Acrosome Reaction and Ca$^{2+}$ Imaging in Single Human Spermatozoa: New Regulatory Roles of [Ca$^{2+}$]$_i$1

Claudia Sánchez-Cárdenas,3 Martha Rocío Servín-Vences,3 Omar José,3 Claudia Lydia Treviño,3 Arturo Hernández-Cruz,4 and Alberto Darszon2,3

1Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, México
2Departamento de Neurociencia Cognitiva, Instituto de Fisiología Celular, UNAM, Circuito exterior s/n, Ciudad Universitaria, México DF

ABSTRACT

The spermatozoa acrosome reaction (AR) is essential for mammalian fertilization. Few methods allow visualization of AR in real time together with Ca$^{2+}$ imaging. Here, we show that FM4-64, a fluorescent dye used to follow exocytosis, reliably reports AR progression induced by ionomycin and progesterone in human spermatozoa. FM4-64 clearly delimits the spermatozoa contour and reports morphological changes before, during, and after AR. This strategy unveiled the formation of moving tubular appendages, emerging from acrosome-reacted spermatozoa, which was confirmed by scanning electron microscopy. Alternate wavelength illumination allowed concomitant imaging of FM4-64 and Fluo-4, a Ca$^{2+}$ indicator. These AR and intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) recordings revealed that the presence of [Ca$^{2+}$]$_i$ oscillations, both spontaneous and progesterone induced, prevents AR in human spermatozoa. Notably, the progesterone-induced AR is preceded by a second [Ca$^{2+}$]$_i$ peak and ~40% of reacting spermatozoa also manifest a slow [Ca$^{2+}$]$_i$ rise ~2 min before AR. Our findings uncover new AR features related to [Ca$^{2+}$]$_i$.

acrosome reaction, calcium, FM4-64, progesterone, sperm

INTRODUCTION

The acrosome reaction (AR) is a prerequisite for spermatozoa to successfully fertilize the oocyte/egg. This process is strictly dependent on an increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) [1]. Determining the magnitude of the AR and its associated [Ca$^{2+}$]$_i$ changes continuously in time is needed to decipher this fundamental process, as many questions remain regarding the kinetic and location correlations between these two physiological parameters (reviewed in [1–3]). Although [Ca$^{2+}$]$_i$ determinations continuously in time have been performed in both cell populations [4, 5] and single human spermatozoa [4, 6, 7] for quite a while, AR measurements have typically been obtained in fixed spermatozoa previously exposed to the experimental conditions of interest [8]. In an attempt to correlate the AR with its associated [Ca$^{2+}$]$_i$ changes, bovine and human spermatozoa were fixed after the completion of Ca$^{2+}$ recordings [4, 9, 10]. More recently, transgenic expression of fluorescent proteins allowed real-time measurements of AR progression in mouse sperm [11, 12], but this approach is not available for human sperm. Nonetheless, two novel strategies were devised using fluorescent probes externally added to follow the AR in real time in human spermatozoa. One of them used specific fluorescent labeling of two different acrosome components: soybean trypsin inhibitor, which binds to acrosin, an enzyme contained in the acrosome, and an antibody against CD46, a protein localized in the inner acrosomal membrane [13]. The other strategy monitored real-time binding of fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA), a lectin that has been extensively used as an AR reporter in fixed spermatozoa [8, 14]. However, only one study reported simultaneous [Ca$^{2+}$]$_i$ and AR determinations in human spermatozoa using a single dye (Fluo-3) as indicator of both parameters. In spite of this, it was not firmly established how closely Fluo-3 leakage follows AR, and morphological confirmation of the AR process was not provided [15].

The sperm AR is an exocytotic event that shares molecular mechanisms with somatic cell exocytosis [16]. The family of FM dyes displays ideal properties for selective tracking of membrane dynamics during exocytosis and endocytosis in somatic cells [17–20]. Here we found that FM4-64 labeled the sperm plasma membrane, and, interestingly, its fluorescence increased in the acrosome region upon induction of the AR. This increased fluorescence correlated with FITC-PSA staining of the acrosome, suggesting that FM4-64 reliably detects the onset and development of the AR. Also, the delineation of the sperm plasma membrane by FM4-64 allowed us to monitor form and/or size changes of the membrane during and after AR. Interestingly, FM4-64 unveiled the formation of tubular appendages, emerging from different areas of the sperm head as the AR was progressing. The formation of these appendages is not a staining artifact, because they were verified with scanning electron microscopy (SEM). Appendage formation was never observed in non-acrosome-reacted sperm cells. Because of its fluorescence properties, FM4-64 imaging can be simultaneously or alternately (here also referred to as concomitantly) performed together with [Ca$^{2+}$]$_i$ imaging using...
Materials and Methods

Materials

FM4-64, Fluor-4 AM, and Sytox Green (SG) were from Life Technologies Corporation (Invitrogen). Bovine serum albumin, Ham F-10 media, FITC-PSA, and progesterone were purchased from Sigma-Aldrich Chemical Co. Ionomycin was from Alomone Labs. All other chemicals were of reagent grade.

