The Opioid Peptide Beta-endorphin Stimulates Acrosome Reaction in Human Spermatozoa.

Running title: Opioids system and male fertility

Summary sentence: A novel role of the opioid system is demonstrated in the regulation of sperm function; by showing that beta-endorphin is involved in acrosome reaction of human sperm cells.

Keywords: *opioid peptides, sperm, signalling pathways, acrosome reaction, male fertility*

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ABSTRACT

The acrosome reaction occurs *in vivo* following sperm capacitation and is essential for the acquisition of sperm fertilization ability. However, little is known about the molecular identity of the physiological acrosome reaction regulators. In addition to progesterone, which is produced by cumulus oophorus cells and known to regulate acrosome reaction by activating the specific calcium channel CatSper, endogenous opioid peptides such as beta-endorphin and met-enkephalin are present at high concentrations in the follicular fluid suggesting that the opioid system may be involved in the mechanisms regulating the acrosome reaction in humans. By using Reverse Transcription-PCR, western blot and immunofluorescence approaches, we described the presence and localization of the beta-endorphin precursor, pro-opiomelanocortin in the middle section and in flagellum of human spermatozoa, and inside the seminiferous tubules of human testis. Flow cytometry and intracellular calcium analyses showed that beta-endorphin causes an inversely dose-dependent increase of the percentage of acrosome-reacted sperm cells by a calcium-independent protein kinase C pathway. These findings are important for future studies of sperm physiology and provide new insight into the function of the opioid system as a target of fertility management.

INTRODUCTION

After ejaculation, human sperm cells are immature and infertile and must undergo many modifications to become fertilization competent (Suarez 2008). Several morphological and biochemical changes occur during the transit through the female tract. These sperm modifications include different processes such as capacitation (sperm membrane reorganization), hyperactive motility (changes to the motility pattern needed to penetrate oocyte vestments) and acrosome reaction. The acrosome reaction of spermatozoa is a complex calcium dependent process and is essential for the spermatozoa to fertilize an egg. Fusion at multiple sites between the outer acrosomal membrane and the cell membrane causes the release of the acrosomal contents and the loss of the membranes surrounding the acrosome (Florman *et al.* 2008).

Progesterone produced by cumulus oophorus cells is known to be the main physiological regulator of acrosome reaction (Baldi *et al.* 2009) since the binding and the respond of progesterone are compromised in spermatozoa derived from infertile men (Gadkar *et al.* 2002; Smith JF *et al*., 2013). Pregesterone-induced acrosome reaction causes a multicomponent intracellular $Ca2^{+}$ increases (Darszon *et al.* 2011). In mammalian sperm, the progesterone-induced intracellular Ca^{2+} increase is controlled by a sperm-specific Ca^{2+} channel called CatSper (cation channel of sperm) (Tamburrino L *et al.*, 2014; Quill *et al.*, 2001; Lishko *et al.*, 2010). However, several chemical molecules including vitamin D, chemokines, small peptides, the gas NO, neurotransmitters, analogues of cyclic nucleotides, and odorants can also affect acrosomal exocytosis *in vitro* (Eisenbach and Giojalas, 2006; Florman *et al*., 2008; Brenker *et al*., 2008; Suarez, 2008). To date, the underlying signalling mechanisms of acrosome reaction are ill-defined.

Endogenous opioid peptides (EOPs) are a type of small peptides known to participate in the regulation of reproductive physiology at multiple sites and, particularly, the opioid system seems to be involved in the regulation of sperm physiology (Subirán *et al*. 2011). Previously, we described the presence of three types of opioid receptors (mu, delta, kappa) and other components of the opioid system in human sperm cells and we described its role in sperm motility. (Fernandez *et al.* 2002, Agirregoitia *et al.* 2006, Subiran *et al.* 2008, 2012). Nevertheless, the role of the opioid system in acrosome reaction is poorly understood and to date, there have been no relevant *in vivo* studies. β-endorphin immunoreactivity has been detected in spermatozoa but the main role of this peptide in human spermatozoa is completely unknown. Together with progesterone, beta-endorphin is secreted in the oviduct (Petraglia *et al.* 1986, 1986), raising the possibility that EOPs may be involved in human acrosome reaction regulation. Here, we describe for the first time that the EOP beta-endorphin precursor, pro-opiomelanocortin (POMC), is present in human testis and sperm cells and that beta-endorphin regulates human acrosome reaction by specific calcium -independent protein kinase C (PKC) pathway.

