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Expression of a mitochondrial progesterone receptor in human spermatozoa correlates with a progestin-dependent increase in mitochondrial membrane potential

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SUMMARY

The hyperactivation of human sperm necessary for fertilization requires a substantial increase in cellular energy production. The factors responsible for increasing cellular energy remain poorly defined. This paper proposes a role for a novel mitochondrial progesterone receptor (PR-M) in modulation of mitochondrial activity. Basic science studies demonstrate a 38 kDa protein with western blot analysis, consistent with PR-M; while imaging studies with confocal and immunoelectron microscopy demonstrate a PR on the mitochondria. Treatment with a PR-specific progestin shows increased mitochondrial membrane potential, not related to induction of an acrosome reaction. The increase in mitochondrial membrane potential was inhibited by a specific PR antagonist but not affected by an inhibitor to the progesterone-dependent CatSper voltage-activated channel. In conclusion, these studies suggest expression of a novel mitochondrial PR in human sperm with a progestin-dependent increase in mitochondrial activity. This mechanism may serve to enhance cellular energy production as the sperm traverse the female genital tract being exposed to increasing concentrations of progesterone.

Keywords

Mitochondrial progesterone receptor; mitochondrial membrane potential; immunocytochemistry; immunoelectron microscopy; human sperm

INTRODUCTION

A role for progesterone in sperm function has been investigated for many years with in vitro data supporting chemo-attraction (Teves, et al., 2006), capacitation, modulation of hyperactivated motility and the acrosome reaction (AR). These processes differ in the required concentration of progesterone and the percentage of sperm responding. Progesterone concentrations to induce capacitation are typically 1–30 μ M (Baldi, et al., 2009, Yamano, et al., 2004). (Delbaere, et al., 1996). Increased hyperactivated motility has

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also been shown by some investigators (Calogero, et al., 1996, Contreras and Llanos, 2001) at doses varying from a low of 0.1 μM (Uhler, et al., 1992) to a high of 31 μM (Yang, et al., 1994) while not observed by others (Luconi, et al., 2004). Induction of the AR has been most extensively studied (Baldi, et al., 1991, Blackmore, et al., 1991, Bronson, et al., 1999, Kirkman-Brown, et al., 2002).

Progesterone induced AR requires an influx of extracellular Ca^{2+} and is seen at concentrations of 10 nM to 100 μM (Luconi, et al., 1998). Although the majority of sperm show a progesterone-induced increase in Ca^{2+} , less than 50% undergo an AR (Herrero, et al., 1997) for unclear reasons. The role of a progesterone receptor (PR) in this process is supported by inhibition by an antibody (C262) directed to the hormone-binding domain (HBD) of PR (Luconi et al., 1998, Sabeur, et al., 1996). More recent data suggests that progesterone acts via the CatSper Ca^{2+} channel to induce extracellular Ca^{2+} influx (Strunker, et al., 2011).

Presence of a PR is further supported by immunofluorescent antibody and ligand staining (Sabeur et al., 1996, Tesarik, et al., 1992), both revealing head staining. Western blot analysis with the C262 antibody and ligand blot analysis reveal proteins of 54 and 57 kDa (Luconi et al., 1998). These proteins are not seen with antibodies to the amino-terminus or DNA-binding domain (DBD) of PR. Identification of a PR in human sperm has remained elusive. An attempt at proteomic identification after immunoprecipitation with the C262 antibody and 2-D gel electrophoresis was unsuccessful (Luconi, et al., 2002). Studies to identify transcripts reveal RT-PCR products consistent with nuclear PR despite the lack of protein detection on western analysis (Luconi et al., 2002, Sachdeva, et al., 2000).

We have previously identified a novel, truncated PR localized to the outer membrane of the mitochondrion, named PR-M (Dai, et al., 2013). Originally cloned from human adipose and aortic cDNA libraries (Saner, et al., 2003), transcript analysis shows a novel sequence derived from the distal 3rd intron of the PR gene, consistent with a mitochondrial localization signal (MLS), followed by the same sequence for exons 4 through 8 of nuclear PR. Thus, the predicted protein structure consists of an amino-terminus MLS followed by the hinge and HBD of PR. RNAi studies in T47D breast cancer cells and overexpression in TET-On HeLa cells reveal a ligand-dependent control of cellular respiration. Progesterone treatment shows an increase in mitochondrial membrane potential (ψ_m) and oxygen consumption, consistent with increased cellular respiration (Dai et al., 2013).