Ejaculates and Sperm Swim-up Preparation

The use of human sperm in this study was approved by the Bioethics Committee of the Instituto de Biotecnología, Universidad Nacional Autónoma de México. All healthy donors filled and signed a written informed consent. Semen samples used in this study fulfilled the requirements defined by the World Health Organization for human samples. After liquefaction, 1 ml of Ham F-10 culture medium was applied above 1 ml of semen and sperm were allowed to swim up into the upper layer of the medium for 1 h in an incubator at 37 °C, saturated with 5% CO2-95% air. Then, the upper layer was separated and the spermatozoa that will acrosome react display a second [Ca2+]i peak just before the reaction starts. Some of these spermatozoa also exhibit a slow Ca2+ rise around 2 min before AR initiation.

Imaging of Individual Human Spermatozoa

After capacitation, spermatozoa were centrifuged and resuspended in recording medium (in mM: NaCl 120, KCl 4, NaHCO3 15, MgCl2 1, CaCl2 2, HEPES 10, d-glucose 5, sodium pyruvate 1, lactic acid 10, pH 7.4). Spermatozoa were immobilized on polylysine (100 µg/ml)-coated 25-mm-diameter coverslips, unattached spermatozoa were removed by gentle washing, and the chamber was filled with the recording medium. Before starting FM4-64 experiments, spermatozoa were incubated in recording medium containing 10 µM FM4-64 during 5 min; FM4-64 was present in the extracellular medium throughout the experiments. For alternate FM4-64 and Fluor-4 imaging, spermatozoa were incubated during 30 min with 2 µM Fluor-4-AM dissolved in dimethyl sulfoxide and 0.05% pluronic acid, and the cells were then centrifuged and incubated with recording medium containing 10 µM FM4-64. For alternate FM4-64-FITC-PSA and FM4-64-SG imaging, the dyes were added together in the recording medium. Final concentrations of FITC-PSA and SG were 5 µg/ml and 100 nM, respectively. For viability experiments we used Triton X-100 (0.1%) to permeabilize (kill) cells at the end of each experiment. We considered cells dead when they were stained at least 30% of the SG fluorescence intensity reached after Triton X-100 treatment. Recordings were performed at 37°C using a temperature controller model 202A (Harvard Apparatus). Spermatozoa were viewed with an inverted microscope (Eclipse Ti-U, Nikon) and an oil immersion fluorescence objective (Plan APO TIRF DIC H/N2 60x/1.45 NA, Nikon). A precentered fiber illuminator, Nikon Intensilight-CHGFL, was used as light source. For excitation and emission collection of Fluor-4, FITC-PSA, and SG the filter set GFP 96343, dichroic mirror (D): 495, excitation 470/40, barrier 525/50 (Nikon) was used. For excitation and emission collection of FM4-64, the filter set Wide Green 11007v2, D: 565 dexc, excitation 535/50, emission 590 lpv2 (Chroma Technology Corporation) was used. Fluorescence images were acquired with an Andor iXon 3 EMCCD camera model DU-8970-C008B (Andor Technology) or Hamamatsu Orca-100 CCD model C4742-95 (Hamamatsu Photonics) under protocols written in Andor iQ 1.10.2 software Version 4.0. The motorized filter cube exchanger of our imaging system alternates Fluor-4 and FM4-64 filter cubes according to a preestablished protocol. The shortest interval possible for filter cube exchange is approximately 400 msec, and this time is the delay between each pair of Fluor/FM4-64 images. However, because our experiments last up to 30 min, and we wished to prevent cellular damage and bleaching due to overexposure to light, a pair of images (Fluor-FM4-64) was taken every 2–5 sec. This is the time resolution of our data. Images were acquired at 0.2-0.5 Hz with an exposure/illumination time of 50 msec for periods of 5–30 min. Sixteen-bit images were obtained and movies were processed and analyzed with macros built into NIH ImageJ Version 1.43.67 (National Institutes of Health). Regions of interest were drawn on each spermatozoon in the movie for quantification. A plot of the fluorescence intensity of each spermatozoon versus time was generated in Origin 6.0 (Microcal Software). Fluorescence is expressed as (F - F0)/F0. When brightness and contrast were adjusted, this was done equally in all images or movies taken under the same conditions.

Scanning Electron Microscopy

Capacitated human spermatozoa were prepared and immobilized on polylysine-coated (100 µg/ml) glass coverslips as indicated below. Spermatozoa exposed to progesterone during 15 or 30 min were fixed using Karnovsky fixative and maintained at 4°C for 2 h. Then they were exposed to 1% buffered osmium tetroxide, dehydrated using graded ethanol series, and dried by the critical point technique [22]. Spermatozoa were then coated using gold particles by evaporated in vacuum and observed with a scanning electron microscope (JSM-5410LV, JEOL). Images were obtained using Digital Scan Generator 1-plus 1.47 software. Amplification of images was between 2000× and 15000×. The images were obtained in bmp format and then opened in Image J Version 1.4.3.67.

Statistical Analysis

All numerical data are presented as ± standard error of the mean and in a few instances we include the standard deviation. Statistical tests were performed using Student’s t test and ANOVA test.