MATERIALS AND METHODS

Samples and Isolation of Spermatozoa

Ethical approval for this study was obtained from the Ethics Committee of the University of the Basque Country (CEISH/61/2011). Freshly ejaculated semen was collected from 80 donors (18–35 years old) with normal sperm parameters according to World Health Organization standards (WHO, 2010). Samples were obtained by masturbation after 3–4 days of sexual abstinence and processed immediately upon liquefaction (at 37ºC for 30 min). Spermatozoa were capacitated by a swim-up

procedure (Cejudo-Roman *et al.*, 2013) and resuspended in G-IVF (Vitrolife, Göteborg, Sweden) supplemented with 1% bovine serum albumin (BSA) for 3 h at 37°C under 5% CO_2.

Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA was extracted from a sperm pool containing sperm from eight different donors using TriReagent (Sigma, San Luis, MO, Estados Unidos) and cDNA was synthesized using the Quantitect Reverse Transcription kit (Qiagen, Venlo, The Netherlands). Specific oligonucleotide primer pairs used for PCR were synthesized and purified by Sigma Genosys (Cambridge, UK) and their sequences were as follows: human *Pomc*, forward 5′-CTCACCACGGAAAGCAACC -3′ and reverse 5′-ATCGGTCCCAGCGGAAGT -3′ (151-bp product); and human *Actb* (β-actin), forward 5′-TCCCTGGAGAAGAGCTACGA-3′ and reverse 5′-ATCTGCTGGAAGGTGGACAG-3′ (362-bp product; exon spanning), used as an internal control.

A pool of cDNAs from 20 different human tissues (human total RNA master panel, BD Biosciences, Clontech, Palo Alto, CA, USA) was used as a positive control of amplification. Amplification was carried out in 25 µl of PCR buffer containing 3 µl of cDNA reaction mixture, 2.5 mM $MgCl_2$, 0.2 µM primers, 200 µM dNTPs and 1.5 U of heat-activated thermostable DNA polymerase (Immolase, Bioline, London, UK). PCR was performed for 35 cycles with cycling parameters being: 15 s at 94°C, 20 s at 60°C and 20 s at 72°C. The primers for *hPomc* were located on the same exon of each respective gene (i.e. they did not span introns). Thus, we verified the possible carryover of genomic DNA during the extraction process by performing PCR in the absence of reverse transcriptase. Expression of *CD4* and *acrosin* was also analysed to exclude the

presence of leukocyte contamination and to verify the presence of sperm complementary DNA, respectively (data no shown). The RT-PCR products were separated by 2.5% gel electrophoresis. The amplicon sizes were verified by comparison with a DNA size-ladder and the identity of the products was established by sequencing of amplicons.

Western blotting

Sperm proteins were prepared as described elsewhere (Subiran *et al*., 2012), modifying the lysis buffer (phosphate-buffered saline [PBS] and 1% [v/v] Triton-X100, with protease inhibitor cocktail). Membrane pellets were suspended in lysis buffer, and then protein extracts were diluted in Laemmly sample buffer containing β-mercaptoethanol (5% vol/vol) and boiled for 5 min. Proteins (50 µg sperm protein; 30 µg human kidney´s cells´s protein) were loaded onto 12% resolving gels and separated by one-dimensional SDS-PAGE. Proteins were then transferred to polyvinylidene fluoride membranes using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA). After transfer, the membrane was blocked with Blotto (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100) containing 5% nonfat dry milk (blocking buffer) for 1 h and then incubated with a dilution of polyclonal rabbit anti-POMC antibody (1:200) After washing (3 x 5 min) in Blotto buffer, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG antibody (1:3000) (Goat anti-rabbit IgG HRP, abcam, ab6112). Blots were revealed for peroxidase activity by enhanced chemiluminiscence (ECL).

