Characterization of the kisspeptin system in human sperm

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Summary

Kisspeptin, the product of the *KISS1* gene, plays an essential role in the regulation of spermatogenesis acting primarily at the hypothalamic level of the gonadotropic axis. However, the presence of kisspeptin and its canonical receptor, KISS1R, in sperm has not been explored nor the direct effects of kisspeptin on sperm function have been studied so far. In the present study, we analyzed the expression of kisspeptin and its receptor in sperm cells by western blot and immunocytochemistry assays and evaluated the effects of exposure to kisspeptin on sperm intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, sperm motility, sperm hyperactivation and the acrosome reaction. Changes in $[Ca^{2+}]_i$ were monitored using Fura-2, sperm kinematic parameters were measured using computer-assisted sperm analysis (CASA), and the acrosome reaction was measured using fluorescein-isothyocianate-coupled *Pisum sativum* agglutinin lectin (FITC-PSA method). We found that kisspeptin and its receptor are present in sperm cells, where both are mainly localized in the sperm head, around the neck and in the flagellum midpiedce. Exposure to kisspeptin caused a slow, progressive increase in $[Ca^{2+}]_{i.}$ which reached a plateau about 3-6 min after kisspeptin exposure. In addition, kisspeptin modulated sperm progressive motility causing a biphasic (stimulatory and inhibitory) response and also induced transient sperm hyperactivation. The effects of kisspeptin on sperm motility and hyperactivation were inhibited by the antagonist of KISS1R, peptide 234. Kisspeptin did not induce the acrosome reaction in human sperm. These data show for the first time that kisspeptin and its receptor are present in human spermatozoa and modulate key parameters of sperm function. This may represent an additional mechanism for their crucial function in the control of male fertility.

Keywords: kisspeptin, kisspeptin receptor, neurokinin B, human sperm.

Introduction

Mammalian sperm acquire the ability to fertilize an egg during their transit through the female reproductive tract (Chang, 1951, Suarez & Pacey, 2006; Visconti, 2009). During this time, sperm cells undergo a series of morphological and functional modifications leading to activation of sperm motility, development of hyperactivated motility, binding to oocyte zona pellucida and the acrosome reaction (AR) (Roldan *et al.*, 1994; Flesch & Gadella, 2000; Visconti *et al.,* 2002; Grasa *et al.,* 2006; Bedu-Addo *et al.,* 2008). For this capacitation to take place, sperm must send and receive specific signals from the environment, which must be properly decoded under a precise spatio-temporal regulation. This complex process involves activation of multiple signal transduction mechanisms which lead to increases in sperm $[Ca^{2+}]_i$, cAMP, pH_i, and phosphorylation of some proteins at serine/threonine and tyrosine residues (Rossato *et al.,* 2001; Visconti *et al.,* 1999, 2002; Darszon *et al.,* 2006; Lamirande & O'Flaherty, 2008; Publicover *et al.,* 2008). However, the precise nature of sequentially activated receptors and channels in the sperm plasma membrane remains incompletely understood.

During the past years, kisspeptins have emerged as essential regulators of reproductive function (de Roux *et al.,* 2003; Seminara *et al.,* 2003). Kisppeptins are primarily synthesized in discrete neuronal populations within the hypothalamus where they modulate GnRH secretion and, thereby, gonadotropin release (Navarro *et al.,* 2004; Colledge, 2008; Tena-Sempere, 2010). These peptides are encoded by the *KISS1* gene which produces a C-terminal amidated peptide of 54 amino-acids named kisspeptin-54 (formerly, also known as metastin). Kisspeptin-54 is further processed to generate C-terminal peptides of 14

(kisspeptin-14), 13 (kisspeptin-13) or 10 amino-acids (kisspeptin-10), all of them showing similar biological activities (Colledge, 2008; Tena-Sempere, 2010).

Kisspeptin effects are mediated by activation of the KISS1 receptor (KISS1R), also known as GPR54, which is encoded by the *KISS1R* gene (Ohtaki *et al.,* 2001, Colledge, 2008). Mutations of *KISS1R* are associated with hypogonadotrophic hypogonadism in humans (Seminara *et al.,* 2003; de Roux *et al.,* 2003), a phenotype which is also observed in mice carrying inactivating mutations of *Kiss1* or *Kiss1r* genes (Tena-Sempere, 2010).