RESULTS

FM4-64 Distribution Changes in Response to AR Inducers

FM4-64 readily labels the plasma membrane of motile human spermatozoa. It even allows clear visualization of the cytoplasmic droplet (Fig. 1A, white arrows right panel), which is hardly distinguishable by phase-contrast microscopy (Fig. 1A, white arrows left panel). Application of 10 µM ionomycin (Fig. 1B, black arrowhead), a potent AR inducer, promotes the redistribution of FM4-64 staining and increases its fluorescence in the acrosomal region of spermatozoa (Fig. 1B and Supplemental Movie S1; all supplemental material is available online at www.biolreprod.org), suggesting that the dye gains access to the internal acrosomal membrane. Figure 1C illustrates the dynamics of the FM4-64 distribution after ionomycin exposure in a representative experiment. It should be pointed out that because the dye is present in the external media, the addition of progesterone or ionomycin, dissolved in dye-free solution, results initially in FM4-64 fluorescence decrease due to dye dilution. To rule out the possibility that dye uptake could be related to cell damage, viability experiments were performed using SG, a cell death reporter, after exposing spermatozoa to either ionomycin or progesterone in the presence of FM4-64 (see Materials and Methods). Figure 1D (lower panel) shows that few cells display SG fluorescence even after 30 min of treatment with these AR inducers, whereas a significant proportion of them increase their FM4-64 fluorescence (upper panel). In contrast, many cells increased their SG fluorescence after Triton-induced sperm death (lower panel). Analysis of these experiments reveals that ~80% and 67% of sperm showed no SG staining even after 30 min exposure to ionomycin or progesterone, respectively (Fig. 1E). Cells were considered dead when SG fluorescence intensity reached >30% of that shown by Triton-treated spermatozoa.
FM4-64 Labels the Acrosome at the Beginning of the AR

To confirm that the FM4-64 fluorescence increase observed in the acrosome of spermatozoa is a good indicator of AR, we used FITC-PSA, which in unfixed cells can gain access into the acrosome only when spermatozoa react [14]. In these experiments spermatozoa were incubated in the presence of both FITC-PSA and FM4-64 (Fig. 2). When the AR was induced either with progesterone or ionomycin (Fig. 2, A and B arrowhead at ~80°), spermatozoa labeling progressed in parallel with both dyes. Sometimes the FM4-64 fluorescence increase in the acrosome region preceded FITC-PSA staining (Fig. 2, A–C), but the difference in latency was not statistically significant. The percentage of acrosome-reacted sperm stimulated by ionomycin was 79.8% ± 5.9% when detected by FM4-64 fluorescence and 71.2% ± 8.2% when using FITC-PSA; the difference between these AR detection assays was not significant (Fig. 2D). Comparison of the time course of fluorescence staining with both dyes during the AR revealed gradual increases often reaching a plateau level (Fig. 2E; red, upper trace, FM4-64; green, lower trace, FITC-PSA). However, Figure 2F shows that FITC-PSA fluorescence reached a plateau more frequently, whereas in general, FM4-64 staining kept increasing or displayed an increase, a transitory plateau, and then a sustained increase (see Supplemental Fig. S1).

The AR is an exocytotic process that depends on the presence of extracellular Ca2+. If FM4-64 is truly reporting the...
FIG. 2. Evidence that FM4-64 acrosome staining corresponds to the onset of the AR. A) Concomitant imaging of FM4-64 and FITC-PSA during ionomycin application to human spermatozoa. Ionomycin applied at ~80 sec (black arrowhead indicates its addition) increases FM4-64 fluorescence in the sperm acrosomal region (red images, 160°–165°; left column). In contrast FITC-PSA labeling is not noticeable before 170° (white arrow, middle column). Merged images on the right column show both FM4-64 and FITC-PSA fluorescence in the acrosome region, though FM4-64 also labels the neck and midpiece. Bar = 3 μm. B) Time course of the fluorescence changes of FM4-64 and FITC-PSA (red and green traces respectively) in the acrosome of human spermatozoa after ionomycin and progesterone treatment (IONO N Ca²⁺, IONO L Ca²⁺, PROG N Ca²⁺, PROG L Ca²⁺, respectively).
occurrence of the AR, the intensity of fluorescence staining should depend on the amount of Ca\(^{2+}\) present in the medium.

FM4-64 staining in the acrosome region induced by ionomycin/progesterone application was significantly reduced when extracellular Ca\(^{2+}\) was diminished from 2 mM to 100 nM\((^{*}P<0.05\) and \(*^{*}P<0.01\), Fig. 2G; n = 5; 402 and 352 cells analyzed respectively). These findings corroborate that FM4-64 acrosome staining depends on external Ca\(^{2+}\), and therefore it is a potential indicator of the extent of AR. Measurements of FM4-64 fluorescence quantified during 5-min-long time windows showed that about 10 min after ionomycin application, nearly half of the cells were already reacted, whereas in the case of progesterone, spermatozoa continued reacting even after 20 min of its application (Fig. 2H). Notably, three distinct patterns of FM4-64 fluorescence increase (AR initiation) were observed: 1) apical (AP), characterized by the initial uptake of FM4-64 in the AP zone of the head; 2) lateral (LAT), in which the uptake of FM4-64 started on the side of the acrosome; and 3) ascending (ASC), where the uptake of FM4-64 began at the postacrosomal area or below, continuing upwards, towards the acrosome (Fig. 2I). The majority of spermatozoa displayed the AP pattern of AR initiation upon progesterone stimulation, whereas after ionomycin the AP and LAT patterns were similarly frequent (Fig. 2J and Table 1).

