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Loss of Calcium in Human Spermatozoa via EPPIN, the Semenogelin Receptor[1]

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ABSTRACT

The development of a new male contraceptive requires a transition from animal model to human and an understanding of the mechanisms involved in the target's inhibition of human spermatozoan fertility. We now report that semenogelin (SEMG1) and anti-EPPIN antibodies to a defined target site of 21 amino acids on the C terminal of EPPIN cause the loss of intracellular calcium, as measured by Fluo-4. The loss of intracellular calcium explains our previous observations of an initial loss of progressive motility and eventually the complete loss of motility when spermatozoa are treated with SEMG1 or anti-EPPIN antibodies. Thimerosal can rescue the effects of SEMG1 on motility, implying that internal stores of calcium are not depleted. Additionally, SEMG1 treatment of spermatozoa decreases the intracellular pH, and motility can be rescued by ammonium chloride. The results of this study demonstrate that EPPIN controls sperm motility in the ejaculate by binding SEMG1, resulting in the loss of calcium, most likely through a disturbance of internal pH and an inhibition of uptake mechanisms. However, the exact steps through which the EPPIN-SEMG1 complex exerts its effect on internal calcium levels are unknown. Anti-EPPIN antibodies can substitute for SEMG1, and, therefore, small-molecular weight compounds that mimic anti-EPPIN binding should be able to substitute for SEMG1, providing the basis for a nonantibody, nonhormonal male contraceptive.

calcium, contraception, EPPIN, male contraception, semen, semenogelin, sperm, sperm motility and transport, spermatozoa

INTRODUCTION

A new male contraceptive will require a transition from animal model to human and an understanding of the mechanisms involved in the target's inhibition of human spermatozoan fertility. In 2004, O'Rand et al. [1] reported that male monkeys immunized with the male-specific protein EPPIN (official symbol, SPINLW1) could develop high titers of anti-EPPIN antibodies and become reversibly infertile. Subsequent work characterized EPPIN as a receptor for the seminal plasma protein semenogelin (SEMG1) [2, 3] and detailed the inhibition of human sperm motility by anti-EPPIN antibodies [4]. Anti-EPPIN antibodies, when bound to the sperm surface, mimic SEMG1 binding, effectively inhibiting progressive sperm motility and rendering the spermatozoa infertile [5]. To make the transition from the immunization of male monkeys to an effective human male contraceptive in which immunization is not required, we have sought to define the mechanism through which anti-EPPIN antibodies and SEMG1 inhibit sperm motility. Consequently, EPPIN's ability to inhibit motility when bound by antibody or SEMG1 is of interest for contraceptive development and for a better understanding of sperm physiology.

EPPIN coats the surface of human testicular and epididymal spermatozoa as part of a protein complex containing lactotransferrin and clusterin [3, 6]. During ejaculation, SEMG1 binds to EPPIN in the complex [3, 5], inhibiting the progressive motility of ejaculate spermatozoa. Subsequently, SEMG1 is hydrolyzed by the serine protease prostate specific antigen (PSA) [7], and EPPIN modulates PSA hydrolysis of SEMG1 on the sperm surface [3], resulting in forwardly motile spermatozoa. Treatment of motile spermatozoa with recombinant SEMG1 fragments containing cysteine residue 239 inhibits forward motility, which can be reversed with PSA treatment [8]. EPPIN's function is to bind SEMG1, modulate PSA activity on the sperm surface, and provide antimicrobial protection [5, 9]. In this study, we have demonstrated that one mechanism through which EPPIN is able to bind either SEMG1 or anti-EPPIN antibody and inhibit motility is the loss of intracellular calcium.

MATERIALS AND METHODS

Reagents and chemicals were molecular biology grade, purchased from Sigma (St. Louis, MO). Fluo-4 acetoxymethyl (AM) and BCECF-AM (2,′ 7′-bis-[2-carboxyethyl]-5-[and-6]-carboxyfluorescein; AM ester derivative) were obtained from Molecular Probes (Eugene, OR). M16-modified medium without Phenol Red (EmbryoMax; M16M) was obtained from Millipore (Billerica, MA). Human semen samples were obtained from the Department of Obstetrics and Gynecology, University of North Carolina Memorial Hospital, Chapel Hill, NC, and the use of samples in this study was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina, School of Medicine Chapel Hill, NC. Informed consent was not required for these samples. Monkey anti-EPPIN antibodies were obtained from a previous study [1] and were affinity purified as described previously [2]. As indicated in our previous study [1], these antibodies were obtained following protocols approved by our local Institutional Animal Care and Use Committee and the Animal Ethics Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (India). Affinity purified rabbit antibodies to the C terminal of EPPIN (amino acids 103–123) were produced by Bethyl Laboratories, Inc. (Montgomery, TX) to the peptide SMFVYG-GAQGNNNNFQSKANC (anti-S21C antibody), in which alanine was substituted for cysteine 110. The Student *t*-test was used as a test of significance to test the null hypothesis that the means of the two populations are equal. A P value of ≤ 0.05 was considered significant.

