A caged progesterone analog alters intracellular Ca^{2+} and flagellar bending in human sperm

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Abstract

Progesterone is a physiological agonist for mammalian sperm, modulating its flagellar movement and facilitating the acrosome reaction. To study the initial action of progesterone, we developed a caged analog with a photosensitive group: nitrophenylethanol, at position 20. Using this compound combined with stroboscopic illumination, we performed Ca^{2+} imaging of human spermatozoa and analyzed the effects of progesterone on the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of beating flagella for the first time. We observed a transient $[\text{Ca}^{2+}]_i$ increase in the head and the flagellum upon photolysis of the caged progesterone and an increase in flagellar curvature. Detailed kinetic analysis revealed that progesterone elicits an increase in the $[\text{Ca}^{2+}]_i$ immediately in the flagellum (mid-piece and principal piece), thereafter in the head with a short time lag. This observation is different from the progesterone-induced Ca^{2+} mobilization in mouse spermatozoa, where the Ca^{2+} rise initiates at the base of the sperm head. Our finding is mostly consistent with the recent discovery that progesterone activates CatSper channels in human spermatozoa, but not in mouse spermatozoa.

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Introduction

Progesterone is a steroid hormone involved in the regulation of the female reproductive cycle in mammals. This steroid binds to a nuclear receptor to regulate the expression of target genes (Aranda & Pascual 2001), its so-called genomic action. On the other hand, it is also known that exposure to progesterone increases the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of mammalian sperm through an unidentified receptor in its plasma membrane (Blackmore *et al.* 1991), referred to as its nongenomic action (Losel *et al.* 2003).

Progesterone was isolated as an active component of the human follicular fluid (Osman *et al.* 1989). Its physiological function was initially attributed mainly to its ability to induce the acrosome reaction in sperm. However, this steroid is now known to modulate sperm flagellar beating through the induction of hyperactivated motility (Parinaud & Milhet 1996, Jaiswal *et al.* 1999). Kirkman-Brown *et al.* (2004) and Harper *et al.* (2004) reported that progesterone induces $[\text{Ca}^{2+}]_i$ oscillations in a fraction of human sperm (~9 and ~30%, respectively), which is accompanied with the increase

in the amplitude of the flagellar bend (Harper *et al.* 2004). It has been proposed that this $[\text{Ca}^{2+}]_i$ oscillation is due to Ca^{2+} release from a Ca^{2+} store localized at the base of the flagellum, rather than Ca^{2+} influx through the plasma membrane. On the other hand, the initial transient increase in the $[\text{Ca}^{2+}]_i$ induced by progesterone mainly arises by Ca^{2+} influx (Blackmore *et al.* 1990). Studying how this fast Ca^{2+} increase modifies the flagellar bend has been hampered by liquid flow perturbations caused by mechanically adding progesterone.

The favorable properties of caged compounds have significantly contributed to study chemotaxis and flagellar modulation in sea urchin sperm (Tatsu *et al.* 2002, Kaupp *et al.* 2003, Bohmer *et al.* 2005, Wood *et al.* 2005, 2007, Guerrero *et al.* 2010). In particular, uncaging by a u.v. light pulse creates a near-instantaneous step increase in the concentration of a bioactive molecule without mechanical perturbation of the medium. Actually, an immediate (initial 5 s) effect of progesterone on human sperm motility is difficult to study by conventional methods (Gakamsky *et al.* 2009).

Figure 1 Structure of caged analogs of progesterone. Chemical structure of progesterone (A) and its caged analogs (B, C and D) are illustrated. The P1 has two nitrophenylethanediole groups, indicated in red, at C3 and C20 (B), and P2 (C) and P3 (D) have only one caging group at C3 and C20, respectively. The modification of each nitrophenylethanediole group resulted in two chiral centers indicated by an asterisk, which implies that each caged compound consists of multiple diastereomers. In this study, we used the diastereomer mixture.