**FM4-64 Staining Revealed Novel Sperm Membrane Structures**

FM4-64 staining also revealed a novel and unique phenomenon. A few minutes\((2.65 \pm 2.5)\) after AR initiation with progesterone, striking tubular structures that moved vigorously appeared from the head in approximately 36% ± 5% of spermatozoa. These structures varied in size and number; they sometimes emerged from a LAT region of the head whereas in other instances they developed from the AP zone of the head (Fig. 3A and Supplemental Movies S2–S4). SEM further confirmed the formation of these structures upon AR stimulation, ruling out that they represented an artifact or damage from FM4-64 staining and/or illumination with excitation light (Fig. 3B). For SEM observations, experiments were carried out omitting the FM4-64 staining. Briefly, spermatozoa were exposed to progesterone for 15 or 30 min and then fixed and processed as required (see material and methods). It is worth mentioning that SEM underestimates the percentage of AR spermatozoa displaying appendages because the technical procedure required often results in their breakage (Supplemental Fig. S2). For these reasons, we did not attempt a systematic quantification of these structures. Also in accordance with our previous observations, these appendages arose from different head areas. A comparison of appendages seen using FM4-64 fluorescence and SEM is shown in Figure 3, C–E. The similarities demonstrate not only that these structures are genuine, but that they do not depend on the presence of the dye or its excitation. Similar appendages were also observed when the AR was induced with ionomycin.

**Alternate Fluorescence Monitoring of AR (FM4-64) and \([Ca^{2+}]_i\) in Single Human Spermatozoa**

Spermatozoa were loaded with FM4-64 and the Ca\(^{2+}\) sensitive dye Fluo-4 to monitor the occurrence of the AR and the associated Ca\(^{2+}\) dynamics in real time. A representative fluorescence image of nonstimulated cells loaded with both dyes is illustrated in Figure 4A. As shown earlier, FM4-64 labels the contour of non-acrosome-reacted spermatozoa, whereas Fluo-4 fluorescence is observed homogeneously distributed inside the sperm head and midpiece, the regions usually in focus. In these experiments, ionomycin and progesterone promoted a \([Ca^{2+}]_i\) increase that led to the AR in 75.9% ± 14% and 12.1% ± 2.7% of the spermatozoa, respectively (Fig. 4, B and C, and Supplemental Movies S5 and S6). Right-side panels in Figure 4, B and C, illustrate representative fluorescence traces from FM4-64 and Fluo-4 labeled single spermatozoa (white arrows) during and after ionomycin and progesterone application, respectively. As previously shown (Figs. 1B and 2, A and B), the onset of the AR, revealed by FM4-64, is characterized by an increased staining in the acrosome region and a smaller one in the midpiece (Fig. 4D, FM4). FM4-64 fluorescence increase is observed on average 114 ± 17 and 539 ± 47 sec after the \([Ca^{2+}]_i\) increase induced by ionomycin and progesterone, respectively. With the time resolution of these experiments (one image every 2.5 sec), the \([Ca^{2+}]_i\) increase induced by progesterone appears to begin close to the base of the head. Faster measurements (10 images/sec) performed in spermatozoa immobilized with concanavalin A (not shown) revealed that \([Ca^{2+}]_i\) increases initiate in the sperm principal piece, as previously reported [7]. Notably, the majority of cells that displayed FM4-64 fluorescence increase experienced a late decrease of Fluo-4 fluorescence (Fig. 4D, 172\(^{+}\)), which is likely because of the leakage of intracellular dye resulting from the formation of multiple fenestrations associated with the AR. Similar results were reported previously, and in fact, the fall in Fluo-3 fluorescence was used as a reporter of the AR [15]. The full loss of Fluo-4 fluorescence (Fig. 4D [EF4] and E and Fig.

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**TABLE 1. FM4-64 fluorescence initiation sites during spermatozoa AR.**

<table>
<thead>
<tr>
<th>FM4-64 fluorescence patterns</th>
<th>Ionomycin(^a)</th>
<th>Progesterone(^b)</th>
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<tr>
<td>Apical pattern</td>
<td>42.3 ± 7</td>
<td>59.6 ± 8</td>
</tr>
<tr>
<td>Lateral pattern</td>
<td>39.4 ± 6</td>
<td>27.2 ± 9</td>
</tr>
<tr>
<td>Ascendant pattern</td>
<td>18.1 ± 6</td>
<td>15.8 ± 6</td>
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\(^a\) n = 7 independent experiments, 95 analyzed cells. Results are expressed as % of spermatozoa.

