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Potential role of αv and β1 integrins as oocyte adhesion molecules during fertilization in pigs

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Integrin molecules are cell adhesion molecules that are thought to be involved in sperm–oocyte interaction in rodents and humans. The objective of this study was to evaluate whether integrin molecules were present on the surface of pig oocytes, consistent with involvement in sperm–oocyte interaction in this species. Immunocytochemistry and confocal microscopy were used to evaluate the presence of β1, and α1, α2, α3, α4, α5, α6 and αv integrin subunits on the plasma membrane of pig oocytes. The β1 and αv integrin subunits were present consistently at the surface of pig oocytes; however, the remaining α integrin subunits evaluated were not routinely detected. The antibodies to the β1 and αv integrin subunits recognized appropriately sized protein bands on western blots of partially purified oocyte plasma membrane. These two antibodies also recognized oocyte plasma membrane protein isolated from a sperm plasma membrane affinity column. Sperm plasma membrane proteins of 137 and 93 kDa appeared to be the ligands for the β1 integrin subunit as revealed by a western sandwich blot. Antibody to an extracellular domain of the β1 integrin subunit reduced pig sperm–oocyte binding ($P < 0.05$), also indicating an assisting role for a β1 oocyte integrin subunit in sperm–oocyte interaction in pigs. These results are consistent with an αvβ1 pig oocyte integrin interacting with a ligand on the sperm plasma membrane during fertilization.

Introduction

Fertilization in mammals is a multi-step process (Yanagimachi, 1994). After passage of acrosome-reacted spermatozoa through the zona pellucida, the fertilizing spermatozoon initially attaches to the oocyte plasma membrane via the inner acrosomal membrane (Koehler *et al.*, 1982; Talbot and Chacon, 1982). Subsequently, binding and fusion with the oocyte plasma membrane occur first with the equatorial segment of the spermatozoon followed by fusion in the post-acrosomal region (Yanagimachi, 1994). Molecules on the mammalian oocyte plasma membrane that interact with the sperm plasma membrane are not well understood, but an integrin heterodimer is proposed as one such receptor in rodents (Almeida *et al.*, 1995).

The discovery that the potential guinea-pig sperm binding molecule fertilin was a disintegrin has contributed significantly to the hypothesis that complementary binding molecules on the oocyte surface are integrins (Blobel *et al.*, 1992; Myles *et al.*, 1994; Wolfsberg *et al.*, 1995a; de Nadai *et al.*, 1996). The discovery of fertilins in other rodents and primates and reports of integrin molecules on the oocyte surface provided further support for the idea that one or more integrins may function as receptors for spermatozoa on the mammalian oocyte surface (Tarone *et al.*, 1993; Evans *et al.*, 1995; Wolfsberg *et al.*, 1995b; Hardy and Holland, 1996; McLaughlin *et al.*, 1997; Capmany *et al.*, 1998; Evans, 1999). Although integrins have been associated with the plasma membrane of rodent oocytes, the presence of these molecules on the surface of oocytes from the Order Artiodactyla has not been reported. The objective of this study was to examine whether integrins are present in pig oocytes, consistent with a function in sperm–oocyte plasma membrane interaction.

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Materials and Methods

Antibodies

Rabbit polyclonal antibodies to the exterior portion of β1 or α5 or to the C-terminal cytoplasmic domain of β1, α1, α2, α3, α4, α5 and αv were obtained from Chemicon Int., Inc. (Temecula, CA; catalogue numbers AB1937, AB1921, AB1952, AB1934, AB1944, AB1948, AB1924, AB1949 and AB1930). A rat monoclonal antibody to α6 (GoH3, catalogue number 33771A) was obtained from PharMingen (San Diego, CA). Crossreactivity of these antibodies (except AB1937 (β1), AB1921 (α5) and AB1924 (α4)) with pig integrins was reported by the manufacturer. The reactivity of AB1921 with pig white blood cells or marrow cells or with the corresponding mouse cells (which should have been a positive control) was not conclusive. This finding prompted the use of the second antibody to the α5 subunit (C-terminal moiety), which had known crossreactivity with the pig

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molecule. The crossreactivity of the α6 monoclonal antibody with pig α6 integrin subunit indicated by the supplier was verified in this laboratory with pig white blood cells, and the crossreactivity of AB1924 with pig white blood cells was demonstrated (data not shown).