In this study, we report the expression of PR-M in human sperm and a progesterone-dependent increase in ψ_m . These observations suggest a new mechanism whereby progesterone may increase sperm energy production to facilitate fertilization.

MATERIALS AND METHODS

Subjects

The Duke University Institutional Review Board approved this study. Semen specimens were obtained from men who were evaluated for infertility at the Duke Fertility Center and from healthy volunteers. All specimens were collected by masturbation after a minimum of

48 hrs of abstinence. Sperm was processed differently according to the procedure as described below.

Sperm Protein Preparation

Semen was collected directly into warm modified Human Tubal Fluid medium (mHTF, Irvine Scientific, Santa Ana, CA, USA) containing Protease Inhibitor Cocktail (PIC, Calbiochem, San Diego, CA, USA) and liquefied at 37°C for 10 minutes. Sperm were subjected to two-step ISolate-gradient centrifugation (Irvine Scientific) to select a motile population enriched in normal morphology. For total protein, sperm were washed twice in ice-cold PBS containing PIC and 6×10^5 sperm were solubilized in Laemmli buffer. Samples were boiled for 5 min and sperm pellets resuspended in RIPA protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.4) supplemented with PIC and briefly sonicated for 15 sec. After the sonication process, the suspension was incubated at 4°C for 20 min on a rotating platform. The suspension was then centrifuged at $13,000 \times g$ for 15 min at 4°C, and the supernatant was collected. For membrane protein extraction, 5×10^6 sperm or T47D cells were prepared in the same fashion and extracted using Mem-PER Plus Membrane protein extraction Kit (Thermo Scientific, Waltham, MA, USA) with minor modifications. Protein concentration of solubilized membrane protein and total protein was determined using a DC Protein Assay (Bio-Rad, Hercules, CA, USA) per manufacturer's directions.

Western Analyses

Samples were separated by electrophoresis on 10% Ready Tris-HCl gels (Bio-Rad) under reducing conditions. Briefly, the samples were heated to 95°C for 5 min with Laemmli sample buffer (Bio-Rad). The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad), followed by blocking in 5% nonfat dried milk powder in TBS-T (0.05% Tween 20, 20 mmol/L Tris pH 7.4, and 500 mmol/L NaCl) at RT for 1 hr.

The sperm total protein blots were incubated with primary antibodies overnight, 4°C. Primary antibodies included C19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a polyclonal antibody directed to the hormone-binding domain of the PR, at a dilution of 1:500 in 5% milk/TBS-T; or 6A1 (Cell Signaling Technology, Inc., Danvers, MA, USA), a monoclonal antibody directed to the amino-terminus of the PR, at the dilution of 1:1000 in 5% milk/TBS-T; or HDAC1 (Cell Signaling Technology), a monoclonal antibody directed to endogenous levels of total HDAC1 protein, at the dilution of 1:1000 in 5% milk/TBS-T, or GAPDH (Cell Signaling Technology), a polyclonal antibody directed to endogenous levels of total GAPDH protein, at the dilution of 1:1000 in 5% milk/TBS-T, or alpha-tubulin (EMD Millipore Corporation, Billerica, MA, USA), a polyclonal antibody directed to the detyrosinated form (Glu tubulin) of the tubulin alpha chain, at a dilution of 1:2000 in 5% milk/TBS-T. Normal serum rabbit IgG was used as a negative control for C19 analysis. Anti-mitochondria antibody (1:1000) (MTC02, Abcam, Cambridge, MA, USA) was used as mitochondrial marker. The blots were washed with TBS-T and incubated with secondary antibodies, peroxidase conjugated goat anti-rabbit IgG (for C19, GAPDH and α -tubulin) at 1:2000 dilution for 1 hr at RT; or peroxidase conjugated goat anti-mouse IgG (for 6A1,

HDAC 1 and anti-mitochondrial antibody) at 1:5,000 dilution for 1 hr at RT. Proteins were detected using an ECL Western blotting detection system (Amersham, Buckinghamshire, UK) according to the manufacturer's directions.

