Progesterone induces the release of bull spermatozoa from oviductal epithelial cells

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The mechanism that causes the detachment of spermatozoa from the oviductal reservoir around the time of ovulation remains to be elucidated. Because the cumulus cells of the bovine oocyte are known to secrete progesterone (P4), and P4 has been shown to act upon cation channels of spermatozoa (CatSper) in human spermatozoa, it was hypothesised that P4 could induce hyperactivation due to an influx of extracellular calcium, and this would facilitate detachment of spermatozoa from oviductal epithelial cells. Therefore, this study aimed to investigate the role and mechanism of action of P4 in the release of spermatozoa from bovine oviduct epithelial cells (BOEC).

Initial dose–response assessments on sperm hyperactivation determined the optimum concentration of P4 (10 nM), mibefradil (a non-specific Ca²⁺ channel antagonist; 5 µM), NNC 55-0396 dihydrochloride (NNC; a CatSper antagonist; 2 µM), mifepristone (a classical and membrane P4 receptor antagonist; 400 nM) and AG205 (a membrane P4 receptor antagonist; 10 μM). BOEC explants were incubated with frozen–thawed bovine spermatozoa for 30 min, following which loosely bound spermatozoa were removed. Two experiments were completed. In Experiment 1, BOECs were treated for 30 min with either no treatment, P4, NNC, mibefradil, P4 + mibefradil, P4 + NNC, P4 + mibefradil + NNC or P4 + EGTA. In Experiment 2, BOECs were treated for 30 min with either no treatment, P4, mifepristone, AG205, mifepristone + AG205, P4 + mifepristone, P4 + AG205 or P4 + mifepristone + AG205. The number of spermatozoa remaining bound per square millimetre of BOEC explant was determined. Progesterone stimulated the release of bound spermatozoa from BOEC explants, whereas NNC, mibefradil and EGTA inhibited this release. The release of spermatozoa by P4 was inhibited in the presence of both mifepristone and AG205, whereas the combination of both had the greatest inhibitory action on P4 release of spermatozoa. These findings suggest the presence of a P4 membrane receptor on bovine spermatozoa and that P4-induced release of spermatozoa from BOECs is likely mediated by extracellular Ca²⁺.

Additional keywords: membrane progesterone receptor, oviduct, progesterone receptor membrane component 1 (PGRMC1), unbinding.

J. Romero-Aguirregomezcorta et al.

Membrane P4 receptor and bull sperm function

This work offers a novel insight into the effect of progesterone (P4) on bovine sperm hyperactivation and associated release from the oviductal epithelium. Through investigation of the molecular mechanism of action of P4 we have demonstrated that hyperactivation induced by P4 plays a role in the detachment of bovine sperm from oviductal epithelial cells and that the presence of extracellular Ca²⁺ is essential in this response to P4. These findings increase our understanding of how sperm are released from reservoirs in the upper female reproductive tract.

Introduction

Formation of a sperm reservoir in the isthmus of the oviducts plays a critical role in maintaining the fertile lifespan of spermatozoa. Uncapacitated spermatozoa preferentially bind by the plasma membrane overlying the acrosome to the ciliated surface of epithelial cells (Yanagimachi and Chang
1963; Lefebvre and Suarez 1996; Fazeli et al. 1999), and bound spermatozoa have been shown to have low intracellular \( \text{Ca}^{2+} \) content, low tyrosine phosphorylation (Gualtieri et al. 2005), intact acrosomes (Gualtieri and Talevi 2000) and normal chromatin structure (Ellington et al. 1999). Their gradual release in response to the oviductal microenvironments surrounding bound spermatozoa supplies the ampulla with a steady supply of functional spermatozoa while minimising the incidence of polyspermy. The role of sperm capacitation in the detachment of spermatozoa from the oviductal epithelium has been well described (Gadella and Boerke 2016; Miller 2018). Once capacitated spermatozoa unbind, they lose their affinity for the oviductal epithelium (Gualtieri et al. 2005).

Moreover, it has been demonstrated that hyperactivation can induce the release of uncapacitated spermatozoa, and these spermatozoa maintain the ability to rebind (Chang and Suarez 2012). Indeed, only mouse spermatozoa that exhibit hyperactivated motility detached from the oviductal epithelium (Demott and Suarez 1992), and those lacking the cation channel of spermatozoa (CatSper) were unable to hyperactivate and failed to progress beyond the oviductal sperm reservoir because they remained bound to the oviductal epithelium in vivo (Ho et al. 2009). Hyperactivated free-swimming spermatozoa demonstrate an asymmetric waveform that results in a helical or curved trajectory (Yanagimachi 1970), whereas a tethered spermatozoan generates tugging forces to enable it to detach from a surface (Curtis et al. 2012). Moreover, the attaching and detaching sequence has not been witnessed with symmetrical flagellar beating spermatozoa (Chang and Suarez 2012). Thus, it remains unclear whether hyperactivation-induced detachment is the same in other species and, if so, what triggers the sequential release of spermatozoa through hyperactivation.