Preparation of Spermatozoa

Semen samples collected from fertile donors at the UNC North Carolina Memorial Hospital infertility clinic were allowed to liquefy for 30 min and subjected to standard semen analysis. Acceptable samples were either used fresh or stored in liquid nitrogen. Samples for study were prepared as previously described [2]. For some preparations, an isolate gradient (Irving Scientific, Irving, CA) was used to prepare spermatozoa. All experiments in

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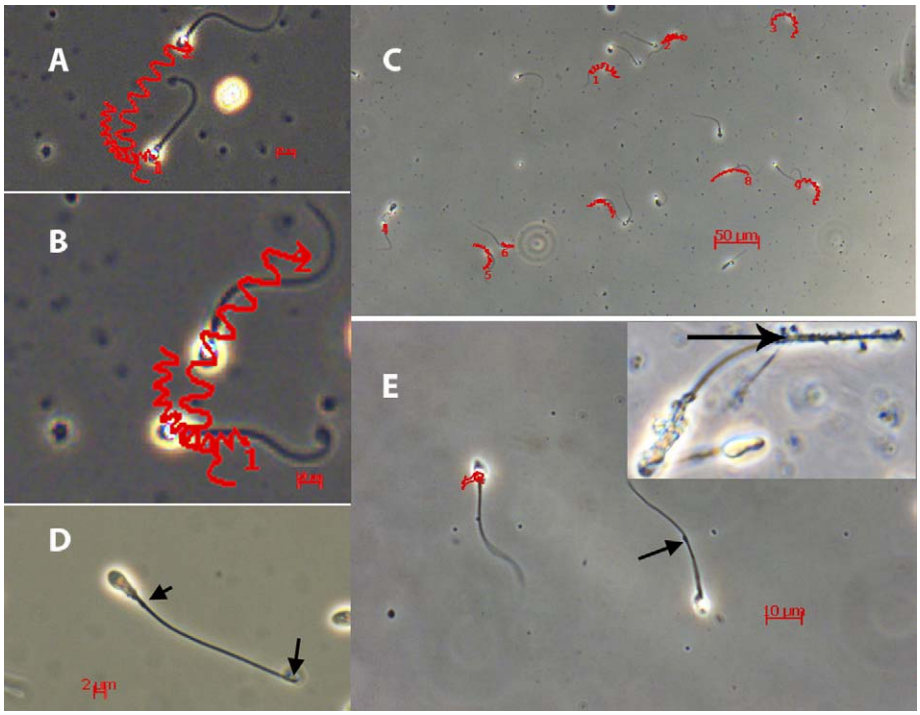

FIG. 1. Inhibition of human sperm motility by semenogelin-coated beads. **A** and **B**) Computer tracks of control spermatozoa at the beginning of their tracks (**A**) and the same spermatozoa (**B**) in frame 51 of 103 frames taken in 1 sec.; 20× objective; 103 frames/sec; pixel window, 328 × 248. Note that the centroid is in the posterior head region. Bar = 10 µm. **C**) Video frame of computer tracks of control spermatozoa (Supplemental Movie S1) treated with EPPIN-coated beads. The sperm bind very few beads, which appear as black dots in the frame, and their motility is not affected; 10× objective; 58 frames/sec; pixel window, 660 × 492. Bar = 50µm. **D**) Video frame of spermatozoon (Supplemental Movie S2) treated with SEMG1-coated beads; 63× objective; 57 frames/sec; pixel window, 660 × 492. Beads are bound to the sperm in the neck middle piece region and the distal end of the flagellum (arrows). Bar = 2 µm. **E**) A computer track of a nonprogressive spermatozoon treated with SEMG1-coated beads, only lateral head movement is detected by the computer. The other spermatozoon is not motile. Beads can be seen bound to the principal piece of the tail (arrow) and in the neck posterior head region; 40× objective; 53 frames/sec; pixel window, 660 × 492. Bar = 10 µm. Inset: high magnification of a single spermatozoon (63× objective) with bound beads along the principal piece of the tail (arrow) and in the neck posterior head region.

this study were carried out with ''swim up'' spermatozoa in M16M buffer. Incubation of spermatozoa with various concentrations of SEMG1 (0–14.4 µM), immunoglobulin G (IgG; 0–0.15 mg/ml), Fab (0–0.1 mg/ml), or monkey anti-EPPIN (0–1 mg/ml) was carried out in 96-well plates as described below or in 12- × 75-mm glass tubes at 37°C. Each experiment reported was repeated with spermatozoa from at least three different ejaculate samples.

Fluo-4 AM Loading

Fluo-4 AM was dissolved in dimethyl sulfoxide and dispersed in 10% Pluronic F-127 in H_2O to make a stock solution of 1 mM. Human spermatozoa were loaded with 10 µM Fluo-4 AM for 30 min at 37°C in a shaking water bath, diluted with 5 ml of M16M, and centrifuged at 300 × *g* for 5 min. Spermatozoa were resuspended in M16M or medium required for experimental conditions and incubated for an additional 10–15 min before use. Aliquots were taken to determine percentage of motility and sperm concentration.

