A reappraisal of the factors involved in *in vitro* initiation of the acrosome reaction in chicken spermatozoa

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Abstract

Chicken spermatozoa may remain in the female oviduct for a prolonged period before induction of the acrosome reaction on contact with the inner perivitelline layer (IPVL). By contrast, the acrosome reaction may be induced very rapidly *in vitro* in the presence of IPVL and Ca^{2+} . In the present study, we examined the extent to which the chicken acrosome reaction can be induced in media of various compositions in the presence or absence of IPVL and/or Ca^{2+} and other factors known to be efficient in mammals. We also compared the efficacy of perivitelline layer (PL) taken at various states of oocyte maturation in initiating the reaction. The acrosome reaction was induced in less than 5 min in the presence of Ca^{2+} and IPVL. Incubation of spermatozoa in different saline media (Beltsville poultry semen extender (BPSE); Dulbecco's modified eagle medium; NaCl-TES buffer) without IPVL showed a significant induction of acrosome reaction in BPSE supplemented with 5 mM Ca^{2+} and in the three media after supplementation with Ca^{2+} and Ca^{2+} ionophore A23187. By contrast, the acrosome reaction was never induced without Ca^{2+} . BSA, NaHCO_3 , and progesterone did not stimulate the acrosome reaction. Ca^{2+} plus PL taken at various physiological states (follicle IPVL, ovulated IPVL, oviposited IPVL, and/or outer perivitelline layer) strongly stimulated the acrosome reaction, the latest states being the most efficient. Although PL induced the acrosome reaction in the presence of extracellular Ca^{2+} , it was not possible to induce hyperactivation in chicken spermatozoa. Taken together, these results emphasize the central role of Ca^{2+} in the *in vitro* initiation of the acrosome reaction in chickens and show specific features of this induction in birds.

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Introduction

In animal species with internal fertilization, spermatozoa must undergo the acrosome reaction to penetrate and fertilize the egg (Olsen 1942). The acrosome is a Golgi-derived secretory vesicle located in the anterior region of the spermatozoa. The acrosome reaction involves fusion between the spermatozoon plasma membrane and the underlying outer acrosomal membrane and results in the release of the content of the acrosome (Oura & Toshimori 1990). The enzymes released are required to allow the chicken spermatozoa to hydrolyze the inner perivitelline layer (IPVL) surrounding the oocyte, and this results in holes that permit the passage of one or several spermatozoa through the IPVL (Bakst & Howarth 1977, Steele *et al.* 1994, Robertson *et al.* 1997). The megalecithal oocyte of birds is surrounded by an extracellular matrix, the perivitelline layer (PL) composed of the IPVL at the state of ovulation and fertilization, IPVL then being very rapidly surrounded by an outer perivitelline layer (OPVL) in the infundibulum (Bellairs *et al.* 1963, Kido & Doi 1988). The IPVL can be considered to a certain extent as analogous to the

mammalian zona pellucida (ZP; Waclawek *et al.* 1998). It consists of ZP glycoproteins that are homologous to the mammalian ZP proteins known to have a key role in sperm binding to the ZP and in the induction of acrosomal exocytosis (Waclawek *et al.* 1998, Takeuchi *et al.* 2001, Bausek *et al.* 2004, Okumura *et al.* 2004).

A period of capacitation of spermatozoa is a prerequisite for the initiation of the acrosome reaction in many mammalian species (Zaneveld *et al.* 1991, Yanagimachi 1994). Capacitation occurs *in vivo* in the female genital tract, involves different signaling pathways, and results in membrane destabilization that facilitates acrosome exocytosis and the hyperactivation of motility (Visconti & Kopf 1998, Visconti *et al.* 1998, Baldi *et al.* 2000, Breitbart 2003). It can also be achieved *in vitro* by incubation of the spermatozoa in a capacitating medium. The capacitating medium depends on the species and, in most cases, contains appropriate ions, including Ca^{2+} and NaHCO_3 , energy substrates, and albumin (Yanagimachi 1994). Capacitated spermatozoa undergo the acrosome reaction *in vivo* when they bind to the ZP that surrounds the mammalian oocyte. The mammalian acrosome reaction

can be induced *in vitro* by other inducers such as Ca^{2+} ionophore A23187 and progesterone (Pg). In addition, semen storage methodology has long been recognized to stimulate a capacitation-like process and *in vitro* induction of the acrosome reaction (Cormier *et al.* 1997, Bedford *et al.* 2000).