The influx of \( \text{Ca}^{2+} \) triggers multiple physiological events in spermatozoa, including hyperactivated motility (Ho et al. 2002), capacitation and the acrosome reaction (Breitbart 2002; Kirkman-Brown et al. 2002), each of which are essential for fertilisation. Similar to somatic cells, \( \text{Ca}^{2+} \) is sourced either extracellularly or released from intracellular stores in ejaculated spermatozoa (Publicover et al. 2007), but the mechanisms by which intracellular \( \text{Ca}^{2+} \) is released from its stores remain to be elucidated. Unlike most somatic cells, mature spermatozoa do not possess an endoplasmic reticulum (ER), which is the chief source of \( \text{Ca}^{2+} \), hence \( \text{Ca}^{2+} \) is thought to be held by a redundant nuclear envelope (RNE), which colocalises with the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) in the region, and the tightly packed mitochondria in the midpiece (for a review, see Costello et al. 2009). Both structures contain calreticulin, a \( \text{Ca}^{2+} \) storage protein, and \( \text{Ca}^{2+} \) mobilisation from these stores involves the IP\(_3\)R and the \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release (CICR) of the ryanodine receptor (RyR).

Conversely, in spermatozoa of many mammals, the sperm-specific \( \text{Ca}^{2+} \) channel CatSper controls the entry of \( \text{Ca}^{2+} \) into the spermatozoa (for a review, see Singh and Rajender 2015). However, the mechanism of CatSper activation in different species has yet to be elucidated.

In human spermatozoa, CatSper can be activated through intracellular alkalisation and progesterone (P4; Lishko and Kirichok 2010; Tamburrino et al. 2014). P4 is secreted by the cumulus cells of the oocyte in vivo and in vitro (Mingoti et al. 2002), and has been demonstrated to stimulate biological responses of human spermatozoa, including \( \text{Ca}^{2+} \) influx, tyrosine phosphorylation, extracellular signal-regulated kinases, chloride efflux and increases in cAMP levels (Falkenstein et al. 1999; Lösel et al. 2003). Studies suggest that the actions of P4 in inducing hyperactivation and the acrosome reaction can occur independent of each other through different intracellular pathways (Marquez and Suarez 2004). P4 stimulation of rapid activities in human spermatozoa, at nanomolar to micromolar concentrations, is proposed to originate from the cell surface and has been seen, in many cells, to be mediated through non-classical progestin membrane receptors (Thomas et al. 2009). P4 stimulates \( \text{Ca}^{2+} \) entry in human spermatozoa (Falkenstein et al. 1999) by activating CatSper, and these channels have been associated with stimulation of hyperactivated motility (Lishko et al. 2011). In porcine
species, CatSper is present in ejaculated boar spermatozoa, being implicated in the regulation of sperm motility during capacitation (Vicente-Carrillo et al. 2017), and it has been demonstrated that sperm release from oviduct epithelial cells is triggered specifically by P4 and involves the action of CatSper (Machado 2013). In the bull, the mechanism of activation of CatSper remains unclear.

Our group has recently demonstrated that hyperactivation of bovine spermatozoa is dependent on Ca\(^{2+}\) influx through CatSper (Johnson et al. 2017), but whether the release of bovine spermatozoa from the oviductal epithelium can be induced by hyperactivation and the role, if any, that P4 may play in the mechanism by which CatSper activation in bovine spermatozoa induces hyperactivation and the release of spermatozoa from the oviductal epithelium remain unknown. To this end, the aim of this study was to investigate the role and mechanism of action of P4 in the release of spermatozoa from bovine oviduct epithelial cells.

Materials and methods

Chemicals and reagents

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich Ireland.

Sperm preparation

Frozen–thawed straws from three different Holstein–Friesian bulls were thawed at 37°C and pooled to minimise interbull variability. Only samples with a post-thaw motility >60% were used. Sperm concentration was determined using a Neubauer chamber and samples were diluted to 40 × 10^6 spermatozoa mL\(^{-1}\) in Tyrode’s albumin lactate pyruvate (TALP) medium. This medium consists of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO\(_3\), 0.4 mM NaH\(_2\)PO\(_4\), 1.1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM HEPES, 25.4 mM Na lactate, 1 mM Na-pyruvate and 6 mg mL\(^{-1}\) bovine serum albumin (BSA).