Indirect Immunofluorescence

Isolated spermatozoa obtained after swim-up and human frozen testis slides provided by Ziagen Company (Maryland, USA) were used to identify the localization of POMC in human sperm cells and testis, and to analyze the effect of beta-endorphin on PKC-signalling pathways.

Cells were fixed in 4% paraformaldehyde for 10 min, permeated in 0.5% Triton X-100 for 10 min and blocked for 30 min with 10% (v/v) fetal bovine serum in PBS. For immunofluorescence staining, samples were incubated overnight at 4ºC with different primary antibodies. We used rabbit polyclonal anti-proopiomelanocortin (1:500) (Santa Cruz Technologies, California, USA) and rabbit anti-phospho-PKC (Cell Signalling) antiserum at a dilution of 1:500. Secondary antibody incubations involved Alexa Fluor 488 donkey anti-rabbit IgG (1:2000) (Molecular Probes, Oregon USA). Nuclei were stained with Hoechst 33342 at 10μg/ml (spermatozoa) and propidium iodide at 100μg/ml (testis), and slides were assembled with Fluoromount G (Molecular Probes). The specificity of the primary antibody was verified by using negative unspecific rabbit immunoglobulin fraction (normal) (Dako) in the same concentration as the primary antibody, and pre-absorbing primary antibody immunoreactivity with beta-endorphin (10^{-5}M) for 2 h at room temperature before incubation. At the same time, controls for the specificity of the secondary antisera were performed by omitting the primary antiserum before addition of the secondary antisera. Finally, the samples were examined using confocal microscopy (Olympus Fluoview FV500, Tokyo, Japan).

Corrected total cell fluorescence (CTCF) per area was measured by ImageJ software using the following equation: CTCF = [Integrated density – (Area of selected cell × Mean fluorescence of background readings)] / Area of selected cell. We measured the green fluorescence of at least 200 cells.

Incubation Media and Treatments

Isolated spermatozoa were treated at 37°C under 5% CO_2 with different doses of beta-endorphin (10^{-5}, 10^{-7} and 10^{-9} M). Sperm samples were divided into aliquots of 0.1 ml in G-IVF (Vitrolife) and one of the different concentrations of beta-endorphin was added to each aliquot. An equal volume of solvent was used as control. In addition, to ascertain the specificity of the action of the peptide, beta-endorphin (10^{-9} M)-treated sperm cells and control samples were also co-incubated with naloxone, an antagonist of opioid receptors, at high (10^{-5} M) and low (10^{-8} M) concentrations. High naloxone doses (10-5 M) block the three opioid receptors and low doses (10-8 M) are able to block selectively the mu-opioid receptor. Sperm cells were treated with naloxone for 10 min before beta-endorphin addition. In all experiments, sperm cells were incubated with beta-endorphin for 60 min.

Finally, to evaluate the effect of beta-endorphin on progesterone-induced acrosome reaction, samples were also co-incubated with 10^{-9} M beta-endorphin and 10^{-6} M progesterone. After 1 h of incubation with beta-endorphin, samples were treated with progesterone for 15 min.

Treated spermatozoa were used for subsequent experiments.

Flow cytometry

In all experiments, acrosome reaction was measured by flow cytometry. We used Fluorescein IsoTioCyanate (FITC) anti-human CD46 (for 60 min at room temperature; BioLegend, California, USA) and Hoechst 33258 (2 min at room temperature; Sigma-Aldrich, Missouri, USA) as acrosome reaction molecular marker and viability dyes, respectively. Samples were checked visually by confocal microscopy to verify the signal of the dyes. Green positive cells represented acrosome-reacted spermatozoa.

Fluorescence data from at least 100,000 events was analysed in a flow cytometer (FACScalibur, Becton Dickinson, San Jose, CA, USA). To ensure fluorescence data were from live spermatozoa, the percentage of Hoechst 33258-positive events was determined by subtraction of background fluorescence in each histogram.