In addition to their prominent expression at hypothalamic levels, fragmentary evidences suggest that KISS1 and/or KISS1R mRNAs or proteins are also present in several peripheral reproductive tissues including the ovary (Castellano et al., 2006; Gaytan et al., 2009), the oviduct (Gaytán *et al.,* 2007) and the testes (Ohtaki *et al.,* 2001). On the latter, however, the potential effects of kisspeptin on the male gonads and, particularly, its possible role in the regulation of ejaculated sperm function have not been studied to date.

The aim of this work was to examine the presence of the kisspeptin system in human spermatozoa and its potential involvement in the regulation of human sperm function.

Materials and methods

Semen samples and sperm preparation

This study was approved by the Ethics Committee of Consejo Superior de Investigaciones Científicas (CSIC) and informed written consent was obtained from all donors. Freshly ejaculated semen was collected from fifty-six healthy donors (18-35 years old) after 3-4 days sexual abstinence. Samples used in the present study were normozoospermic and displayed the absence of leukocytospermia, abnormal morphology or viscosity. The samples were allowed to liquefy at 37ºC for 30 min and examined for concentration and motility

following World Health Organization (WHO) guidelines (1999). Liquefied semen samples were washed with modified human tubal fluid (mHTF, Irvine Scientific, Santa Ana, CA) supplemented with 10 mM HEPES and 0.5% bovine serum albumin (BSA) and processed as described previously (Ravina *et al.*, 2007; Pinto *et al.* 2009). Briefly, sperm suspensions were centrifuged through a discontinuous density gradient (Spermgrad-125, Vitrolife, Kungsbacka, Sweden), allowed to swim-up for 1 hour at 37ºC and the supernatant carefully aspirated. Sperm motility and concentration were re-examined and the concentration adjusted to 50 x 10^6 cells/ml for subsequent experiments.

Indirect immunofluorescence

Immunolocalization of kisspeptin and its receptor was assessed by fluorescence microscopy. Sperm cells were washed, resuspended in phosphate-buffered saline (PBS) and smeared onto poly-L-lysine-coated slides. Spermatozoa were then fixed by incubation in cold methanol (-20ºC) for 20 min. Slides were washed thoroughly with PBS and incubated for 120 min with 2% casein in PBS to block non-specific binding sites. Test slides were incubated overnight at 4ºC with a primary polyclonal antibody designed to recognize human kisspeptin (either sc-15400, from Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:400, or T-4771, from Bachem, Bubendorf, CH, dilution 1:200), or KISS1R (sc-48220, Santa Cruz, dilution 1:600). The specificity of antibodies was previously established by one of us (Gaytán *et al.*, 2007) or assessed by using sections of human ovary and oviduct as positive controls (Gaytán *et al.*, 2007, 2009). We also found, in experiments performed with the kisspeptin primary antibody T-4771, that preincubation with an excess of kisspeptin immunogenic peptide caused a disappearance of the fluorescent signal. Negative control slides were not exposed to the primary antibody and were incubated with pre-immune

serum or PBS and processed in the same conditions as test slides. Samples were washed three times in PBS and incubated for 60 min with appropriate FITC-conjugated secondary antibodies (Santa Cruz). Slides were mounted using Prolong Gold antifade reagent (Invitrogen, Molecular Probes, Eugene, OR) with or without DAPI (Invitrogen) (for nuclear counterstaining) and examined with a Olympus BX-51 fluorescence microscopy (Tokyo, Japan).

In some experiments we analyzed the immunolocalization of kisspeptin and its receptor, or kisspeptin and the tachykinin neurokinin B (NKB) in the same sperm cells. These double-immunostaining experiments were performed essentially as described above, incubating sperm cells overnight with rabbit anti-human kisspeptin antibody (sc-15400, Santa Cruz, 1:200 dilution) and either goat anti-human KISS1R (sc-48220, Santa Cruz, 1:600 dilution) or goat anti-human NKB antibody (sc-14109, Santa Cruz, 1:200 dilution). Secondary antibodies (1:400 dilutions of chicken anti-rabbit and donkey anti-goat) were conjugated to Tx-red or FITC (Santa Cruz) and used together.