Assessment of hyperactivation

Hyperactivation was assessed subjectively using a phase contrast microscope (CX41; Olympus) fitted with a heated stage (37°C) at a magnification of ×400. Aliquots (200 μL) of treated spermatozoa were incubated for 10 min in 5% CO\(_2\) at 37°C. After incubation, a 10-μL droplet of sample was placed on a prewarmed slide with a coverslip for evaluation. The percentage hyperactivation in a sample was determined by assessing the number of hyperactivated spermatozoa from 100 motile sperm cells assessed. Hyperactivated motility was manifested as characteristic figure-of-eight swimming trajectories with a helical pattern (Ho et al. 2002), compared with the linear movement of non-hyperactivated spermatozoa. Three technical replicates of each sample were completed (total 300 spermatozoa assessed per replicate) before averaging the percentage hyperactivation per replicate. All assessments were performed in a blinded manner. This was the method used for all assessments of hyperactivation in this study. The sperm treatments used are detailed in their relevant experimental sections below.

Bovine oviductal epithelial cell unbinding assay

Non-pregnant heifer reproductive tracts were retrieved from a commercial abattoir immediately after the heifers had been killed. Reproductive tracts from different oestrus cycle stages were used because previous studies have shown that stage does not affect the binding capability of spermatozoa (Lefebvre et al. 1997; Gwathmey et al. 2003). The tracts were transported at 4–5°C to the laboratory in Dulbecco’s phosphate-buffered saline (PBS) with Ca\(^{2+}\) and Mg\(^{2+}\) and supplemented with 0.5% gentamycin. At the laboratory, the oviducts were trimmed free of surrounding connective tissue, ligated using sterile umbilical cord clamps and sterilised in 70% ethanol for 30 s before being.
washed twice in PBS. Under the laminar flow, a transverse incision was made to separate the isthmic portion of the oviduct, after which bovine oviductal epithelial cells (BOECs) were isolated from each oviduct through a mechanical scraping technique using a glass slide (Green et al. 2001). The BOECs were washed, recollected in 1 mL PBS and were centrifuged at 200g for 1 min at 37°C. The supernatant was removed after centrifugation and 1 mL M199 culture medium supplemented with 10% fetal bovine serum and 0.5% gentamycin was added to the pellet. The BOECs were then incubated in a 5% CO₂ incubator at 37.5°C for 1 h to allow the formation of everted vesicles with apical ciliated surfaces orientated outwards (Ignotz et al. 2007).

Sperm samples were stained with a 10 µg mL⁻¹ Hoechst 33342 solution, centrifuged twice at 300g for 5 min at 37°C and diluted to a concentration of 7.14 × 10⁶ mL⁻¹ using prewarmed TALP medium, after which they were added to a 24-well plate in 140-µL aliquots. After incubation with M199 culture medium, the BOEC explants were washed and centrifuged at 200g for 5 min at 37°C with PBS (5 mL). After this centrifugation, the supernatant was discarded, and 20 µL pellet (BOEC explants in a minimum volume of PBS) was added to each aliquot of spermatozoa in the 24-well plate. Spermatozoa and BOECs were coincubated for 30 min at 37.5°C and 5% CO₂, after which loosely bound spermatozoa were removed from the samples by gently pipetting through two 75-µL droplets of TALP. Each sample was assessed for bound sperm density, which confirmed equal binding in all wells. Then, spermatozoa and BOECs were treated with P4 or the range of either agonists and antagonists for 30 min at 37.5°C and 5% CO₂. After incubation, loosely bound and unbound spermatozoa were removed by gently pipetting through two 75-µL droplets of TALP. A 10-µL droplet was then placed on a prewarmed slide and covered with a coverslip. Slides were viewed on a heated stage (37°C) at ×400 under a combination of transmitted and epifluorescence lighting using a fluorescent microscope (BX60; Olympus). Ten fields of view were randomly assessed per slide. Spermatozoa were classified as bound if the sperm head was in contact with the apical surface of the BOEC (Lyons et al. 2018). A micrometer, placed in the eyepiece of the microscope, was used to measure the surface area of each explant. Bound sperm density was calculated by quantifying the number of spermatozoa bound per 0.1 mm² of the explant surface. The assessor was blinded to the treatments. One well was assessed per treatment per replicate, where one replicate (n) was one heifer reproductive tract.

Experiment 1: effects of P4 and extracellular Ca²⁺ on hyperactivation and detachment from BOEC explants

The aim of this experiment was to investigate the effect of P4 on hyperactivated motility and detachment from BOEC explants.