We also analysed the effect of beta-endorphin on PKC-signalling pathways using flow cytometry. Capacitated spermatozoa obtained by Percoll gradient followed by a swim–up procedure were incubated for 3 h in GVI-F® medium. A minimum of 3 x10^6 cell/ml was collected and treated aswe described before. Collected spermatozoa were fixed and permeated in suspension in 0.5% Triton X-100 for 10 min. Samples were washed twice in PBS by centrifugation at 800 g for 5 min and incubated in blocking medium (PBS / 10% (v/v) fetal bovine serum) for 30 min. For immunofluorescence staining, samples were incubated overnight at 4ºC with rabbit anti-phospho-PKC (Cell Signalling) antiserum at a dilution of 1:500. On the next day, the samples were centrifuged in PBS at 800 g for 5 min and incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:2000) (Molecular Probes, Oregon USA) in the dark, at room temperature for 1 h . Nucleus was stained with 0.1 μg/ml Hoechst 33258 for 2 min. Finally, samples were washed twice by centrifugation in PBS at 800 g for 5 min, suspended in PBS and kept in the dark until analysis. Negative controls were performed by omitting the primary antibody before secondary antibody addition and by using negative unspecific rabbit immunoglobulin fraction (normal) (Dako) in the same concentration as the primary antibody. Fluorescence data from at least 100,000 events were analyzed. In order to measure the green fluorescence only from spermatozoa, the percentage of Hoechst 33258-positive was determined by subtraction of background fluorescence in each histogram. Histograms were analyzed using the Summit v4.3 software.

Measurements of Sperm Intracellular Free Ca^{2+} Concentration $[Ca^{2+}]_i$

For measurement of $[Ca^{2+}]_i$, spermatozoa were adjusted to a concentration of 10×10^6 cell/ml in corresponding medium. They were then incubated with the acetoxymethyl ester form of Fura-2 (Fura-2/AM, 8 × 10–6 M, Molecular Probes, Oregon USA) for 60 min at room temperature in the presence of the non-cytotoxic detergent pluronic acid (0.1%, Molecular Probes). After loading, the cells were washed and resuspended in G-IVF solution and used within the next 2-7 hours. Sperm aliquots (1 ml) were placed in the quartz cuvette of a spectrofluorometer (SLM Aminco-Bowman, Series 2, Microbeam, Barcelona, Spain) and magnetically stirred at 37º C. The emitted fluorescence was measured at 510 nm. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 as previously described (Cejudo-Roman *et al.* 2013). To measure $[Ca^{2+}]_i$, samples were alternatively illuminated with two excitation wavelengths (340 nm and 380 nm) and the fluorescence ratio (F340:F380) was recorded continuously. The emitted fluorescent light from the two excitation wavelengths was measured by a photomultiplier through a 510-nm filter. After subtracting the autofluorescence signal, obtained by adding 5 mM $MnCl_2$ at the end of the experiment, the F340/F380 ratio was used as an indicator of $[Ca^{2+}]_i$. The effect of beta-endorphin was studied on sperm aliquots incubated with this peptide at different doses (10^{-6}, 10^{-7}, 10^{-8} or 10^{-9} M). Progesterone 10^{-6} M was added to the same sperm aliquot to analyse the effect of beta-endorphin on the progesterone-induced intracellular Ca^{2+} levels. Calibration of $[Ca^{2+}]_i$ was achieved according to the equation of Grynkiewicz *et al*. (1985) adding Triton X-100 (5%), to obtain the maximal response, followed by addition of EGTA (40 mM) to obtain the minimal response.

Statistics

Acrosome-reacted data were normalized as [(Treatment – Control)/(Control)] × 100. and evaluated using the Kruskal–Wallis non-parametric test followed by Mann-Whitney U tests. These procedures were undertaken using GraphPad PRISM (version 5.0) program. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$. Data are expressed as mean ± SEM.

RESULTS

Expression and Localization of POMC in Human Sperm Cells

POMC transcript was not detected in human spermatozoa using RT-PCR. The expected 151-bp fragment for *POMC* was undetectable in human spermatozoa. We only observed the fragment corresponding to the pool of DNA from 20 different human tissues used as a positive control (pc). The housekeeping gene *ACTB* was detected in all tissues and the absence of amplicons in the retrotranscriptase negative controls confirmed the absence of contaminating genomic DNA in each sample (Fig. 1A). The absence of *CD4* in the spermatozoa preparation indicates no leukocyte contamination, whereas the presence of *ACR* verifies the presence of sperm complementary DNA (data not shown). On the other hand, using western blot a band of 55 kDa was observed in human sperm protein fraction as well as in human kidney protein fraction, which was used as a control (Fig. 1B). The molecular weight corresponds to the theoretical molecular weight of POMC in humans. We did not detect any signal in the absence of primary antibody (data not shown).