Immunofluorescent confocal microscopy

For microscopic studies, semen was collected directly into mHTF medium containing PIC and processed by two-step ISolate-gradient centrifugation. Sperm were incubated with 200 nM Mitotracker 580 (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min, washed twice with mHTF, and plated onto 0.01% (w/v) poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA) precoated slides in a moist chamber. Sperm were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, treated with cold methanol for 3–5 min and then incubated (1 hr, RT) with PBS-3% BSA-10% goat serum to prevent nonspecific binding of antibodies. The sperm were then incubated overnight (4°C) with 2 µg/ml C19, MAB 462 (Chemicon International, Temecula, CA, USA) 6A1, or PGR 636 (Thermo Scientific, Rockford IL, USA) antibodies. Sperm treated with 2 µg/ml rabbit IgG, mouse IgG, and MAB 462 preabsorbed with the antigen served as controls. After overnight incubation, sperm were stained (1 hr, RT) with 8 µg/ml Alexa 488-conjugated goat anti-rabbit or anti-mouse IgG. Slides were then washed several times in PBS, mounted in PBS:glycerol (1:1 :vol:vol) or FluorSave, and a cover slip was placed over the cells. Dual channel images were obtained with Zeiss LSM 410 or 510 confocal microscopy after excitation at 488nm for Alexa 488 and 568 nm for Mitotracker.

Immuno-Electron Microscopy

Motile sperm were prepared as above and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X, and blocked for 1 hr (3% BSA, 10% Goat Serum in PBS). Primary antibody (MAB-462, Preabsorbed MAB-462, or Normal Mouse IgG) was added overnight at 4°C. Ultrathin cryosections were cut from pelleted sperm and the tissue was placed onto carbon-coated Formvar grids. Blocking solution containing BSA and Cold Water Fish Skin Gelatine (Aurion, Wageningen, The Netherlands) was applied for 1 hr at RT. Gold conjugated goat anti-mouse IgG secondary antibody (Aurion) at 1:20 dilution was applied for 1 hr at RT. All tissue sections were analyzed on a Philips CM-12 (FEI, Hillsboro, OR, USA) transmission electron microscope. Location of gold was determined in 23 slides of sperm with MAB-462 staining and 28 slides of sperm with mIgG staining. Approximately 500 sperm were examined in each group.

Sperm mitochondrial membrane potential (ψ_m)

Semen was collected directly into warm mHTF medium with 10% BSA, liquefied at 37°C for 10 minutes and processed by two-step ISolate-gradient centrifugation. Mitochondrial membrane potential (ψ_m) was determined by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolecarbocyanide iodine (JC-1) dye (Invitrogen) (Marchetti, et al., 2004). Three million sperm were treated with different ligands or vehicle in polypropylene tubes. After 90 minutes incubation in modified Krebs-Ringer-HEPES buffer (KRH) containing 25 mM Na-HEPES, 115 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM, MgSO₄, 0.5 mM CaCl₂, and 5 mM glucose at pH 7.4 plus 6.26 mM pyruvate at 37°C with gently shaking, sperm were then treated with 2.5 µg/mL JC-1 in the KRH buffer plus 6.26

mM pyruvate for 10 minute at 37°C. Sperm were then washed three times with pre-warmed Ca^{2+} -free PBS. The sperm were resuspended in 200 μL Ca^{2+} -free PBS and placed into Costar 48-well plates (Corning, Corning, NY, USA). The plate was then read immediately using a Tecan Safire multichannel plate reader pre-heated to 37°C. Sperm were excited at 488 nm (band width 5 nm), and emission was determined at 529 (band width 7.5 nm) and 585 nm (band width 7.5 nm). Fluorescence was read from the bottom. Each treatment was duplicated within an experiment.