Experiment 1a: effects of P4 and extracellular Ca²⁺ on hyperactivation

It was hypothesised that P4 would induce hyperactivation and would act through the influx of extracellular Ca²⁺. Therefore, the aim of this experiment was to quantify the effect of calcium agonists and antagonists on spermatozoa exhibiting hyperactivated motility. Experiments were performed using P4 and the Ca²⁺ channel antagonists mibebradil and NNC 55-0396 (NNC). Mibebradil is a Ca²⁺ channel inhibitor that acts on both L- and T-type channels (Mullins et al. 1998), whereas NNC is a derivative of mibebradil and is a highly selective T-type Ca²⁺ channel inhibitor (Li et al. 2005). Initial dose–response assessments were performed to determine the optimum concentrations of P4, mibebradil and NNC to induce sperm hyperactivation. Vehicle controls were performed for treatments dissolved in ethanol (P4) and dimethylsulfoxide (DMSO; NNC). P4 concentrations of 0, 5, 8, 10, 50, 500 nM and 1, 10 and 50 µM were assessed. After 10 min incubation, hyperactivation was assessed as described above, and three replicates were completed. Because it was hypothesised...
that P4 stimulates hyperactivation through extracellular Ca\(^{2+}\) influx, NNC concentrations were assessed on the basis of their efficiency to antagonise T-type Ca\(^{2+}\) channels in the presence of 10 nM P4. Treatments included a no treatment control, 10 nM P4 alone and 10 nM P4 combined with NNC (500 nM, 1, 2, 10, 20 or 30 μM). Hyperactivation was assessed and three replicates were completed. Similarly, the concentration of mibefradil was determined based on its ability to suppress the proposed influx of Ca\(^{2+}\). Treatments included a no treatment control, 10 nM P4 alone and 10 nM P4 combined with mibefradil (10, 100, 500 nM, 1, 5, 10, 20 or 40 μM). Hyperactivation was assessed, and three replicates were completed.

From each of the three dose–response experiments above, the optimum concentration of each drug was chosen and assessed in one experiment to confirm their effects. The calcium chelator EGTA was also used to chelate the Ca\(^{2+}\) in the media. In this series of experiments, the treatment groups included a no treatment control, 10 nM P4 alone, 2 μM NNC alone, 5 μM mibefradil alone, 10 nM P4 + 5 μM mibefradil, 10 nM P4 + 2 μM NNC, 10 nM P4 + 5 μM mibefradil + 2 μM NNC and 10 nM P4 + 2.5 mM EGTA.

**Experiment 1b: effects of P4 and extracellular Ca\(^{2+}\) on the detachment of bound spermatozoa from BOECs**

It was hypothesised that the incubation of bound spermatozoa with P4 would stimulate a release of spermatozoa from the BOEC explants due to an influx of extracellular Ca\(^{2+}\). NNC and mibefradil were used as antagonists of Ca\(^{2+}\) channels. Therefore, the aim of this experiment was to investigate the action of P4 on the release of bound spermatozoa from BOECs. P4 has been proposed to act upon CatSper in human spermatozoa (Strünker et al. 2011; Alasmari et al. 2013; Tamburrino et al. 2014) and because CatSper is a T-type Ca\(^{2+}\) channel in spermatozoa (Darszon et al. 2006), NNC is an effective antagonist. The following treatments were assessed: a no treatment control, 10 nM P4 alone, 2 μM NNC alone, 5 μM mibefradil alone, 10 nM P4 + 5 μM mibefradil, 10 nM P4 + 2 μM NNC, 10 nM P4 + 5 μM mibefradil + 2 μM NNC and 10 nM P4 + 2.5 mM EGTA. All treatments were assessed for bound sperm density after treatment as described above, and three replicates were completed.

**Experiment 1c: effects of P4 and extracellular Ca\(^{2+}\) on hyperactivation after detachment from BOECs**

This experiment was performed to determine the percentage hyperactivation of spermatozoa detached from BOECs after the following treatment: no treatment control, 10 nM P4 alone, 2 μM NNC alone, 5 μM mibefradil alone, 10 nM P4 + 5 μM mibefradil, 10 nM P4 + 2 μM NNC, 10 nM P4 + 5 μM mibefradil + 2 μM NNC and 10 nM P4 + 2.5 mM EGTA. The treatments chosen were the same as those assessed in Experiments 1a and 1b. For this experiment, after the 30 min incubation of sperm bound to BOECs with P4 and Ca\(^{2+}\) channel antagonists, a 10-μL aliquot was collected from each well and the percentage hyperactivation of spermatozoa detached from BOECs was determined as described above. The assessor was blinded to the treatments being assessed, and four replicates were completed.

**Experiment 2: effects of P4 receptor antagonists on the action of P4**

The aim of this experiment was to investigate the mechanism of P4 action on bovine spermatozoa. It was hypothesised that P4 acts on spermatozoa through non-genomic pathways to initiate the influx of Ca\(^{2+}\). AG205 is a P4 receptor membrane component 1 (PGRMC1) antagonist (Guo et al. 2016) and has been used to investigate the involvement of PGRMC1 in numerous cell types, predominantly from the liver and kidneys and cancerous cells (Peluso et al. 2008; Mir et al. 2012). Mifepristone (RU-486) is an antiprogestin that exerts inhibitory effects on membrane P4 receptor (mPR), as seen in T
To examine the possible role of both membrane receptors on bovine spermatozoa, the effects of P4 receptor antagonists in the presence of P4 were assessed on hyperactivated spermatozoa and on detached spermatozoa from BOECs.