In contrast to mammals, the acrosome reaction of chicken spermatozoa may be induced very rapidly *in vitro* after incubation of spermatozoa in the presence of IPVL or IPVL-derived N-linked glycans and extracellular Ca^{2+} (Horrocks *et al.* 2000), contrasting with the long stay of spermatozoa in the hen oviduct before initiation of the reaction (reviewed by Blesbois & Brillard 2007).

Despite these breakthroughs, current understanding of the various factors inducing the acrosome reaction in chicken spermatozoa subjected to *in vitro* conditions remains limited due to poor information on the possible role exerted by the milieu in which spermatozoa have been suspended. Such studies are indeed of major interest for the understanding of spermatozoon biology and the fertilization process, and for the development of semen quality parameters and methods of semen storage that does not stimulate spontaneous acrosome reactions.

The first aim of the present study was to examine the extent to which the acrosome reaction could be induced *in vitro* after incubation of chicken spermatozoa in different saline media in the absence of the IPVL. We also measured the effectiveness of modulation of the acrosome reaction by the presence of PL taken from oocytes at various physiological states and by the addition of components to the suspending medium such as albumins, NaHCO_3 , Ca^{2+} , Ca^{2+} ionophore A23187, and Pg known to affect capacitation or acrosome reactions in mammals.

Fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA, *Arachis hypogaea*) (FITC-PNA) has been used to study acrosomal status (Horrocks *et al.* 2000, Ashizawa *et al.* 2004, 2006a, 2006b). Motility was evaluated by computer-assisted semen analyses (CASA) to measure any motility hyperactivation related to the initiation of acrosome reaction.

Results

Time needed to initiate chicken acrosome reaction *in vitro*

The results reported in Fig. 1 show that the acrosome reaction can be observed within 1 min of incubation of spermatozoa in the presence of Ca^{2+} and IPVL. However, the highest percentages of acrosome-reacted spermatozoa were obtained after 4–10 min of incubation. Longer exposure to Ca^{2+} and IPVL resulted in decreasing percentages of spermatozoa stained with FITC-PNA. All subsequent measurements of induction of

acrosome reaction were therefore evaluated after 5 min of incubation at 40 °C.

Effects of incubation media on *in vitro* induction of chicken acrosome reaction

The presence of Ca^{2+} was needed in every medium tested to induce the acrosome reaction, Dulbecco's modified eagle medium (DMEM) containing over 2 mM Ca^{2+} (Fig. 2a–c). The addition of 5 mM Ca^{2+} was sufficient to induce the presence of small percentages (5%) of acrosome reactions when spermatozoa were incubated in Beltsville poultry semen extender (BPSE) or DMEM. On the other hand, there was no acrosome reaction with spermatozoa incubated in NaCl-TES supplemented with Ca^{2+} . The addition of BSA (Fig. 2), or its homolog in the hen oviduct, ovalbumin, or other components known to stimulate capacitation or acrosome reaction in mammals such as NaHCO_3 or Pg did not stimulate acrosome reaction when spermatozoa were incubated with or without Ca^{2+} (data not shown).

The addition of the Ca^{2+} ionophore A23187 (Fig. 2) had no effect on spermatozoa incubated without Ca^{2+} , while its addition to spermatozoa incubated with Ca^{2+} initiated a mean acrosome reaction of 10% with every medium studied.

The addition of IPVL to the different media without Ca^{2+} did not induce any acrosome reaction. With the DMEM that already contained 2 mM Ca^{2+} , the addition of IPVL without further addition of Ca^{2+} induced 5–10% acrosome reaction.

