal transduction factors such as protein kinases (PKA, PKG and PKC) and G-proteins. In particular, PKC has been found to play an important role in the human AR (De Jonge *et al*., 1991; Breitbart *et al*., 1992, 1997; De Jonge, 1995; Doherty *et al*., 1995; Liu and Baker, 1997). We have also found that actin polymerization is critical in the human ZP-induced AR (Liu *et al*., 1999). This cytoskeletal protein is present mainly in the monomeric form in many mammalian sperm (Clarke *et al*., 1982; Ochs and Wolf, 1985; Flaherty *et al*., 1988). In the pig, actin polymerization during capacitation and the AR is essential for fertilization (Camatin *et al*., 1986; Castellani-Ceresa *et al*., 1993). Inhibition of actin polymerization with cytochalasin D blocks guinea pig and human sperm penetration of hamster oocytes and the human ZP-induced AR (Rogers *et al*., 1989; Liu *et al*., 1999).

We report here that an anti-actin monoclonal antibody (mAb) inhibits hyperactivation and the ZP-induced AR *in vitro* and present studies to determine how the mAb enters live human sperm during capacitation, ZP-binding and initiation of the ZP-induced AR.

Materials and methods

Chemicals and culture medium

The mouse anti-actin mAb of IgG_1 class was purchased from ICN Pharmaceuticals Inc. (Catalogue number 691002; Costa Mesa, CA, USA). This antibody reacts specifically with all known isoforms. Cytochalasins B and D, dimethyl sulphoxide (DMSO), calcium ionophore A23187, $(NH_4)_2SO_4$ and *Pisum Sativum* agglutinin (PSA) labelled with fluorescein isothiocyanate (PSA-FITC) or tetramethyl-rhodamine B isothiocyanate (PSA-TRITC), anti-mouse IgG (anti-mIgG) conjugated with 5 µm gold, anti-mIgG labelled with FITC and sodium azide were purchased from Sigma Chemical Company (St Louis, MO, USA). Human tubal fluid (HTF; Irvine Scientific, Irvine, CA, USA) medium supplemented with 5 mg/ml bovine serum albumin (BSA; Commonwealth Serum Laboratory, Melbourne, Australia) (HTF-BSA) was used as a culture medium for all the experiments. Dynabeads coated with anti-mIgG were purchased from Dynal (Oslo, Norway). An irrelevant monoclonal antibody (4C7) with the same subclass (mouse IgG_1) and protein concentration (1 mg/ml) as the anti-actin mAb was used as a negative control wherever the anti-actin mAb was used.

Cytochalasins B and D (2 mmol/l) and A23187 (5 mmol/l) stock solutions were prepared in DMSO and stored in aliquots at –70°C. On the day of the experiment, the cytochalasin stock solution was diluted twice with HTF medium and then 20 µl of the diluted cytochalasin was added to 0.5 ml sperm suspension to achieve final a concentration of 40 µmol/l. The ionophore stock solution was diluted 10 times with HTF medium and then 3 µl of the diluted ionophore was added to 0.5 ml sperm suspension to achieve a final concentration of 3 µmol/l. The same dilutions of DMSO alone were used for controls.

Western blot

Western blotting was performed to confirm the specificity of the anti-actin mAb in of human sperm. Washed motile sperm (~10^8) suspensions selected by swim-up from 3–5 normospermic semen samples were extracted with 1% Triton X-100 in Tris-HCl containing a protease inhibitor cocktail (ICN Pharmaceutical Inc., CA, USA). The protein was concentrated by methanol precipitation at –20°C, resuspended in sample buffer and boiled in sample buffer before sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) membrane. Non-specific protein binding to the membrane was blocked by incubation with 5% skim milk powder in Tris-buffered saline (TBS) overnight at 4°C. The PVDF membrane was incubated first with the anti-actin mAb (3 µg/ml) for 2 h and then with a secondary antibody, peroxidase-conjugated goat anti mouse IgG (0.5 µg/ml, Dako, Denmark) for 90 min. Excess antibody was removed by washing the membrane in 0.05% Tween-20 in TBS for 1 h. The membrane was finally incubated in Western Blot Chemiluminescence Reagent (NEN Life Science Products) for 1 min before exposure to X-OMAT Blue XB-1 (Kodak, Australia). Mouse brain extract was used as a positive control for actin in each blotting experiment.

Human oocytes

Oocytes that showed no evidence of two pronuclei or cleavage at 48–60 h after insemination in a clinical IVF programme were used. Most of the oocytes displayed regular shape and had lost the cumulus and corona cells. In the remainder, the cumulus and corona cells were removed by aspiration with a glass pipette. If the oocyte had sperm bound to the ZP from the IVF insemination, these were removed by repeated aspiration of the oocyte using a small glass pipette with an inner diameter (120 µm) slightly smaller than the oocyte diameter. Most of the oocytes were obtained from patients with partial failure of fertilization and >50% of these unfertilized oocytes had a few sperm penetrating the ZP from the IVF insemination. However, we have shown previously that oocytes with sperm in the ZP have a similar capacity for sperm–ZP binding and ZP-induced AR as those without sperm penetrating the ZP (Liu and Baker, 1996a). Some of the oocytes were stored in 1 mol/l ammonium sulphate at 4°C (Yanagimachi *et al*., 1979; Liu and Baker, 1996a). The salt-stored oocytes were washed in HTF-BSA medium with four changes of the medium at least 4 h before being used for the sperm–ZP interaction tests. For each experiment, the same type of oocytes (fresh or salt-stored) were used for both test and control.

All patients signed consent forms permitting use of their gametes for research. The Royal Womens Hospital Research and Ethics Committees approved the project.

Sperm samples and preparation

Semen was obtained by masturbation after 2–3 days abstinence, from men with normal semen analysis according to World Health Organization criteria (World Health Organization, 1992) together with strict sperm morphology assessment (normal >15%) and normal sperm–ZP binding (Liu and Baker, 1992). Motile sperm were selected by a swim-up technique with 1 h incubation as described previously (Liu and Baker, 1996a,b). The motile sperm were washed with fresh HTF-BSA medium by centrifugation and then the sperm pellet was resuspended in fresh HTF-BSA medium to a sperm concentration of 4×10^6/ml for subsequent experiments.