**Experiment 2a: effects of P4 receptor antagonists on P4-induced hyperactivation**

The aim of this experiment was to investigate whether P4-induced hyperactivation is mediated through a membrane-bound P4 receptor. An initial dose–response experiment was performed for both mifepristone and AG205 and the optimum concentration with the greatest effect on counteracting the action of P4 on sperm hyperactivation was chosen. A vehicle control was performed for mifepristone because it was dissolved in DMSO. In the case of mifepristone, the following treatments were assessed: no treatment control, 10 nM P4 alone and 10 nM P4 + mifepristone (40 and 400 nM and 4 and 400 μM). In the case of AG205, the following treatments were assessed: no treatment control, 10 nM P4 alone and 10 nM P4 + AG205 (5, 10, 20, 30 and 40 μM). Hyperactivation responses of spermatozoa were analysed using the methods described above.

From each of the two dose–response experiments above, the optimum concentration of each drug was chosen and assessed in one experiment to confirm their effects. Treatments included a no treatment control, 10 nM P4 alone, 400 nM mifepristone alone, 10 μM AG205 alone, 400 nM mifepristone + 10 μM AG205, 10 nM P4 + 400 nM mifepristone, 10 nM P4 + 10 μM AG205 and 10 nM P4 + 400 nM mifepristone + 10 μM AG205. All treatments were assessed in a blinded manner, and three replicates were completed.

**Experiment 2b: effects of P4 and P4 receptor antagonists on the detachment of spermatozoa from BOECs**

It was hypothesised that the action of P4 in the detachment of spermatozoa from BOECs would be suppressed through the use of P4 receptor antagonists. Bound spermatozoa were treated with a no treatment control, 10 nM P4 alone, 400 nM mifepristone alone, 10 μM AG205 alone, 400 nM mifepristone + 10 μM AG205, 10 nM P4 + 400 nM mifepristone, 10 nM P4 + 10 μM AG205 and 10 nM P4 + 400 nM mifepristone + 10 μM AG205. The number of bound spermatozoa remaining after each treatment was assessed. The assessor was blinded to the treatments being assessed, and three replicates were completed.

**Experiment 2c: effects of P4 and P4 receptor antagonists on hyperactivation after the detachment of spermatozoa from BOECs**

This experiment was performed to determine the percentage hyperactivation of spermatozoa detached from BOECs after the following treatments: no treatment control, 10 nM P4 alone, 400 nM mifepristone alone, 10 μM AG205 alone, 400 nM mifepristone + 10 μM AG205, 10 nM P4 + 400 nM mifepristone, 10 nM P4 + 10 μM AG205 and 10 nM P4 + 400 nM mifepristone + 10 μM AG205. The treatments chosen were the same as assessed in Experiments 2a and 2b. For this experiment, after the 30 min incubation of spermatozoa bound to BOECs with P4 and P4 receptor antagonists, a 10-μL aliquot was collected from each well and the percentage hyperactivation of the sperm detached from BOECs was determined as described above. All treatments were assessed in a blinded manner, and four replicates were completed.

**Statistical analysis**

All data were examined for normality of distribution and were analysed using IBM SPSS Software, Version 22 (IBM Corp.). All data were normally distributed and thus univariate analysis of variance
(ANOVA) was used. Post hoc tests were conducted using the Bonferroni test and two-sided \( P < 0.05 \) was considered significant. All results are reported as the mean ± s.e.m.

**Results**

**Experiment 1: effects of P4 and extracellular \( \text{Ca}^{2+} \) on hyperactivation and release of bound spermatozoa from BOECs**

**Experiment 1a: effects of P4 and extracellular \( \text{Ca}^{2+} \) on hyperactivation**

Hyperactivated motility developed rapidly when spermatozoa were treated with P4, and there was an effect of P4 treatments on hyperactivated motility (\( P < 0.001 \); Fig. 1). Increasing the concentration of P4 increased sperm hyperactivation, with peak hyperactivation achieved at a concentration of 10 nM P4 (76.8 ± 3.0%; \( P < 0.001 \)). The \( \text{Ca}^{2+} \) channel antagonists NNC and mibefradil successfully suppressed P4-induced hyperactivation, with concentrations of 2 \( \mu \text{M} \) NNC and 5 \( \mu \text{M} \) mibefradil having the greatest antagonistic effect (\( P < 0.001 \); Fig. 2); these concentrations were used for subsequent experiments.

**Experiment 1b: effects of P4 and extracellular \( \text{Ca}^{2+} \) on hyperactivation and the release of bound spermatozoa from BOEC explants**

P4 increased sperm hyperactivation (\( P < 0.001 \); Fig. 3) and stimulated the release of bound spermatozoa from BOEC explants (\( P < 0.001 \); Fig. 3). Incubation of spermatozoa with the \( \text{Ca}^{2+} \) channel antagonists NNC and mibefradil alone had no effect on sperm hyperactivation (\( P > 0.05 \)) compared with control. However, incubation of spermatozoa with NNC and mibefradil alone had an effect on the release of spermatozoa from BOEC explants (\( P < 0.01 \)). NNC, mibefradil and EGTA inhibited P4-induced hyperactivation (\( P < 0.001 \)) and the release of spermatozoa from BOEC explants induced by P4 (\( P < 0.001 \)). The mean size of the vesicles was 0.00453 ± 0.000333 mm\(^2\). There were no significant differences in the size of the vesicles among the different treatment groups (\( P = 0.176 \)).