\(^b\) n = 10 independent experiments, 395 analyzed cells. Results are expressed as % of spermatozoa.
FIG. 3. FM4-64 reveals structural details during AR. A) Selected images of an FM4-64 stained human spermatozoon undergoing AR. Progesterone addition is indicated by an arrowhead; time in seconds is shown in each figure. Upper row: example of a tubular appendage emerging from the head of a spermatozoon (arrow; Supplemental Movie S2). Middle row: a spermatozoon with several appendages evolving from its head during the AR (arrows; Supplemental Movie S3). Lower row: human spermatozoon with a moving appendage emerging from its head (arrow; Supplemental Movie S4). Bars = 3 μm. B) SEM micrographs of human spermatozoa incubated with progesterone displaying tubular appendages similar to those observed during FM4-64.
S3 in supplementary material [Empty F4]), could be associated with the emptying of the acrosome content and therefore the termination of the AR. This Fluo-4 fluorescence loss is delayed by approximately 254 ± 4 and 224 ± 8 sec relative to the FM4-64 fluorescence increases induced by progesterone and ionomycin respectively. This delay may be considered as the duration of the AR, which accordingly lasts on average approximately 4 min (see Table 2 and EF4-FM4, Supplemental Fig. S3). Interestingly, closer inspection of FM4-64 and [Ca\(^{2+}\)]\(_i\) signals in spermatozoa that reacted revealed that the typical progesterone-induced [Ca\(^{2+}\)]\(_i\) transient increase is followed by a second Ca\(^{2+}\) peak (indicated as P in Fig. 4) that immediately precedes AR initiation (pattern 4; Fig. 4, F–H). Practically all spermatozoa that acrosome reacted (98%) showed this pattern. Figure 4F shows Fluo-4 and FM4-64 fluorescence images of a representative spermatozoon during and after progesterone stimulation (black arrowhead). The second Ca\(^{2+}\) peak occurred in the panel labeled 731%. The images illustrate that AR initiates concomitantly with this second Ca\(^{2+}\) peak. In fact, the Fluo-4 and FM4-64 fluorescence traces in Figure 4G corresponding to the same spermatozoon shown in Figure 4F reveal that the second Ca\(^{2+}\) peak slightly precedes the peak FM4-64 increase. The mean time that the second Ca\(^{2+}\) peak took to reach maximum fluorescence from the baseline was ~40.9 ± 5.9 sec. Interestingly, ~40% of spermatoza that acrosome react show a slow Ca\(^{2+}\) rise (indicated as R in Fig. 4) that on average started 111.9 ± 13.1 sec before the second Ca\(^{2+}\) peak. After the progesterone initial [Ca\(^{2+}\)]\(_i\) increase (panel labeled 50° in Fig. 4F) a plateau was reached (Fig. 4F, panel labeled 243°; see Fig. 4G) and then a slow Ca\(^{2+}\) rise triggered the second Ca\(^{2+}\) peak, (Fig. 4F, panel labeled 625°) indicated by an arrow in Figure 4G. The remaining spermatozoa that reacted displayed three different [Ca\(^{2+}\)]\(_i\) patterns before AR (see Fig. 4H). “Single peak” refers to spermatozoa that undergo AR after the first progesterone-induced [Ca\(^{2+}\)]\(_i\) peak. “Two peaks” refers to spermatozoa that react after two consecutive progesterone-induced [Ca\(^{2+}\)]\(_i\) peaks. These two [Ca\(^{2+}\)]\(_i\) patterns were observed in 23% and 18%, respectively of spermatoza undergoing AR. The third [Ca\(^{2+}\)]\(_i\) pattern, called “slow Ca\(^{2+}\) rise absence,” corresponded to spermatozoa that lacked the slow Ca\(^{2+}\) rise and was present in 20% of acrosome-reacted spermatozoa (115 analyzed sperm, n = 9).

Next we evaluated if the AR was correlated with a certain [Ca\(^{2+}\)]\(_i\) pattern induced by progesterone. We first identified four general patterns of progesterone-induced [Ca\(^{2+}\)]\(_i\) changes in spermatozoa: 1) transient, 2) transient-sustained, 3) transient-oscillatory, and 4) double transient and recovery (Fig. 5 and Table 3). Analysis of these results indicated that the reduction of AR observed in spermatozoa that manifested transient-oscillatory [Ca\(^{2+}\)]\(_i\) responses promoted by progesterone was statistically significant (P = 0.013). Consistent with previous reports [21, 23], we noticed that spermatozoa showed spontaneous [Ca\(^{2+}\)]\(_i\) oscillations of different amplitude and frequency (see Fig. 6, A–C, and Supplemental Movie S7). We quantified this behavior and found that 35% ± 6% of capacitated spermatozoa displayed this behavior and wondered if the presence or absence of spontaneous [Ca\(^{2+}\)]\(_i\) oscillations at rest could influence the ability of sperm to undergo the progesterone-induced AR. Thus, we performed 10-min-long recordings of Fluo-4 and FM4-64 fluorescence signals prior to progesterone stimulation and continued recording for at least another 10 min afterwards. Interestingly, 97.6% ± 2% of the spermatozoa that displayed spontaneous [Ca\(^{2+}\)]\(_i\) oscillations at rest failed to undergo AR upon the addition of progesterone (only 2.3% ± 2% reacted) (Fig. 6F). The difference between these two preconditions is significant (P = 0.013). Conversely, 32% ± 8% of spermatozoa lacking spontaneous [Ca\(^{2+}\)]\(_i\) oscillations at rest underwent AR in response to progesterone treatment. Given that [Ca\(^{2+}\)]\(_i\) oscillations, both spontaneous as well as those promoted by progesterone, inhibited AR, possibly the spontaneous [Ca\(^{2+}\)]\(_i\) oscillations conditioned spermatozoa to respond to progesterone with an oscillatory [Ca\(^{2+}\)]\(_i\) pattern. However, after analyzing 132 cells (n = 5 donors) displaying spontaneous [Ca\(^{2+}\)]\(_i\) oscillations and determining the percentage of cells displaying transient, transient sustained, and transient oscillatory responses to progesterone, we found no correlation between spontaneous Ca\(^{2+}\) oscillations and an oscillatory Ca\(^{2+}\) component in response to progesterone.