Pig oocyte maturation

Pig ovaries were obtained approximately 5 min after exsanguination at the university abattoir. Oocytes were aspirated from antral follicles (2–3 mm diameter) into warm PBS (Table 1) and transported to the laboratory (30–37°C). Cumulus-intact oocytes were removed from the follicular aspirate and washed through three 100 µl drops of PBS, two 100 µl drops of HEPES–Tyrode's albumin lactate pyruvate (Table 1) and three 100 µl drops of maturation medium (Table 1). A total of 10–15 oocytes were transferred to each 50 µl drop of maturation medium and matured under oil for 24 h at 39°C in 5% CO_2 in air (Zheng *et al.*, 1992). Cumulus masses were removed from these partially matured oocytes during an approximate 5 min exposure to 1 mg hyaluronidase (500 U) ml^{-1} in saline–BSA (0.9% (w/v) NaCl, 1 mg BSA ml^{-1}) followed by rinsing through three drops of saline–BSA. The zona pellucida was removed by brief exposure to 0.25 mg protease (3.75 U) ml^{-1} in saline-BSA (catalogue number P5147, a type XIV protease from *Streptomyces griseus*; Sigma, St Louis, MO). When the zona pellucida showed some deformity, oocytes were transferred to saline–BSA and rinsed through at least two additional drops of saline–BSA until the zona pellucida was completely removed.

Antibody localization of integrin subunits on the oocyte surface

β1 and α5 integrin subunits. Zona-free pig oocytes were incubated for 1 h with a 1:300 dilution of normal rabbit serum as a control or rabbit serum containing polyclonal antibodies to the β1 or α5 integrin subunits. These oocytes were rinsed through three drops of saline–BSA to remove unbound serum components followed by a 30 min incubation with a biotinylated goat anti-rabbit IgG (15 µg ml^{-1}). Unbound IgG was removed during transfer of oocytes through three drops of saline–BSA. The oocytes were incubated for 30 min with 20 µg tetramethylrhodamine isothiocyanate (TRITC)-tagged avidin, and rinsed three times to remove unbound avidin. The oocytes were fixed for 15 min in 3% (w/v) paraformaldehyde in PBS, rinsed, transferred to a drop of mounting medium (95% (v/v) glycerol, 5% (v/v) PBS containing 9.2 mmol *p*-phenylenediamine l^{-1} and 15.38 mmol sodium azide l^{-1}, pH 8) and loaded into square glass capillary tubes (Vitro Dynamics, Inc., Rockaway, NJ; catalogue number 8320).

β1, α1, α2, α3, α4, α5 and αv integrin subunits. Zona-free pig oocytes were fixed for 15 min in 3% (w/v) paraformaldehyde in PBS, rinsed through three drops of saline–BSA, and incubated with 2% (v/v) normal goat serum for 1 h to block non-specific binding. Oocytes were incubated with a 1:100 dilution of the rabbit polyclonal antisera to an integrin subunit or normal rabbit serum (all in the presence of 0.4% (v/v) normal goat serum). Oocytes were rinsed through three drops of saline–BSA and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated second antibody (5 µg goat anti-rabbit IgG ml^{-1}). Oocytes were rinsed through three drops of saline–BSA, transferred to mounting medium and loaded into square capillary tubes.

α6 subunit. The presence of the α6 subunit on fixed zona-free pig oocytes was evaluated using procedures similar to that described above. Normal rabbit serum (10% v/v) was used as the initial blocking serum, and oocytes were incubated with a 1:1000 dilution of rat monoclonal IgG to the α6 subunit or normal rat serum in the presence of 0.4% (v/v) normal rabbit serum. FITC-conjugated rabbit anti-rat IgG (31 µg rabbit anti-rat IgG ml^{-1}) was used.

Visualization of bound antibody. Oocytes were scanned individually with a Zeiss LSM410 confocal microscope using the × 40 objective to evaluate fluorescence on the plasma membrane (Wheeler *et al.*, 1996). The FITC fluorescence was observed using a 488 nm excitation wavelength, a 510 nm