**Experiment 1c: effects of P4 and extracellular \( \text{Ca}^{2+} \) on hyperactivation after detachment from BOECs**

We also determined hyperactivation of the spermatozoa detached from BOECs after the different treatments. P4 alone significantly increased the percentage hyperactivation of spermatozoa released from BOECs compared with the control group (52.7 ± 5.5% vs 16.0 ± 3.2% respectively; \( P < 0.001 \)). In the absence of P4, mibefradil and NNC did not affect hyperactivation (17.7 ± 4.7% and 20.7 ± 3.9% respectively; \( P > 0.05 \)). The hyperactivation shown by the spermatozoa released by P4 was inhibited in the presence of EGTA (21.3 ± 2.2%; \( P < 0.001 \)), mibefradil (27.0 ± 4.0%; \( P < 0.001 \)), NNC (17.7 ± 2.3%; \( P < 0.001 \)) and the combination of both \( \text{Ca}^{2+} \) channel antagonists (14.0 ± 4.9%; \( P < 0.001 \)).

**Experiment 2: effects of P4 receptor antagonists on the action of P4**

**Experiment 2a: effects of P4 receptor antagonists on P4-induced hyperactivation**

Both P4 receptor antagonists (AG205 and mifepristone) had an effect on P4-induced hyperactivated motility (\( P < 0.001 \) and \( P < 0.001 \) respectively; Fig. 4). All concentrations of AG205 inhibited P4-induced hyperactivation (\( P < 0.001 \)). Mifepristone (400 nM) had the greatest inhibitory effect against P4 (\( P < 0.0001 \)). Therefore, the lowest, most-effective concentrations of 10 \( \mu \text{M} \) AG205 (\( P < 0.0001 \)) and 400 nM mifepristone (\( P < 0.0001 \)) were deemed optimum for use in subsequent experiments.

**Experiment 2b: effects of P4 and P4 receptor antagonists on hyperactivation and the detachment of spermatozoa from BOECs**
There was an inhibitory effect of P4 receptor antagonists on sperm hyperactivation ($P < 0.001$) and release of spermatozoa from BOEC explants ($P < 0.01$; Fig. 5). In the absence of P4, mifepristone and AG205 alone or in combination did not affect hyperactivation ($P > 0.05$) or the release of spermatozoa from BOECs ($P > 0.05$). Hyperactivation and the release of spermatozoa from BOEC explants induced by P4 were inhibited in the presence of mifepristone ($P < 0.001$ and $P < 0.05$ respectively) and AG205 ($P < 0.001$ and $P < 0.05$ respectively). The combination of AG205 and mifepristone had the highest significant effect in inhibiting the actions of P4 on both hyperactivation and the release of spermatozoa from BOEC explants ($P < 0.001$ and $P < 0.01$ respectively). Bound sperm density was not affected by P4 when incubated with both AG205 and mifepristone compared with the control ($P > 0.05$). The mean size of the vesicles was $0.00371 \pm 0.00020 \text{mm}^2$. There were no significant differences in the size of the vesicles among the different treatment groups ($P = 0.956$).

Experiment 2c: effects of P4 and P4 receptor antagonists on hyperactivation after the detachment of spermatozoa from BOECs

Hyperactivation of spermatozoa released from BOECs after the different treatments was determined. P4 alone significantly increased the percentage hyperactivation of spermatozoa released from BOECs compared with the control group (55.3 ± 7.4% and 13.8 ± 3.4% respectively; $P < 0.001$). In the absence of P4, mifepristone and AG205 alone or in combination had no effect on hyperactivation (11.5 ± 3.0%, 12.0 ± 1.7% and 17.0 ± 4.3% respectively; $P > 0.05$). The hyperactivation shown by spermatozoa released by P4 was inhibited in the presence of mifepristone (18.3 ± 8.1%; $P < 0.001$), AG205 (11.8 ± 3.6%; $P < 0.001$) and the combination of both P4 receptor antagonists (19.3 ± 2.2%; $P < 0.001$).

Discussion

The sequential release of fertile spermatozoa from the oviductal reservoir around the time of ovulation helps deliver a supply of fertile spermatozoa to the ampulla and increases the chances of successful fertilisation. Most studies to date have focused on the role of capacitation in the mediation of sperm release and, to the best of the authors’ knowledge, the present study is the first published study to assess the effects of P4 on bovine sperm hyperactivation and associated unbinding from the oviductal epithelium. The main findings of this study were that: (1) hyperactivation stimulates a significant release of spermatozoa from BOECs; (2) hyperactivation of bovine spermatozoa can be induced by P4 by a rapid non-genomic action; (3) P4 elicits its effect by directly or indirectly acting on Ca$^{2+}$ channels, which facilitates the influx of extracellular Ca$^{2+}$ into the cell; and (4) the action of P4 on spermatozoa, which was suppressed by P4 receptor antagonists in this dataset, infers the presence of a P4 membrane receptor.