Sperm acrosome reaction

Sperm acrosome reaction was determined by the pisum sativum-FITC staining method with minor modifications (Cross, et al., 1986). Semen was processed exactly as described above for the ψm assay. The sperm were treated with three different groups: R5020 (10^{-6} M), Ionomycin (20 μM), ethanol plus DMSO as vehicle. After 90 minutes incubation with the above treatments in KRH plus 6.26 mM pyruvate medium at 37°C, samples were fixed in 95% ethanol at 4°C for 30 minutes. An aliquot of fixed sperm was placed on a poly-L-lysine (Sigma) coated slide and dried thoroughly on a slide warmer. Finally, the slides were stained with pisum sativum agglutinin-FITC (100 $\mu\text{g/mL}$ in PBS) at RT for 5 min in a dark, moist chamber followed by rinsing with ddH₂O, then were mounted with FluorSave (Calbiochem) immediately. In each treatment group, ten fields were randomly selected, and pictures were captured by fluorescent microscopy (magnification X 400), for sperm acrosome reaction analysis. Acrosome reacted sperm were defined as a completely absent acrosomal membrane. Four replicate experiments were performed, and two readers (TP and QD) were both blinded to image acquisition and sperm analysis. In all 4 experiments, 535 sperm were counted for Ionomycin, 430 for R5020 and 456 for EtOH treatments.

Statistical analysis

Statistical analysis was performed with IBM SPSS Analysis version 20 with an $\alpha = 0.05$ defined as significant. Data for ψm were analyzed with univariate ANOVA using Tukey HSD post hoc testing. Non-normal ψm data were $\log(2)$ transformed prior to analysis. For the acrosome reaction, univariate ANOVA with Tukey HSD post-hoc testing was performed. For ANOVA, results are reported as ($F(\text{dFB-G}, \text{dFW-G}) = \text{value}, p = \text{value}$), where F is the F statistic, dFB-G is the between-groups degrees of freedom, dFW-G is the within-groups degrees of freedom and p is the probability.

RESULTS

Identification of PR-M in human spermatozoa

Figure 1 shows western blot analyses for PR-M. A total protein preparation of sperm (panel A) shows a 38 kDa protein consistent with PR-M with hybridization with a polyclonal antibody (C19) directed to the HBD of PR. A non-specific smear is noted at ~75 kDa. There is no evidence of nuclear (n) PR bands. Replacing the primary antibody with normal rabbit IgG showed no binding. Panel B shows hybridization with the C19 antibody comparing the sperm reaction to nPR expressing T47D breast cancer cells. Analysis of both total protein (TP) and membrane protein (MP) was performed. In T47D cells, the TP preparation shows a 116 kDa band consistent with PR-B, a 81 kDa band consistent with PR-A and the 38 kDa

band consistent with PR-M. The bands at 51 and 60 kDa are commonly seen with PR antibodies directed to the HBD (Samalecos and Gellersen, 2008). Whether these bands represent undiscovered PR isoforms or are non-specific, continues to be debated. With enrichment of membrane proteins, the nPR bands in T47D cells are greatly diminished, while the 38 kDa band persists. With membrane protein enrichment in sperm, the 38 kDa band persists as seen in the total protein sperm preparation. The lower blot shows mitochondrial content of the preparations. Panel C shows total protein western blot analyses with a monoclonal antibody (6A1) directed to the N-terminus of nPR. Protein from an equal number of sperm and T47D cells was loaded instead of equal protein quantity by optical absorbance. This was performed as there is no known protein in sperm and somatic cells with equivalent abundance to serve as a loading control. Hybridization shows PR-B and A in T47D cells but no evidence of the 38 kDa PR-M, which should not be recognized by this antibody. Nuclear PR is not seen in sperm. Other blots are shown as control reactions. Expression of the nuclear protein, histone deacetylase 1 (HDAC1), is seen in T47D cells but is known to be absent in mature spermatozoa (Omisano, et al., 2007). α -tubulin is very abundant in sperm flagella. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is less abundant in spermatozoa compared to T47D cells. In the majority of cells GAPDH is located in the cytoplasm, whereas in spermatozoa GAPDH is bound to the fibrous sheath in the principal piece of the tail (Westhoff and Kamp, 1997).