Sperm–ZP interaction test

Sperm suspensions of 0.5 ml in 4-well culture plates (Nunc, Rosilde, Denmark) were mixed with the test or control agents and incubated

for 10 min for equilibration before four oocytes were added to each culture well and incubation was continued for 2 h at 37°C in 5% CO_2 in air. After this 2 h incubation period, each group of four oocytes was transferred to phosphate-buffered saline (PBS) containing 2 mg/ml BSA. The oocytes were then washed by aspiration with a glass pipette (inside diameter ~250 μm) to dislodge sperm loosely adherent to the surface of the ZP. The number of sperm bound tightly to the ZP was counted and usually found to be >100 per oocyte.

All sperm bound to the surface of the ZP of the four oocytes were then removed by repeated vigorous aspiration with a narrow gauge glass pipette with an inner diameter (~120 μm) slightly smaller than that of the oocyte (Liu and Baker, 1996a,b). This was performed on a glass slide with ~5 μl PBS containing 0.2% BSA and the ZP-bound sperm were smeared in a limited area (~16 mm^2) which was marked with a glass pen to help find the sperm under the microscope for acrosome assessment. After the pipetting there were no sperm remaining bound on the surface of the ZP. Usually >500 ZP-bound sperm were obtained from each group of four oocytes. Our previous studies confirmed that this pipetting procedure does not affect acrosome status or damage the sperm severely, as many remain motile after removal from the ZP (Liu and Baker, 1994, 1996a).

As well as sperm ZP-binding and the ZP-induced AR, the motility and the AR of sperm remaining in the medium were also determined. After 2 h incubation, the percentage motility of sperm in the medium was assessed manually by counting 200 sperm per sample using phase contrast microscopy at ×400 magnification. For each experiment, several replicates were performed using sperm from different men.

Assessment of acrosome status

The acrosome status of sperm was determined with fluorescein labelled *Pisum Sativum* agglutinin (PSA; Sigma) as described previously (Cross *et al*., 1986; Liu and Baker 1996a). Sperm smears were fixed in 95% ethanol for 30 min after air-drying and then stained in 25 μg/ml PSA in PBS for 2 h at 4°C. The slides were washed and mounted with distilled water and 200 sperm per sample were counted with a fluorescence microscope using excitation wavelengths of 450–490 nm and oil immersion at a magnification of ×400. When more than half the head of a sperm was brightly and uniformly fluorescing, the acrosome was considered intact. Sperm with a fluorescing band at the equatorial segment or without fluorescence (a rare pattern) were considered acrosome reacted. One technician scored acrosome status blind to the experimental group of the sperm.

Effect of actin monoclonal antibody on ZP-induced AR

Inhibition of the ZP-induced AR by various concentrations of the anti-actin mAb was performed as follows: motile sperm (2×10^6) selected by swim-up in 0.5 ml HTF-BSA medium were mixed with 1:50 to $1:10^5$ dilutions of anti-actin mAb or control mAb (4C7) for 10 min for equilibration. A group of four oocytes were then added to each culture well containing the various dilutions of the anti-actin mAb and incubated for 2 h at 37°C in 5% CO_2 in air. The anti-actin mAb was present in the medium all the time during the 2 h incubation of sperm with oocytes. Sperm bound to the ZP of the four oocytes for each antibody dilution were recovered and the AR was assessed. Five replicates were performed using sperm samples from five different men.

To compare the inhibitory effects of anti-actin mAb and cytochalasins B or D on the ZP-induced AR, motile sperm (2×10^6) selected by swim-up in 0.5 ml HTF-BSA medium were mixed with the anti-actin mAb (1:100 dilution), cytochalasins B or D (40 μmol/l) or control media and incubated for 10 min for equilibration. Four oocytes were then added to each of the test or control sperm suspensions and incubation was continued for 2 h. The AR of sperm bound on the ZP, and the motility and AR of sperm in the medium were assessed.

Effect of sodium azide and dialyzed anti-actin mAb on the ZP-induced AR

Because the commercial anti-actin mAb contains 0.1% sodium azide as preservative and may contain other small molecules such as salts that could affect the ZP-induced AR, the effects of sodium azide and dialysis of the mAb on the ZP-induced AR and motility of sperm in the medium were tested. The ZP-induced AR was performed on five sperm samples with 0.2% sodium azide in the test medium. To check the effect of dialysis, 3 ml of 1:50 diluted anti-actin mAb was dialyzed against 1 l of HTF medium for 24 h at 4°C using cellulose tubing with a molecular weight pore size of 12 kDa (Sigma). Dialyzed anti-actin mAb, undialyzed anti-actin mAb and control mAb (4C7) were diluted to 1:100 and tested for effect on the ZP-induced AR.

Effect of actin monoclonal antibody on ionophore A23187-induced AR

Motile sperm (2×10^6) selected by swim-up in 0.5 ml HTF-BSA medium, were pre-incubated with 1:100 anti-actin mAb or control antibody (4C7) for 10 min. Then, 3 μl of diluted A23187 stock solution was added to each test sperm suspension to achieve a final concentration of 3 μmol/l. A similar dilution of DMSO alone was added to the control sperm suspension. After mixing well, both test and control sperm were incubated for 1 h at 37°C in 5% CO_2 in air. After incubation, sperm were washed with 5 ml 0.9% NaCl. The sperm pellet was resuspended in ~50 μl of 0.9% NaCl and ~5 μl of the sperm suspension was used to make a smear on a glass slide for assessment of acrosome status. The ionophore-induced AR was calculated from the percentage of acrosome-intact sperm exposed to DMSO alone, minus the percentage of acrosome-intact sperm exposed to ionophore A23187. Six replicates were performed using sperm samples from different men.

Because swim-up was used to select sperm with motility >90% before exposure to ionophore A23187, the viability of acrosome-reacted sperm was not assessed. We have previously shown that similar results are obtained when ionophore A23187-induced AR is assessed in either viable or total (viable and non-viable) populations when a motile sperm suspension prepared by swim-up is used for the test (Liu and Baker, 1998).

Assessment of sperm motility, velocity and hyperactivation

Sperm motility and hyperactivation were measured after 2 h incubation with the actin or control mAb by the Hamilton-Thorn Motility Analyzer (IVOS 10, 60 Hz; Hamilton-Thorn Research, Danvers, MA, USA). Because the sperm concentration was only 2×10^6/ml when the sperm were incubated in culture medium, the sperm concentration was adjusted to ~20×10^6/ml by centrifugation at 800 *g* for 5 min and resuspended in 100 μl of the same medium. A sample of 5 μl was placed in a Microcell (20 μm depth) for computer-assisted semen analysis (CASA) assessment. Progressive motility was defined as the percentage of sperm with average path velocity (VAP) >20 μm/s. The Burkman criteria (Burkman, 1991) for hyperactivation were used as follows: curvilinear velocity (VCL) ⩾100 μm/s, linearity (LIN) <65% and amplitude of lateral head displacement (ALH) ⩾7.5 μm. For each sperm sample, four to six fields with mean total number of 340 (range 217–460) sperm were assessed. The percentage motility was also assessed manually by counting 200 sperm per sample using phase contrast microscopy at ×400 magnification.