The presence of IPVL and 5 mM Ca^{2+} induced acrosome reactions in every medium tested. The inductions obtained in BPSE supplemented with IPVL and 5 mM Ca^{2+} were equivalent to those obtained without IPVL. In DMEM, they were not significantly different from those obtained with IPVL and 2 mM Ca^{2+} . The combination of IPVL and 5 mM Ca^{2+} was the most effective combination to obtain acrosome reactions in NaCl-TES.

Figure 1 Evolution over time of percentage of acrosome-reacted spermatozoa incubated at 40 °C in NaCl-TES containing 5 mM Ca^{2+} and IPVL. Values represent the mean of five samples $\pm$ S.E.M. Super-scripts indicate significant differences ($P < 0.05$).

Figure 2 Effects of BSA, Ca²⁺, and A23187 on induction of acrosome reaction in chicken spermatozoa incubated with or without IPVL at 40 °C in (a) BPSE, (b) DMEM, and (c) NaCl-TES. Values represent the mean of five samples $\pm$ S.E.M. Superscripts indicate significant differences ($P < 0.05$).

Addition of Ca²⁺ ionophore A23187 to IPVL and 5 mM Ca²⁺ did not increase further the percentage of acrosome-reacted spermatozoa in any of the media tested.

Pre-incubation of spermatozoa for 1 h in NaCl-TES with different factors believed to facilitate induction of the acrosome reaction, including NaHCO₃, albumin, Ca²⁺, and different combinations of these factors before the addition of IPVL, A23187, or Pg, confirmed that with this medium, the combination of Ca²⁺ and A23187 or Ca²⁺ and IPVL was the only inducers of acrosome reaction (Fig. 3). The addition of A23187 to IPVL did not increase the percentage of reacted spermatozoa.

Finally, the viability (measured by propidium iodide (PI) staining) of the acrosome-reacted spermatozoa did not significantly differ from that of the non-reacted spermatozoa, irrespective of the media (data not shown, $P > 0.05$).

Figure 3 Effects of pre-incubation of spermatozoa in NaCl-TES supplemented with molecules believed to facilitate induction of acrosome reaction. Spermatozoa were pre-incubated at 40 °C in NaCl-TES in the presence or absence of IPVL, Ca²⁺, BSA, and/or NaHCO₃. After 1 h of incubation, A23187 or Pg was added to the medium. Values represent the mean of five samples $\pm$ S.E.M. Superscripts indicate significant differences ($P < 0.05$).

Effects of in vitro induction of acrosome reaction on motility parameters

The results reported in Table 1 show that the addition of 5 mM Ca²⁺ to the NaCl-TES medium significantly increased ($P < 0.05$) many motility parameters, including percentage of motile spermatozoa (17% increase), rapid spermatozoa (48% increase), progressive cells (37% increase), and two parameters of spermatozoa velocity, VCL (15% increase) and VAP (11% increase). The addition of IPVL to NaCl-TES showed the same stimulating effect as Ca²⁺ on the percentage of motile cells, but did not significantly increase the other parameters. There was no additional increase in the percentage of motile cells after the incubation of spermatozoa in the medium containing Ca²⁺ and IPVL. None of the other parameters of motility were significantly changed by the addition of Ca²⁺ and IPVL, although acrosome reactions were observed only with this last treatment.

Effects of different physiological states of PLs on the in vitro induction of the acrosome reaction

In order to evaluate the specificity of the PL physiological state to stimulating the induction of acrosome reactions, spermatozoa were incubated in NaCl-TES containing PLs isolated from eggs taken at different physiological states. The results reported in Table 2 show that all the physiological states of PL studied significantly stimulated the initiation of the chicken acrosome reaction ($P < 0.05$). However, the PL taken from the oviposited eggs or one of its two components, IPVL or OPVL, was the most effective state. The IPVL taken at the ovulation state was less effective than the oviposited untreated PL or the OPVL isolated by HCl incubation. In addition, the IPVL taken on the F1 follicle

Table 1 Effects of *in vitro* induction of acrosome reaction on motility parameters of chicken spermatozoa.