The physiological factors that trigger the sequential release of spermatozoa from oviductal reservoirs are not well understood. The attachment of spermatozoa to the lower region of the isthmus maintains sperm viability and delays sperm capacitation in vivo (Pollard et al. 1991; Chian et al. 1995). It has been shown that the release mechanism does not affect the number of carbohydrate binding sites that are present in the oviductal epithelium (Lefebvre et al. 1995); therefore, the key to release mechanisms is through an alteration to the sperm surface in response to tract components. The release of spermatozoa from the isthmic epithelium has been reported to be dependent on sperm changes associated with capacitation (Bosch et al. 2001). In the present study, hyperactivation alone was sufficient for sperm detachment from the isthmic portion of the oviduct, indicating that hyperactivation is an important sperm release mechanism, in addition to capacitation. This supports the suggestion that sperm transport involves a cycle of detachment and attachment in the oviduct before capacitation but, once capacitated, spermatozoa lose their affinity.
for the oviductal epithelium (Lefebvre and Suarez 1996; Gualtieri et al. 2005). Studies of tethered spermatozoa show that hyperactivated spermatozoa can exert enough propulsive force to penetrate through viscoelastic fluids and detach from a surface (Ishijima 2011; Curtis et al. 2012). P4 is known to be released by the cumulus cells of the bovine oocyte (Mingoti et al. 2002), therefore spermatozoa are exposed to a P4 gradient with maximal concentrations in close proximity to the oocyte. In the present study a concentration of 10 nM P4 was used, which may be higher than physiological concentrations to which spermatozoa are exposed to in the female reproductive tract. This and similar concentrations have been used in functional studies of human spermatozoa (Luconi et al. 1998; Strünker et al. 2011; Sumigama et al. 2015), with picomolar concentrations used to demonstrate chemotaxis (Villanueva-Díaz et al. 1995; Teves et al. 2009), whereas the induction of the acrosome reaction in human spermatozoa has been performed using micromolar concentrations (Blackmore et al. 1991; Bronson et al. 1999; Kirkman-Brown et al. 2002; Baldi et al. 2009). To date, much of the focus of the effect of P4 on bovine spermatozoa has been on its ability to induce chemotaxis (Gil et al. 2008), yet the effect of P4 in the induction of bovine sperm release from the oviductal reservoir has not been assessed.

This study has shown the positive effect of P4 in the induction of hyperactivation of bovine spermatozoa, which enabled us to test the hypothesis that hyperactivated motility is responsible for sperm detachment from oviductal epithelial cells. It has been shown in human spermatozoa that P4 activates a biphasic response, involving repeated intracellular Ca$^{2+}$ oscillations of a rapid Ca$^{2+}$ rise, followed by a sustained increase that is thought to be a result of intracellular Ca$^{2+}$ store mobilisation (Harper et al. 2004; Kirkman-Brown et al. 2004). The intracellular Ca$^{2+}$ concentration is approximately 30–50 nM in a normal motile bovine spermatozoon, and increases to 200–1000 nM in a hyperactivated sperm cell (Ho et al. 2002). We demonstrated that hyperactivation induced by P4 occurs rapidly and is reliant on the function of the primary Ca$^{2+}$ channel in spermatozoa, CatSper. The importance of Ca$^{2+}$ influx via CatSper has been illustrated by CatSper-null mice, which are unable to progress beyond the sperm reservoir (Ho et al. 2009) and have been found to be infertile (Ren et al. 2001; Carlson et al. 2005; Qi et al. 2007). CatSper has been reported in human (Strünker et al. 2011), mouse (Ren et al. 2001), stallion (Loux et al. 2013) and bovine (Johnson et al. 2017) spermatozoa and can be antagonised by mibebradil (a non-specific Ca$^{2+}$ channel blocker) and more specifically by its derivative NNC, which is less cytotoxic to the spermatozoa (Bui et al. 2008). These antagonists suppress the effect of P4, demonstrating that P4 acts by stimulating an influx of extracellular Ca$^{2+}$ into the cell. When Ca$^{2+}$ was removed from the medium through the use of EGTA, the action of P4 was also nullified. Therefore, the presence of extracellular Ca$^{2+}$ may be essential for P4 to elicit its effect on hyperactivation and its resultant release of spermatozoa from BOEC explants.