Our successful experience with a caged peptide for the study on sea urchin sperm responses (Tatsu *et al.* 2002, Nishigaki *et al.* 2004, Wood *et al.* 2007, Guerrero *et al.* 2010) motivated us to develop a caged derivative of progesterone. Not long ago, the group of Kaupp developed a chemically distinct caged progesterone and demonstrated its utility to study the nongenomic action of progesterone (Kilic *et al.* 2009).

Just recently, two groups independently reported, using whole-cell patch-clamp recording, that progesterone activates CatSper channels in human sperm, but not in mouse sperm (Lishko *et al.* 2011, Strunker *et al.* 2011). Electrophysiological and stopped-flow fluorometry experiments suggest that progesterone directly activates CatSper in human sperm (Kilic *et al.* 2009, Strunker *et al.* 2011). One of the auxiliary subunits of the CatSper channel, CatSper- β (Liu *et al.* 2007), - γ (Wang *et al.* 2009), or - δ (Chung *et al.* 2011), may function as a progesterone receptor as they have large extracellular domains.

In this work, we stimulated human sperm by uncaging our newly synthesized caged progesterone and captured fluorescence images (~ 15 images/s) of Fluo-3-loaded human sperm by stroboscopic excitation. With this time resolution we observed a rapid increase in $[Ca^{2+}]_i$ in both the sperm head and flagellum, and a concomitant increase in flagellar curvature, particularly in the distal region. We discuss the significance of our findings considering the recent discovery that CatSper is a progesterone receptor in human sperm (Kilic *et al.* 2009, Strunker *et al.* 2011).

Results

Development of caged progesterone (P3)

Progesterone has two ketone groups at carbon positions 3 and 20 (C3 and C20). We introduced a photosensitive group, 2-nitrophenylethanediole, at these positions and prepared the three derivatives of progesterone (Fig. 1). Although the biological activity of P3 (nitrophenylethanediole group at position C20) was only slightly reduced (1/8 of progesterone as shown in Supplementary Figure S1, see section on supplementary data given at the end of this article), its uncaging elicited the largest sperm response (Fig. 2). This result was supported by chemical analysis, in which several unexpected byproducts were detected together with progesterone from P1 and P2 after u.v. irradiation (Supplementary Figure S2, see section on supplementary data given at the end of this article). Therefore, we used P3 as our caged progesterone for further experiments.

Ca^{2+} imaging of human sperm with motile flagella

Although the effect of progesterone on sperm $[Ca^{2+}]_i$ has been intensively studied by Ca^{2+} imaging (Kirkman-Brown *et al.* 2000, 2003, 2004, Harper *et al.* 2003, 2004), most studies focused only on the sperm heads and did not record $[Ca^{2+}]_i$ in the beating flagellum due

Figure 2 The increase in the sperm $[Ca^{2+}]_i$ induced by photolysis of caged analogs. The changes in the $[Ca^{2+}]_i$ of human sperm were measured upon the photolysis of caged analogs of progesterone. Panel A shows the representative traces of the $[Ca^{2+}]_i$ changes in the presence of 1 μ M of P1, 320 nM of P2, and 320 nM of P3 (left to right). Each arrow indicates the u.v. light irradiation period (500 ms). Panel B shows the amplitude of fluorescence changes induced by the first u.v. irradiation in the presence of different concentration of caged analogs. Changes of fluorescence intensities were normalized as Supplementary Figure S2. White, black, and striped bars correspond to P1, P2, and P3, respectively. Each value is a mean of at least three experiments with $\pm$ S.E.M. Asterisk indicates statistically significant difference (ANOVA, $P < 0.05$) and all other category comparisons are not significant.

to technical limitations. In this study, we performed Ca^{2+} imaging of human sperm using stroboscopic illumination with LEDs, which allowed us to acquire clear fluorescence images of the moving sperm flagellum while minimizing phototoxicity (Nishigaki *et al.* 2006).