Motility parameters	Incubation medium			
	Control	Ca ²⁺	IPVL	IPVL, Ca ²⁺
Motile cells (%)	70.9±3.6 ^b	82.6±2.6 ^a	84.5±2.3 ^a	82.8±2.8 ^a
Rapid cells (%)	37.1±1.22 ^c	55.5±4.2 ^{a,b}	45.7±3.8 ^{b,c}	50.6±3.2 ^{a,b}
Prog cells (%)	20.2±2.8 ^b	27.8±2.4 ^a	23.1±2.6 ^{a,b}	24.2±1.7 ^{a,b}
VCL (µm/s)	125.5±5.5 ^b	144.1±6.2 ^a	126.5±5.8 ^b	134.3±4.1 ^{a,b}
VSL (µm/s)	48.7±3.7	57.3±2.9	48.1±3.7	53.1±2.4
VAP (µm/s)	65.7±3.9 ^b	77.4±3.4 ^a	65.4±4.2 ^b	72.5±2.7 ^{a,b}
Lin (%)	37.1±1.2	38.7±0.9	37.3±0.9	38.8±0.8
STR (%)	68.4±1.4	69.5±1.1	69.2±0.6	69.0±0.9
Acrosome reaction (%)	0 ^b	0 ^b	0 ^b	33.4±2.5 ^a

Spermatozoa were incubated for 5 min at 40 °C in NaCl-TES in the presence or absence of Ca²⁺ and/or IPVL. Values represent the mean of five samples ± S.E.M. Superscripts indicate significant within line differences ($P < 0.05$).

was the least effective state and showed significantly less induction of acrosome reactions than the different PL fractions taken on the oviposited egg ($P < 0.05$).

Discussion

Since Howarth (1970) showed that the ovulated oocyte of the hen could be activated *in vitro* after a short-incubation time with spermatozoa, different studies have attempted to define the conditions of penetration of chicken spermatozoa into the oocyte. Okamura & Nishiyama (1978) described the ultrastructure of the acrosome reaction when spermatozoa are in contact to the IPVL of the ovulated oocyte. Horrocks *et al.* (2000) suggested that the IPVL inducers of acrosome reaction could be N-linked oligosaccharides with terminal N-acetyl-glucosamine residues, while Ashizawa *et al.* (2004, 2006a, 2006b) started to describe the signaling pathways involved in the chicken acrosome reaction and Rabbani *et al.* (2006, 2007) suggested the involvement of sperm-associated bodies in the interaction of spermatozoa and IPVL. However, the understanding of the factors involved in the induction of the acrosome

reaction is still very incomplete, despite its importance for the understanding of the process of fertilization itself and the need to counteract induction of the acrosome reaction when it occurs spontaneously after *in vitro* storage for example.

We showed in the present study that extracellular Ca²⁺ appears to be the factor that is absolutely necessary to initiate the chicken acrosome reaction. The addition of Ca²⁺ ionophore can increase the Ca²⁺ effect. However, the presence of PL or one of its subfractions greatly increases the reaction. We also showed that other classical inducers of the mammalian capacitation and acrosome reaction have no stimulating effect in chickens and that, unlike mammals, there is no sign of motility hyperactivation to accompany the preparation of the chicken acrosome reaction. Finally, the stimulating effect of PL taken at different physiological states led us to question the specificity of IPVL to initiate the process.

Ca²⁺ is the most widely used intracellular messenger in cell signaling and is involved in virtually all spermatozoon functions, including capacitation, hyperactivation, and acrosome reaction (reviewed by Tomes 2007). Ca²⁺ influx from the extracellular medium to the cytosol through voltage channels is believed to be the first event that initiates the successive signaling pathways required for the acrosome exocytotic secretory response. The concomitant need of two factors, IPVL components and Ca²⁺, for the *in vitro* induction of the chicken acrosome reaction has already been reported by different authors (Horrocks *et al.* 2000, Ashizawa *et al.* 2004, 2006a, 2006b). However, the present study showed that a small percentage of acrosome-reacted spermatozoa may be obtained with the presence of Ca²⁺ without IPVL in the BPSE and DMEM media, and in the three media studied after the addition of Ca²⁺ ionophore. BPSE is a phosphate-buffered, mainly glutamate-based, organic salt solution while DMEM is a medium containing different inorganic salts including NaHCO₃, glucose, a wide range of amino acids, and vitamins. The acrosome reaction can thus be modulated in the absence of IPVL according to medium composition. On the other hand, the third medium used in the present study, NaCl-TES

Table 2 Effects of perivitelline layers taken from oocytes at various physiological states on *in vitro* induction of acrosome reaction.