PR-M localizes to the mitochondria

Figure 2 shows green (G) immunofluorescent staining of permeabilized sperm with multiple PR antibodies, and red (R) immunofluorescent staining for mitochondria with Mitotracker. Panels A-B demonstrate mid-piece staining with a monoclonal antibody (MAB 462) directed to the HBD of PR. Panel C shows absence of staining when the antibody was preabsorbed with the specific antigen. Panel D shows lack of mid-piece green staining with a monoclonal antibody directed to the N-terminus of PR (PGR 636) while panels E-F show absence of staining with controls lacking primary antibody or replacing the primary antibody with mouse IgG. Red mitochondrial staining is seen in all 3 panels. Panel G shows the green mid-piece staining pattern with a polyclonal rabbit antibody directed to the HBD of PR (C19), while panel H shows mitochondrial staining. Panel I shows diffuse, minimum non-specific staining with rabbit IgG. Panel J shows absence of green staining with another monoclonal antibody directed to the N-terminus of PR (6A1), while panel K shows red mitochondrial staining. Panel L shows a control reaction of the 6A1 antibody staining the nucleus of PR expressing T47D breast cancer cells. In summary, both antibodies directed to the HBD (MAB 462 and C19), showed specific staining of the mid-piece, co-localizing with the mitochondrial staining, while no staining was seen with antibodies directed to the N-terminus of PR.

The mid-piece staining showed a consistent pattern of high intensity at the most proximal aspect. To determine if this was mitochondrial staining verses staining of redundant nuclear membrane of the connecting piece immunoelectron microscopy was performed. Figure 3A shows mitochondrial staining in an intact mid-piece of a sperm while panel B shows mitochondrial staining in a disrupted mid-piece. Panel C shows no staining after pre-absorption of the antibody and panel D shows no staining when the primary antibody was

replaced with mouse IgG. Table 1 summarizes the location of gold staining in the images. The majority of staining was found on the mitochondrial membrane within the mid-piece. Essentially no staining was seen with mIgG (1 gold particle in 28 examined slides).

Progestin increases ψ_m in human sperm

Figure 4A shows an increase in ψ_m with a 90 min treatment with R5020 ($F(3,12) = 5.4$, $p = .014$), with the 10^{-6} M dose being greater than the EtOH vehicle, $p = .010$; while the comparison of 10^{-6} M dose to the 10^{-8} M dose showed a $p = .069$. There was a significant linear trend increase in ψ_m among the treatments, $p = 0.002$. Treatment with caffeine served as a positive control. Figure 4B shows the R5020-dependent increase in ψ_m inhibited with a specific PR antagonist, RTI-6413-049b, $F(2,15) = 4.8$, $p = .024$. In comparison to RU-486, RTI-6413-049b does not bind estrogen receptor-alpha ($ER\alpha$), estrogen receptor-beta ($ER\beta$) and androgen receptor, lacks PR agonist effect and has much lower anti-glucocorticoid effect (Sathya, et al., 2002). A possible interaction of the progestin with the CatSper T-type Ca^{2+} channel protein, explaining the change in ψ_m , was investigated with results shown in figure 4C. Treatment with the CatSper inhibitor NNC 55-0396 resulted in an overall decrease in ψ_m compared to vehicle ($F(3,8) = 27.3$, $p < .001$) but had no effect on the progestin-induced increase in ψ_m , (panel A).

We next sought to determine if the increase in ψ_m was secondary to an AR induced by the progestin treatment. Figure 5 shows no difference in the percent of sperm undergoing an AR with the same treatment parameters using R5020 (10^{-6} M) compared to vehicle control. The Ca^{2+} ionophore, Ionomycin, was used as a positive control, $F(2,9) = 8.2$, $p = .010$.

DISCUSSION

Years of research support a role for progesterone in sperm function without conclusive identification of a functional specific receptor. Nuclear PR A and B are not expressed in mature human spermatozoa leaving the option of progesterone interaction with a novel, non-nuclear PR or with proteins not homologous to the established nuclear PR. In this study, we focus on the former possibility after previous identification of a mitochondrial progesterone receptor (PR-M) (Dai et al., 2013, Saner et al., 2003), shown to increase cellular respiration.

The novel structure of PR-M makes conclusive identification separate from nuclear PR (nPR) difficult. The 16 unique N-terminal amino acids, consistent with a transmembrane domain, are highly hydrophobic with poor antigenicity, precluding specific antibody creation. For this reason, we used the technique of selective antibody testing with commercial antibodies directed to the HBD of PR, which should recognize PR-M, and antibodies directed to the N-terminus of PR, which should not recognize PR-M. Only antibodies directed to the HBD identified PR-M on Western blot analysis and immunofluorescent staining. This result agrees with other reports using a similar technique (Castilla, et al., 1995, Luconi et al., 2002).