DISCUSSION

Here we report a new, simple, and practical technique to monitor the progression of AR in human sperm in real time. The use of the fluorescent dye FM4-64 allows monitoring of morphological membrane changes during the AR and has revealed the appearance of tubular structures in the sperm head along this reaction. Furthermore, the combined use of FM4-64 and Fluo-4 allows the concomitant recording of AR and [Ca\(^{2+}\)]\(_i\) in a single human spermatozoon. Our findings suggest that spontaneous and progesterone-induced [Ca\(^{2+}\)]\(_i\) oscillations determine AR potential in human spermatozoa. Notably, our alternate observations of the progesterone-induced AR and [Ca\(^{2+}\)]\(_i\) changes have revealed that a second [Ca\(^{2+}\)]\(_i\) increase precedes AR and the loss of Fluo-4 fluorescence (see Fig. 4, B–D).

FM4-64 has been used extensively to monitor secretion in several cell types [17–20], but had not been conceived as a reporter of AR. Validation was accomplished by correlating its fluorescence with that of FITC-PSA, and by their dependence on the presence of extracellular Ca\(^{2+}\), which is anticipated in all eucaryotic processes, such as the AR. FITC-PSA uptake appears to be slightly delayed with respect to FM4-64 fluorescence increase, suggesting that the latter is able to enter the acrosome through the first fenestrations generated at the beginning of the AR. FITC-PSA diffuses and binds to glycosylated proteins contained in the acrosome [8], whereas FM4-64, in addition to diffusing, associates to membranes in general [20], so it is likely labeling the inner plasma membrane and acrosomal membranes as fusion begins during AR. Part of the inner acrosomal membrane becomes the plasma membrane of the acrosome-reacted spermatozoon. In this respect, it could be considered as a region equivalent to the secretion vesicle in somatic cells. FM4-64 can be used to label and observe human spermatozoa for up to 30 min, with minimal effects on sperm viability evaluated with SG. The percentage of viable human sperm after exposure to FM4-64 during AR-inducing experimental conditions is similar to that reported in other studies [13, 14].

Our findings with FM4-64 indicate that AR does not initiate in the same region in every spermatozoon, as previous reports of real-time AR detection have revealed in human and mouse spermatozoa [13, 24]. With the strong AR inducer ionomycin, this reaction initiates with no preference regarding the AP or
FIG. 4. AR and \([\text{Ca}^{2+}]_i\) imaging in single human spermatozoa exposed to ionomycin and progesterone. A) Nonstimulated human spermatozoa labeled with FM4-64 (red) and Fluo-4 (green). FM4-64 labels the plasma membrane, and Fluo-4 is distributed mainly in the cytoplasm. Bar = 5 µm. B, C) Time-lapse fluorescence images of Fluo-4 \([\text{Ca}^{2+}]_i\) and FM4-64 (AR) in human spermatozoa before and after ionomycin (B; Supplemental Movie S5) or progesterone (C; Supplemental Movie S6) treatment. After the stimulus, green fluorescence increases, reporting a rise in \([\text{Ca}^{2+}]_i\). FM4-64 fluorescence also increases, but with a delay, and only in acrosome-reacted cells. Right side: traces of FM4-64 and Fluo-4 fluorescence from the spermatozoon indicated by traces of FM4-64 and Fluo-4 fluorescence from the spermatozoon indicated by traces of FM4-64 and Fluo-4 fluorescence from the spermatozoon indicated by traces of FM4-64 and Fluo-4 fluorescence from the spermatozoon indicated by.
**TABLE 2.** Timing (expressed in seconds) of Fluo-4 and FM4-64 parameter changes in response to ionomycin and progesterone.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Ionomycin(^{a})</th>
<th>Progesterone(^{b})</th>
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<tbody>
<tr>
<td>Fluo-4</td>
<td>61.68 ± 2</td>
<td>53.9 ± 6</td>
</tr>
<tr>
<td>FM4-64</td>
<td>175.8 ± 17</td>
<td>593.3 ± 47</td>
</tr>
<tr>
<td>Fluo-4 empty</td>
<td>183.1 ± 13</td>
<td>601.89 ± 49</td>
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<tr>
<td>Fluo-4 drop</td>
<td>390.6 ± 6</td>
<td>847.6 ± 43</td>
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\(^{a}\) n = 8 independent experiments, 256 analyzed cells. Results expressed in seconds.  
\(^{b}\) n = 13 independent experiments, 74 analyzed cells. Results expressed in seconds.

LAT sperm head region. In contrast, AR induced by a more physiological stimulus like progesterone typically starts at the AP region of the sperm head. A similar conclusion was reached in mouse spermatozoa regarding the starting point of AR promoted by either zona pellucida (ZP) or a Ca\(^{2+}\) ionophore [24].

FM4-64 imaging lacks enough spatial resolution to discern acrosomal membrane vesiculation, as it has been observed with electronic microscopy [25]. This is probably due to spatial limitations of conventional optical microscopy. However, we were able to discern novel structural changes in the plasma membrane during and after the triggering of AR, which were corroborated using SEM. Particularly interesting was the emergence of tubular membrane structures (resembling filopodia) from different areas of the sperm head in response to progesterone after AR initiated. Previous studies in guinea pig spermatozoo using electron microscopy reported the presence of sheets of hybrid membranes forming tubules or filaments associated with the luminal surface of the residual outer acrosomal membrane during the AR [26]. Moreover, an earlier study reported the presence of filaments closely resembling the membrane appendages observed in our study, which extended from the head of pig spermatozoa and were observed by phase-contrast microscopy. Interestingly, these structures were observed in spermatozoa embedded in the ZP layer, suggesting they may have a role in approaching the egg and penetrating it [27]. We believe that these tubular membrane structures have seldom been reported because they are difficult to observe with light microscopy, and because a membrane dye such as FM4-64 has not been used before to monitor AR in real time. The resemblance and coincidences between our results and previous studies lead us to think that these appendages are not an artifact and could have a physiological role during spermatozoa-egg interactions [27]. We believe that FM4-64 represents an excellent tool to further examine the involvement of these structures in sperm progression and navigation towards the egg in future studies.