Anti-mouse IgG Dynabead test

Dynabeads coated with anti-mIgG were used to detect anti-actin mAb on the surface of sperm in the culture medium and after removal

from the ZP. The Dynabeads were washed with 10 ml HTF-PBS medium and resuspended in the same medium at a concentration of ~4×10^8/ml. After 2 h incubation with either the anti-actin mAb or control 4C7 mAb plus the sperm suspension, the oocytes were transferred to HTF-BSA medium and washed with four changes of medium. Sperm bound to the surface of the ZP were removed by aspiration with a small-bore pipette into ~4 µl of medium on a glass slide. Then, 3 µl of anti-mIgG bead (1.2×10^6) suspension was added, mixed with the sperm and covered with a small coverslip (~4 mm^2). After 3 min at room temperature, the proportion of sperm bound with one or more beads was assessed by counting 100 motile sperm using phase-contrast microscopy at ×400 magnification. Sperm recovered from the medium were also washed three times with 10 ml HTF-BSA medium and 10 µl of the resuspended pellet (0.2×10^6 sperm) was mixed with 5 µl of the bead suspension (2×10^6) and covered with a coverslip. The percentage of sperm bound with beads was determined by counting 200 motile sperm. In some experiments, the same sperm samples were used for both the bead binding test and the measurement of the ZP-induced AR in the absence of the anti-actin mAb.

Immunofluorescent detection of anti-actin mAb on live capacitated sperm

To show that anti-actin mAb can enter live capacitated sperm, motile sperm (2×10^6 in 0.5 ml HTF-BSA) were incubated with actin or control mAb (1:100) for 2 h and the sperm suspension centrifuged and the supernatant removed. The sperm pellet was washed three times with 10 ml PBS with 2 mg/ml BSA, resuspended in 40 µl of PBS-BSA, smeared on a glass slide and allowed to air-dry. The slides were fixed in 95% ethanol for 30 min. The fixed sperm smear was placed in PSA-TRITC for 1 h to assess the status of the acrosome. After rinsing the slide with distilled water, the slides were incubated with 10 mg/ml BSA in PBS for 10 min and then anti-mIgG (secondary antibody) labelled with FITC in PBS containing 10 mg/ml BSA for 2 h in a humidified box at 37°C. Finally, the slide was rinsed and mounted with distilled water. Immunofluorescent patterns of the sperm, double-labelled with the anti-actin mAb and PSA-TRITC for acrosomal status, were examined under a fluorescence microscope (Dialux 20; Leitz, Wetzlar, Germany) using excitation wavelengths of 450–490 nm for FITC (green) and 546 nm for TRITC (red) at a magnification of ×400. The proportion of green staining sperm was calculated by counting 200 acrosome intact sperm.

Immunogold electron microscopy (EM)

Pre-embedded immunogold EM was performed to confirm entry of the anti-actin mAb into live sperm and was similar to the above immunofluorescent study. Motile sperm (6×10^6 in 1.5 ml HTF-BSA) were incubated with anti-actin mAb or control mAb (1:100) for 2 h and the sperm suspension was centrifuged and the supernatant removed. The sperm pellet was washed three times with 10 ml PBS containing 2 mg/ml BSA and then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 30 min. Sperm were embedded in L.R.White resin after serial dehydration through 70–100% ethanol. EM sections were incubated with anti-mouse IgG (secondary antibody) conjugated with 5 µm gold in PBS containing 10 mg/ml BSA for 2 h at 37°C.

Post-embedded immunogold EM was also performed on motile sperm without pre-exposure to anti-actin mAb. For this experiment, only EM sections of the untreated sperm were exposed to anti-actin mAb and then the gold-labelled secondary antibody to demonstrate the distribution of actin in sperm.

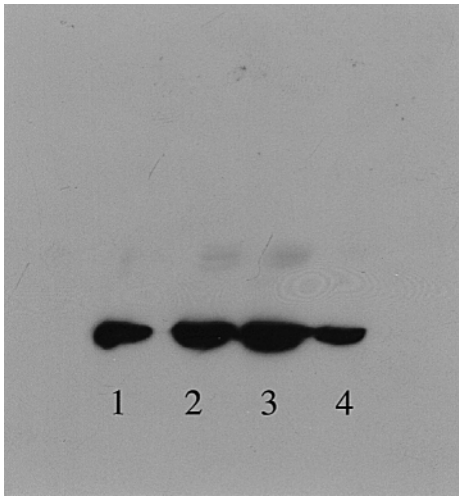

Figure 1. Western blots of human sperm membrane extracts with the anti-actin mAb. Lane 1, control actin from mouse brain extract; lanes 2–4, three different sperm extracts from groups of 3–5 men. The blots for the control and each sperm extract are similar and the molecular weight is ~43 kDa.

Transmission EM

EM was performed on two sperm samples with eight oocytes to determine the effect of the anti-actin mAb on acrosome morphology of sperm bound to the ZP. After 2 h incubation of oocytes with sperm in the presence of anti-actin mAb or control 4C7 mAb, oocytes were transferred to fresh medium and washed by repeated aspiration with a fine glass pipette (inner diameter ~250 µm) to dislodge sperm loosely bound to surface of the ZP. Oocytes with many sperm tightly bound to the ZP were fixed in 4% glutaraldehyde, post-fixed with 1% osmium and embedded in resin (Liu and Baker, 1994). Acrosome status and morphology were examined in EM photographs of sections of sperm heads bound to the ZP.

Statistical analysis

The dose–response of the anti-actin mAb on the ZP-induced AR was examined by log linear regression analysis. The significance of differences between test and control percentages of motility, acrosome-reacted sperm, hyperactivation, sperm bound with beads and sperm velocity were examined by analysis of variance or paired *t*-tests. The significance of the correlation between the ZP-induced AR and the percentage of sperm bound with anti-mIgG beads was examined by Spearman's non-parametric test.

Results

Confirmation of anti-actin mAb specificity

Western blots of sperm membrane extracts with the anti-actin mAb confirmed specificity for human sperm actin (Figure 1). Both control actin (rat brain extract) and sperm actin had a molecular weight of ~43 kDa.