We captured Fluo-3 fluorescence of sperm attached to poly-lysine-coated coverslips by the head with their flagella beating freely. In most of the cells (52/59, three donors), we observed an increase in the $[\text{Ca}^{2+}]_i$ in the head following photolysis of the caged progesterone (Fig. 3). We then carried out detailed analysis of eight individual cells that satisfied the following criteria: i) the flagellum was beating in the same focal plane of the head (49%, 29/59), and ii) it did not crossover with another flagellum (14%, 8/59).

Figure 4A shows a representative series of Ca^{2+} images before and after photolysis of the caged progesterone, indicating that the flagellar beat became more vigorous with an increase in the flagellar curvature at the same time as the increase in the $[\text{Ca}^{2+}]_i$ in the flagellum was observed (Supplementary Movie 1, see section on supplementary data given at the end of this article). As a consequence, the orientation of sperm was also altered in this case (Fig. 4C). The changes in flagellar curvature were quantified after semiautomatic tracing of the flagellar form in every image (BohBoh Software, Tokyo, Japan). Figure 4D shows that the curvatures of the flagellum increased after the stimulus of progesterone. Figure 4E indicates the mean absolute values of the curvature, showing that the curvature increased as the $[\text{Ca}^{2+}]_i$ elevated. The increment in the flagellar curvature induced by progesterone is more notable in the distal part of flagellum than in the basal part.

We also observed different types of sperm responses to progesterone; some flagella did not change orientation even though their curvature changed in the distal part

(Supplementary Figure S3A and B, see section on supplementary data given at the end of this article), another one started turning around by asymmetric flagellar bending (Supplementary Figure S3C). Some spermatozoa did not show a significant increase in flagellar curvature even though their $[\text{Ca}^{2+}]_i$ clearly increased (Supplementary Figure S3D, Supplementary Table S1, see section on supplementary data given at the end of this article). One of those spermatozoa (Supplementary Figure S3D) had a high flagellar curvature even before u.v. stimulation, which may partially explain the insignificant curvature change induced by progesterone. These types of sperm responses may reflect in part the heterogeneity of human sperm but are also probably due to the high basal activity of our caged progesterone (Supplementary Figure S1). Namely, sperm $[\text{Ca}^{2+}]_i$ seems slightly elevated in the presence of P3 before u.v. irradiation. In control experiments, without the caged progesterone, u.v. illumination did not induce any notable effects on either sperm $[\text{Ca}^{2+}]_i$ or flagellar curvature (Supplementary Figure S4, see section on supplementary data given at the end of this article and Supplementary Table S1).

Figure 5A shows the representative spatiotemporal changes of fluorescence intensity of Fluo-3 along the entire flagellum induced by progesterone. The fluorescence intensity of the basal region of the flagellum (mid-piece, mp) is always higher than that of the distal part probably because of the larger cytoplasmic volume at that region. Therefore, we normalized the fluorescence intensity using the mean resting fluorescence intensity (F_0) of each point of the flagellum. As seen in Fig. 5B, the kinetics of the fluorescence changes show no obvious regional variation along the entire flagellum and no apparent propagation of Ca^{2+} wave from a particular site. To confirm this perception,

Figure 3 Ca^{2+} images of Fluo-3-loaded human spermatozoa stimulated by photolysis of the caged progesterone (P3). Fluorescence images of human sperm loaded with Fluo-3 were captured at 15 ips using the system described in Materials and Methods section. Representative fluorescence images before (A) and after (B) photolysis (320 nM P3 and 200 ms u.v. irradiation) are shown using pseudo color. The lower panels are the magnified head images from the upper panels. Color bar indicates fluorescence intensities. Scale bars indicate 10 μm . Panel C shows kinetics of fluorescence changes in sperm around head. The upper trace is the average of normalized fluorescence (F/F_0) from all sperm. The lower traces show the kinetics of individual cells indicated by Roman numerals in the panel A: i, iii, v, and vii (black line) and ii, iv, vi, and viii (red line). As the image (i) includes two cells, one of them is displayed by gray line. Purple lines indicate the u.v. illumination. Arrows indicate the time points, in which the images (A and B) were selected.