Western Blot experiments

Western blotting was used to analyze the presence of KISS1R in spermatozoa and to assess the specificity of the KISS1R antibody. This procedure was performed as described previously, with slight modifications (Ravina *et al.*, 2007; Pinto *et al.*, 2009). We also studied the presence of KISS1R in human placenta, used as a positive control (Ohtaki *et al.*, 2001). For extraction of total proteins, the sperm cells were subjected to sonication in urea extraction buffer (1% w/v SDS, 9 M Urea, 1 mM EDTA, 0.7 M mercapto-ethanol, in 25 mM Tris-HCl, pH 6.8), boiled and processed by polyacrylamide gel electrophoresis (PAGEprep Advance kit, Pierce, Rockford, IL). The protein content was measured using a

bicinchoninic acid (BCA) protein assay kit (Pierce) and 40 μg sperm or placenta protein were loaded on 10% sodium dodecyl sulphate (SDS)-PAGE gels. Proteins were separated by electrophoresis, transferred to polyvinyldifluoride (PVDF) membranes and processed according to the Amersham advance enhanced chemiluminescence (ECL) kit (Buckinghamshire, UK). Primary antibody dilution was 1:10000 and for the secondary antibody was 1:100000.

$[Ca^{2+}]_i$ Measurements

Changes in $[Ca^{2+}]_i$ were measured in sperm suspensions according to previously published procedures (Bedu-Addo *et al.*, 2005; Pinto *et al.*, 2009). Following swim-up, spermatozoa were incubated in mHTF with the acetoxymethyl ester form of Fura-2 (Fura-2/AM, 10 μM, Invitrogen) for 60 min at room temperature. After loading, the cells were washed and resuspended in mHTF or in a solution of the following composition (mM): NaCl 140; KCl 4.7; $CaCl_2$ 2.0; $MgCl_2$ 0.3; glucose 10 and HEPES 10 (pH 7.4). Sperm aliquots (1 ml, 10 x 10^6 cells/ml) were placed in the cuvette of a spectrofluorometer (SLM Aminco-Bowman, Series 2, Microbeam, Barcelona, Spain) and continuously stirred at 37ºC. The sperm suspension was alternatively illuminated with two excitations wavelengths (340 nm and 380 nm) and the emitted fluorescence was measured at 510 nm. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 (F340/F380) fluorescence ratio as previously described (Pinto et al., 2009).

Sperm Motility Studies

Motility kinematics parameters were evaluated by computer analysis with Sperm Class Analyzer (SCA, Microptic, Barcelona, Spain) following WHO recommendations (1999). Aliquots of semen samples (5 μL) were placed into a Makler counting chamber (Sefi

Medical Instruments, Haifa, Israel) and at least 200 sperm cells and 5 fields were evaluated at each incubation time by phase contrast microscopy. Twenty-five consecutive digitalized images were analyzed for each single field. The movement of every encountered sperm was graded as: *a*: rapid progressive motility; *b*: slow progressive motility; *c*: non-progressive motility and *d*: immotility (WHO, 1999).

Individual sperm samples were divided in several aliquots and each aliquot was treated with a single concentration of kisppeptin (kisspeptin-13, Bachem) (0.01-10 μM) or its solvent. In parallel experiments, we observed that the solvent does not alter sperm motility, in comparison with untreated, time-matched paired aliquots. Sperm motility was measured 5 min before agent addition (initial value) and after 2, 15, 30, 60 and 120 min contact time periods. In additional experiments, the effect of kisspeptin (10 μM) or its solvent was investigated in aliquots pretreated for 15 min with the KISS1R-selective antagonist peptide 234 (p234, Sigma) (0.3 μM) (Roseweir *et al.*, 2009) or its solvent.

Progressive motility (*a*+*b*), non-progressive motility (*c*) and immotility (*d*) were measured as percentage of the total (*a*+*b*+*c*+*d*), which was considered as 100%. Values of progressive motility, non-progressive motility and immotility were then expressed as the positive or negative increment produced by kisspeptin relative to the value observed at the same time in solvent-treated controls using the formula: (kisspeptin-treated at time x-initial value)-(solvent-treated at time x-initial value).

We also analysed the effects of kisspeptin or solvent on hyperactivation in sperm capacitated in vitro by incubation for 6 h in a humidified incubator at 37ºC in 5% CO_2. The following kinematics parameters were measured: hyperactive motility; curvilinear velocity (VCL , μm/s); straight-line velocity (VSL, μm/s), average-path velocity (VAP , μm/s);

amplitude of lateral head displacement (ALH, μm); beat-cross frequency (BCF, Hz); linearity (LIN=VSL/VCL x 100) and straightness (STR=VSL/VAP x 100). These values were then expressed as percentage changes induced by kisspeptin using the formula: kisspeptin treated-solvent-treated/solvent-treated x 100.