Inhibition of human ZP-induced AR by anti-actin mAb

Initial experiments showed the anti-actin mAb at 1:50 and 1:100 dilutions significantly ($P < 0.01$) inhibited ZP-induced AR and were equally effective for both concentrations of the antibody (control 56 ± 17.4%, anti-actin mAb dilution 1:50 18 ± 5.6% and dilution 1:100 18 ± 6.1%, $n = 4$). In this experiment, significant inhibition of the ZP-induced AR was

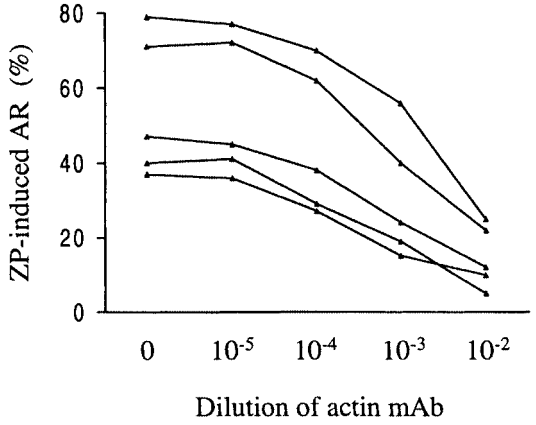

Figure 2. Dose–response effect of the actin monoclonal antibody on inhibition of the human ZP-induced AR. Five experiments were performed using semen from different normospermic men indicated by each line. Each point represents the AR of sperm removed from a group of four oocytes. The anti-actin mAb significantly ($P < 0.01$) inhibited the ZP-induced AR in a dose-dependent manner.

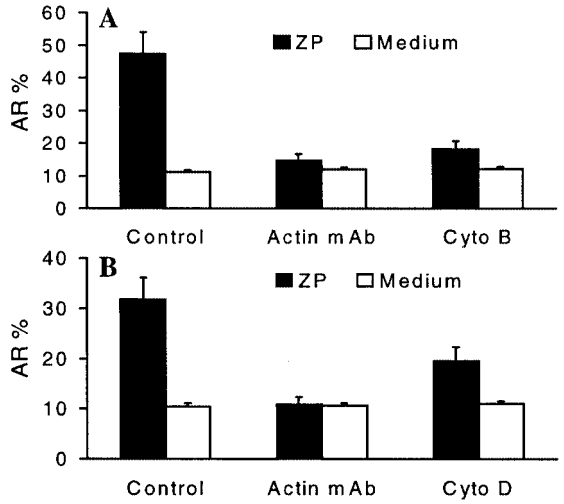

Figure 3. Comparison of the inhibitory effects of the anti-actin monoclonal antibody (Actin mAb) with cytochalasin B (Cyto B) or D (Cyto D) on the ZP-induced AR (ZP) and the spontaneous AR of sperm in the culture medium (Medium). The bars represent mean + SEM from (**A**) 10 and (**B**) six sperm samples. The ZP-induced AR was significantly lower with the anti-actin mAb ($P < 0.001$), Cyto B ($P < 0.001$) or Cyto D ($P < 0.01$) than with the control mAb (4C7) and there was no significant difference between the anti-actin mAb and Cyto B ($P > 0.05$) and Cyto D ($P > 0.05$). There was no significant difference in spontaneous AR of sperm in the culture medium between control, anti-actin mAb, Cyto B or Cyto D.

observed at antibody concentrations higher than 0.1 μg/ml ($1:10^4$ dilution) (Figure 2). There was no inhibitory effect on the ZP-induced AR by the anti-actin mAb at a concentration of 0.01 μg/ml ($1:10^5$ dilution). The 1:100 dilution of the anti-actin mAb had a similar inhibitory effect on the ZP-induced AR, as did 40 μmol/l cytochalasins B or D (Figure 3A,B). Both anti-actin mAb and cytochalasins B and D had no effect on sperm–ZP binding (data not shown).

Table I. Mean (± SD) percentage total motility, progressive motility and hyperactivation of sperm after incubation with anti-actin or control (4C7) monoclonal antibodies (1:100 dilution) for 2 h with 10 normospermic semen samples

	Control 4C7 mAb	Anti-actin mAb	*P*-value
Total motility (%)	91 ± 3.1	90 ± 3.0	> 0.05
Progressive motility (%)	82 ± 8.2	75 ± 9.7	> 0.05
Hyperactivation (%)	13 ± 4.8	5 ± 3.9	< 0.01

Effect of sodium azide and dialyzed anti-actin mAb on the ZP-induced AR

Because the commercial anti-actin mAb contains 0.1% sodium azide as a preservative, the effect of sodium azide (0.2%) on the ZP-induced AR and sperm motility was determined for five different samples. Sodium azide had no effect on the ZP-induced AR [mean ± SD, test 40 ± 15.1% versus control 40 ± 16.5%, not significant (NS)], the spontaneous AR (test 8 ± 3.3% versus control 9 ± 4.0%, NS) or motility (test 90 ± 2.0% versus control 90 ± 1.5%, NS) of sperm in the medium.

Dialysis of the mAb had no significant effect. The anti-actin mAb with and without dialysis had a similar inhibitory effect on the ZP-induced AR ($n = 4$, mean ± SD, control 61 ± 17%, dialyzed anti-actin mAb 24 ± 13% versus anti-actin mAb without dialysis 26 ± 13%, NS).

Inhibition of ionophore A23187 induced AR by anti-actin mAb

The anti-actin mAb (1:100) significantly decreased ionophore A23187-induced AR (mean ± SD, anti-actin mAb 27 ± 9.1% versus control 4C7 mAb 48 ± 7.4%, $n = 6$, $P < 0.01$). In contrast, the anti-actin mAb had no effect on the spontaneous AR of sperm in the culture medium after 2 h incubation (anti-actin mAb 11 + 5.6% versus control 4C7 mAb 12 ± 5.6%, $n = 19$, not significant).

Effect of anti-actin mAb on sperm motility and hyperactivation

The anti-actin mAb (1:100) had no effect on the percentages of either total sperm motility or progressive motility (VAP >20 μm/s) after 2 h incubation. However, the anti-actin mAb (1:100) significantly decreased percentage hyperactivation (Table I).