Figure 4 Ca^{2+} imaging of human sperm with moving flagellum. Fluorescence images were captured as in Fig. 4. Panel A shows representative series of fluorescence images of human sperm with a moving flagellum using pseudo color. u.v. irradiation (200 ms) was carried out at $t=0$. Color bar indicates fluorescence intensities. Insets show enlarged head images using a different color scale. Panel B illustrates kinetics of normalized fluorescence (F/F_0) in the sperm head (black line) and the entire flagellum (gray line). The purple vertical line indicates the time of u.v. irradiation. Panel C shows forms of the sperm flagellum superimposed before (-3 to 0 s, black) and after (0 – 3 s, blue; 3 – 6 s, red, 6 – 9 s, green) photolysis. Scale bar indicates $10\text{ }\mu\text{m}$. Panel D displays curvatures of the entire sperm flagellum from the basal to the distal portion before (-3 to 0 s, black) and after (3 – 6 s, red) photolysis. Right side curvature is expressed by positive values and the left one by negative values. Panel E indicates the mean absolute value of curvature of the entire sperm flagellum before (-3 to 0 s, black) and after (0 – 3 s, blue, 3 – 6 s, red, 6 – 9 s, green) photolysis. In Panels D and E, the flagellar axis is displayed from the basal to the distal as left to right.

we divided the flagellum into several sections ($5\text{ }\mu\text{m}$ each) from the basal to the distal section and determined the time to reach 50% of the maximum fluorescent intensity ($t_{1/2}$), of each section from eight cells, and found no statistically significant difference among them (Fig. 5C). It is worth noting that the peak F/F_0 value of the basal region was greater than that of more distal regions, which may indicate an additional minor contribution of Ca^{2+} release from an intracellular Ca^{2+} store in the basal part of the flagellum as previously reported (Bedu-Addo *et al.* 2007).

On the other hand, the kinetics of the progesterone-induced $[\text{Ca}^{2+}]_i$ increase in the head and the entire flagellum in the eight cells show some variation; the increase in flagellar Ca^{2+} preceded the increase in Ca^{2+} in the head in some cells (Fig. 4B, Supplementary Figure S3B and S3C), but not in others (Supplementary Figure S3A and S3D). To clarify which is the dominant sperm response pattern, we performed further experiments and analyzed the kinetics of $[\text{Ca}^{2+}]_i$ changes in the three regions: head (h), flagellar mp, and flagellar principal piece (pp). To improve the kinetic analysis of the $[\text{Ca}^{2+}]_i$, instead of tracking flagellar form in each image, regions of interest (ROIs) for each compartment were defined after integrating fluorescence images with

time (30 s), as shown in Supplementary Figure S5, see section on supplementary data given at the end of this article. Thereafter, fluorescence kinetics of each region was determined. Figure 6 shows representative kinetics of $[\text{Ca}^{2+}]_i$ changes in the three regions and the time to reach 50% of maximum response ($t_{1/2}$) in each region ($t_{1/2}$ of individual cells are shown in Supplementary Table S2, see section on supplementary data given at the end of this article). The $t_{1/2}$ of the flagellar mp and pp are significantly shorter than that of the head, indicating that progesterone initiates the Ca^{2+} rise in the flagellum of human sperm. On the other hand, we could not find a statistical difference between the $t_{1/2}$ of the flagellar mp and pp in this analysis as well as those shown in Fig. 5.