Analysis of Sperm Motility

The analysis of sperm motility was carried out as previously described with either Zeiss Cell Observer time lapse and tracking software (AxioVs40 version 4.6.3.0) [2] or computer-assisted sperm analysis (CASA) (Ceros version 12.3 software; Hamilton-Thorne) [8]. In the Zeiss Cell Observer system, either a Plan-Neofluar 10×/0.3 phase 1, a Plan-Apochromat 20×/0.8 phase 2 (diameter width, 0.55 mm), a Plan-Neofluar 40×/0.75 phase 2, or a Plan-Apochromat 63×/1.4 phase 2 objective on a Zeiss Axiophot microscope was used. At least four random fields were selected, and sperm motility was recorded with an Axiocam HSc high-speed camera. Recordings were made for 1 sec at frame rates varying between 53 and 111 frames/sec with a pixel window of either 660 × 492 or 328 × 248 pixels, depending upon the experiment. Sperm recordings were analyzed with Zeiss Cell Observer time lapse and tracking software (AxioVs40 version 4.6.3.0). To track spermatozoa, we placed the centroid [10] over the posterior aspect of the head, which was tracked by the computer as the spermatozoon moved along its path (Fig. 1, A and B). Parameters measured were curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and beat/cross frequency (BCF) [10]. Measurements were conducted at 24°C. The parameters of the Hamilton-Thorne Ceros 12.3 software system have been described in our previous publication (Table 1 in reference 8).

Measurement of Intracellular Free Calcium

Fluo-4 AM-loaded spermatozoa were pipetted into 96-well black-walled plates (Perkin Elmer, Waltham, MA) at 1.1×10^5–0.9×10^5 spermatozoa/well in 50 µl of M16M at 37°C, and read in a BioTek (Winookski, VT) Synergy2 Multiplatform automated plate reader (with heater and shaker). Wells were excited using a 485/20 filter, and emission was read with a 528/20 filter, and data were acquired using a kinetic modification of the Alexafluor 488 protocol in the Gen5 software program (BioTek). Calibration of Fluo-4 in the plates showed that metal-free Fluo-4 had 1/183 the fluorescence of the calcium-saturated complex. After treatment of spermatozoa in each well with control or test reagents was completed (10–15 min), the Fluo-4 in the spermatozoa in each well was calibrated by adding ionomycin (2.5 µM), followed 15 min later by the addition of Mn^{2+} (2 mM $MnCl_2$) to bring the fluorescence (F) to 30% of that of the saturated dye. Fifteen min later, lysis of the spermatozoa with 1% Triton X-100 gave the background signal. Using these values for each well, the F_{max} and F_{min} were calculated according to the method of Kao et al. [11]. Fluorescence measurements were converted to calcium concentration according to the equation $[Ca^{2+}] = K_d$ ($[F - F_{min}]/[F_{max} - F]$) [11]. The K_d (dissociation constant) for Fluo-4 = 345 nM [12]. Readings for each well were normalized, averaged, and expressed as percentages. Data from three ejaculate samples are reported.

Dye leakage [12] from Fluo-4-loaded spermatozoa was measured by removing spermatozoa every 15 min (from t = 5 to t = 65 min) from the incubation medium by centrifugation and measurement of Fluo-4 fluorescence in the supernatant. There was minimal leakage at 15 min and ~5% leakage after 1 h incubation.

TABLE 1. Recovery of motility after NH_4Cl treatment of SEMG1-treated spermatozoa.*

Treatment†	Motile (%)	Progressive (%)	VCL (μm/sec)	ALH (μm)
Control	79 ±3	65 ± 7	101 ± 11	4.3 ± 0.1
SEMG1	54 ± 6[a]	41 ± 10	78 ± 14	3.1 ± 0.1[b]
SEMG1 + NH_4Cl	80 ± 6[c,d]	60 ± 16[d]	107 ± 7[d]	4.6 ± 0.7[c,d]

* Data are from CASA measurements of three ejaculates ± SD.
[a] $P = 0.03$ versus control.
[b] $P = 0.007$ versus control.
[c] $P = 0.05$ versus SEMG1.
[d] $P =$ not significant versus control.
† SEMG1 = 0.1 mg/ml; 2×10^6 sperm/ml; 50 pg/sperm; 10 mM NH_4Cl.

pH Detection

The AM ester derivative of the pH-sensitive dye BCECF (5 μM; Molecular Probes) was used to detect changes in the pH in spermatozoa treated with SEMG1. Spermatozoa were loaded with BCECF-AM for 30 min in bovine serum albumin-free medium, centrifuged to remove extracellular dye, and resuspended in M16M. Changes to 1.1×10^5–0.9×10^5 spermatozoa/well in 50 μl of M16M at 37°C after treatment with 3.2 μM SEMG1 were monitored in a BioTek (Winooksi, VT) Synergy2 Multiplatform automated plate reader (with heater and shaker); wells were excited using a 485/20 filter and emission read with a 528/20 filter. Fluorescence was read immediately and recorded every 10 sec; readings for each time point were normalized, averaged, and expressed as percentages of decrease in relative fluorescent units. Data from three ejaculate samples are reported.