In contrast, bands found on Western blot analyses by other investigators are disparate from our observation, without obvious explanation. Two groups of investigators demonstrated proteins of 54 and 57 kDa (PR-B = 118 kDa, PR-A = 92 kDa) on western analysis with a

C262 antibody directed to the HBD of nPR, and with ligand binding of peroxidase conjugated progesterone, respectively. These 2 proteins were not identified with antibodies to the DNA-binding domain (DBD) or the N-terminal domains of the nPR (Luconi et al., 1998, Sabeur et al., 1996). In an attempt to isolate these proteins, 2-D gel electrophoresis was performed after immunoprecipitation with the C262 antibody. Bands of 56 and 29 kDa were identified with silver staining and processed for Maldi-ToF analysis. Database search of the peptide masses after trypsin digestion revealed no matches with previously published protein sequences (Luconi et al., 2002).

We sought to perform immunofluorescent staining of sperm with as little post-ejaculatory change as possible. Ejaculated sperm placed in media undergo capacitation with significant protein alterations, especially in the head. Capacitation is dependent upon the presence of Ca^{2+} , bicarbonate and albumin (Visconti and Kopf, 1998). To minimize protein changes, sperm were collected directly into HTF media without BSA containing protease inhibitors. After an ISolate gradient centrifugation, sperm were reacted with Mitotracker and then fixed with paraformaldehyde. Staining with PR antibodies to the HBD showed intense staining of the mid-piece with colocalization of mitochondrial staining. Mid-piece staining had a specific pattern with a bright spot at the proximal end adjacent to the connecting piece. Immunoelectron microscopy, performed to further analyze the staining pattern, showed mitochondrial binding, without substantial binding in the head, connecting piece or tail. The cause of the asymmetrical staining in the mid-piece remains to be determined.

Other investigators have performed immunofluorescent staining with a different PR antibody. Sabeur and colleagues demonstrated a band in the sperm head with immunofluorescent staining with the C262 antibody (directed to the HBD) in uncapacitated sperm. Inspection of the image provided also shows obvious mid-piece staining (Sabeur, et al., 1996). The reason(s) for the difference in observations is unclear, but may lay in the various sperm preparation techniques.

Treatment with the progestin R5020 increased ψ_m which was inhibited by a specific PR antagonist. This was not due to induction of an AR as the progestin treated sperm had no higher AR rate than did vehicle treated. This study did not determine the mechanism of the progestin-mediated increased ψ_m . An increase in mitochondrial membrane potential may be due to; 1) an increase in proton pumping by increased activity of respiratory enzyme complexes and consequential ATP production, 2) a transient block of electron transfer, 3) a transient block of proton use by enzymes within the mitochondria and 4) a change in the mitochondrial pH leading to a change in membrane potential (Zorov, et al., 2006). Previous indirect and direct studies in different cell types suggest the progestin-dependent increase in ψ_m is associated with increased cellular respiration (Behera, et al., 2009, Dai et al., 2013) (Turner, 2003)(Storey and Kayne, 1975, Travis, et al., 2001)(Miki, et al., 2004).

The question remains as to whether the increase in ψ_m is a direct effect via PR-M or secondary to other progesterone receptors. Other candidates for progesterone action in sperm include ligand interaction with CatSper (a voltage-gated Ca^{2+} channel), G-protein-coupled-type progestin receptor (mPR) and progesterone-receptor membrane component (PGRMC). The CatSper channel is activated by progesterone to increase Ca^{2+} influx

(Strunker et al., 2011). As an increase in intracellular Ca^{2+} modulates mitochondrial function (Hajnóczky, et al., 1995), the possibility of our results reflecting involvement of CatSper was considered. This appears unlikely given the lack of inhibition of the progestin-induced increase in ψ_m in the presence of the CatSper inhibitor NNC 55-0396 (Strunker et al., 2011). Activity of NNC 55-0396 was proven by the decrease in ψ_m compared to vehicle control and the inhibition of caffeine action. Although we did not determine $[\text{Ca}^{2+}]_i$ in this study, it is reasonable to presume that treatment with NNC 55-0396 decreased extracellular Ca^{2+} influx resulting in decreased mitochondrial ψ_m (Duchen, 2000).