Discussion

In this study, taking advantage of a caged progesterone analog, we focused on the initial sperm response to this hormone with a relatively high time resolution (15 images/s). Using Ca^{2+} imaging of motile human sperm, we determined for the first time the effect of photoactivating progesterone on flagellar $[\text{Ca}^{2+}]_i$ and motility. As anticipated, flagellar beat became more

Figure 5 Spatiotemporal analysis of the $[\text{Ca}^{2+}]_i$ changes in the sperm flagellum. Panel A shows representative kinetics of fluorescence changes in the entire flagellum (same sperm as Fig. 4). The fluorescence intensities are expressed according to the color scale. Purple arrow indicates u.v. irradiation (200 ms at $t=0$). Panel B displays the same kinetics as panel A using normalized fluorescence intensities (F/F_0). Panel C shows the average of $t_{1/2}$, time to reach 50% of the maximum fluorescent intensity, of seven sections of flagellum from eight cells with $\pm$ s.d. There is no statistically significant difference among the sections by ANOVA analysis.

vigorous with increased curvature when the $[\text{Ca}^{2+}]_i$ was elevated by progesterone (Fig. 4). At the same time, we observed some spermatozoa which did not show a significant increase in flagellar curvature in spite of the apparent increase in the $[\text{Ca}^{2+}]_i$ (Supplementary Figure S3D and Supplementary Table S1). As already mentioned in Results section, this may be partially due to the heterogeneity of human sperm responsiveness and also because of certain undesirable features of the P3 analog as caged progesterone. Namely, P3 has a high basal activity (1/8 of native progesterone) and thus induces a significant $[\text{Ca}^{2+}]_i$ increase at 320 nM (Supplementary Figure S1). Ca^{2+} imaging of individual human spermatozoa has revealed that progesterone induces a biphasic elevation of the $[\text{Ca}^{2+}]_i$ (Kirkman-Brown *et al.* 2000) and some cells show $[\text{Ca}^{2+}]_i$ oscillations after a transient increase in the $[\text{Ca}^{2+}]_i$ (Harper *et al.* 2004, Kirkman-Brown *et al.* 2004). Therefore, in our experiments in the presence of P3 at 320 nM, the $[\text{Ca}^{2+}]_i$ of most spermatozoa might be higher than that in the control groups. This speculation is supported by the fact that we could trace the Fluo-3 fluorescence in the distal part of the flagellum in the presence of P3 even before u.v. irradiation, but we could not in its absence (Supplementary Figure S4). The elevated basal $[\text{Ca}^{2+}]_i$ may modify the flagellar

curvature before u.v. irradiation and/or desensitize flagellar responsiveness against a further increase in the $[\text{Ca}^{2+}]_i$. In this sense, a caged progesterone with bromohydroxycoumarin possibly works better than P3 to study the sperm flagellar responses because of its low basal activity (Kilic *et al.* 2009).

A common feature of sperm chemotaxis in marine animals is an acute highly asymmetric flagellar curvature during the initial phase of the $[\text{Ca}^{2+}]_i$ increase (Bohmer *et al.* 2005, Shiba *et al.* 2008, Guerrero *et al.* 2010). This strong flagellar asymmetry generates an abrupt change in the swimming direction known as a chemotactic turn (Miller 1985). In this study, we did not observe a similar flagellar bending pattern to the chemotactic turn during sperm responses to progesterone. In marine animals, chemoattractants increase the $[\text{Ca}^{2+}]_i$ of the sperm flagellum with significant time lag (Nishigaki *et al.* 2001, 2004) whose regulation is fundamental for sperm chemotaxis (Bohmer *et al.* 2005, Shiba *et al.* 2008, Guerrero *et al.* 2010). On the other hand, progesterone rises the $[\text{Ca}^{2+}]_i$ of human sperm without a time lag (Kilic *et al.* 2009), indicating that sperm chemotaxis by progesterone in human sperm should be completely different from that of marine animals if it exists.