Preparation of Semenogelin-Coated Beads

Streptavidin-coated 0.49-μm microspheres (Bangs Laboratories, Fishers, IN) were bound to biotinylated recombinant SEMG1 at 110% of saturation, blocked, and washed according to the manufacturer's directions. Beads were resuspended at a concentration of 5 mg of beads/ml. Biotinylated recombinant EPPIN was bound to streptavidin-coated 0.49-μm microspheres to serve as control beads because EPPIN will bind to the sperm surface [4] but has no affect on motility. For treatment of spermatozoa, we incubated $\sim 10^4$ spermatozoa with 2 μl of beads in 110 μl of M16M buffer at 37° C for 1–2 h. After a loss of motility was observed, we added thimerosal to a final concentration of 50 μM.

Preparation of Fab Fragments

IgG was purified from preimmune serum on protein A agarose beads (Pierce, Rockford IL). Affinity purified S21C and preimmune IgG were digested on papain-agarose, using a Fab preparation kit (Pierce). Digestion was confirmed by SDS-PAGE, and purified Fab fragments were concentrated and dialyzed against PBS (pH 7.4).

RESULTS

SEMG1 Treatment of Spermatozoa

SEMG1 binds EPPIN on the human sperm surface and inhibits progressive motility [2, 4, 8] in a binding site thought to be similar to that of anti-EPPIN antibody S21C [4] because in vitro assays demonstrate that SEMG1 will compete with the S21C antibody for binding to EPPIN [4, 13]. To study spermatozoa showing decreased progressive motility after treatment with recombinant SEMG1, we tracked individual spermatozoa with 0.49-μm streptavidin-biotinylated recombinant SEMG1 beads attached. This ensured that the SEMG1 was acting on the sperm surface.

Sperm movement can be visualized by computer tracks, which are shown in Figure 1, A, B, C, and E. Control spermatozoa are shown in Figure 1, A, B, and C. Figure 1, A and B, demonstrates the computer tracks of two control spermatozoa in M16-modified medium (M16M), pH 7.4; in Figure 1A, the spermatozoa are at the beginning of their tracks, while in Figure 1B, the same spermatozoa are in frame 51 of 103 frames taken in 1 sec. The spermatozoon on track 1 has a VCL of 86 μm/sec (ALH = 3.8 μm; BCF = 23 Hz), and the spermatozoon on track 2 has a VCL of 125 μm/sec (ALH = 7.8 μm; BCF = 16 Hz). Control spermatozoa are also shown in Figure 1C at lower magnification to demonstrate that treatment with EPPIN-coated control beads does not affect their motility. Figure 1C is a video frame from Supplemental Movie S1 (Supplemental Data are available online at www.biolreprod.org).

Spermatozoa treated with SEMG1-coated beads are shown in Figure 1, D and E. Figure 1D is a video frame from Supplemental Movie S2 and demonstrates the nonprogressive motility of a spermatozoon with bound SEMG1-coated beads (Supplemental Movie S2, arrows). There is no track for this spermatozoon because the centroid placed over the posterior aspect of the head does not move. A nonprogressive spermatozoon treated with SEMG1-coated beads with a computer track is shown in Figure 1E. Only slight lateral head movement was detected by the computer. The other spermatozoon in Figure 1E was not motile, and beads can be seen bound to the principal piece of the tail (Fig. 1E inset, arrow). In the inset at higher magnification a spermatozoon with beads on the neck-posterior head region and principal piece (Fig. 1E, arrow) can be seen. CASA measurements confirmed that the percentages of motile and progressive sperm decreased with SEMG1-bead treatment compared to EPPIN-bead treatment (Fig. 2; data ± SEM are from three ejaculates). We previously established, using CASA, the fact that spermatozoa treated with recombinant SEMG1 begin to lose their progressive motility after 15–30 min [8].

The inositol trisphosphate (IP_3) receptor agonist thimerosal has been shown to induce hyperactivation and an increase in high-amplitude flagella bending in mice [14, 15]. To determine if thimerosal could restimulate motility in SEMG1 bead-treated spermatozoa, we treated spermatozoa with 50 μM thimerosal. As shown in Figure 2, CASA measurements demonstrate that the percentage of motile sperm and the percentage of progressive sperm recover after thimerosal treatment, indicating that flagellar activity can be reactivated in SEMG1-treated spermatozoa. The recovery of VCL and ALH parameters was not significant (Fig. 2). Thimerosal treatment had no significant effect on the percentage of motile sperm when spermatozoa were treated with control EPPIN-coated beads (data not shown).