Acrosome reaction assays

Acrosomal status was assessed with fluorescein isothyocianate-conjugated lectin from *Pisum sativum* (FITC-PSA) following previously described procedures (Mendoza *et al.*, 1992; Bedu-Addo *et al.,* 2005). Following swim-up, sperm cells were adjusted to a concentration of 10 x 10^6 cells/ml and capacitated (6 h at 37ºC in 5% CO_2). Sperm aliquots were then untreated (time-matched paired controls) or treated for different times with kisspeptin (10 μM), the ionophore A23187 (10 μM) or the corresponding solvent at 37ºC, 5% CO_2. At the end of each incubation period, cells were centrifuged, the supernatant removed and spermatozoa resuspended in hypo-osmotic swelling (HOS) medium (0.74% sodium citrate and 1.35% fructose in ultra-pure H_2O) as described by Bedu-Addo *et al.* (2005). Sperm cells were washed (400 g for 5 min) and supernatants partially removed. The remaining pellets were spotted onto poly-L-lysine-coated slides, fixed/permeabilized in methanol and air-dried. The slides were incubated with 50 μl of FITC-PSA (50 μg/ml) for 30 min in a humid chamber, washed, air-dried and mounted with Citifluor Solid Mountant Kit (Agar Scientific, Essex, UK). The acrosomal status was evaluated by fluorescence microscopy. Spermatozoa displaying an intact acrosome are strongly labeled with the fluorescent lectin at the acrosomal region whereas AR reacted cells show no labeling in this region, with or without labeling of the equatorial region. At least 200 cells were counted for each experimental condition and only HOS-positive (viable) cells were scored. Percentage

AR values were calculated by the formula: (%AR reacted spermatozoa in treated aliquots)-(%AR reacted spermatozoa in the corresponding solvent-treated aliquots). The spontaneous AR range in control, untreated aliquots was 10-15% and neither A23187 nor kisspeptin solvent modified the AR status of the samples.

Statistical analysis

Results shown represent means ± SEM and *n* indicates number of experiments in sperm amples from *n* different donors. Kisspeptin responses were observed in 65-70% of sperm preparations assayed and only these samples were considered for mean calculation. Statistical analyses were made using Mann-Whitney's U test (for comparison of mean ranks between two groups) or Kruskal-Wallis test (to compare more than two groups) nonparametric tests. These procedures were undertaken using GRAPHPAD PRISM (version 5.0). $P<0.05$ values were considered significant.

Results

Immunodetection of kisspeptin and its receptor in human sperm

Immunocytochemistry studies showed the presence of kisspeptin and KISS1R immunoreactivity (IR) in human spermatozoa (Figs. 1, 2, 3). For kisspeptin, positive immunolabeling was found in 100% of cells in all samples assayed ($n= 6$). Kisspeptin IR was localized in the post-acrosomal region of the sperm head and, in most cells, it was particularly intense in the equatorial segment (Figs. 1, 3). In many spermatozoa, an additional positive labeling was observed around the neck (Figs. 1, 3). In some sperm preparations ($n=2$), kisspeptin IR was restricted to the equatorial segment and the neck while in other preparations ($n=4$) kisspeptin-positive labeling was also observed in the

flagellum midpiece (Figs. 1, 3). We used two different kisspeptin antibodies and found comparable results with both of them.

Fig. 1

The kisspeptin receptor showed a distribution similar to that of kisspeptin; KISS1R IR was mainly found in the equatorial segment of the sperm head and around the neck (Figs. 2, 3). Strong immunostaining for KISS1R in these two regions was found in approximately 95% of cells (*n*=6, Fig. 2). In addition, approximately a 30-40 % of spermatozoa showed positive immunolabeling in the flagellum midpiece and this was observed in all preparations assayed (*n*=6, Figs. 2, 3). Control assays incubated only with secondary antibodies showed no signal.

Western blot analysis of sperm homogenates confirmed the presence of KISS1R in spermatozoa. The KISS1R antibody recognized a band with the expected molecular weight of approximately 43 kDa (Fig. 2) and also labeled other bands of similar size, which may be the result of post-translational modifications (Fig. 2). The immunoreactive bands for KISS1R were also observed in the human placenta, used as positive control (Fig. 2). No band was observed in control assays, where primary antibodies were omitted (not shown).

Fig. 2

Co-localization of kisspeptin and neurokinin B in human sperm

In a previous study we showed the presence of positive immunoreactivity for NKB in human spermatozoa (Pinto *et al.,* 2010). In this study, we performed double immunolabeling experiments with anti-kisspeptin and anti-NKB antibodies to analyze the localization of NKB and kisspeptin in the same sperm cells. We found, in agreement with our previous results (Pinto *et al.,* 2010), that NKB immunofluorescence was localized in the

sperm head and was particularly intense in the equatorial region (Fig. 1). Double immunofluorescence analysis confirmed the co-localization of both peptides in sperm cells, where kisspeptin and NKB appeared to uniformly merge, particularly over the equatorial segment (yellow signal, Fig. 1).