Table 1. Composition of media used for *in vitro* maturation of pig oocytes

PBS	HEPES–TALP	Maturation medium
137 mmol NaCl l^{-1}	114 mmol NaCl l^{-1}	Medium 199 with
2.7 mmol KCl l^{-1}	9.22 mmol HEPES l^{-1}	L-glutamine, Earle's salts
8.4 mmol $Na_2HPO_4 7H_2O$ l^{-1}	3.2 mmol KCl l^{-1}	25 mmol HEPES l^{-1}
1.5 mmol KH_2PO_4 l^{-1}	0.5 mmol $MgCl_2 6H_2O$ l^{-1}	4.16 mmol $NaHCO_3$ l^{-1}
0.09 mmol $CaCl_2$ l^{-1}	0.655 mmol $NaHCO_3$ l^{-1}	3.05 mmol glucose l^{-1}
5.5 mmol glucose l^{-1}	2.0 mmol $CaCl_2$ l^{-1}	0.9 mmol sodium pyruvate l^{-1}
0.32 mmol sodium pyruvate l^{-1}	5.0 mmol glucose l^{-1}	50 µg gentamycin ml^{-1}
0.05 mmol kanamycin SO_4 l^{-1}	13.2 mmol sodium lactate l^{-1}	5 iu LH per 100 ml
4 mg BSA ml l^{-1}	0.1 mmol sodium pyruvate l^{-1}	0.5 iu FSH per 100 ml
0.014 mmol phenol red l^{-1}	0.168 mmol sodium penicillin l^{-1}	
	3 mg BSA ml^{-1}	
	0.028 mmol phenol red l^{-1}	

TALP: Tyrode's albumin lactate pyruvate.

dichroic mirror and a 515 nm emission filter. The TRITC fluorescence was observed using a 568 nm excitation wavelength, a 580 nm dichroic mirror and a 590 nm emission filter. The binding of β1, α5, αv and α6 antibodies and of respective controls was evaluated in a minimum of three replicates; in each replicate 5–15 oocytes were incubated with a single antibody. The binding of a second β1 and a second α5 antibody (both recognizing the cytoplasmic portions of the integrin subunits) and of α1, α2, α3 and α4 antibodies to fixed oocytes was examined on 10–15 zona-free pig oocytes in conjunction with the αv antibody as a positive control and normal rabbit serum as a negative control.

Western blotting

Partially purified pig oocyte plasma membrane was separated from an oocyte homogenate by density gradient centrifugation (Ash *et al*., 1994b) after isolation of zona pellucida intact oocytes from pig ovaries and removal of zona pellucida fragments. Oocyte plasma membrane proteins (20 μg per well) were solubilized under reducing conditions, separated on 10 cm × 12 cm tricine SDS polyacrylamide gels (Schagger and von Jagow, 1987) and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Blots were incubated for 2 h with 5% (v/v) normal goat serum in Tris-buffered saline (TBS; 100 mmol Tris l^{-1}, 0.9% (w/v) saline, pH 7.5) containing 0.4 % (v/v) Tween 20 (TTBS) to block non-specific binding sites. Blots were rinsed three times for 5 min each in TTBS. Individual lanes were incubated for 1 h with rabbit polyclonal antiserum to β1 integrin subunit or normal rabbit serum (1:1000) or with rabbit polyclonal antiserum to αv integrin subunit or normal rabbit serum (1:300). Blots were again rinsed three times in TTBS followed by incubation with goat anti-rabbit IgG linked to horseradish peroxidase (Sigma, St Louis, MO). Unbound second antibody was removed by three successive rinses in TTBS and bound antibody was visualized with chemiluminescence (Renaissance, NEN, Boston, MA).

Western sandwich blots

Sperm plasma membrane proteins were solubilized under reducing conditions, separated by SDS-gel electrophoresis on Tris–glycine or Tris–tricine gels, and transferred to Immobilon-P membrane. Designated lanes were blocked for 1 h with TTBS followed by incubation with 40 μg oocyte plasma membrane ml^{-1} for 2–3 h at room temperature in CTBS (TBS containing 16 mmol 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS) l^{-1} and 2 mmol $CaCl_2$ l^{-1}). These lanes were rinsed, incubated with a 1:300 dilution of primary antibody (αv or β1) in 1% (v/v) normal goat serum in TTBS containing 2 mmol $CaCl_2$ l^{-1} or a 1:500 dilution in TTBS containing 5–10% (v/v) normal goat serum and 2 mmol $CaCl_2$ l^{-1} with or without an additional blocking incubation in 5–10% (v/v) normal goat serum. Other lanes (not incubated with oocyte plasma membrane) were blocked with 5–10% (v/v) normal goat serum in TTBS for 1 h and incubated with 1:500 dilutions of normal rabbit serum, or α1, α2, α3, α4, α5 or αv antiserum, or a 1:300 dilution of β1 antiserum in TTBS containing 1% (v/v) normal goat serum and 2 mmol $CaCl_2$ l^{-1}. Blot strips were rinsed and incubated for 1 h with a 1:80 000 dilution of peroxidase-labelled goat anti-rabbit antiserum, rinsed, incubated with chemiluminescence substrate threefold more dilute than recommended by the manufacturer, and bound antibody was observed on X-ray films. Lanes of sperm plasma membrane were also incubated with a 1:300 dilution of normal rat serum or α6 antibody as described for the polyclonal rabbit antibodies, followed by a peroxidase-labelled sheep anti-rat antibody.