Calcium Changes in Spermatozoa Treated with SEMG1

To study changes in calcium concentration in human spermatozoa, we first established the fact that there was no difference between the motility of sperm loaded with the calcium indicator Fluo-4 in M16-modified medium (M16M) and unloaded sperm in wells of a single 96-well black-walled plate in the automated plate reader. The VCL of human

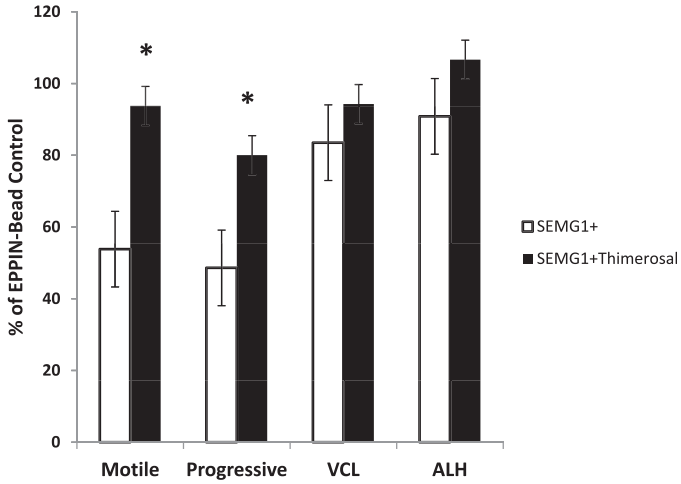

FIG. 2. Thimerosal treatment. Recovery of motility in SEMG1 bead-treated spermatozoa after treatment with 50 μM thimerosal. Data are from three ejaculates; normalized data are presented as the mean ± SEM. Motile, percentage of motile; Progressive, percentage of progressive motility; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement. *SEMG1 + thimerosal was significantly different from SEMG1+.

spermatozoa loaded with the calcium indicator Fluo-4 in M16M (156 ± 65 μm/sec) did not differ from that of unloaded spermatozoa (156 ± 35.5 μm/sec; n = 6 replicate wells; >25 sperm tracks) when incubated in a 5% CO_2 incubator at 37°C. After 1 h, the motility characteristics of Fluo-4-loaded spermatozoa in a 5% CO_2 incubator at 37°C (123 ± 8 μm/sec) were not significantly different ($P = 0.59$ for VCL; n = 6 replicate wells; >25 sperm tracks) from those of Fluo-4-loaded spermatozoa incubated for 1 h in M16M in the multiwell plate at 37°C (116 ± 45 μm/sec).

As described in Materials and Methods, spermatozoa in each well were treated with control or test reagents, and the Fluo-4 in the spermatozoa in each well was calibrated. A representative trace of the fluorescence signal is shown in Figure 3A. After 15 min of treatment, ionomycin was added (2.5 μM) (Fig. 3A, arrow), causing an immediate spike in the fluorescence signal. Fifteen minutes later, $MnCl_2$ (2 mM) (Fig. 3A, arrowhead) was added, which caused an immediate drop in the fluorescence signal. Fifteen minutes later, spermatozoa were lysed with 1% Triton X-100 to be sure that background readings had been obtained.

Spermatozoa treated with SEMG1 (or SEMG1-coated beads, data not shown) showed a significant decrease in Fluo4 signal compared to spermatozoa treated with buffer or control beads. Figure 3B shows a dose-response curve demonstrating that as little as 3 μM of SEMG1 can decrease the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) by approximately 30%. As previously reported [8], spermatozoa treated with SEMG1 lose their motility in a time- and concentration-dependent manner. As shown in Figure 3B, as the $[Ca^{2+}]_i$ decreases, sperm motility, measured by CASA, also decreases. Spermatozoa treated with SEMG1-coated beads showed a similar decrease in intracellular $[Ca^{2+}]_i$ compared to spermatozoa treated with control beads (22.4%, with a pool of four donors, n = 4 experiments, $P = 0.02$, at 30 min). Calcium levels after thimerosal treatment could not be measured because thimerosal interferes with the calibration steps (maximum and minimum cannot be accurately measured).

Calcium levels are affected by intracellular pH [16, 17]; therefore, we asked whether treatment of spermatozoa with SEMG1 changed the intracellular pH. Spermatozoa were loaded with BCECF and treated with 3.2 μM SEMG1, and the fluorescence was monitored in 96-well plates. Within 1 min, there was a 15% decrease in relative fluorescent units; with a plateau after 5–6 min (Fig. 3C). To determine if motility could be reversed by intracellular alkalization, 30 min after SEMG1 treatment, we treated spermatozoa with 10 mM NH_4Cl. CASA measurements demonstrated that after treatment with NH_4Cl, the spermatozoa recover to pre-SEMG1 treatment levels (Table 1). Similar to thimerosal, calcium levels after NH_4Cl treatment could not be measured because NH_4Cl interferes with the calibration steps (maximum and minimum cannot be accurately measured).