Co-localization of kisspeptin and its receptor in human sperm

Double immunolabeling experiments showed co-expression of kisspeptin and KISS1R in human sperm cells (Fig. 3). Fluorescence images show that co-localization of both proteins was mostly confined to the equatorial segment (Fig. 3).

Fig. 3

Effects of kisspeptin on intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$

The presence of kisspeptin and its cognate receptor in human spermatozoa strongly suggests a local role of kisspeptin system in the modulation of sperm functions, many of which are associated with changes in $[Ca^{2+}]_i$ levels. Therefore, we analyzed the effects of kisspeptin on $[Ca^{2+}]_i$ in sperm cells. Kisspeptin (≥ 1 μM) caused a slowly developed, progressive increase in $[Ca^{2+}]_i$ in Fura-2-loaded human spermatozoa (Fig. 4). This increase was initiated rapidly and reached a plateau about 3-6 min after kisspeptin exposure. The mean resting $[Ca^{2+}]_i$ was 104 ± 2 nM in the absence of kisspeptin (n=16) and raised to 108 ± 3 nM (n=3, P>0.05), 114 ± 4 nM (n=5 , P<0.01) and 124 ± 8 nM (n=8, P<0.01) in the presence of kisspeptin 1, 10 and 20 μM, respectively. The peptide solvent had no effects (not shown). In parallel sperm aliquots, progesterone (1 μM) caused the typical biphasic $[Ca^{2+}]_i$ response consisting in a rapid transient peak that raised $[Ca^{2+}]_i$ from a basal value of 101 ± 1 nM to 232 ± 16 nM (n=11 , P<0.001). This was followed by a decay to $[Ca^{2+}]_i$ levels slightly over basal levels and a lower, sustained plateau phase (141 ± 5 nM, n=11, P<0.001) which

persisted during the time of stimulation with progesterone. The sex steroid induced virtually identical responses when added to sperm suspensions previously exposed to kisspeptin (Fig. 4) or its solvent (not shown). Similarly, kisspeptin induced a full $[Ca^{2+}]_i$ response in the presence of progesterone (not shown).

Fig. 4

The $[Ca^{2+}]_i$ response was observed in 3/8 sperm suspensions with 1 μM kisspeptin, in 5/9 with 10 μM and in 8/9 with 20 μM kisspeptin. In contrast, the response to progesterone was observed in 100% of samples assayed. Kisspeptin and progesterone induced $[Ca^{2+}]_i$ responses of similar kinetics in sperm suspensions maintained in HTF or HEPES-based medium.

Effects of kisspeptin on human sperm motility

Kisspeptin (0.01 and 0.1 μM) did not modify sperm motility. At 1 μM, kisspeptin caused a transient increase in the percentage of *a* grade spermatozoa and a concomitant decrease in the percentage of *b* grade spermatozoa, but failed to produce a net modification in the percentage of progressive (*a*+*b* grades) motile spermatozoa (Fig. 5A). At a higher concentration (10 μM), kisspeptin caused a rapid stimulation of sperm progressive motility and increased both *a* grade and *b* grade spermatozoa (Fig. 5B), which result in a net increase in sperm progressive motility (Fig. 5C). This lasted for approximately 15 min, and was followed by a decrease in the percentage of *a*+*b* grade sperm cells (Fig. 5C). A new phase of stimulation of progressive motility was observed at more prolonged times of incubation (60-120 min, Figs. 5B and 5C). The effects of kisspeptin on sperm motility were inhibited in the presence of the KISS1R antagonist, p234 (Fig. 5C).

Fig. 5

With regard to sperm kinematics parameters, kisspeptin increased ALH and BCF causing a parallel decrease in straightness and linearity ($P<0.05$ *vs*. solvent-treated aliquots, Fig. 6). This led to a motility pattern characteristic of hyperactivated sperm which was transiently maintained (Fig 6). The effects of kisspeptin on hyperactive motility were reduced in the presence of the antagonist p234 (Fig. 6).