Another important finding in this study is that progesterone induces an increase in $[\text{Ca}^{2+}]_i$ first at the flagellum (the mp and the pp) of human sperm and with a short time lag in the head. The spatiotemporal pattern of $[\text{Ca}^{2+}]_i$ changes observed in this study is apparently different from the one reported in mouse (Fukami *et al.* 2003), though as yet the $[\text{Ca}^{2+}]_i$ in the entire flagellum of mouse sperm has not been reported. Fukami *et al.* (2003) showed that progesterone initiates an increase in the

Figure 6 Kinetics of the $[\text{Ca}^{2+}]_i$ increase induced by progesterone in the three sperm compartments. Panel A shows representative normalized fluorescence changes of Fluo-3 in the three sperm compartments: head (h; black), mid-piece (mp; red), and principal piece (pp; blue). Regions of interest (ROIs) of three compartments were defined as described in Supplementary Figure S5. Mean fluorescent intensities of each ROI were obtained and normalized. Due to a large noise in the pp, fluorescent signals of only this compartment were processed with three-point rolling average filter (67×3 ms). Panel B shows the averages of the time to reach 50% of the maximum fluorescent intensities ($t_{1/2}$). Error bars indicate $\pm$ s.d. (ten cells $\times$ three donors). Two-tailed ANOVA analysis (Tukey–Kramer) indicates the $t_{1/2}$ of the head is statistically different from those of two compartments of the flagellum (* $P < 0.01$, ** $P < 0.001$).

$[Ca^{2+}]_i$ at the base of the mouse sperm head, which spreads to its apical part. This Ca^{2+} wave propagation is relatively slow and can be visualized in slow Ca^{2+} imaging experiments (1 image/2 s). Very recently, it was finally revealed that CatSper is a Ca^{2+} channel that mediates the progesterone-induced Ca^{2+} influx in human sperm (Lishko *et al.* 2011, Strunker *et al.* 2011). Interestingly, mouse CatSper does not seem to be activated by progesterone (Lishko *et al.* 2011). Therefore, the difference in the spatiotemporal pattern of the progesterone-induced Ca^{2+} transient between the two species could be now reasonably accounted by the distinct regulation of CatSper channels. Considering that CatSper is localized in the pp of the flagellum and indispensable for the physiological hyperactivated motility of mouse sperm (Carlson *et al.* 2003), it is reasonable that we observed that progesterone rapidly increases the flagellar $[Ca^{2+}]_i$ and flagellar curvature in human sperm. On the other hand, we did not observe a slow Ca^{2+} wave from the sperm flagellar pp to the head as observed in mouse sperm upon CatSper channel activation (Xia *et al.* 2007). Instead, we recorded a rapid and simultaneous Ca^{2+} increase in the flagellar mp and the pp, which is propagated into the entire cell body within a few seconds. We can think of four possible explanations for this observation: i) there may be another progesterone receptor in the mp, which induces an increase in $[Ca^{2+}]_i$ by a different mechanism; ii) Ca^{2+} diffusion from the pp to the mp in human sperm should be faster than that reported in mouse sperm (Xia *et al.* 2007) because of the difference of the size of mp between two species and experimental conditions (at 37 °C in this work and at room temperature in the work of Xia *et al.* (2007)); iii) the use of the time to reach 50% of maximum response ($t_{1/2}$) could lead to a wrong interpretation of the real Ca^{2+} propagation pattern; iv) human CatSper channel may be localized in the mp besides the pp.

Although CatSper is not activated by progesterone in mouse sperm, progesterone somehow elicits the Ca^{2+} transient in these cells even from CatSper1-null mice (Ren *et al.* 2001, Fukami *et al.* 2003). This fact suggests that progesterone might activate another Ca^{2+} influx pathway besides CatSper in mammalian sperm. Zhu *et al.* (2003a, 2003b) discovered a new type of progesterone receptor in fish oocytes that has typical features of G-protein coupled receptors, and they also found its mammalian homolog (mPR α) in human testis. The same group determined the localization of the mPR α protein in the mp of human sperm by immunocytochemistry (Thomas *et al.* 2008). These results indicate mPR α is a candidate for the progesterone receptor, which contributes a rapid Ca^{2+} influx in the flagellar mp.