Affinity chromatography

Isolated pig sperm plasma membrane proteins (Peterson *et al*., 1980; Ash *et al*., 1994a) were solubilized with CHAPS and attached to AminoLink gel (Pierce, Rockford, IL). Partially purified oocyte plasma membrane proteins (Ash *et al*., 1994b) were incubated with the sperm plasma membrane affinity matrix for 3 h in CTBS. The unbound protein was removed by washing with 10 volumes of TTBS medium. Bound oocyte plasma membrane proteins were eluted with 0.5% (w/v) SDS in TBS and dialysed extensively against 10 mmol $NaHCO_3$ l^{-1}.

Slot blots

Approximately 1 μg of affinity isolated oocyte plasma membrane was applied to each slot in a slot blot apparatus. Slot blots were probed essentially as described for western blots using a 1:1000 dilution of the polyclonal β1 antiserum or normal rabbit serum and a 1:300 dilution of the polyclonal αv antiserum or normal rabbit serum. Each preparation of affinity-isolated oocyte plasma membrane was analysed twice.

In vitro *sperm–oocyte interaction*

Ejaculated pig spermatozoa were washed on a Percoll gradient and resuspended to a concentration of 4×10^6 spermatozoa ml^{-1} in Tris-buffered medium containing 10 mmol $CaCl_2$ l^{-1} (Berger and Horton, 1988). Spermatozoa were pre-incubated for 1 h at 39°C with 5% CO_2 before insemination of partially matured zona-free oocytes. Polyclonal antiserum (β1 or α5 antibodies) was added to the oocytes before addition of the sperm suspension to provide a 1:300 final dilution of the serum. Gametes were co-incubated for 5 h at 39°C in 5% CO_2 in air, rinsed free of loosely attached spermatozoa and incubated overnight. Oocytes were placed on slides and compressed gently between slide and coverslip resting on ribbons of a mixture of vaseline and paraffin wax. After fixation and clearing for 3 days in 75% (v/v) methanol:25% (v/v) acetic acid, oocytes were stained with 1% (v/v) aceto-orcein and the stain was removed with 45% (v/v) acetic acid. The number of bound spermatozoa and number of penetrated spermatozoa were evaluated for each oocyte.

Since the polyclonal antibody to the extracellular domain of α5 did not bind to the oocyte surface, this antibody was chosen as the negative control. The number of spermatozoa bound per oocyte was evaluated in three separate replicates, in which the replicate was a random factor and the mean number of bound spermatozoa in each treatment within each replicate was weighted by the number of oocytes evaluated (SAS statistical programs, Cary, NC). The percentage of oocytes fertilized and the number of spermatozoa that penetrated per oocyte were similarly analysed.

Results

The antibody to the extracellular portion of the β1 integrin subunit bound to the surface of unfixed oocytes (Fig. 1). Binding of this antibody to the oocyte surface showed variability among oocytes within each replicate. In some oocytes, the binding appeared uneven around the periphery of the oocyte, but the most typical pattern was a beaded one. Neither normal rabbit serum nor the polyclonal α5 antiserum bound to the periphery of unfixed oocytes. The αv antibody recognized the cytoplasmic tail of its integrin subunit and labelled the periphery of fixed pig oocytes (Fig. 2). Similarly, the binding of the αv antibody was uneven on some pig oocytes, but the binding of the αv antibody to the fixed oocytes appeared more evenly distributed than the binding of the β1 antibody to unfixed oocytes. Both β1 antibodies (one to the cytoplasmic portion and one to the extracellular domain) bound to fixed pig oocytes, similar to the pattern observed for the αv antibody. Fixed oocytes were not labelled by the rat monoclonal antibody to α6, by rabbit polyclonal antibodies to the cytoplasmic portions of the α1, α2 and α5 integrin subunits, or by normal rabbit or rat serum. Most of the fixed oocytes incubated with the polyclonal antibodies to cytoplasmic portions of α3 and α4 integrin subunits were unlabelled, but a few (one-quarter or less) were very faintly labelled.

