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Clinical Research Article

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The Calcium-Sensing Receptor Is Essential for Calcium and Bicarbonate Sensitivity in Human Spermatozoa

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Abbreviations: ADH1, autosomal dominant hypocalcemia type 1; BSA, bovine serum albumin; Ca^{2+}, free ionized calcium; $[Ca^{2+}]_i$, intracellular calcium concentration; cAMP, 3′,5′-cyclic adenosine 5′-monophosphate; CaSR, calcium-sensing receptor; EC_{50}, median effective concentration; FHH1, familial hypocalciuric hypercalcemia type 1; FITC, fluorescein isothiocyanate; HTF, human tubal fluid; mRNA, messenger RNA; PI, propidium iodide; PSA, *Pisum sativum* agglutinin; PTH, parathyroid hormone; qRT-PCR, quantitative reverse-transcriptase–polymerase chain reaction; RT, room temperature; sAC, soluble adenylyl cyclase; TBS, Tris-buffered saline.

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Abstract

Context: The calcium-sensing receptor (CaSR) is essential to maintain a stable calcium concentration in serum. Spermatozoa are exposed to immense changes in concentrations of CaSR ligands such as calcium, magnesium, and spermine during epididymal maturation, in the ejaculate, and in the female reproductive environment. However, the role of CaSR in human spermatozoa is unknown.

Objective: This work aimed to investigate the role of CaSR in human spermatozoa.

Methods: We identified CaSR in human spermatozoa and characterized the response to CaSR agonists on intracellular calcium, acrosome reaction, and 3′,5′-cyclic adenosine

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5′-monophosphate (cAMP) in spermatozoa from men with either loss-of-function or gain-of-function mutations in *CASR* and healthy donors.

Results: CaSR is expressed in human spermatozoa and is essential for sensing extracellular free ionized calcium (Ca^{2+}) and Mg^{2+}. Activators of CaSR augmented the effect of sperm-activating signals such as the response to HCO_3^- and the acrosome reaction, whereas spermatozoa from men with a loss-of-function mutation in *CASR* had a diminished response to HCO_3^-, lower progesterone-mediated calcium influx, and were less likely to undergo the acrosome reaction in response to progesterone or Ca^{2+}. CaSR activation increased cAMP through soluble adenylyl cyclase (sAC) activity and increased calcium influx through CatSper. Moreover, external Ca^{2+} or Mg^{2+} was indispensable for HCO_3^- activation of sAC. Two male patients with a *CASR* loss-of-function mutation in exon 3 presented with normal sperm counts and motility, whereas a patient with a loss-of-function mutation in exon 7 had low sperm count, motility, and morphology.

Conclusion: CaSR is important for the sensing of Ca^{2+}, Mg^{2+}, and HCO_3^- in spermatozoa, and loss-of-function may impair male sperm function.

Key Words: calcium, fertility, reproduction, CaSR, bicarbonate

Fertilization is the last step in a complex series of events starting with spermatogenesis and sperm maturation in the testis and epididymis. Ejaculation introduces the sperm to the female environment that activates the sperm to swim up and bind to the oocyte (1). The haploid spermatozoa are formed during spermatogenesis, where most of the DNA is packed with protamines making the spermatozoa virtually transcriptionally silent. Protein synthesis and processing are hampered by a lack of functional ribosomes, endoplasmic reticulum, and Golgi apparatus. Spermatozoa therefore critically depend on second messenger systems, particularly changes in intracellular calcium concentration $[Ca^{2+}]_i$ (2). Several factors can induce changes in $[Ca^{2+}]_i$, but progesterone is the best characterized and activates the sperm-specific calcium channel CatSper (3). CatSper may be responsible for all calcium influxes from the extracellular compartment, and several activators have been suggested for the promiscuous CatSper (4). Previous studies in rodents have shown that spermatozoa are exposed to huge changes in extracellular concentrations of calcium, magnesium, phosphate, and pH during transit from the seminiferous tubules through the rete testis to the caput, corpus, and cauda epididymis, where the spermatozoa reside until they are mixed with the secretions from the prostate and seminal vesicle during ejaculation (5, 6). The new environment in the female reproductive tract induces an increase in $[Ca^{2+}]_i$, and the spermatozoa undergo capacitation, hyperactivation, and the acrosome reaction—all processes required for fertilization. Especially the concentration of HCO_3^- is markedly higher in the female reproductive tract (7) and essential for activation of the spermatozoa through an extracellular calcium-dependent activation of the soluble adenylyl cyclase (sAC) by an unknown mechanism (8). Furthermore, impaired calcium signaling in human spermatozoa leads to low motility, sperm survival, and inability to undergo the necessary activation steps to fertilize the oocyte (9).