Incubation medium	Acrosome reactions (%)
Control without PL	0.9±0.1 ^e
IPVL follicle	13.1±0.9 ^d
IPVL ov	19.9±1.2 ^{c,d}
PL laid	27.5±3.5 ^{a,b}
IPVL laid in HCl	25.6±4.4 ^{a,b,c}
OPVL laid in HCl	30.6±3.0 ^a
PL laid in HCl	27.0±2.8 ^{a,b}

Spermatozoa were incubated for 5 min at 40 °C in the presence of 5 mM Ca²⁺ and perivitelline layer taken at different physiological states. IPVL follicle, inner perivitelline layer taken on F1 follicle; IPVL ov, IPVL taken at the time of ovulation; PL laid, perivitelline layers taken at time of laying. IPVL laid in HCl, IPVL originating from laid eggs incubated 50 min in HCl 1 M; OPVL laid in HCl, OPVL originating from laid eggs incubated 50 min in HCl. Values represent the mean of six samples ± S.E.M. Superscripts indicate significant within line differences ($P < 0.05$).

buffer contains only sodium chloride and TES. Despite large differences of composition between DMEM and BPSE (BPSE being less complete than DMEM, containing fructose instead of glucose, no bicarbonate ions, etc.), factors stimulating Ca^{2+} influx seem to be present in these two media. However, preliminary experiments in our laboratory involving supplementation or deprivation of these different solutions indicate that stimulation does not seem to arise from a simple factor. The role of Ca^{2+} as the inducer of the acrosome reaction is emphasized by the response of chicken spermatozoa to the Ca^{2+} ionophore A23187. Indeed, we showed that A23187 plus millimolar concentrations of Ca^{2+} induced acrosome reactions with every medium tested. This means that when spermatozoa are made fully permeable to Ca^{2+} , a mean of 10% do not require IPVL to undergo the acrosome reaction.

The use of millimolar concentrations of extracellular free calcium is generally required for the stimulation of acrosome reaction because Ca^{2+} seems to be needed at different steps of the process. Contributions of possible internal acrosomal stores of Ca^{2+} (micromolar) are also needed for the accomplishment of the reaction, but would not be sufficient for the realization of the whole process (reviewed by Roldan & Shi 2007, Tomes 2007). In our study, the use of Ca^{2+} ionophore was effective in inducing the acrosome reaction in the presence of 5 mM Ca^{2+} . However, it is possible that a micromolar concentration of extracellular Ca^{2+} could be sufficient to induce the acrosome reaction in chicken spermatozoa and further work is needed to explore this hypothesis. Among the other possible facilitators of the acrosome reaction, albumin and NaHCO_3 have previously been described as stimulating the capacitation process that confers the ability to undergo the acrosome reaction in many mammals (Yanagimachi 1994). Capacitation can be achieved *in vitro* in balanced salt solutions containing appropriate concentrations of electrolytes and albumin as a primary source of protein. NaHCO_3 seems to play a key role in this process. It is believed to activate signaling pathways through adenylate cyclase activation and regulation of intracellular cAMP (Visconti *et al.* 1999, Gadella & Harrison 2000, Visconti *et al.* 2002, Salicioni *et al.* 2007). It is also believed to facilitate lipoprotein-mediated cholesterol efflux, to induce lateral redistribution in low cholesterol containing spermatozoa, which in turn facilitates cholesterol extraction by albumin, and to activate scramblases that move phospholipids in both directions across the membrane (Flesch *et al.* 2001, Harrison & Gadella 2005). However, our results support the assumption that NaHCO_3 is not involved in preparing the acrosome reaction in birds. Another important activator of the acrosome reaction in mammals is Pg. Mammalian spermatozoa, such as other mammalian cells, possess Pg receptors on the plasma membrane. Pg induces the acrosome reaction by raising the intracellular Ca^{2+} levels (Flesch & Gadella

2000, Naz & Sellamuthu 2006), possibly via the γ -aminobutyric acid receptor. Pg and ZP seem to induce the acrosome reaction in a synergistic and comparable way. Despite this situation in mammalian species, our results showed that the use of Pg at a dose that induces the acrosome reaction in mammalian species (Wu *et al.* 2006) has no effect in chickens. This questions the existence of Pg receptors on the chicken spermatozoa plasma membrane and/or the existence in bird spermatozoa of the pathways leading to Pg-dependent increases in intracellular Ca^{2+} .