Anti-actin mAb-binding to the sperm head during capacitation and ZP interaction

After 2 h incubation with 1:100 anti-actin mAb, anti-mIgG beads bound to the heads of 10% (range 1–27) of motile sperm in the culture medium and 50% (range 6–81) of sperm removed from the ZP (Figure 4). From examination of >1000 sperm bound with beads it appeared that the beads preferentially bound to the middle of the sperm head in the region of the equatorial segment. Anti-mIgG beads did not bind to sperm incubated with control mAb (4C7). After exposure to anti-actin mAb, a significantly higher percentage sperm removed from the ZP bound anti-mIgG beads than did those in the culture medium ($P < 0.001$), and there was a significant

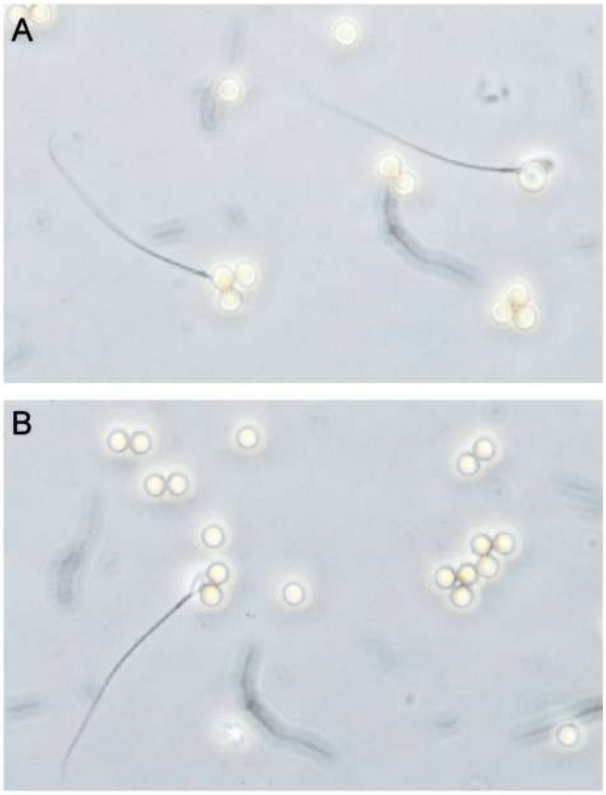

Figure 4. Anti-mIgG beads binding to the head of motile sperm removed from the ZP surface after exposure to anti-actin mAb. (**A**) Two sperm one with one and the other with two beads bound to the head; (**B**) one sperm with two beads bound to the head. (Phase-contrast microscopy, ×400 magnification).

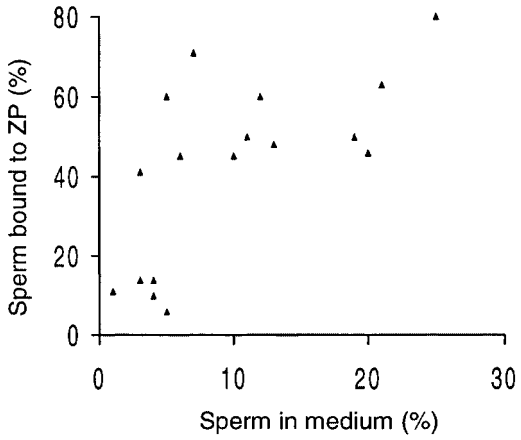

Figure 5. Correlation between the percentages of sperm bound with anti-mIgG beads following recovery from the culture medium or from the surface of the ZP after 2 h incubation with the anti-actin mAb ($n = 17$, Spearman $r = 0.734$, $P < 0.01$).

correlation between the percentages (Figure 5). That is, when sperm were incubated with the anti-actin mAb and oocytes for 2 h, the percentage that would bind anti-mIgG beads after recovery from the surface of the ZP was higher, but also related to the percentage from the medium that would bind anti mIgG beads.

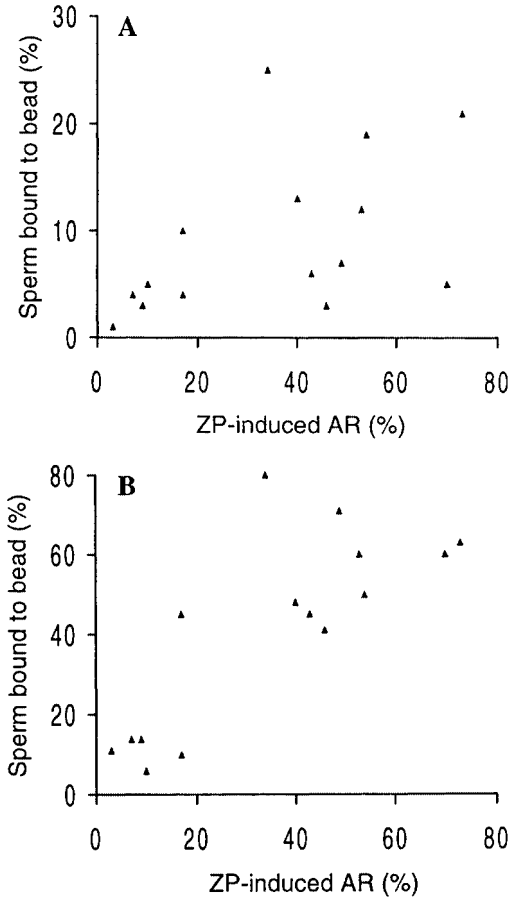

Figure 6. Correlations between the percentages of sperm incubated with the anti-actin mAb for 2 h and which bound anti-mIgG beads after: (**A**) recovery from the culture medium ($n = 15$, Spearman $r = 0.582$, $P < 0.05$) or (**B**) removal from the ZP ($n = 15$, Spearman $r = 0.732$, $P < 0.01$), and the ZP-induced AR of the same sperm not incubated with the anti-actin mAb.

Relationship between anti-actin mAb binding to sperm and the ZP-induced AR

There was a significant (Spearman $r = 0.582$, $P < 0.05$) correlation between the percentage of sperm binding anti-mIgG beads after exposure to the anti-actin mAb in the culture medium and the ZP-induced AR in the absence of the antibody (Figure 6A). Similarly, but more highly significant (Spearman $r = 0.732$, $P < 0.01$) was a correlation between the percentage of anti-mIgG bead-positive sperm recovered after binding to the ZP in the presence of the anti-actin mAb, and the ZP-induced AR in the absence of the antibody (Figure 6B).

Immunofluorescence of anti-actin mAb exposed sperm

Dual labelling immunofluorescence indicated that anti-actin mAb entered some of the live acrosome-intact sperm during the incubation period (Figure 7). There were two main patterns observed for the anti-actin mAb: equatorial segment only or the whole acrosome. Sperm incubated with the control mAb were negative. In three sperm samples assessed with both techniques, the proportion of acrosome-intact sperm with positive fluorescence was similar to the proportion of sperm with anti-mIgG beads bound to the head (10 ± 2.6% versus 11 ± 2.2%, $n = 3$).