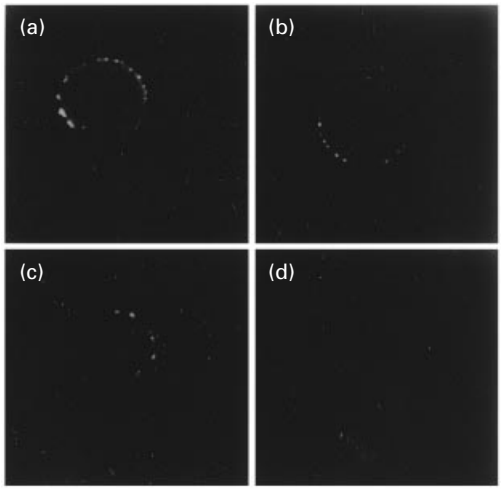

Fig. 1. Pig oocytes incubated with an antibody to the β1 integrin subunit (a–c) or a control oocyte incubated with normal rabbit serum (d). Bound antibody was visualized with a biotinylated goat anti-rabbit second antibody followed by incubation with 20 μg tetramethylrhodamine isothiocyanate (TRITC)-tagged avidin ml^{-1}.

Western blotting

The oocyte plasma membrane preparations contained three bands (estimated M_r of 63, 87, and 126 kDa) recognized by the β1 polyclonal antibody (Fig. 3). The αv antibody

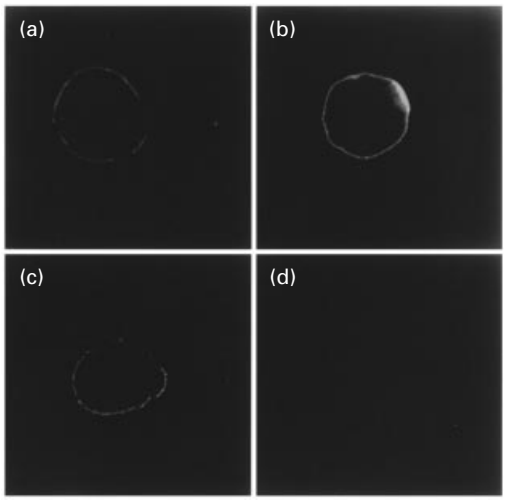

Fig. 2. Pig oocytes incubated with an antibody to the αv integrin subunit (a–c) or a control oocyte incubated with normal rabbit serum (d). Bound antibody was visualized with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit second antibody.

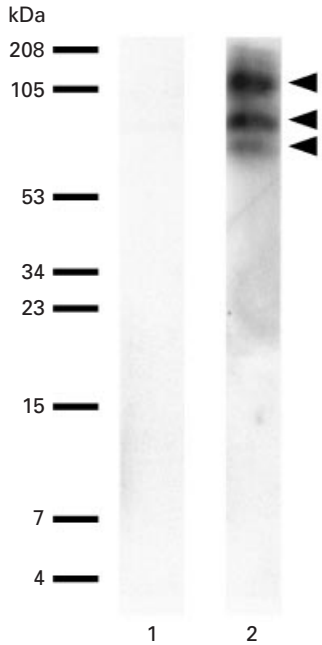

Fig. 3. Western blot of pig oocyte plasma membrane proteins. Lane 1 was probed with normal rabbit serum and lane 2 was probed with the β1 integrin subunit antibody. The M_r standards are indicated on the left-hand side.

recognized a single band with an estimated M_r of 124 kDa (Fig. 4).

Slot blots

The affinity-isolated oocyte plasma membrane proteins included proteins recognized by the αv and β1 polyclonal antibodies (Fig. 5). Reactivity with the β1 antibody differed between preparations of affinity-isolated oocyte plasma membrane: one preparation reacted strongly whereas a second reacted weakly (only slightly above the control reaction). Analysis of β1 antiserum binding was repeated on these preparations and the same results were observed. The reactivity with the αv antibody was consistently strong.

Western sandwich blots

The β1 antibody bound to two bands (137 and 93 kDa) when sperm plasma membrane proteins were first incubated with oocyte plasma membrane (Fig. 6); the β1 antibody did not bind to sperm plasma membrane proteins in the absence of oocyte plasma membrane. The αv antibody did not bind specifically to the sperm plasma membrane protein blot pre-incubated with oocyte plasma membrane. Antibodies to α integrin subunits did not bind to these sperm plasma membrane protein blots, indicating that the 137 and 93 kDa sperm plasma membrane proteins were ligands for the β1 integrin subunit in the oocyte plasma membrane rather than α subunits interacting with a β subunit.