In accordance with the lack of stimulating effect on the chicken acrosome reaction of compounds known to be involved in the process in mammals, we found no sign of motility hyperactivation in chicken spermatozoa incubated to induce the acrosome reaction. Motility hyperactivation involves changes that prime sperm to respond to the mammalian ZP (Suarez & Ho 2003). It consists of exaggerated, large amplitude flagellar movements characterized in CASA by low linearity together with high velocity and strength. Hyperactivation is thought to be important for spermatozoon progression through the highly viscous environment of the mammalian oviduct (Yanagimachi 1994, Darszon *et al.* 2007). The viscosity of the hen oviduct may be also very high, especially with the albumen secretions. However, the hen oocyte is not surrounded by investments such as cumulus cells that would require a different way of motility in mammals. It may therefore be suggested that there is no need for motility hyperactivation to prepare for the acrosome reaction in the chicken and that this special motility pattern has not been developed in birds.

Due to the use of CASA methodology and to the standardization at 35 °C of the final observation of motility (Blesbois *et al.* 2008), we did not find the classical lack of motility of chicken spermatozoa suspended in NaCl-TES at 40 °C (Ashizawa & Nishiyama 1977, 1978, Ashizawa *et al.* 1989). However, in agreement with previous reports (Wishart & Ashizawa 1987, Ashizawa *et al.* 1994), we found a clear stimulating effect of Ca^{2+} on motility. We showed that Ca^{2+} increased the number of motile spermatozoa and the velocity of the cells. This could mean that Ca^{2+} may act simultaneously on the recruitment of previously immotile spermatozoa and the acceleration of motility of previously active spermatozoa. It could thus be hypothesized that the stimulating action of Ca^{2+} on chicken spermatozoa may correspond to the simultaneous opening of previously closed Ca^{2+} channels and the stimulation of already opened channels.

The presence of IPVL also increased the percentage of motile spermatozoa but not the other parameters of motility. It should be noticed that IPVL components increase the viscosity of the medium surrounding spermatozoa (unpublished observations), possibly limiting the velocity of gametes and masking of other effects. The increase in the proportion of motile cells

induced by IPVL observed here, added to the potential presence of Ca^{2+} bodies embedded in the IPVL of quails and hens (Rabbani *et al.* 2006, 2007) lead us to question the IPVL Ca^{2+} content and its possible Ca^{2+} -like effect on motility.

The present study also agrees with findings concerning the respective roles of Ca^{2+} and IPVL. Previous reports have indicated the necessity of IPVL components for activation of the acrosome reaction (Horrocks *et al.* 2000, Ashizawa *et al.* 2004). We showed that the need for IPVL may at least to a certain degree be bypassed by favorable *in vitro* conditions of Ca^{2+} influx. We also showed that every physiological state of PL studied, from the F1 follicle to the laid egg, stimulates the acrosome reaction. This is a very interesting feature since the composition of the PL is different at each state. At the state of the F1 follicle, the PL is composed of the IPVL in construction (Elis *et al.* 2008), very closely surrounded (tight junctions) by granulosa cells. At the ovulation state, the PL is composed of IPVL mainly comprising ZP proteins (Waclawek *et al.* 1998, Takeuchi *et al.* 2001, Bausek *et al.* 2004, Okumura *et al.* 2004). At the oviposition state, a mean of 24 h after ovulation and possible *in vivo* fertilization, the PL is composed of two layers, the IPVL and the OPVL. The latter is thought to be secreted just after fertilization in the upper part of the oviduct, i.e., the infundibulum. After OPVL deposition, the two membranes evolve concomitantly during the 24 h of deposition of the other components of the egg in the oviduct (white and shell) up to the oviposition. The composition of the OPVL is different from the composition of the IPVL. Its main components are ovomucine, lysozyme, and two specific proteins, VMO1 and -2 (vitelline membrane outer layer protein 1 and 2; Kido & Doi 1988). Despite these main differences in composition, we showed that IPVL taken at the F1 state or the ovulation state and IPVL or OPVL or whole PL taken at the oviposition state stimulated the chicken acrosome reaction. Previous reports had suggested a low specificity of the IPVL physiological state for the fabrication of holes in the presence of spermatozoa (Steele *et al.* 1994, Robertson *et al.* 1997). The present study, focusing on an earlier state in the initiation of the fertilization process, supports this hypothesis. We also went further, as we showed that the replacement of the IPVL by the OPVL stimulates the induction of the acrosome reaction effectively.