Calcium Changes in Spermatozoa Treated with anti-EPPIN Antibodies

Previously [1], the contraceptive C-terminal epitope of EPPIN was identified as amino acids 101–125 (TCSMFVYGGCQGNNNNFQSKANCLN). Affinity purified rabbit antibodies (anti-S21C antibodies) to the EPPIN S21C epitope (SMFVYGGAQGNNNNFQSKANC) had effects similar to those of recombinant SEMG1, namely, the dose-dependent inhibition of the progressive motility of spermatozoa [4]. To determine if anti-EPPIN treatment would result in loss of $[Ca^{2+}]_i$, we treated spermatozoa loaded with the calcium indicator Fluo-4 in M16-modified medium (M16M) in wells of a single 96-well black-walled plate in the automated plate reader with 150 μg/ml of anti-S21C antibody. Treatment of spermatozoa with affinity purified S21C antibodies (IgG) resulted in decreased levels of intracellular $[Ca^{2+}]_i$, which differed significantly from those seen in control rabbit IgG-treated samples or M16M buffer controls. Data from five ejaculate samples demonstrated a 52% decrease in $[Ca^{2+}]_i$ after treatment with anti-S21C (Fig. 3D).

The two antigen binding sites of anti-S21C IgG molecules could influence the lateral mobility of the eppin protein complex in the plane of the membrane and thereby disrupt calcium uptake into the cell. To address this possibility, Fab fragments with only a single antigen binding site were tested for their effect on the levels of intracellular $[Ca^{2+}]_i$. Similar to the treatment with anti-S21C IgG, treatment of spermatozoa with anti-S21C Fab fragments (100 μg/ml) resulted in a 66% decrease in the level of intracellular calcium (Fig. 3D; data from eight ejaculates). Moreover, treatment with S21C Fab resulted in a 58% loss of VCL after 10 min (115 μm/sec vs. 49 μm/sec, $P = 5.7 \times 10^{-8}$; n = 3 ejaculates) and a reduction of ALH from 3.7 ± 1 μm to 1.9 ± 0.9 μm ($P = 3.7 \times 10^{-6}$; n = 3 ejaculates). After 20 min of treatment with S21C Fab, there was a complete loss of progressive motility such that spermatozoa were twitching or vibrating in place, whereas spermatozoa treated with control Fab were unaffected.

To confirm that antibodies to the S21C binding site had effects similar to those of contraceptive antibodies from infertile male monkeys, Fluo-4-loaded spermatozoa were incubated in affinity purified anti-EPPIN antibodies obtained from infertile male monkeys [1]. Antibodies from three male monkeys, d523/602, d523/619, and d523/625, depressed the $[Ca^{2+}]_i$ normally seen in control samples by 33%, 33%, and 38%, respectively (Fig. 3D; data from three ejaculates). Control spermatozoa treated with M16M or adjuvant control IgG (data not shown) did not show decreased levels of $[Ca^{2+}]_i$ over the same incubation time period.