The calcium-sensing receptor (CaSR) is a G protein–coupled receptor expressed on the surface of renal and parathyroid gland cells, where it regulates calcium excretion and suppresses parathyroid hormone (PTH) production/release in response to high serum calcium, respectively. Loss-of-function mutations in *CASR* have demonstrated its essential role for sensing the free ionized calcium concentration, urinary excretion, and mineral ion homeostasis (10). However, CaSR also responds to multiple other factors such as Mg^{2+} and spermine (11). Notably, the concentration of spermine is higher in seminal plasma than in any other body fluid or tissue, and Mg^{2+} concentrations are higher in the male reproductive tract than in serum (12, 13). The calcium concentration in the male reproductive tract, seminal fluid, and female reproductive tract varies dramatically from very low to much higher than the corresponding serum concentrations (5, 6, 14, 15). Interestingly, human spermatozoa are exposed to very low calcium concentrations in the cauda epididymis, where they are stored until ejaculation, whereas total calcium in seminal fluid is several fold higher than in serum (16, 17). However, citrate concentrations are also very high in the seminal fluid, making calcium lower than the corresponding serum level, whereas the concentration in the female reproductive tract is comparable with serum levels (18). It is not known how spermatozoa sense these changes in the

extracellular calcium concentration and the possible impact on sperm function. However, it has been shown that low Ca^{2+} in the cauda epididymis is crucial for sperm motility and male fertility potential because ablation of the calcium transient receptor potential vanilloid 6 transporter (*TRPV6*) in mice leads to high extracellular Ca^{2+} concentration in the epididymis and infertility (19). To investigate the role of CaSR in spermatozoa, we used several ligands and a negative allosteric modulator of CaSR supported by blockers of putative factors in the downstream signaling pathways in spermatozoa from healthy donors and patients with a loss-of-function mutation in *CASR* leading to familial hypocalciuric hypercalcemia type 1 (FHH1) or with gain-of-function mutation leading to autosomal dominant hypocalcemia type 1 (ADH1).

Materials and Methods

Participants and specimens

Testis and kidney samples were obtained from specimens removed because of cancer. The nonmalignant tissue was used for these studies. Three out of 6 testis samples used for quantitative reverse-transcriptase–polymerase chain reaction (qRT-PCR) were purchased from BioChain, Ambion, and Clontech, whereas the rest were from patients. Follicular fluids were from 5 women undergoing multiple in vitro fertilization or intracytoplasmic sperm injection treatments (NCT No.: NCT02437578). Informed consent was obtained from all men and women participating in the study, and the study protocol was approved by the regional ethics committee of the Capital Region of Denmark before study start (H-15000931, H-17004362). All human semen samples from healthy volunteers were obtained with their prior consent. The donors were recruited from the semen donor corps, which routinely donates samples for semen analyses at the Department of Growth and Reproduction. After delivery, the samples were anonymized, and no data on the fertility status or general health of donors are provided. Each donor received a fee of kr 500 per sample for their inconvenience. Patients with FHH1 or ADH1 were recruited from endocrine departments at Rigshospitalet, Hvidovre Hospital, and Skejby Hospital.