Analysis by immunofluorescence confirmed that POMC was present in human sperm cells (Fig. 1C) and in human testis (Fig. 1D). We found a strong immunoreactivity of POMC in the middle section and in the tail of the sperm cells (Fig. 1C). In human frozen testis, POMC immunoreactivity was also detected inside the seminiferous

tubules, where spermatogenic cells are present (Fig. 1D). In both cases, no fluorescent signal was detected using pre-absorbing primary antibody and non specific rabbit immunoglobulin confirming the specificity of primary antibody. When the primary antibodies were omitted before secondary antibody addition, the fluorescent staining pattern was also abolished.

Effect of Beta-endorphin on Acrosome Reaction

Beta-endorphin induced an inversely dose-dependent increase of acrosome-reacted spermatozoa in capacitated samples (Fig. 2A). The incubation with 10^{-9} M beta-endorphin caused the highest increase in the percentage of acrosome-reacted cells ($P < 0.01$). Incubation with higher doses (10^{-7} M) led to a smallerd increase in the percentage of acrosome-reacted cells ($P < 0.05$) and beta-endorphin 10^{-5} M caaused no significant effect.

To further analyze the specificity of the beta-endorphin effect, we co-incubated this pentapeptide with the opioid receptor antagonist naloxone. After pre-incubation with naloxone the effect of beta-endorphin on the percentage of acrosome-reacted cells was blunted by the high dose of naloxone (10^{-5} M, $P < 0.05$), but not by the low doses (10^{-8} M, Fig. 2B). The co-incubation of beta-endorphin with 10^{-8} M naloxone caused a partial non-significant reversion of the acrosome reaction. High or low doses of naloxone, added alone, had no effect on the acrosome reaction.

To evaluate the effect of beta-endorphin on progesterone-induced acrosome reaction, we co-incubated spermatozoa with beta-endorphin and progesterone. As expected, progesterone increased the percentage of acrosome-reacted sperm cells ($P < 0.05$, Fig. 2C)., Additional incubation of this samples with Beta-endorphin (10^{-9} M) caused a greater stimulation ofthe the acrosome reaction ($P < 0.01$). The percentage of

297 acrosome-reacted sperm cells was higher after the treatment of both substances
298 compared to progesterone exposure, being the difference statistically significant ($P < 0.05$).

300 *Effects of Beta-endorphin on Intracellular Free Ca^{2+} Concentration $[Ca^{2+}]_i$*

301 Beta-endorphin (10^{-9}M) did not modify $[Ca^{2+}]_i$ in Fura-2-loaded human sperm cells
302 (Fig. 3A). Higher doses of beta-endorphin assayed (10^{-8}, 10^{-7} or 10^{-6} M) neither caused
303 any effects, even after prolonged periods of incubation (30 min, not shown). Subsequent
304 addition of 10^{-6} M progesterone to the same sperm aliquot caused a typical biphasic
305 $[Ca^{2+}]_i$ progesterone response, consisting of a rapid transient peak followed by a decay
306 to $[Ca^{2+}]_i$ levels slightly above basal and a lower sustained plateau phase. Beta-
307 endorphin was not able to modify the progesterone-induced intracellular Ca^{2+} response
308 (Fig. 3A). The area of the progesterone-induced $[Ca^{2+}]_i$ signal was not modified in
309 sperm aliquots pre-incubated with 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M beta-endorphin (Fig. 3B).