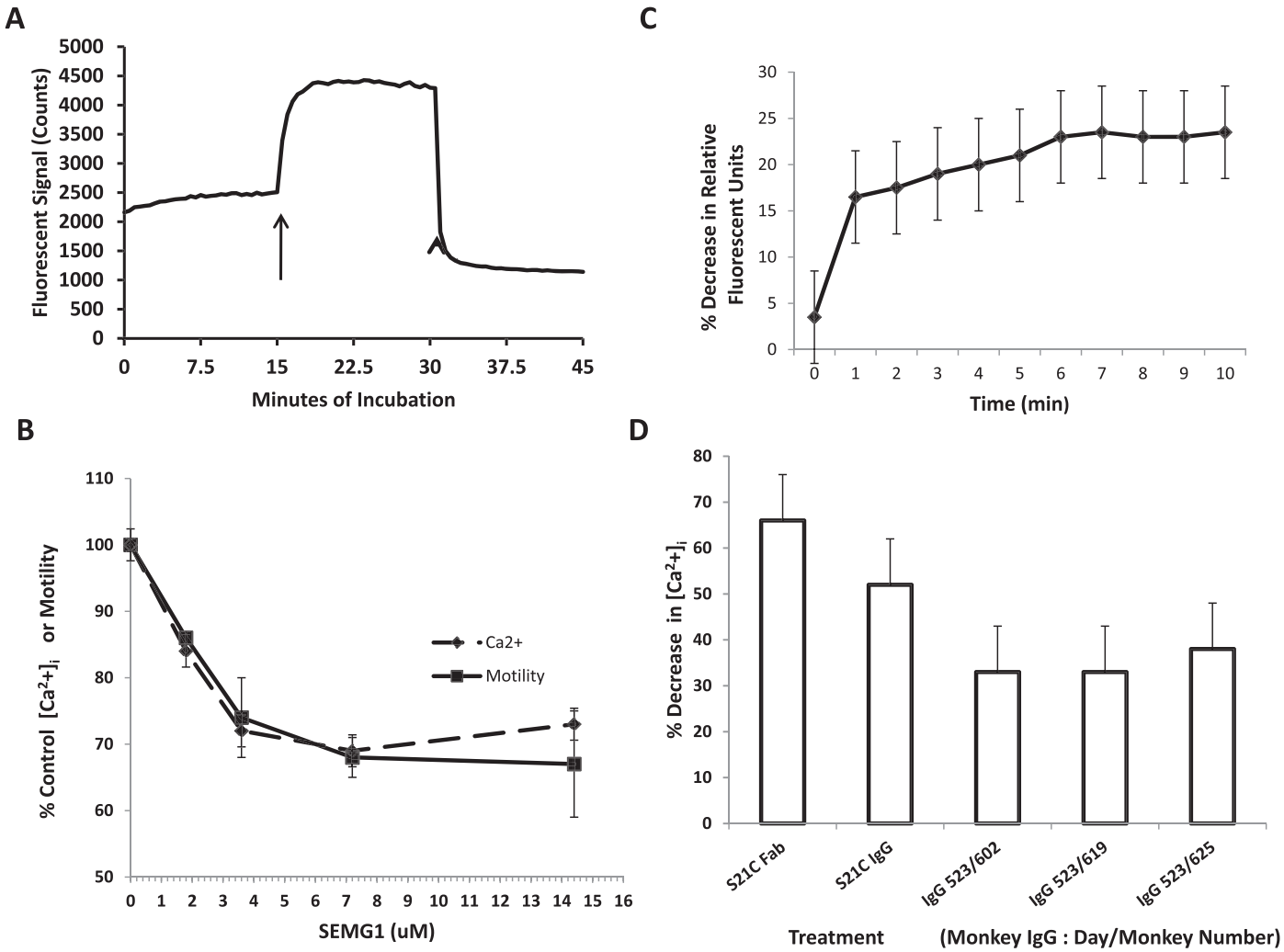

FIG. 3. **A**) Time plot of Fluo-4 fluorescence intensity. A representative trace is shown of the fluorescence signal from a single well of a 96-well plate containing Fluo-4-loaded spermatozoa in M16M. After 15 min, ionomycin was added (2.5 μM [arrow]), causing an immediate spike in the fluorescence signal. Fifteen minutes later, $MnCl_2$ (2 mM [arrowhead]) was added, which caused an immediate drop in the fluorescence signal. **B**) Loss of Intracellular $[Ca^{2+}]$ in human spermatozoa treated with SEMG1. Fluo-4-loaded spermatozoa were washed by centrifugation and resuspended in M16M and pipetted into a 96-well plate containing either control M16M medium or SEMG1. A dose-response curve of spermatozoa treated with increasing concentrations of SEMG1 (1.8, 3.6, 7.2, 14.4 μM). Data are from three ejaculates after 15 min of treatment; normalized data are presented as the means ± SD. Motility measurements were made by CASA. **C**) Fluorescence of the pH indicator BCECF in 1.1×10^5–0.9×10^5 spermatozoa/well in 50 μl of M16M at 37°C after treatment with 3.2 μM SEMG1. Spermatozoa were monitored in a BioTek Synergy2 multiplatform automated plate reader. Data are from three ejaculates; normalized data are presented as the means ± SD. **D**) Loss of $[Ca^2+]i$ in human spermatozoa treated with affinity purified anti-EPPIN antibodies. Fluo-4-loaded spermatozoa were washed by centrifugation and resuspended in M16M and pipetted into a 96-well plate containing either control M16M medium or antibodies. S21C Fab, affinity purified anti-EPPIN epitope Fab antibodies, 100 μg/ml, eight ejaculates; S21C IgG, affinity purified anti-EPPIN epitope IgG antibodies, 150 μg/ml, five ejaculates; IgG 523/602; 523/619; 523/625, affinity purified monkey anti-EPPIN IgG antibodies from three different monkeys (day/monkey number), 1mg/ml, three ejaculates. Normalized data are presented as the means ± SD. All samples are significantly different from controls ($P < 0.05$).

DISCUSSION

During ejaculation, SEMG1 binds to EPPIN [3, 5], inhibiting the progressive motility of ejaculate spermatozoa. The present study has demonstrated that when SEMG1binds to human spermatozoa, intracellular calcium levels decrease (Fig. 3B). During semen liquefaction, activated PSA in the ejaculate hydrolyzes SEMG1 [7], removing it from the sperm surface and resulting in the activation of motility. As motility begins, intracellular calcium levels in the noncapacitated spermatozoa are at basal levels, usually <100 nM (range 40–100 nM) [18, 19], and increase over time with incubation under capacitating conditions [20]. Significant increases in intracellular calcium are then required for hyperactivation and subsequent fertilization [21]. The normal motility of ejaculate human spermatozoa can be reversed by SEMG1 and again reactivated by PSA treatment [8]. We conclude from the results reported here that under normal physiological conditions, when seminal plasma is added to spermatozoa during ejaculation, the observed inhibition of sperm motility before liquefaction is most likely caused by the lack of intracellular calcium brought about by the binding of SEMG1 to EPPIN.