The membrane progestin receptor, mPR α , has been identified in the plasma membrane of human sperm with progesterone treatment causing G-protein activation (Thomas, et al., 2009). As the progestin R5020 shows very low binding affinity for mPR α (Thomas, et al., 2007) it is unlikely that the increase in ψ_m involves this protein.

Less research has been done with PGRMC 1 and 2, with identification of transcripts (Lösel, et al., 2005) and protein in human sperm (Falkenstein, et al., 1999). Treatment with a rabbit derived antiserum against a recombinant protein showed inhibition of progesterone-induced increase in $[\text{Ca}^{2+}]_i$ (Falkenstein et al., 1999). As PGRMC also binds R5020, although 20 to 40-fold less than progesterone (Falkenstein et al., 1999), we cannot totally exclude involvement of this protein in the observed increase in ψ_m .

Our study did not explore an interaction of estrogen with PR-M. Binding of estradiol to a plasma membrane protein in human sperm results in inhibition of progesterone-induced Ca^{2+} influx and progesterone-dependent AR (Baldi, et al., 2000). Whether estradiol would also have an effect of progesterone-dependent ψ_m remains to be investigated.

The requirement for ATP for cellular processes including capacitation, AR, and motility from flagella movement is obvious, but the main source of ATP is debated. ATP production occurs in the mid-piece via mitochondrial oxidative phosphorylation and in the principal piece of the flagellum via glycolysis (Turner, 2003). The principal piece is enriched in glycolytic enzymes with plasma membrane glucose transporters. Bicarbonate (HCO_3), necessary for in vitro capacitation, increases glycolysis (Hereng, et al., 2014).

Compartmentalization is substantial in sperm and studies suggest that diffusion of ATP produced in the mid-piece into the tail is insufficient to support motion (Storey and Kayne, 1975, Travis et al., 2001). In a knock-out mouse ablating sperm glycolysis, sperm motility was lacking with ATP levels that were only 10% of that of wild-type mice (Miki et al., 2004). (Amaral, et al., 2013, Narisawa, et al., 2002)

In contrast, evidence also supports a role for mitochondrial activity in human sperm function. Oxidative phosphorylation, as shown by oxygen consumption, dramatically increases after a swim-up preparation of sperm correlating with capacitation and hyperactivation (Piomboni, et al., 2012, Stendardi, et al., 2011). There is a correlation between increased mitochondrial membrane potential and motility and fertilization; whereas a decrease in mitochondrial number and proteins of the electron transport chain are found in poor quality sperm (Amaral et al., 2013).

One question is whether the role of glycolysis versus mitochondrial ATP production changes with nutrient availability? Levels of lactate, pyruvate and glucose vary according to time in the cycle and the presence of a cumulus-oocyte-complex (COC). At ovulation, compared to the follicular phase, levels of lactate increase while levels of glucose decrease in tubal fluid (Gardner, et al., 1996). The presence of a COC may further increase pyruvate and lactate in tubal fluid due to metabolism by cumulus cells (Gardner and Leese, 1990). Thus, the contribution of glycolysis compared to oxidative phosphorylation may change as the sperm progresses in the genital tract toward the COC, with the latter becoming predominant (Miki, 2007).

In conjunction, a progesterone concentration gradient within the fallopian tube likely exists originating from the COC. Thus, we hypothesize that the increasing concentration of progesterone, as the sperm approaches the COC, results in increasing mitochondrial ATP production via PR-M concordant with a change in nutrient availability away from glucose to lactate.

Future research will seek to understand the mechanism of PR-M action within the mitochondrion with possible techniques of high resolution respirometry and metabolomic analyses. Previous studies support localization of PR-M to the outer mitochondrial membrane (OMM) (Dai et al., 2013). Although varied in function, OMM proteins are involved in ion transport, mitochondrial fusion, and protein sorting (Harbauer, et al., 2014). Spermatoocytes provide an attractive model for these studies.

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Figure 1.