310

311 *Effect of Beta-endorphin on Sperm PKC-signalling Pathways*

312 Figure 4A shows PKC-substrate phosphorylation using an anti-phospho-PKC. In
313 control samples, immunofluorescence was detected along the tail and we observed an
314 increase in the phospho-PKC immunoreactivity after progesterone treatment, as we
315 expected. CTCF analysis showed also a significant increase in PKC induced
316 phosphorylated substrates after progesterone treatment ($P<0.05$) (Figure 4B). Beta-
317 endorphin also increased significantly the staining of the phospho-PKC substrates in the
318 tail and induced appearance of positive immunoreactivity over the acrosome region
319 (Figure 4A, 4B). The co-incubation of beta-endorphin with progesterone caused a
320 stronger increase in the immunoreactivity of phosphorylated substrates induced by PKC
321 (Figure 4A). Measured by CTCF, beta-endorphin caused a 1.7-fold increase in the

phosphorylation of PKC substrates respect to progesterone (Figure 4B). Experiments were repeated at least five times and non-specific binding was not observed in the negative controls that were not exposed to the primary antibody.

Flow cytometry analysis was carried out to verify the positive effect of beta-endorphin on phosphorylation of PKC substrates. As we expected, the intensity of fluorescence of PKC-induced phosphorylated substrates (Figure 4C) increased after progesterone treatment ($P < 0.05$). Compared with controls, beta-endorphin caused also a positive effect on the phosphorylation of PKC substrates. The fluorescence of phospho-PKC substrates increased in beta-endorphin-treated semen samples ($P < 0.05$). A synergic effect was observed after the co-incubation of beta-endorphin and progesterone on the phosphorylation of PKC substrates status. The fluorescence intensity of PKC-induced phosphorylated substrates was significantly higher (1.2-fold) compared to progesterone ($P < 0.05$) and controls ($P < 0.01$) (Figure 4C).

DISCUSSION

Endogenous opioid peptides participate in the regulation of reproductive physiology at multiple sites and appear to be increasingly important in the regulation of sperm physiology. In this study, we showed that beta-endorphin exerted a regulatory effect on sperm function.

Expression and Localization of POMC in Human Spermatozoa and Testis

Beta-endorphin has been described over the acrosome reaction of human spermatozoa (Fraioli *et al.* 1984). However, the presence of its protein precursor -pro-opio-melanocortin (POMC)- was completely unknown. RT-PCR revealed the absence of

POMC mRNA in human spermatozoa, consistent with the fact that mature mammalian sperm are not transcriptionally active because of their highly condensed chromatin and the scarcity of cytoplasm capable of supporting translation (Miller and Ostermeier 2006, Ostermeier *et al.* 2004). However, recent findings have shown that a limited pool of RNA could be selectively maintained in mature sperm cells to be subsequently translated into protein upon fertilization. The absence of POMC mRNA in human sperm cells suggests that the transcript of the precursor may not be important during the first steps of embryogenesis as reported for other sperm transcripts (Agirrergoitia *et al.* 2010, Ravina *et al.* 2007). Despite this, immunoblooting and immunofluorescence analysis revealed the presence of POMC protein in human sperm cells – specifically, there was immunoreactivity in the tail of spermatozoa. In agreement with previous studies (Kilpatrick *et al.* 1987, Garrett *et al.*1989), we showed the presence of POMC inside of human seminiferous tubules, where spermatogenic cells are present. Cathepsin L, the major proteolytic enzyme for the production of POMC-derived peptides (Funkelstein *et al.* 2008), is also present in mice male germ cells and haploid cells (Wright *et al.* 2003). This suggests that spermatozoa may be able to synthesize POMC-derived peptides *de novo*, such as beta-endorphin, through processing their precursor POMC.

Beta-endorphin stimulates acrosome reaction by PKC Pathway

The acrosome reaction is an exocytosis process triggered by very complex signalling pathways involving the activation of protein kinases, intracellular protein activation and the activation of ionic channels (Ickowicz *et al.* 2012). Progesterone, ZP3, prostaglandins, sterol sulphates and glycosaminoglycans are some inductors of the acrosome reaction and are found in the cumulus oophorus cells and in the follicular fluids (Vigil *et al.* 2011). These inductors promote the sperm penetration and a rise in calcium

concentration of the cytosol that is required for the acrosomic reaction (Ickowicz *et al.* 2012; Vigil *et al.* 2011).