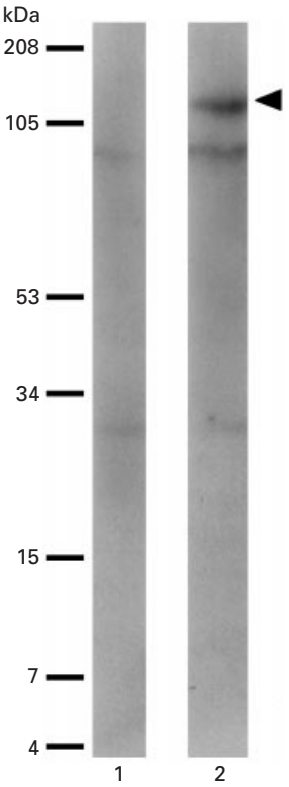

Fig. 4. Western blot of pig oocyte plasma membrane proteins. Lane 1 was probed with normal rabbit serum and lane 2 was probed with the αv integrin subunit antibody. The M_r standards are indicated on the left-hand side.

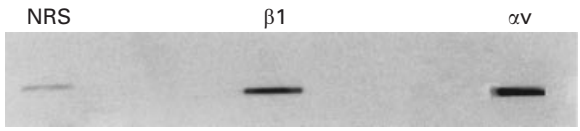

Fig. 5. Slot blots of pig oocyte plasma membrane proteins eluted from a sperm plasma membrane affinity column. Slots were incubated with normal rabbit serum (NRS), β1 or αv integrin subunit antibodies as indicated.

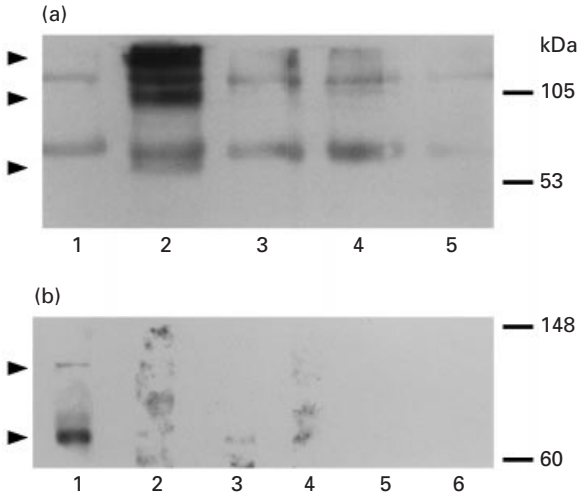

Fig. 6. Sandwich blots of pig sperm plasma membrane proteins. Integrin subunits were detected with specific anti-integrin subunit antibodies followed by a peroxidase-labelled second antibody. (a) Sperm plasma membrane proteins blocked once before incubation with the oocyte plasma membrane preparation. Lanes 1, 2 and 3 were incubated with oocyte plasma membrane proteins followed by αv or β1 antiserum, or normal rabbit serum, respectively. Lanes 4 and 5 were incubated with the β1 antiserum or normal rabbit serum, respectively (no oocyte plasma membrane proteins). Arrowheads indicate 137 and 93 kDa sperm proteins (and a minor band at 65 kDa) in lane 2 binding β1 integrin subunits in the oocyte plasma membrane. (b) Sperm plasma membrane proteins blocked before and after incubation with the oocyte plasma membrane proteins. Lane 1 shows the 137 and 93 kDa sperm plasma membrane proteins binding β1 integrin subunits in the oocyte plasma membrane. Lanes 2, 3, 4, 5 and 6 illustrate the absence of binding of normal rabbit serum and antiserum specific to α1, α2, α3 and α5 integrin subunits to the sperm plasma membrane protein. The M_r standards are indicated on the right-hand side.

Inhibition of sperm–oocyte interaction

Pig sperm–oocyte binding was reduced by the β1 antibody (1:300 dilution) compared with the control antibody (α5; Fig. 7). Population distributions of spermatozoa binding to oocytes