Interestingly, hyperactivation and the release of spermatozoa from BOEC explants was induced by P4 but was suppressed by P4 receptor antagonists mifepristone and AG205 when used alone or in combination. The rapid action of P4 on spermatozoa indicates that P4 acts through a non-genomic mechanism on the transcriptional and translationally silent cell. The action of AG205 against a non-classical P4 receptor PGRMC1 in this study suggests the presence of this receptor in bovine spermatozoa. This receptor, part of the membrane-associated progesterone receptor (MAPR) family, has been located in human and porcine spermatozoa with a molecular mass of approximately 28 kDa (Lösel et al. 2005; Thomas 2008). In addition, treatment with mifepristone blocked the action of P4 in spermatozoa. Mifepristone has been shown to antagonise both nuclear and membrane P4 receptors (Chabbert-Buffet et al. 2005; Chien et al. 2009), but because the classical P4 receptors have not been detected in spermatozoa (Baldi et al. 2009) the suppression of P4 hyperactivation by mifepristone must occur through the inhibition of an mPR on bovine spermatozoa.
This study, as well as previous work in human spermatozoa (Lishko et al. 2011; Strünker et al. 2011), suggests that P4 acts through non-genomic pathways by stimulating CatSper. The fast action of P4 on sperm hyperactivation and the demonstrated requirement of extracellular Ca\(^{2+}\) to elicit the response strongly suggest a direct extracellular effect of P4 on a sperm membrane protein. Interestingly, it has been found that P4 is more effective on the cell when it is present in the extracellular milieu than when P4 is microinjected into the cytoplasm (Maller and Krebs 1977). It is therefore proposed that the action of P4 on a surface receptor may trigger an influx of extracellular Ca\(^{2+}\) through CatSper (rapid rise), followed by a further CICR from intracellular stores (transient rise) and stimulation of an intracellular pathway (i.e. phosphatidylinositol 3-kinase/AKT), meaning phosphorylation of proteins in the sperm cell and thus leading to hyperactivated motility and the development of the propulsive force to detach from the epithelial cells of the oviduct. Through this proposed mechanism, the sperm cell still holds the essential surface molecules to reattach to the oviduct cells on its journey to the oocyte.

In conclusion, this novel study has demonstrated that hyperactivation induced by P4 plays a role in the detachment of bovine spermatozoa from BOEC explants, which is an indication of events occurring in vivo. These findings indicate that the presence of extracellular Ca\(^{2+}\) would be essential for the sperm response to P4. In addition, we have presented evidence that the action of P4 is mediated by a membrane P4 receptor, but the underlying mechanism that transduces the action of P4 to intracellular pathways remains to be elucidated. Dissecting the physiological components of the oviductal environment and their effects on sperm function will expand our understanding of sperm binding, sequential release and their availability in a sufficient number at the site of fertilisation close to the time of ovulation. This will lead to an optimisation of the use of semen during AI, for example by reducing the sperm concentration per straw and thus increasing the availability of straws of elite bulls.

Conflicts of interest

The authors declare no conflicts of interest.

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References


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doi:10.1530/reprod.0.1220305


doi:10.1095/biolreprod62.6.1754


doi:10.1038/srep36869


doi:10.1074/jbc.M401194200


doi:10.1006/dbio.2002.0797


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**Fig. 1.** Proportion of spermatozoa exhibiting hyperactivated motility following incubation with progesterone (P4). Data are the mean ± s.e.m. (n = 3). **P < 0.01, ***P < 0.001 compared with control.

**Fig. 2.** Percentage of hyperactivated spermatozoa following incubation with 10 nM progesterone (P4) alone or in the presence of different concentrations of (a) NNC 55-0396 dihydrochloride (NNC) or (b) mibefradil. Data are the mean ± s.e.m. (n = 3). Different letters above columns indicate significant differences (P < 0.05).

**Fig. 3.** (a) Hyperactivated motility and (b) release of spermatozoa from bovine oviduct epithelial cell explants of frozen–thawed bovine spermatozoa treated with 10 nM progesterone (P4) alone or in the presence of the calcium channel antagonists NNC 55-0396 dihydrochloride (NNC; 2 µM) and mibefradil (5 µM) or the calcium chelator EGTA (2.5 µM). Data are the mean ± s.e.m. (n = 3). Different letters above columns indicate significant differences (P < 0.05).
**Fig. 4.** Sperm hyperactivation following incubation with 10 nM progesterone (P4) alone or in the presence of different concentrations of the P4 receptor antagonists (a) AG205 or (b) mifepristone. Data are the mean ± s.e.m. (n = 3). Different letters above columns indicate significant differences (P < 0.05).

**Fig. 5.** (a) Hyperactivated motility and (b) release of spermatozoa from bovine oviduct epithelial cell explants of frozen–thawed bovine spermatozoa treated with 10 nM progesterone (P4) alone or in the presence of the P4 receptor antagonists AG205 (10 µM) and mifepristone (400 nM). Data are the mean ± s.e.m. (n = 3). Different letters above columns indicate significant differences (P < 0.05).