There were considerable variations between samples with respect to i) their ability to respond to kisspeptin and ii) the time needed to reach maximal responses. Hence, the effects of kisspeptin 1 μM on sperm motility were observed in 4 of 5 experiments. The effects of 10 μM kisspeptin were observed in 5 of 7 sperm samples in experiments performed in the absence of the antagonist, and in 8 of 14 experiments in the presence of p234 or its solvent. Sperm hyperactivation was observed in 9 of 14 experiments. Within these 9 responsive samples, 5 showed hyperactivation at 2 min, all were hyperactivated at 15 min, 7 remained hyperactive at 30 min and 4 displayed hyperactive motility at 60 min.

Fig. 6

Effect of kisspeptin on the acrosome reaction

We then analyzed whether kisspeptin was able to induce the AR in human sperm capacitated for 6 h and then exposed to kisspeptin (10 μM) for different times. As shown in Fig. 7, kisspeptin did not induce the AR at any of the times assayed (2-120 min, Fig. 7). Conversely, A23187 (10 μM) induced the AR in time-matched paired sperm aliquots (Fig. 7) while its solvent had no effect.

Fig. 7

DISCUSSION

To date, the effects of kisspeptin on reproduction have been mainly studied at the hypothalamic-pituitary levels (Colledge, 2008, Tena-Sempere, 2010). In this sense, it remains undisputed that kisspeptin act through its receptor, KISS1R, in hypothalamic neurons and play an essential role in the regulation of sexual maturation, gametogenesis and fertility (de Roux *et al.,* 2003; Seminara *et al.,* 2003; Navarro *et al.,* 2004, Colledge, 2008, Tena-Sempere, 2010). Evidence for the expression of *KISS1* and *KISS1R* genes in the human testis had been presented preliminarily (Ohtaki *et al.,* 2001), suggesting that kisspeptin could exert direct actions in the male gonads. Our present data are fully compatible with this possibility as we show, for the first time, that kisspeptin and its receptor are present in mature human spermatozoa, with specific patterns of cellular distribution. The strongest immunolabeling for both kisspeptin and KISS1R was found in the equatorial segment, a region with an important role in oocyte-sperm fusion (Bedford *et al.,* 1979; Flesch & Gadella, 2000). In most cells, kisspeptin and its receptor were also localized around the neck, a region that participates in the regulation of flagellar activity (Publicover *et al.,* 2008; Bedu-Addo *et al.,* 2008). In a smaller population of spermatozoa, kisspeptin and KISS1R were also found in the midpiece, a region involved in the control of energetic requirements, Ca^{2+}-buffering and motion (Suarez *et al.,* 2007; Publicover *et al.,* 2008).

Kisspeptin is expressed in discrete populations of neurons within the hypothalamus, including the arcuate nucleus, where it is co-expressed with two other bioactive peptides, the tachykinin NKB and the opioid dynorphin A (Rance, 2009; Lehman *et al.,* 2010; Tena-Sempere, 2010). These are named KNDy neurons, and play a crucial role in the pathway

through which the brain regulates reproduction (Topaloglu *et al.,* 2009; Lehman *et al.,* 2010). The present data show that NKB co-localizes with kisspeptin also in human spermatozoa, particularly in the equatorial region. Dynorphin is also present in human sperm and is mainly localized in the equatorial segment (N. Subiran, personal communication). Thus, spermatozoa represent the first known type of non-neuronal KNDy cell. In hypothalamic neurons, kisspeptin, NKB and dynorphin appear to act in a coordinated manner to modulate pulsatile GnRH secretion and hence gonadotropin release (Lehman *et al.,* 2010). It may thus be tempting to propose that some of the effects of kisspeptin in sperm, modulated by the concerted actions of NKB and dynorphin, may stem from its ability to locally modulate GnRH secretion. Previous studies have demonstrated the presence of GnRH and the GnRH receptor in many extrahypothalamic tissues including the human oviduct, ovarian granulosa cells and spermatozoa (Izumi *et al.,* 1985; Morales, 1998; Lee *et al.,* 2000). Experimental data have shown that GnRH increases the ability of sperm to bind to the zona pellucida, whereas it is unable to induce the AR or to modify the motility pattern in human sperm (Morales, 1998). In any event, our current data suggest that kisspeptin, alone or in concert with NKB (and dynorphin) is capable to modulate important sperm functions. The physiological relevance of such a local role of kisspeptin, as well as NKB and dynorphin, in human sperm awaits further investigation.