Thimerosal, an inositol triphosphate (IP_3) receptor agonist that induces hyperactivation and an increase in high-amplitude flagella bending [14, 15], has been shown to release calcium from internal stores [22, 15]. Our finding that thimerosal will stimulate motility in spermatozoa treated with SEMG1 (Fig. 2) indicates that the binding of SEMG1 to the sperm plasma membrane most likely did not deplete internal calcium stores.

The low-bend amplitude of flagella (ALH), characteristic of SEMG1 treatment ($<3.6 \pm 0.8$ μm), is indicative of low intracellular calcium and has been shown to be increased after thimerosal treatment [15]. Although we favor the conclusion that SEMG1 treatment blocked calcium uptake, we cannot rule out the possibility that calcium loss from the spermatozoon was accelerated via either plasma membrane Ca^{2+}-ATPase or Na^{+}/Ca^{2+} exchanger [23, 24]. Further experiments will be necessary to test these possibilities.

The effects of pH on sperm motility are well documented [25, 26], and increased intracellular calcium levels are known to depend upon an internal alkaline pH [22, 27, 28]. In order to determine whether SEMG1 binding changed the internal pH of spermatozoa, we tested spermatozoa loaded with BCECF and monitored fluorescence changes (Fig. 3C). The rapid decrease in relative fluorescent units most likely indicates that there was a drop in intracellular pH and that SEMG1 binding may have changed the polarity of the plasma membrane. The low-bend amplitude of flagella (ALH), characteristic of SEMG1 treatment, is characteristic of low intracellular pH (pH 6.6) [22] (ALH = 1.6 ± 0.6 μm at pH 6.7 for human spermatozoa [O'Rand and Widgren unpublished observations]). To test whether the loss of motility in SEMG1-treated spermatozoa could be reversed by intracellular alkalization, we treated spermatozoa with NH_4Cl to raise the pH. NH_4Cl has been shown to increase intracellular pH and calcium levels [22]. Our results indicate that motility parameters characteristic of activated spermatozoa were restored to SEMG1-treated spermatozoa (Table 1). This result may be interpreted to mean that the loss of calcium by SEMG1 treatment is from an upstream effect on the intracellular pH rather than a direct blocking effect of a calcium channel.

Several different plasma membrane calcium channels exist in human spermatozoa, including transient receptor potential channels (TRPM8) [29] and the CATSPER calcium channel that is activated by intracellular alkalinization [28, 30]. Whether the EPPIN protein complex on the surface of spermatozoa, in the presence of SEMG1, interacts with the CATSPER channels is unknown; however, it has been reported that sperm from *Catsper*$^{-/-}$ null mice show motility as if they have low intracellular calcium levels [15], and the motility can be restored by release of calcium stores [15]. Although the mechanism through which SEMG1 and anti-EPPIN antibodies exert their effectiveness might be a disruption of CATSPER, alternatively, our BCECF and NH_4Cl data indicate that it might be a disruption of intracellular pH. The voltage-gated HVCN1 proton channel [17] is present in human spermatozoa [28], and the presence of anti-EPPIN antibodies or SEMG1 bound to the EPPIN protein complex on the sperm surface could inhibit or reverse intracellular alkalinization, leading to an inhibition of calcium uptake and a loss of progressive motility. The motility characteristics of spermatozoa at low pH are similar to those observed in the presence of anti-EPPIN or SEMG1, suggesting that an inhibition of calcium uptake could be occurring through a change in activity of a proton channel.

Treatment of human spermatozoa with anti-EPPIN antibodies (anti-S21C) specific to the C terminal of EPPIN (Fig. 3D) or from infertile male monkeys (Fig. 3D) produced a significant loss of calcium that is accompanied by the previously reported significant decrease in progressive motility [4]. Interestingly, the dual binding sites of IgG, which might induce lateral association of plasma membrane surface molecules, were not responsible for the loss of calcium. The significant loss of calcium by the S21C Fab antibody (Fig. 3D) indicates that binding directly to the EPPIN epitope perturbs the surface enough to affect calcium levels, velocity, and flagella amplitude. Decreases in flagella beat amplitude and progressive motility resulting from decreased intracellular calcium have been reported previously [16] when measured using ethylene glycol tetraacetic acid (EGTA) to decrease external calcium concentrations.

The results of this study demonstrate that the male contraceptive target EPPIN controls sperm motility in the ejaculate by binding SEMG1, resulting in a loss of intracellular calcium. Anti-EPPIN antibodies can substitute for SEMG1, and, therefore, small-molecular weight compounds that mimic anti-EPPIN binding should be able to substitute for SEMG1, providing the basis for a nonantibody, nonhormonal male contraceptive.

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