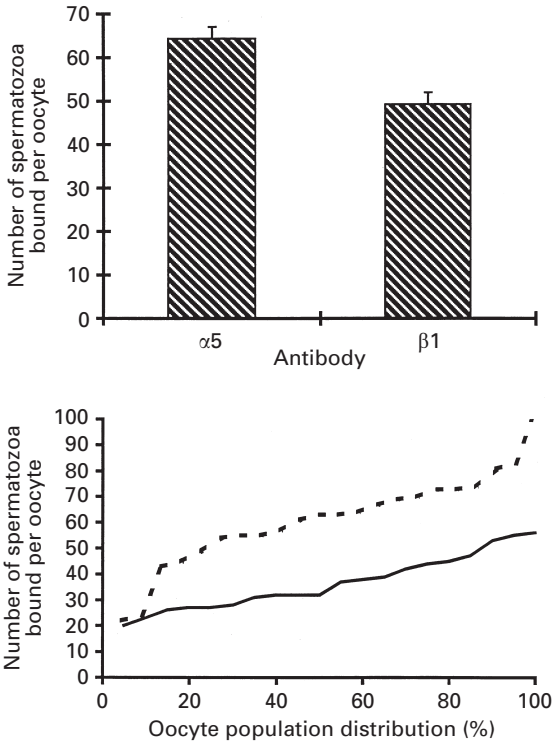

Fig. 7. Inhibition of sperm–oocyte plasma membrane interaction by antibody to the β1 integrin subunit. (a) Reduction in the number of spermatozoa bound to the oocyte plasma membrane. Bars represent means from three replicates with standard error bars (total of 181 oocytes examined). (b) Population distribution of oocytes by number of spermatozoa bound. Data from the two treatments in a single replicate are represented: α5 control (---) and β1 (—).

in each replicate demonstrated a reduction of sperm binding to all oocytes in the presence of the β1 antibody rather than a large change at one end of the distribution. In a preliminary experiment, there was no difference in sperm binding in the presence of a 1:300 dilution of control antiserum (α5) or in the absence of serum (102 versus 117). The reduction in sperm binding was not sufficient to reduce fertilization rates (62% versus 63% for β1 and the control α5 antiserum, respectively, SEM = 3) or spermatozoa that penetrated per oocyte (2.8 versus 2.3, SEM = 0.8).

Discussion

The binding of polyclonal antibodies to αv and β1 integrin subunits to the surface of pig oocytes indicates that these integrin subunits are part of the pig oocyte plasma membrane. Binding of a polyclonal antibody that recognizes the extracellular portion of the β1 integrin subunit to unfixed oocytes supports the contention that this subunit is exposed on the surface of the pig oocyte. The available polyclonal antibody to the αv integrin subunit was to the cytoplasmic tail of the molecule, hence an exposed surface localization of the αv subunit could not be verified. Identification of proteins by these integrin subunit antibodies on western blots is further evidence that these two integrin subunits are part of the oocyte plasma membrane. Although the estimated M_r values of oocyte plasma membrane proteins recognized by these two integrin subunit antibodies are only approximations from mini-gels, the values are in the appropriate range for integrin subunits (Hemler, 1990). Recognition of three distinct bands by the β1 antibody was unexpected, but a similar result was observed in testicular tissue (Giebel *et al.*, 1997).

In contrast to murine oocytes (Almeida *et al.*, 1995), the α6 subunit did not appear to be a prominent component of the pig oocyte plasma membrane since no labelling was observed. The very weak reactivity observed in a small fraction of oocytes incubated with the α3 and α4 antibodies and the absence of labelling with the α1, α2 and α5 antibodies indicates that these subunits are not present or are present in very small numbers on the surface of the pig oocyte. The presence of very small numbers of some integrin subunits on rodent and human oocytes is implied by discrepancies among studies in the ability to detect specific subunits, in conjunction with differences in the sensitivity of different detection systems (Campbell *et al.*, 1995; Evans *et al.*, 1995; de Nadai *et al.*, 1996; Capmany *et al.*, 1998).

The inhibition of sperm–oocyte plasma membrane binding by an antibody recognizing an extracellular portion of the β1 integrin subunit implies involvement of the β1 moiety in sperm–oocyte interaction. The small but significant reduction in binding is consistent with β1 integrin having an ancillary rather than an essential role in sperm–oocyte interactions. However, there are alternative explanations for the antibody inhibition of sperm–oocyte binding, such as an effect of antibody binding on membrane fluidity since even monovalent antibodies can occasionally affect membrane fluidity (Chasis and Mohandas, 1992). These trials were done with a divalent antibody since fragments with antigen binding (FABs) were not readily available. The presence of the β1 integrin subunit in the oocyte plasma membrane proteins eluted from the sperm plasma membrane affinity column also indicates that the β1 integrin subunit may be a receptor for a sperm plasma membrane protein. Binding of the β1 integrin antibody to oocyte plasma membrane bound to sperm plasma membrane proteins with estimated M_r values of 137 and 93 kDa on a sandwich blot also supports a role for a β1 integrin subunit in sperm–oocyte interaction. This observation indicates that these two sperm plasma membrane proteins of high M_r are the sperm ligands for the oocyte β1 integrin subunit. Neither sperm plasma membrane protein band bound any of the polyclonal antibodies to seven common α integrin subunits, indicating that the interactions between the β1 integrin unit in the oocyte plasma membrane and the sperm plasma membrane proteins were ligand– receptor interactions rather than associations of integrin subunits. Although both the αv and the β1 integrin subunits could be detected in the oocyte plasma membrane fraction bound to the sperm plasma membrane affinity column, only the β1 subunit appeared to

bind to a sperm plasma membrane in the sandwich blot. The binding of a β integrin subunit to a sperm plasma membrane protein is consistent with the study of Evans *et al.* (1997), in which the β1 integrin subunit appeared to bind to the sperm fertilin β in mice. However, the α integrin subunit has also been proposed as the binding molecule for fertilin β (Almeida *et al.*, 1995; Chen and Sampson, 1999; Chen *et al.*, 1999).