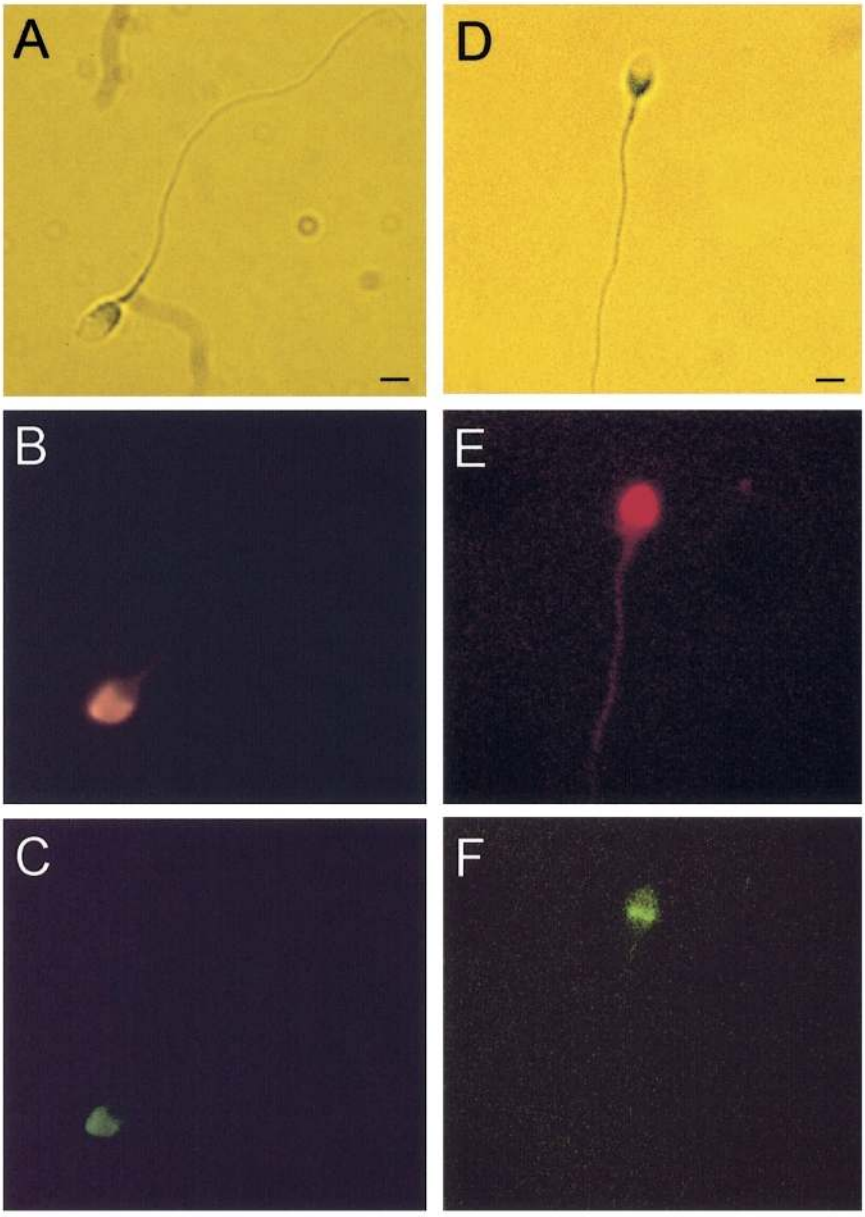

Figure 7. Immunofluorescent demonstration of the anti-actin mAb in live sperm. Sperm were incubated with the anti-actin mAb for 2 h, washed three times to remove unbound antibody and the sperm pellet was smeared on a glass microscopy slide, fixed with 95% ethanol and stained with PSA-TRITC for 1 h and anti-mouse lgG (secondary antibody) labelled with FITC. Two sperm (**A**,**B**,**C** and **D**,**E**,**F**) are shown by light microcopy (**A**,**D**), PSA-TRITC, to show acrosome intact sperm (**B**,**E**) and second antibody-FITC to demonstrate immunolocalization of the anti-actin mAb (**C**,**F**). There were two main fluorescent patterns: either the whole acrosomal region was stained (**C**), or only the equatorial segment (**F**) of acrosome-intact sperm was stained. Staining was observed in ~10% of sperm in the medium. Original magnification ×600, scale bar = 2.5 μm.

Localization of anti-actin mAb in sperm

Immunogold EM demonstrated in both pre- and post-embedded samples that anti-actin mAb bound to the acrosome, inner acrosomal membrane, nucleus and tail structures (Figure 8b,c). Sperm incubated with control 4C7 mAb were negative (Figure 8a). Usually the sperm plasma membrane was partially damaged during processing with L.R.White, so that surface localization of actin was not clearly demonstrated. This was a common technical problem for immunogold EM.

For post-embedded samples in which live sperm were not incubated with anti-actin mAb, but the EM sections were exposed to both anti-actin mAb (primary) and the secondary antibody (mIgG-gold), all sperm were positive with gold labelling. In contrast, pre-embedded samples in which live sperm were incubated with anti-actin mAb and the EM sections were subsequently exposed to the secondary antibody (mIgG-gold), only a proportion (10%) of sperm were positive with gold labelling. This further confirmed the results of the immunofluorescence study, that the anti-actin mAb enters some live sperm during incubation.