The presence of kisspeptin and its receptor in spermatozoa strongly suggest a regulatory role of the kisspeptin system. Because $[Ca^{2+}]_i$ signaling plays a central role in the control of many different sperm functions (Rossato *et al.,* 2001; Darszon *et al.,* 2006; Publicover *et al.,* 2008), we analyzed the ability of kisspeptin to induce changes in sperm $[Ca^{2+}]_i$. Our data show that kisspeptin induced a slowly developed, sustained increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$

response induced by kisspeptin was very different in time-course and kinetics to that induced by progesterone. Moreover, both kisspeptin and progesterone were able to induce a full response in sperm preparations previously exposed to the other compound (see Fig. 4), suggesting that kisspeptin and progesterone increase $[Ca^{2+}]_i$ acting by different mechanisms. The observed modifications in $[Ca^{2+}]_i$ prompted us to investigate the effect of kisspeptin on motility, hyperactivation and the acrosome reaction, since all these functions have been associated to changes in $[Ca^{2+}]_i$ levels in sperm. We found that kisspeptin induced small, but significant changes in sperm motility. These effects were reduced in the presence of the KISS1R antagonist, p234, providing further support for the presence of functional kisspeptin receptors in human sperm. Kisspeptin, acting via KISS1R, could activate different signal transduction pathways leading to its modulatory role on sperm movement. Sperm motility is essential for natural reproduction, and spermatozoa show different motility patterns at different functional states. Initially, sperm cells develop a progressive motility with relative regular and linear trajectories, which is necessary for swimming and transport through the female reproductive tract (Mortimer, 1997; Turner, 2006; Subiran *et al.,* 2008). When sperm cells arrive to the oviduct, they develop a hyperactivated motility characterized by the appearance of asymmetric trajectories, which is necessary for sperm to leave their reservoirs in the oviductal isthmus, in order to find and fuse with the oocyte (Mortimer, 1997; Suarez & Pacey, 2006; Chang & Suarez, 2010). Previous studies have shown that kisspeptin is present in the oviduct, it being particularly abundant in the luminal surface of the isthmus segment (Gaytan *et al.,* 2007). Kisspeptin could thus participate in some of the important processes that occur in the oviduct, such as sperm hyperactivation, the acrosome reaction and/or oocyte fertilization (Roldan *et al.,* 1994; Visconti,. 2009;

Chang & Suarez, 2010). The present data show that the effects of kisspeptin on motility were characterized by an increase in flagellar beating and in ALH with a concomitant decrease in straightness and linearity, leading to motility trajectories that are characteristics of hyperactivated spermatozoa. The transient nature of kisspeptin-induced hyperactivation is consistent with the hypothesis that sperm flagellar beat pattern must turn intermittently between asymmetrical and symmetrical swimming patterns in order to reach the oocyte (Chang & Suarez, 2010). On the contrary, kisspeptin was unable to induce the AR, which is consistent with the very different $[Ca^{2+}]_i$ response elicited by kisspeptin in comparison with that of progesterone, a well-known AR inducer (Roldan *et al.,* 1994; Bedu-Addo *et al.,* 2005). Further studies will help to clarify the role of kisspeptin and KISS1R in oocyte fertilization.

In this study, kisspeptin immunoreactivity was found in 100% of sperm cells and KISS1R immunoreactivity was found in approximately 95% of cells, indicating a wide distribution of both kisspeptin and its receptor in sperm cells. In addition, there were differences in subcellular immunolocalization between sperm cells, which is an usual fact when working with spermatozoa (see Agirregoitia *et al.,* 2006; Subiran *et al.,* 2008; Colas *et al.,* 2009). At the moment, the most accepted explanation is that these differences may reflect changes in the capacitation stage of the sperm cell (Colas *et al.,* 2009). KISS1R, the protein that would mediate functional responses to kisspeptin, was found in both the equatorial segment and around the neck in more than 95% of cells and was additionally found in the flagellum midpiece in about 30-40% of cells, and this occur in all preparations assayed. With respect to functional experiments, we found that approximately a 30% of sperm preparations did no

respond to kisspeptin. These data do not permit actually to establish a correlation between changes in immunolocalization and the functional responses to kisspeptin.

In conclusion, this study shows for the first time that kisspeptin and its receptor are present in human spermatozoa. Moreover, we document that kisspeptin, acting through KISS1R, is able to modulate some human sperm functions. The physiological significance of this novel facet of kisspeptin action upon the male reproductive axis warrants further investigation.