The involvement of integrins in sperm–oocyte interaction was initially supported by the presumed essential role of the complementary molecule fertilin. The observation that spermatozoa from mice lacking fertilin have some ability to fuse with the oocyte plasma membrane (Cho *et al.*, 1998) indicates a facilitatory rather than an essential role for fertilin and its complementary receptor (Fenichel and Durand-Clement, 1998). However, the presence of cyritestin provides another ADAM family member to function as an integrin ligand in mouse sperm adhesion, and other less well characterized ADAM family members might also function in this role (Wolfsberg *et al.*, 1995b; Yuan *et al.*, 1997). The presence of fertilin α and cyritestin as pseudogenes in primates may be compensated by the presence of other related proteins that are complementary to integrin receptors (Jury *et al.*, 1997; Frayne and Hall, 1998; van Huijsduijnen, 1998). However, Ji *et al.* (1998) suggested that the β1 integrin subunit is a facilitatory rather than an essential receptor in human gamete interaction.

The M_r values of the sperm plasma membrane proteins binding the β1 oocyte integrin subunit are higher than would be expected for fertilin; the bovine sperm fertilin heterodimer is approximately 80 kDa under similar electrophoretic conditions (Waters and White, 1997). Alternatively, osteopontin is known to bind to the αvβ1 integrin (Hu *et al.*, 1995). Osteopontin is a component of bovine seminal plasma and is located on rat sperm plasma membrane (Siiteri *et al.*, 1995), but there is no evidence to indicate that it is present on the plasma membrane in pig spermatozoa.

It is unclear whether an interaction with an integrin receptor is a major contributor to sperm–oocyte plasma membrane interaction of intact pig gametes. In the initial western ligand blots, sperm plasma membrane proteins with similar M_r values to those binding the oocyte β1 integrin subunit appeared as minor rather than predominant binders of the biotinylated oocyte plasma membrane, and the extent of binding was not greater than that demonstrated in the most prominent non-tissue-specific proteins (Ash *et al.*, 1995). It is possible that this amount of binding on western ligand blots is unimportant, or alternatively the extent of binding may have been reduced compared with other proteins due to a greater reduction in biological activity during the harsh conditions of gel electrophoresis and blotting (Ocrant *et al.*, 1992).

It is likely that some, but not all, of the receptor–ligand interactions involved in sperm–oocyte interaction are tissue specific and specific to mammalian orders. Differences among species in integrin subunits may contribute to species-specific binding. However, the widespread presence of integrin molecules on cell surfaces contributes to the question of tissue specificity. Alternative splicing as proposed for the α6 and β1 integrin subunits might provide a tissue-specific mechanism for involvement (Altruda *et al.*, 1990; Zucotti *et al.*, 1998). An oocyte-specific sequence would support the hypothesis that an integrin was involved in tissue-specific sperm–oocyte interaction. Polyclonal antibodies, as were used in the present study (and many monoclonal antibodies), would be expected to recognize the similarities in the sequences rather than detect potentially limited but functionally important differences. Alternatively, an integrin moiety may be involved in sperm–oocyte interaction after a tissue-specific step, which might modify the ligand or receptor. Interaction of an integrin with another oocyte molecule in a gamete-specific manner might also modify binding affinity.

The results of the present study indicate that the β1 and αv integrin subunits are present in the plasma membrane in pig oocytes and that at least the β1 subunit is exposed on the surface of the oocyte. These subunits showed an affinity for sperm plasma membrane proteins and the β1 subunit recognized two sperm plasma membrane proteins of 137 and 93 kDa. Antibody binding to the extracellular portion of the β1 subunit inhibited sperm–oocyte plasma membrane binding. Although the role of the β1 integrin subunit on the oocyte plasma membrane remains to be determined, these observations are consistent with a role for β1 integrin in sperm–oocyte interaction in pigs, possibly as a facilitating rather than an essential molecule.

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