Our results suggest that the opioid peptide beta-endorphin can be a physiological inductor of the acrosome reaction. We found an inverse dose-dependent activation of acrosome reaction induced by beta-endorphin. In fact, the physiological doses of beta-endorphin (10^{-9} M) caused the most potent effect on acrosome reaction. High doses (10^{-5} M) of the specific antagonist, naloxone, blunted the activation of acrosome reaction, suggesting that the effect of beta-endorphin is specifically mediated by activation of the opioid receptors. However, low doses of naloxone (10^{-8} M) -at which this compound acts selectively on the mu-opioid receptor- only partially blocked the effect of beta-endorphin on acrosome-reacted spermatozoa, raising the possibility that more than one receptor might be involved in this process The activation of more than one type of opioid receptor also can explain the inverse dose-dependent inhibition, since the mu- and delta-opioid receptors can activate opposite responses, as we observed in human sperm motility (Agirregoitia *et al.* 2006).

Together with progesterone, beta-endorphin is present at high concentrations in the follicular fluid and in the vicinity of the egg (Petraglia *et al.* 1985, 1986). To elucidate whether beta-endorphin modulates progesterone action, we co-incubated sperm cells with both beta-endorphin and progesterone. Beta-endorphin modified the progesterone-response. The percentage of acrosome-reacted sperm cells in samples co-incubated with beta-endorphin and progesterone was 1.5-fold higher that in samples with only progesterone.

Owing to the fact that the progesterone response is totally dependent on Ca^{2+}/PKC pathways (O'Toole *et al.* 1996; Chen *et al*., 2000; Rathi *et al.,* 2003), we investigated whether beta-endorphin may stimulates acrosome reaction by activation of the

Ca^{2+}/PKC pathway. A common and fundamental feature of physiological and pharmacological acrosome reaction inducers is that they provoke intracellular multicomponent Ca^{2+} increases (Darszon *et al.* 2011). Thus, we investigated whether beta-endorphin stimulates an increase in intracellular Ca^{2+}. Progesterone caused a typical biphasic wave of intracellular Ca^{2+} stimulation in sperm, composed of a transient increase followed by a sustained elevation as previously reported (Baldi *et al.* 2009, Gadkar *et al.* 2002). We failed to detect any change in Ca^{2+} after addition of beta-endorphin. Beta-endorphin did not cause any effect on spermatozoa $[Ca^{2+}]_i$ in Fura-2-loaded sperm suspensions and none of the doses assayed was able to modify the progesterone-induced calcium response. In spite of that, beta-endorphin caused an activation of the PKC-induced substrates phosphorylation. By immunofluorescence and flow cytometry approaches, we observed an increase in the phosphorylation of PKC-induced substrates after beta-endorphin exposure. In addition, we also reported a further activation of the PKC-signalling pathway in semen samples co-incubated simultaneously with beta-endorphin and progesterone. Compared to progesterone alone, the co incubation of beta-endorphine and progesterone caused a 1.7-fold and 1.2-fold increase in the phosphorylation of PKC substrates, measured by CTCF and flow cytometry respectively. This result was also consistent with the increase observed in the percentage of acrosome reacted sperm cells. Thus, beta-endorphin may stimulate the acrosome reaction via PKC-signalling pathway activation, as have been reported for other inductors (Vigil *et al.* 2011, O'Toole *et al.* 1996). Moreover, our data suggest that beta-endorphin can activate the PKC-signalling pathways through a Ca^{2+}-independent pathway. Mouse and rat eggs can express the atypical Ca^{2+}-independent PKC isoforms ζ and λ, (Pauken *et al.* 2000, Page *et al.* 2004) but further analyses will be necessary to analyze the presence of Ca^{2+}-independent PKC isoforms in human sperm cells.

In conclusion, the present data allow us to identify a new physiological acrosome-reaction inductor and described its signalling pathways in human sperm. Beta-endorphin may be involved in the regulation of acrosome reaction by a Ca^{2+}-independent PKC pathway in humans. These findings are important for future studies of sperm physiology and provide new insight into the function of the opioid system as a target for fertility management.

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Disclosures

The authors have nothing to disclose

Author's contributions

I.U., H.E., and I.M carried out and analyzed the experiments, F.M.P. and L.C. carried out the experiments and provided conceptual support, R.M and A.E evaluated the samples., A.V and J.I. provided conceptual support N.S. designed the study, analyzed the experiments and wrote the manuscript.