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FIGURE LEGENDS

Fig. 1. Immunofluorescence and corresponding differential interference contrast images of human sperm cells stained with anti-kisspeptin and/or anti-neurokinin B antibodies. (A) Immunolocalization of kisspeptin (green staining). (B) Double immunofluorescence analysis of kisspeptin (red signal), neurokinin B (green signal) and merged image showing the co-localization of kisspeptin and neurokinin B (yellow signal). Experiments were performed at least six times with similar results. Scale bar, 20 μm.

Fig. 2. Analysis of the presence of the kisspeptin receptor (KISS1R) in human sperm. (A) Immunofluorescence and corresponding differential interference contrast images of human sperm cells stained with a primary antibody against the kisspeptin receptor, KISS1R. Sperm nuclei were stained with DAPI. Experiments were performed at least six times with similar results. Scale bar, 20 μm. (B) Western Blot analysis showing the presence of KISS1R in sperm homogenates (SPZ) and in human placenta (PLC), used as a positive control. Results are representative of at least five separate protein preparations, each from 5 different donors.

Fig. 3. Immunofluorescence and corresponding differential interference contrast images of human sperm cells stained with primary antibodies against kisspeptin and its receptor, KISS1R. Double immunofluorescence analysis of kisspeptin (green signal), KISS1R (red signal) and merged image showing the co-localization of kisspeptin and its receptor (yellow signal). Experiments were performed at least six times with similar results. Scale bar, 20 μm.

Fig. 4. Effects of kisspeptin (10 μM) and progesterone (1 μM) on intracellular free Ca^{2+} levels, $[Ca^{2+}]_i$, in human sperm loaded with Fura-2. The trace is representative of typical results obtained in 6 different experiments. The X Axis shows time in seconds with respect

to addition of kisspeptin and progesterone and the Y axis shows $[Ca^{2+}]_i$ data expressed by the ratio of F340/F380 signals.

Fig. 5. Time- and concentration-dependent effects of kisspeptin on human sperm motility. (A) Effects of kisspeptin (1 μM) on rapid progressive (*a* grade) and slow progressive (*b* grade) motility at different times of incubation. (B) Effects of kisspeptin (10 μM) on rapid progressive (*a* grade) and slow progressive (*b* grade) motility at different times of incubation. (C) Effects of kisspeptin (10 μM) on sperm progressive motility (*a*+*b* grades) in the presence of the selective KISS1R antagonist peptide 234 (p234, 0.3 μM) or its solvent at different times of incubation. Progressive motility (*a* and *b*), non-progressive motility (*c*) and immotility (*d*) were measured as percentage of the total (*a*+*b*+*c*+*d*) that was considered as 100%. Values of *a*, *b*, *c*, *d* or *a*+*b* were then expressed as the positive or negative increment produced by the drug relative to the value observed at the same time in solvent-treated paired controls Bars are means with SEM of 4-8 different experiments. $^{*}P<0.05$, significant difference *vs.* values of progressive motility (grade *a*, *b* or *a*+*b* sperm) at time=0, Kruskal-Wallis test. $^{a}P<0.05$, significant difference *vs.* kisspeptin responses at times 2, 15 and 60, Kruskal-Wallis test. $^{b}P<0.05$, significant difference *vs.* kisspeptin responses in the presence of p234 solvent, Mann-Whitney U test.

Fig. 6. Effects of kisspeptin (10 μM) on sperm kinematic parameters at 2, 15, 30, 60 and 120 min of incubation. (A) Effects on linearity index (LIN) and on the amplitude of lateral head displacement (ALH). (B) Effects on straightness index (STR) and on beat cross frequency (BCF). (C) Effects on hyperactivated motility. Values are means with SEM of 9 experiments and represent percentage changes relative to the response observed at the same time in paired, solvent-treated controls. $^{*}P<0.05$, significant difference *vs.* responses in

solvent-treated controls at the corresponding time, Mann-Whitney U test. [a]$P<0.05$, significant difference *vs.* kisspeptin responses in the presence of p234 solvent, Mann-Whitney U test.

Fig. 7. Effects of kisspeptin on the acrosome reaction (AR). Capacitated sperm samples were treated with kisspeptin (10 μM) or A23187 (10 μM) for 2, 15, 30, 60 or 120 min and the acrosomal status was assessed by staining with fluorescein isothyocianate-conjugated lectin from Pisum sativum (FITC-PSA). Bars are means with SEM of 6 different experiments and were calculated as: (%AR reacted spermatozoa in kisspeptin- or A23187-treated aliquots)-(%AR reacted spermatozoa in the corresponding solvent-treated aliquots at the same time). [*]$P<0.05$, significant difference *vs.* values at time=0, Kruskal-Wallis test.