For many years ZP has been considered the physiological inducer of the AR [28]. However, recent studies in transgenic mice have shown that spermatozoa underwent AR before contacting the ZP during in vitro fertilization of cumulus-egg complexes [12]. Alternatively, progesterone, which exists at micromolar concentrations in the cumulus oophorus, has been proposed as a physiological inducer of AR. This is consistent with the notion that the AR is strictly Ca\(^{2+}\)-dependent and that progesterone directly activates the cation channels of sperm (CatSper), the only Ca\(^{2+}\)-permeable channel recorded by patch clamp in human spermatozoa so far [5, 29]. Progesterone may stimulate further Ca\(^{2+}\) uptake by alternate pathways [30] because the human sperm AR is only partially inhibited by CatSper blockers [31] and spermatozoa from CatSper knockout mice are still able to undergo this reaction [32]. Taking into account these considerations, we used progesterone to induce AR in human spermatozoa.

When the time courses of the progesterone-induced AR detected with FM4-64 or using fixed spermatozoa are compared, important differences are revealed. From the sperm undergoing AR, the FM4-64 signal indicates ~40% of the cells reacted 5 min after progesterone addition. In contrast, in this time, close to 80% of sperm underwent AR in fixed spermatozoa assayed with FITC-PSA staining [4]. This discrepancy could indicate an overestimation of AR, possibly due to difficulties establishing the condition of the acrosome and the extent of cell death in fixed cells. Differences also exist regarding the correlation between the type of [Ca\(^{2+}\)]\(i\) increase pattern and the progesterone-induced AR. In our experiments ~20% of the reacting sperm underwent AR after the first [Ca\(^{2+}\)]\(i\) transient increase whereas close to 40% acrosome reacted when fixed spermatozoa were examined at different times after progesterone application [4]. In contrast, our results are in agreement with a previous report where the sudden loss of Fluo-3 fluorescence was used as a real-time AR indicator [15]. Our data show that unreacted spermatozoa that sometime later will undergo AR display a transitory-sustained response pattern (also called biphasic response). This observation fits well with a previous quantification of AR in real time [15]. The discrepancies discussed above indicate that it is preferable to measure continuously in time both the AR and [Ca\(^{2+}\)]\(i\) to adequately examine any possible correlation between them.

It is well established that a [Ca\(^{2+}\)]\(i\) increase is necessary for the spermatozoa to achieve AR [1]. Previous efforts to correlate [Ca\(^{2+}\)]\(i\) changes and AR were carried out in bovine spermatozoa using time-lapse phase-contrast microscopy.
following stimulation with solubilized ZP [9]. In human spermatozoa, AR was elicited and then quantified in fixed spermatozoa [4, 9]. However, to our knowledge, no previous study succeeded in concomitantly and independently measuring \([\text{Ca}^{2+}]_i\) changes and AR extent continuously in time in individual spermatozoa. A previous investigation determining \([\text{Ca}^{2+}]_i/\text{AR}\) in real time using Fluo-3 [15] did not reveal details about the relation between \([\text{Ca}^{2+}]_i\) and the AR because Fluo-3 fluorescence was used for both purposes.

Concomitant analysis of the progesterone-induced \([\text{Ca}^{2+}]_i\) and AR changes determined here independently in single human spermatozoa indicates that neither the amplitude of the first \([\text{Ca}^{2+}]_i\) transient nor its total area under the curve resolves whether or not AR is triggered. These observations are similar to those obtained in human spermatozoa where AR was measured using fixed cells [4]. However, analysis of the correlation between \([\text{Ca}^{2+}]_i\) patterns and AR reveals that the transitory oscillatory \([\text{Ca}^{2+}]_i\) responses promoted by progesterone inhibits AR. Notably, closer examination of these recordings also unveiled the presence of a second \([\text{Ca}^{2+}]_i\) increase (named “second \([\text{Ca}^{2+}]_i\) peak”) occurring after the well-known progesterone transient \([\text{Ca}^{2+}]_i\). The presence of the second \([\text{Ca}^{2+}]_i\) peak is the signal to initiate the AR in >90% of reacting spermatozoa. The sperm second \([\text{Ca}^{2+}]_i\) peak resembles the \([\text{Ca}^{2+}]_i\) increase reported in neuroendocrine cells, where \([\text{Ca}^{2+}]_i\) reaches a ~10 μM threshold for triggering hormonal secretion [33]. An alternative explanation for the second \([\text{Ca}^{2+}]_i\) peak could be the influx of external \([\text{Ca}^{2+}]_i\) through fusion pores generated during AR initiation. The time resolution of our experiments allowed determining that the second \([\text{Ca}^{2+}]_i\) peak

TABLE 3. \([\text{Ca}^{2+}]_i\) change patterns promoted by progesterone and their relation to the AR.*