Western blot analyses for PR-M. Panel A: Hybridization of total protein with an antibody to the HBD of PR (C19) shows a 38 kDa protein consistent with PR-M, while no binding is seen with a rIgG control. Panel B: Analysis of total protein (TP) from T47D breast cancer cells shows nuclear PR at 116 and 81 kDa while PR-M is seen at 38 kDa (Lane 1). Bands of unclear identification are seen at 51 and 60 kDa (see results). Enrichment of membrane proteins (MP) resulted in a dramatic decrease in nuclear PR bands with persistence of PR-M in T47D cells, (Lane 2), while sperm show the 38 kDa PR-M band with no evidence of nuclear PR, (Lane 3). Panel C: Analyses with a monoclonal antibody directed to the N-terminus of PR (upper blot), showing no evidence of binding in sperm and clear binding of nuclear PR in T47D cells. Control reactions show lack of expression of histone deacetylase

1 (HDAC 1) in sperm (second blot), abundant expression of α -tubulin in sperm (third blot) and expression of GAPDH in sperm (bottom blot).

Figure 2.

Immunofluorescent staining with PR antibodies. Row 1: MAB 462 (directed to the HBD) shows green staining (G) of the mid-piece co-localizing with red mitochondrial staining (R) (panels A-B). Staining is not seen with pre-absorption of the primary antibody (panel C). Row 2: PGR 636 (directed to the N-terminus of PR) shows no staining of the mid-piece (panel D), as do control reactions (panels E and F). Red mitochondrial staining is seen in each panel. Row 3: C19 (directed to the HBD of PR) shows green staining of the mid-piece (panel G) co-localizing with mitochondrial staining (panel H). Rabbit IgG control shows low intensity non-specific staining of head, mid-piece and tail, (panel I). Row 4: 6A1 (directed to the N-terminus of PR) shows no staining of sperm (panel J), with mitochondrial staining seen (panel K). Nuclear staining of T47D cells serves as a positive control (panel L). Original magnification: 100X for panels A-F and 63X for G-L. Images have been enlarged for easier viewing.

Figure 3. Immunoelectron microscopy. Hybridization with the MAB 462 antibody directed to the HBD of PR shows gold particles on the mitochondria of an intact mid-piece (panel A), and on a disrupted mid-piece (panel B). No staining was seen with antigen pre-absorption (panel C) or with mouse IgG (panel D). The bar in each figure represents 500 nm. Also see Table 1.

Figure 4.

Determination of ψ_m . Panel A shows an increase in ψ_m comparing different concentrations of R5020. Pairwise comparisons of a difference in ψ_m include: $p = 0.010$, 10^{-6} M R5020 vs EtOH; $p = 0.069$, 10^{-6} M R5020 vs 10^{-8} M R5020. Panel B shows inhibition of the R5020-dependent increase in ψ_m by the PR antagonist, RTI-6413-049b. Pairwise comparisons include: $p = 0.035$, 10^{-6} M R5020 vs 10^{-6} M RTI; $p = 0.050$, 10^{-6} M R5020 vs 10^{-6} M R5020 + 10^{-6} M RTI. Panel C shows a lack of effect of the Catsper inhibitor, NNC 55-0396, on the R5020-dependent increase in ψ_m . Pairwise comparisons include: $p = .003$, NNC vs EtOH; $p < .001$, NNC vs NNC+R5020; $p < .001$ NNC vs R5020; $p = .076$, R5020 vs EtOH. All values are mean $\pm$ SEM. Values with different superscript letters are significantly different.

Figure 5.

Acrosome reaction induction by progestin. A: The identical R5020 treatment protocol used for the determination of ψ_m showed no increase in AR. Pairwise comparisons of a difference in AR include: $p = 0.023$, 10^{-6} M R5020 vs Ionomycin; $p = 0.929$, 10^{-6} M R5020 vs EtOH. All values are mean $\pm$ SEM. Values with different superscript letters are significantly different. B: The left image shows acrosome reacted sperm, while the right image shows non-reacted, acrosome intact sperm, (original magnification 400 X).

Table 1

Summary of Gold Staining in Immunoelectron Microscopy

Antibody	Location in sperm with staining	# gold particles (% of total)
MAB 462	Mid-piece	45 (70)
	Connecting piece	7 (11)
	Non specific	12 (19)