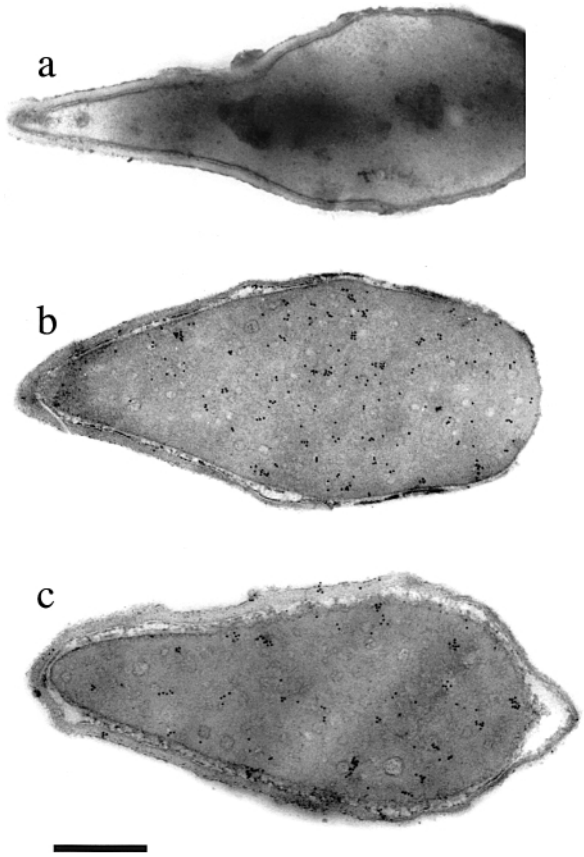

Figure 8. Immunogold labelling of anti-actin mAb in the sperm head. (**a**) Control mAb 4C7 was negative. (**b**) Pre-embedded sperm (live sperm were pre-incubated with actin mAb and EM sections were directly exposed to the secondary antibody) with positive gold labelling. (**c**) Post-embedded sperm (live sperm were not pre-incubated with actin mAb and only EM sections were exposed to both actin mAb and the secondary antibody) with positive gold labelling. Both pre-and post-embedded sperm had a similar gold labelling pattern (scale bar = 1 μm).

Effect of the anti-actin mAb on ultrastructure of human sperm bound to the ZP

EM examination of sperm bound to the ZP in the presence of the anti-actin mAb showed that most sperm were acrosome-intact. Each showed a ballooning and separation of the plasma membrane from the outer acrosomal membrane. Some also had parts of the plasma membrane broken (Figure 9). With the control 4C7 mAb, most sperm were acrosome-reacted, but the acrosome-intact sperm showed no similar separation of the plasma membrane from the outer acrosomal membrane.

Discussion

Immunohistochemistry indicates that localization and distribution of actin in sperm varies between species (Clarke *et al*., 1982; Virtanen *et al*., 1984; Ochs and Wolf, 1985; Camatini *et al*., 1986, 1988; Flaherty *et al*., 1988; Fouquet and Kann, 1992). In rabbit sperm, actin has been identified between the inner acrosomal membrane and the nuclear envelope and also in the post-acrosomal region (Camatini *et al*., 1988). In boar sperm ~1% of the total plasma membrane protein is actin (Peterson *et al*., 1990). Peterson *et al*. have found that most actin associated with boar sperm plasma membranes is monomeric and actin filaments or protofilaments are attached to the outer acrosomal membrane (Peterson *et al*., 1990). These filaments may also be associated with the plasma membrane overlying the acrosome. In human sperm, actin has been identified in various regions, specifically the acrosome, post-acrosomal area, neck and principal tail piece (Virtanen *et al*., 1984; Ochs and Wolf, 1985; Fouquet and Kann, 1992).

The subcortical actin network has been implicated in the regulation of exocytosis in endocrine and secretory cells (Koffer *et al*., 1990; Burgoyne *et al*., 1991; Dudani and Ganz, 1996). The AR involves multiple fusions between plasma and outer acrosomal membranes resulting in vesiculation of the membranes and release of acrosomal enzymes. This has been regarded as equivalent to exocytosis and should therefore have similar mechanisms. Actin polymerization is likely to be involved in the AR. Its role in membrane fusion and vesiculation has been studied with bovine sperm membrane preparations (Spungin *et al*., 1995; Spungin and Breitbart, 1996). Our previous work using dual-fluorescent stains for actin and PSA (for acrosome status) on the same human sperm has shown that actin was present in the acrosomal region and is lost following the AR (Liu *et al*., 1999). We showed that inhibition of actin polymerization with cytochalasin B or D strongly inhibits the ZP-induced AR (Liu *et al*., 1999). On the other hand, inhibition of actin depolymerization with phalloidin had no effect on the ZP-induced AR (Liu *et al*., 1999). In the present study, the anti-actin mAb at a 1:100 dilution had a similar inhibitory effect on the ZP-induced AR, as did 40 μmol/l cytochalasin B or D. It is possible that the anti-actin mAb could block the secondary binding of the acrosome-reacted sperm to the ZP so that the reacting sperm are lost from the ZP. However, this is unlikely because >50% of sperm (motile) bound to the ZP showed positive immunobead binding and the antibody could be demonstrated on the surface of acrosome-intact sperm. Furthermore, the anti-actin mAb also blocks the calcium ionophore-induced AR. Additionally, we have shown previously that AR sperm are less capable of initiating ZP binding than are acrosome-intact sperm (Liu and Baker, 1990)

The anti-actin mAb also decreased hyperactivation without affecting percentage motility. At a 1:100 dilution it has an inhibitory effect on hyperactivation similar to that of 40 μmol/l cytochalasin B (Liu *et al*., 1999). It is known that the plasma membrane of the sperm neck and tail contain actin. The effects of the anti-actin mAb on ZP and calcium ionophore-induced AR and hyperactivation are specific to the antibody and are not explained by small molecules contaminating the antibody solution. The anti-actin mAb probably inhibits actin polymerization as it has the same effect as cytochalasins on both hyperactivation and the ZP-induced AR without affecting total sperm motility or sperm–ZP binding. Hyperactivation is a marker for capacitated sperm, which may be more responsive to AR induction after binding to the ZP.

Whilst the anti-actin mAb and cytochalasins are similarly effective in inhibiting hyperactivation and the AR, the mechanisms by which they act may differ. Cytochalasins are small molecules that are cell membrane permeable; however,