<table>
<thead>
<tr>
<th>Change pattern</th>
<th>AR</th>
<th>NAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Transient-sustained</td>
<td>32.8 ± 5</td>
<td>67.1 ± 5</td>
</tr>
<tr>
<td>Transient-oscillatory</td>
<td>8.3 ± 8.3</td>
<td>91.6 ± 8.3</td>
</tr>
<tr>
<td>Transient-recovery</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results are expressed as % of spermatozoa (100% = AR + no acrosome reaction (NAR). n = 6 independent experiments, 111 analyzed cells.
occurs several seconds before the AR, as assayed by FM4-64 fluorescence. However, faster [Ca\(^{2+}\)] oscillation imaging acquisition will be necessary to examine in detail the time delay, the path through which Ca\(^{2+}\) enters causing the second peak, and the molecular events that precede AR. It is worth pointing out that we also found that an important proportion of spermatozoa (~40%) show a slow Ca\(^{2+}\) rise ~2 min before the progesterone-induced AR. In neuroendocrine cells, a sub-threshold [Ca\(^{2+}\)] facilitates the proper docking of secretory granules before their fusion [34]. Given the similarity between some molecular events that occur during AR spermatozoa and exocytosis in somatic cells [16, 35] one can imagine that the slow Ca\(^{2+}\) rise could facilitate AR. However, some spermatozoa do not display a slow Ca\(^{2+}\) rise prior to reacting. Perhaps their [Ca\(^{2+}\)] reaches the threshold needed to induce AR after one or two [Ca\(^{2+}\)] peaks. As the [Ca\(^{2+}\)] signal that precedes AR is a necessary step for the reaction to occur, it will be important to determine the molecular nature of the transport systems involved, to further elucidate how the AR is triggered.

Our findings showed that close to 30% of human spermatozoa display spontaneous [Ca\(^{2+}\)] oscillations. We found that the majority (98% ± 2%) of spermatozoa undergoing spontaneous [Ca\(^{2+}\)] oscillations prior to the progesterone addition fail to progress to the AR. These observations lead us to consider that perhaps some unknown cell condition associated with these [Ca\(^{2+}\)] oscillations could preclude untimely AR. Thus the mechanisms that control Ca\(^{2+}\) oscillations in human sperm could be crucial to determine the state of the spermatozoa previous to the AR. As spermatozoa have to deal with [Ca\(^{2+}\)] increases during their preparation to fertilize the egg, such as those occurring during capacitation and hyperactivation [36–38], it is important that these [Ca\(^{2+}\)] increases do not trigger AR prematurely. Therefore, the generation of spontaneous [Ca\(^{2+}\)] oscillations and their termination could be a signal to regulate when the AR must occur. The [Ca\(^{2+}\)] oscillations triggered by progesterone in human spermatozoa have been well characterized [21, 39, 40], and are not drastically altered by Ca\(^{2+}\) channel blockers [21], but modified by ryanodine [39], suggesting that a capacitative Ca\(^{2+}\) entry could be involved in their regulation. Capacitative Ca\(^{2+}\) entry sensitive to ryanodine results from Ca\(^{2+}\) emptying of intracellular stores in various somatic cell types [41]. Considering that the presence of both spontaneous and progesterone-induced [Ca\(^{2+}\)] oscillations reduces AR, it will be necessary in future studies to perform their detailed analysis and compare their pharmacology to determine if they reduce AR probability by the same mechanism(s).

We recently reported that mouse spermatogenic cells undergo spontaneous [Ca\(^{2+}\)] oscillations that are abolished when the extracellular Ca\(^{2+}\) concentration is low (~5 μM), and partially inhibited by Ca\(^{3+}\) Ca\(^{2+}\) channel blockers and Ni\(^{2+}\) [42]. However, in mature spermatozoa, the spontaneous [Ca\(^{2+}\)] fluctuations have not been well characterized [21, 23]. In hamster sperm their presence has been related to flagellar beating [43]. It would be very important to fully characterize these [Ca\(^{2+}\)] signals in human sperm to determine not only the underlying Ca\(^{2+}\) mobilization mechanisms but also their modes of regulation and how they behave during sperm capacitation.

In summary, here we describe and validate a new methodology that allows the concomitant and independent recording in real time of the dynamics of [Ca\(^{2+}\)] and the
structural membrane changes that accompany the AR in live single human spermatozoa. This methodology can also be applied to study these phenomena in mouse spermatozoa (not shown). Improvements in spatial and temporal resolution of this technique make it an attractive tool to broaden our understanding of the mechanisms involved and the physiological ligand(s) of the AR, matters that are currently debated. FM4-64 staining unveiled the emergence of tubular appendages in the human sperm head during the AR. These structures had never been observed in real time before, and, together with the AR, they could participate in the final steps of sperm-egg interaction. Future studies should examine in detail the role of these structures in fertilization. Finally, our recordings of AR and [Ca\(^{2+}\)]\(_i\) in real time indicate that a second [Ca\(^{2+}\)]\(_i\) increase induced by progesterone usually occurs in human spermatozoa that will undergo AR and that the presence of [Ca\(^{2+}\)]\(_i\) oscillations, both spontaneous as well as induced by progesterone, may prevent untimely AR. It now becomes of utmost importance to understand the nature of this second [Ca\(^{2+}\)]\(_i\) elevation; might it be related to the crucial efflux of Ca\(^{2+}\) from internal stores proposed to be necessary for AR [16, 44]?

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