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FIGURE LEGENDS

FIG. 1. Expression of beta-endorphin in human spermatozoa. A) RT-PCR analysis of proopiomelanocortin (POMC) precursor in human spermatozoa (sp1 and sp2); nc: primers without cDNA were used as negative control and pc: pool of DNA from 20 different human tissues used as a positive control. **B)** Western blotting analysis of POMC in human spermatozoa (Sp) and kidney (kd) using a rabbit anti-POMC polyclonal antiserum. The molecular mass markers (kDa) are indicated on the left. Molecular weights of pre-stained markers proteins are indicated. Representative blot obtained from four normozoospermic donors is shown **C)** Immunofluorescence analysis of POMC in human sperm cells (panel 1). Negative controls incubating with unspecific rabbit immunoglobulin fraction (panel 2) and preadsorbing the anti-POMC antibody with beta-endorphin (panel 3). Incubation with secondary antibody alone (panels 4). DNA of controls was stained with Hoechst 33342. Representative photomicrographs are shown; n = 5. Scale bar for all panels, 1 □m. **D)** Immunofluorescence analysis of POMC in human testis (panel 1). Negative controls incubating with unspecific rabbit immunoglobulin fraction (panel 2) and preadsorbing the anti-POMC antibody with beta-endorphin (panel 3). Incubation with secondary antibody alone (panels 4). DNA of controls was stained with Propidium Iodide. Representative photomicrographs are shown; n = 3. Scale bar for all panels, 50 μm.

FIG. 2. Effect of beta-endorphin on human acrosome reaction. **A)** Dose-dependent effect of beta-endorphin on the percentage of acrosome-reacted sperm cells for 1 h. **B)** Percentage of CD46-positive sperm cells after co-incubation with beta-endorphin (10^{-9} M) and high (10^{-5} M) and low doses (10^{-8} M) of naloxone for 1 h. **C)** Percentage of CD46-positive sperm cells after co-incubation with beta-endorphin (10^{-9} M) and progesterone (10^{-6} M) for 1 h. * $P < 0.05$, significant difference vs control responses; ** $P < 0.01$, significant difference vs control

responses; and + $P < 0.05$ significant difference vs beta-endorphin responses. (n = 12). Normalized data as [(Treatment – Control)/(Control)] × 100

FIG.3. Effects of beta-endorphin on intracellular free Ca^{2+} ($[Ca^{2+}]_i$). A) Intracellular free Ca^{2+} measurement in human sperm cells loaded with Fura-2 in response to beta-endorphin (10^{-9} M) (red line) and control (black line). Subsequent addition of 10^{-6} M progesterone to the same sperm aliquot caused a typical biphasic $[Ca^{2+}]_i$ progesterone response that had not been modified by beta-endorphin. The X axis shows time in seconds and the Y axis shows $[Ca^{2+}]_i$ data expressed by the F340/F380 ratio. TX= Triton X-100. Traces are representative of typical results obtained in five different experiments for each blocker. **B)** Dose-dependent effect of beta-endorphin on progesterone-induced intracellular Ca^{2+} response. Data expressed the area of the progesterone-induced $[Ca^{2+}]_i$ signal measured by the ratio of F340/F380 signals. Calibration of [Ca2+]i was achieved adding Triton X-100 (TX), to obtain the maximal response, followed by addition of EGTA to obtain the minimal response. n=5.

FIG. 4. Effect of beta-endorphin on Ca^{2+}/protein kinas C (PKC)-signalling pathway. A) Immunofluorescence analysis of the PKC-induced substrate phosphorylation in samples treated with beta-endorphin, beta-endorphin and progesterone, and progesterone. DNA of controls was stained with Hoechst 33342. Representative photomicrographs are shown; n = 5. Scale bar, 2 μm. **B**) Percentage of phospho-PKC substrates positive spermatozoa and **C)** fluorescence intensity measured by flow cytometry in samples treated with beta-endorphin, beta-endorphin and progesterone, and progesterone. Fluorescence data from at least 100,000 events was analyzed. *$p < 0.05$, significant difference vs control responses; **$p < 0.01$, significant difference vs control responses; and + $p < 0.01$, significant difference vs progesterone responses.