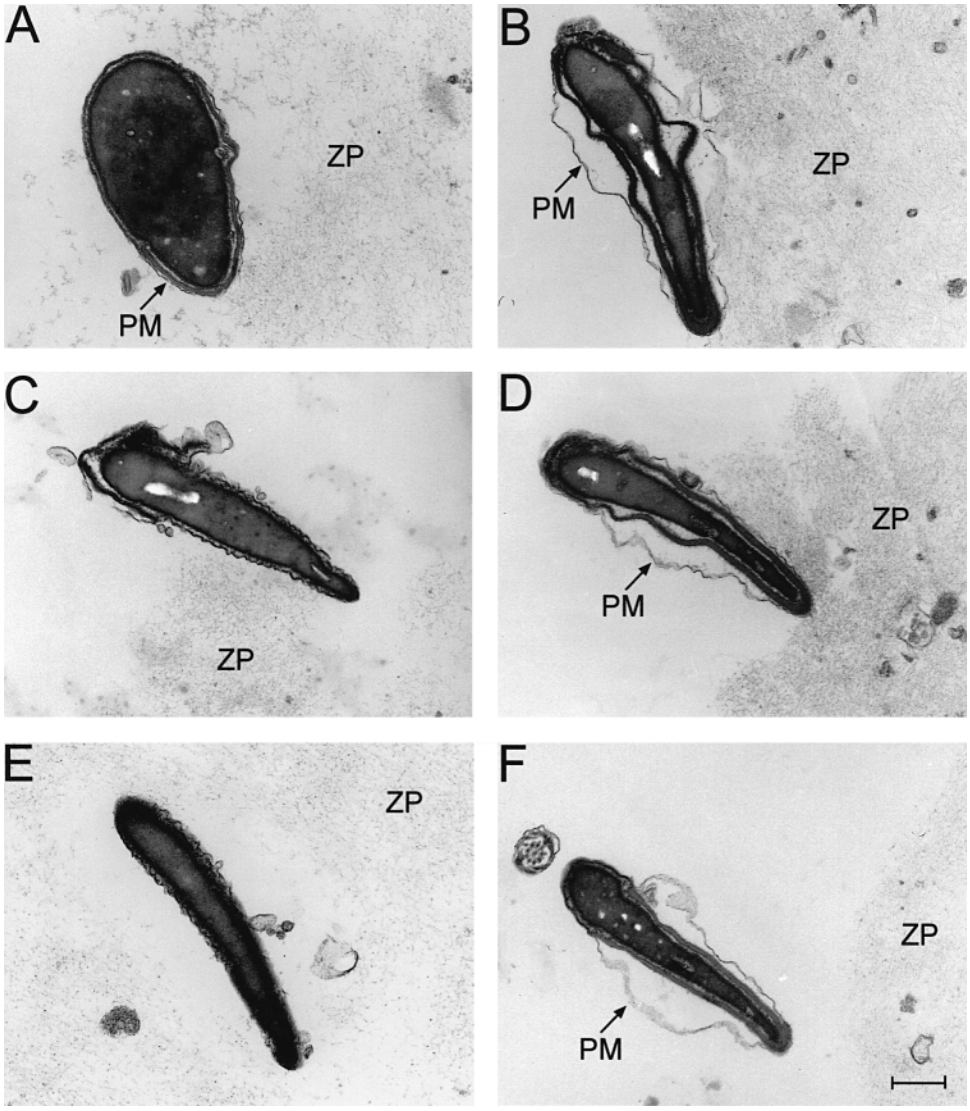

Figure 9. Electron microscopy of sperm bound to the zona pellucida (ZP) after incubation with control mAb 4C7 (**A**, **C**, **E**) or with anti-actin mAb (**B**, **D**, **F**). (**A**) An acrosome intact sperm with closely apposed plasma membrane (PM) and outer acrosomal membrane; (**C** and **E**) acrosome-reacted sperm; (**B**, **D** and **F**) acrosome-intact sperm with ballooning and separation of the plasma membrane from the outer acrosomal membrane (scale bar = 1 μm).

immunoglobulins are large molecules not usually considered able to enter live cells with intact plasma membranes, and actin is an intracellular protein. However, sperm are different from somatic cells. The sperm plasma membrane undergoes complicated modifications during sperm–ZP interaction, including changes in membrane fluidity, which may allow entry of antibodies or expose the actin-binding epitope to the extracellular medium. There are precedents for actin being exposed on the plasma membrane of somatic cells. For example, endothelial cell surface actin serves as a binding site for plasminogen, tissue plasminogen activator and lipoprotein (Dudani and Ganz, 1996). Owne *et al*. have reported the presence of actin on the surface of human B lymphocytes (Owne *et al*., 1978). There are also reports of antibodies entering live cells and of antibodies to intracellular proteins influencing sperm function. Alarcon-Segovia *et al*. have shown that anti-ribonucleoprotein IgG can penetrate live human T gamma lymphocytes (Alarcon-Segovia *et al*., 1979). Antibodies to SNARE proteins can block the ionomycin-induced AR in bovine sperm *in vitro* (Ramalho-Santos *et al*., 2000). Also, a mAb to the intra-acrosomal protein SP-10 reduces completion of the AR of capacitated bovine sperm without affecting motility (Coonrod *et al*., 1996).

The anti-mIgG bead and immunofluorescence studies showed that the anti-actin mAb binds to the head of ~10% of sperm in the culture medium and 50% of sperm bound to the ZP. This indicates that the membranes of some sperm in the medium, and many sperm interacting with the ZP, change to allow interaction with the anti-actin mAb. Samples with high proportions of sperm capable of binding to the ZP and undergoing the ZP-induced AR also have high proportions of sperm reacting with the anti-actin mAb during both incubation and binding to the ZP. This relationship between binding of the anti-actin mAb and the ZP-induced AR suggests that at the initiation of the AR, more actin binding sites may be exposed on the outer leaflet of the plasma membrane, or that

there is an increase in the permeability of the membrane to the anti-actin mAb. While the total amount of actin (G+F) does not change during capacitation, our data suggest that the capacitation process increases the number of sperm that are exposing their actin binding sites. This was particularly well demonstrated by sperm removed from surface of the ZP. We believe this is an important observation of sperm membrane changes during capacitation.

The immunogold EM results indicate that anti-actin mAbs enters the sperm and becomes widely dispersed. The observation that anti-mIgG beads bind to the sperm head near the equatorial segment suggests that the anti-actin mAb enters the sperm in this region, possibly associated with a partial disruption of the membrane. The EM of acrosome-intact sperm bound to the ZP also showed a substantial separation between the plasma membrane and outer acrosomal membranes when the AR was inhibited by the anti-actin mAb. This ballooning of the membrane may result from an inability of the actin to polymerize and hold the plasma membrane close to the outer acrosomal membrane. It may alternatively be an osmotic effect of the entry of the mAb. The negative findings with the control antibody indicate that a specific interaction between the anti-actin mAb and actin is necessary for the inhibitory effect on the AR and that the anti-actin antibody does not bind to or enter the sperm by some general mechanism for the passage of immunoglobulins into sperm. Our findings indicate there is specificity for the antibody and that the actin probably becomes accessible to the antibody at the sperm surface.

In conclusion, this study shows that an anti-actin mAb can enter capacitating sperm and sperm interacting with the human ZP to inhibit hyperactivation and the AR. These results also confirm that actin plays an important role in the human AR.

Acknowledgements

We thank Mingli Liu for technical assistance, the scientists in both the Royal Women's Hospital and Melbourne IVF Laboratories for collecting the oocytes, the scientists in the Andrology Laboratory for collecting sperm samples and Kim Williams and Nadine Watson in the Department of Anatomy and Cell Biology for helping with EM.

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Received on June 29, 2001; accepted on October 23, 2001