Just recently, it was reported that the prostasomes supply some membrane proteins to human sperm that modify their function (Park *et al.* 2011). Although mouse sperm are usually obtained from the epididymis, human

sperm are normally obtained from ejaculated semen. Thus, human spermatozoa are already exposed to the prostasomes and other active compounds of seminal fluids in our experiments. This difference in the preparation of the cells may lead to functional variations in the spermatozoa of the two species (Ren 2011). Further investigations, such as biochemical identification of progesterone receptor(s) in each species and Ca^{2+} imaging in the presence of CatSper channel blockers in human sperm, are required for better understanding of the action of progesterone in mammalian sperm.

Materials and Methods

The use of human sperm in this study was approved by the Bioethics Committee at the Institute of Biotechnology, National Autonomous University of Mexico. All healthy donors gave written informed consent. Qualities of semen used in study were as follows: semen volume (2–5 ml), sperm density ($20\text{--}95 \times 10^6$ spermatozoa/ml), viability (74–98%), motility (54–85%), and normal morphology (15–18%), which are slightly better than normal semen quality defined by WHO (Cooper *et al.* 2010). Fluo-3 AM was from Invitrogen. Other reagents, unless indicated, were from Sigma–Aldrich.

Synthesis of caged analogs of progesterone

2-Nitrophenylethanediol and progesterone were obtained from Aldrich. Progesterone (1.57 g, 5 mmol), 2-nitrophenylethanediol (3.66 g, 20 mmol), and *p*-toluenesulfonic acid (75 mg) were dissolved in benzene (70 ml) and heated under reflux through anhydrous $MgSO_4$ for 16 h (Gravel *et al.* 1983). The solution was washed with 10% $NaHCO_3$ aq and saturated $NaCl$ aq, and the organic layer was dried over anhydrous $MgSO_4$ and evaporated to give the mixture of isomers of caged progesterone (3.1 g). The products were separated by gel permeation chromatography (GL Sciences, Inc., Tokyo, Japan, PU714), using TSKgel G1000H_{HR} (TOSOH Corp., Tokyo, Japan, 21.5 mmID $\times$ 600 mm) and TSKgel G1000H₆ (TOSOH Corp., 21.5 mmID $\times$ 1200 mm) columns at a flow rate of 7 ml/min. The elution containing the target was recycled from the detector at 275 nm into the columns. The u.v. monitor was not used at the final recycling and three fractions were obtained. The first fraction, P1 (1.3 g), was assigned to the di-caged progesterone derivative of which C3 and C20 of progesterone were protected with 2-nitrophenylethanediol: ESI-MS (Thermo Electron, Waltham, MA, USA; LC deca XP plus; calcd., 644.31 obsd., $([M+H]^+)$ 644.94. The second fraction, P2 (1.1 g), was assigned to mono-caged progesterone derivative at the carbonyl 3: ESI-MS (calcd., 479.27, $([M+H]^+)$ 480.15. The third fraction, P3 (0.7 g), was assigned to mono-caged progesterone derivative at the carbonyl 20: ESI-MS (calcd., 479.27; obsd., $([M+H]^+)$ 480.18. P2 and P3 were identified by 1H NMR. The singlet peak at 5.74 ppm was clearly seen in P3, which can be assigned to the proton at C4, whereas the peak was not observed in P2.