Considerable evidence indicates that carbohydrate recognition plays a key role in the spermatozoa–egg interaction in all species, including birds (Horrocks *et al.* 2000). However, it is now accepted in the mouse that spermatozoa–egg binding may take different ways into account (Clark & Dell 2006). It may be that in birds, common carbohydrate fractions present on the PL at different states share the ability to bind spermatozoa and stimulate the acrosome reaction and/or that there is not a single system of interaction between the extracellular membrane surrounding the

oocyte and the spermatozoon to obtain the induction of the acrosome reaction.

Finally, our results demonstrate the extreme rapidity of initiation of the *in vitro* acrosome reaction in the chicken as we show that it may be induced within 1 min. In combination with our other results and the literature reports, it clearly emphasizes the lack of a capacitation-like process before the initiation of the chicken acrosome reaction. However, this raises questions regarding the physiological regulation that inhibits initiation *in vivo* of acrosome reaction in the lower parts of the oviduct where the free Ca^{2+} content may be much higher (up to 15 mM in the shell gland; Holm *et al.* 2000).

Taken together, the present results show *in vitro* that the initiation of the acrosome reaction in chickens is an original process that shares with many other species the central role of Ca^{2+} as inducer and second messenger. The results also show very specific features including the rapidity of the induction, the lack of sensitivity to compounds usually known to facilitate the induction of the reaction, and the poor specificity of the physiological state of the PL to induce the reaction.

Materials and Methods

Animals

The males used were 28- to 50-week-old adult chickens of the meat type D+ lines (Peron *et al.* 2006). The females were 25- to 60-week-old adult Isa Brown layer type hens (Institut de Sélection Animale, Saint Brieuc, France). All the animals were housed in individual battery cages under a 14-h light:10-h darkness photoperiod and fed a standard diet of 12.5 ml/day supplemented with Ca^{2+} for the females.

Semen collection and preparation

Semen was routinely collected twice a week by the abdominal massage method (Burrows & Quinn 1937). Spermatozoa concentration was determined by light absorption of semen with a photometer (IMV, L'Aigle, France) at a wavelength of 545 nm (Brillard & McDaniel 1985). Each experiment was independently managed and only the ejaculates containing more than 80% motile cells (measured by CASA, paragraph 2.4) were retained. For each experiment, samples of semen pooled from three males were centrifuged at 500 g for 10 min at room temperature. The pellets were resuspended in BPSE to give final spermatozoa concentration of 2×10^9 cells/ml before further dilution and use.

Incubation of spermatozoa

All chemicals were purchased from Sigma. PLs were isolated at different states of the daily reproductive cycle of hens. IPVLs were isolated from pre-ovulatory mature follicles (F1) as described previously by Takeuchi *et al.* (2001) or from just

ovulated eggs (Batellier *et al.* 2003). They were homogenized in 150 mM NaCl with 20 mM TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 (NaCl-TES) as described previously by Horrocks *et al.* (2000). PLs from oviposited eggs were also isolated and then used either without further preparation or after separation of IPVL and OPVL. PLs after 1 h of incubation at 40 °C in HCl 0.01 M as described by Kido & Doi (1988). A 2 cm×2 cm square of IPVL, OPVL, or PL was homogenized in 1 ml of 150 mM NaCl with 20 mM TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 (NaCl-TES), or in DMEM containing 25 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.4); or BPSE according to the medium used for the incubation of spermatozoa.

Aliquots of 20×10^6 spermatozoa were incubated in 1 ml NaCl-TES, or in DMEM or BPSE, with or without 100 µl IPVL preparation, 0.3 g/l BSA, 0.3 g/l ovalbumin, 5 mM CaCl_2 , 20 µM Ca^{2+} ionophore A23187, 25 mM NaHCO_3 (with or without controlled CO_2 atmosphere), and 10 µg/ml Pg. The samples were incubated for 5 min at 40 °C before measurement of the acrosome reaction. For the kinetics of acrosome reactions, incubation times varied between 1 and 30 min at 40 °C. For the experiment of pre-incubation of spermatozoa with 'capacitating factors' before induction of acrosome reaction, spermatozoa were pre-incubated for 1 h at 40 °C before the 5 min of 'induction'.

Semen analysis

Evaluation of acrosome reaction by FITC-PNA

Acrosome-reacted spermatozoa were identified using FITC-conjugated PNA according to an adaptation of the method described by Horrocks *et al.* (2000). PNA binds to spermatozoa that have started the acrosome reaction, but not to acrosome-intact spermatozoa. Briefly, the samples of semen were centrifuged at 400 *g* for 5 min. The pellets were resuspended in 100 µl NaCl-TES with 20 µg/ml FITC-PNA, incubated for 10 min at 4 °C in darkness, then washed with 500 µl NaCl-TES and centrifuged at 400 *g* for 5 min. The pellets containing spermatozoa were resuspended in 500 µl NaCl-TES. The suspensions (10 µl) were examined by phase contrast and fluorescence microscopy (×1000, Zeiss Axioplan 2; Zeiss Gruppe, Jena, Germany). A minimum of 100 spermatozoa were counted by sample. Acrosome-reacted spermatozoa were characterized by green fluorescence of the acrosomal region.

Proportion of viable spermatozoa

Spermatozoa viability was assessed by PI staining (Chalah & Brillard 1998). After incubation, samples were centrifuged at 400 *g* for 5 min. The pellets were resuspended in NaCl-TES with 20 µg/ml PI for 5 min in darkness, and aliquots of the suspensions were examined using fluorescence microscopy (×1000, Zeiss). A minimum of 100 spermatozoa were counted for each sample. Membrane-damaged cells showed red fluorescence.

Objective parameters of motility

CASA of different objective motility parameters was performed with an HTM-IVOS (Hamilton Thorne Biosciences, Beverly, USA) as described previously (Blesbois *et al.* 2008). The parameters measured were percentage of motile sperm, path velocity (VAP=average velocity measured over the actual point-to-point track followed by the cell), progressive velocity (VSL=straight line distance between beginning and end of the track/time elapsed), straightness (STR= $100 \times (\text{VSL}/\text{VAP})$), linearity (LIN=departure of the cell track from a straight line= $100 \times \text{VSL}/\text{VCL}$), proportion of rapid spermatozoa (RAPID=percentage of the sperm moving with $\text{VAP} > 60 \mu\text{m/s}$), and proportion of progressive spermatozoa (PROG=proportion of rapid spermatozoa with straightness > 80%). Other parameters classically measured on mammalian spermatozoa such as the mean amplitude of lateral head displacement and the frequency of head displacement were not retained because they were not relevant for chicken spermatozoa.

Aliquots of 2.5 µl semen diluted 1:200 in their corresponding incubation media at 40 °C were observed in MAKLER chambers maintained at 35 °C (0.01 sq/mm; 10 µm deep; Sefi-Medical Instruments Ltd, Haifa, Israel). Three analyses were performed per sample.

Statistical analysis

Statistical analyses were performed with the Statview software (Abacus Concepts Inc., Berkeley, Canada). Changes in acrosome reactions, viability, and motility of spermatozoa were evaluated by one to three ways ANOVA according to the number of factors involved in each experiment. Analyses of variance were followed by Fisher's protected least significant difference test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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