$[\text{Ca}^{2+}]_i$ measurements in human sperm population

Fluo-3-loaded human sperm was prepared as previously described (Nishigaki *et al.* 2006) with some modifications. We used a noncapacitating medium omitting BSA (Bedu-Addo *et al.* 2005), which consists of (mM) NaCl (120), KCl (4), NaHCO_3 (15), MgCl_2 (1), CaCl_2 (0.3 or 2), HEPES (10), D-glucose (5), sodium pyruvate (1), and sodium lactate (10), pH 7.4 adjusted with NaOH. We used 0.3 mM Ca^{2+} for sperm preparation and Fluo-3 loading, but 2 mM Ca^{2+} for the $[\text{Ca}^{2+}]_i$ measurements. $[\text{Ca}^{2+}]_i$ measurements in human sperm populations were performed using an Aminco SLM 8000 spectrofluorometer upgraded by Olis (Bogart, GA, USA). To quantify the sperm response to progesterone and its caged analogs, we used 600 μl of sperm suspension (2×10^6 cells/ml) in a flat bottom glass tube (ID 8×50 mm) with magnetic stirring at 37 °C and injected 2 μl of test solution (in DMSO) using a Hamilton microsyringe. For photolysis experiments, we used 200 μl of sperm suspension (4×10^6 cells/ml) in a Pyrex glass tube (ID 4×50 mm) without stirring. Uncaging was achieved with a u.v. lamp, UVICO (Rapp Optoelectronic, Hamburg, Germany), which has a built-in shutter combined with UV2 filter (270–380 nm). The Pyrex glass tube was coupled to the UVICO through a liquid light guide. A pulse stimulator, Master-8 A (AMPI, Jerusalem, Israel), was used to the control the u.v. irradiation time of the UVICO.

Ca^{2+} imaging of individual human sperm

Fluorescence images of Fluo-3-loaded human sperm were captured using a LED-based pulsed light excitation system as previously described (Nishigaki *et al.* 2006) with some modifications. As indicated above, u.v. uncaging was achieved using the UVICO system coupled to a spot illumination system (Rapp OptoElectronic) combined with dichroic mirror M40-DC400 and liquid light guide (4 mm $\times$ 2 m). To maintain the sample at 37 °C, an objective warmer system, OWS-2 (Warner Instruments, Hamden, CT, USA), was used; an open perfusion micro incubator, PDMI-2 (Harvard Apparatus, Holliston, MA, USA), was also used. Fluorescence images were collected using a Nikon PlanApo 40 $\times$ (1.4 NA oil immersion; Nikon, Melville, NY, USA) objective and an iXon (512 $\times$ 512 pixels) EMCCD camera (Andor Technology, Belfast, Northern Ireland) with binning 2 $\times$ 2 using AndorIQ software. Fluorescence images were captured at ~ 15 images per second (ips) with 1 ms LED pulse duration.

A poly-L-lysine (100 $\mu\text{g}/\text{ml}$) coated round coverslip (25 mm diameter) was mounted in a chamber of the PDMI-2 incubator and 10 μl human sperm suspensions were deposited on the coverslip and left for a few minutes. Sperm unattached to the coverslip were removed by gentle washing and the chamber was filled with the medium containing caged progesterone, P3. A small cover slip was placed on the sample in some experiments in order to retain the head and the flagellum in the same focal plane. Regions where most of cells were attached to the coverslip only by the head (with the flagellum moving freely) were selected for imaging. For Ca^{2+} imaging, we used human spermatozoa incubated in a capacitating medium

(the medium described earlier with 5 mg/ml BSA and 2 mM Ca^{2+} at final) at least for 4 h at 37 °C with 5% CO_2 .

Image processing

Image J (National Institutes of Health, <http://rsbweb.nih.gov/ij/>) was used to convert monochrome fluorescence images acquired by iQ software (Andor Technology) into pseudo color images. The same software was used to create a movie file. To obtain the flagellar form (series of dots defined by x, y positions), the curvature and the fluorescence intensity as CSV files, the fluorescence image (TIF file) was semiautomatically traced using BohBoh Software. The CSV files were processed by Excel (Microsoft) and SigmaPlot10 (SSI, San Jose, CA, USA) to draw figures. Fluorescence intensity of the head region was obtained using a circular ROI covering the whole area of the moving head instead of tracing the head movement in each image with Image J.

Statistical analysis

All numerical data are presented as mean values $\pm$ S.E.M. unless otherwise mentioned. Statistical tests were performed using two-tailed ANOVA (Tukey–Kramer) with KyPlot (freeware) and two-tailed Student's *t*-test with Excel (Microsoft).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0268>.

Declaration of interest

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

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