Measurements of changes in intracellular calcium in human sperm

Healthy semen donors (n = 12) from the general population without any known diseases or calcium homeostasis–related disorders were used for sperm function tests and calcium measurements. Motile sperm were separated from the raw semen sample by 1-hour swim-up separation at 37 °C in human tubal fluid (HTF) medium containing (in millimolar [mM]): 97.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 Na-lactate, 2.78 glucose, 21 HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid), 4 $NaHCO_3$, and pH was adjusted to 7.3 to 7.4 with NaOH/HCl. After 2 washes in HTF medium, the sperm concentration was adjusted to 10×10^6 cells/mL in HTF medium with 3 mg/mL human serum albumin (Irvine Scientific, No. 9988). The spermatozoa were loaded with the fluorescent Ca^{2+} indicators Fluo-4 (10 µM) (Thermo Scientific, No. F14201) or Fura-2 (3 µM) (Thermo Scientific, No. F1201) for 45 minutes at 37 °C. Fluorescence was measured in a fluorescence plate reader (FLUOstar Omega, BMG Labtech) and was excited at 480 nm and emission was recorded at 520 nm with bottom optics. Fluorescence was measured before and after the addition of 25 µL of treatment in duplicates. Ligands or inhibitors were first dissolved in dimethyl sulfoxide and then in HTF with 4 or 50 mM $NaHCO_3$. HTF medium with 50-mM $NaHCO_3$ was formulated as stated earlier with the addition of more $NaHCO_3$. Spermatozoa were incubated with RU1968, KH7, YM-234890, or NPS 2143 for approximately 5 minutes before the application of ligands. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$, indicating the percentage change in fluorescence (ΔF) to the mean basal fluorescence (F_0) before the addition of treatment. When treating with $CaCl_2$, the measurement of $[Ca^{2+}]_i$ in spermatozoa was performed by resuspending Fluo-4–loaded spermatozoa to 5×10^6 spermatozoa/mL in nominal calcium-free HTF medium, which was formulated as HTF, without the addition of $CaCl_2$. Changes in Fura-2 fluorescence are shown as relative changes to baseline. For changes in $[Ca^{2+}]_i$ measured using Fura-2, spermatozoa were loaded in polylysine-coated chambers to minimize movement of the spermatozoa (Lab-Tek, Nalge Nunc International). Excess dye was removed by washing the well with HTF. A Zeiss Axiovert 135 microscope equipped with a Weiss Achrostigmat 40 × 1.3 NA objective was used to acquire images from the fluorescent probe. Excitation was obtained by a polychrome Villuminator from Till Photonics, and images were acquired using a Cool Snap CCD camera (Photometrics) from Robert Scientific. For measurements of $[Ca^{2+}]_i$, the excitation wavelengths were 338 and 380 nm, measuring emission above 510 nm using a cutoff filter. $[Ca^{2+}]_i$ calculations were conducted by MetaFluor software from Molecular Devices and using a K_d of 160 nM. After background subtraction, ratio images were formed using MetaFluor software.

Sperm handling for measurements of 3′,5′-cyclic adenosine 5′-monophosphate

cAMP was measured in motile spermatozoa (1×10^6 spermatozoa per well) mixed 1:1 with different treatments in HTF medium containing 500 µM 3-isobutyl-1-methylxanthine (IBMX) and no added $CaCl_2$ in a white 384-well microplate (No. GR-784075, Greiner). After stimulation for 30 minutes at room temperature (RT), 10 µL fluorophore solution was added to each well according to the manufacturer's instructions (2.5% anti-cAMP cryptate conjugate and 2.5% d2 dye–labeled cAMP conjugate in lysis and detection buffer). After 1-hour incubation at RT in the dark, the fluorescence was assessed using an EnVision multimode plate reader (PerkinElmer). Emission was measured at 620 nm and 655 nm after excitation at 340 nm. The 620/655-nm emission ratios are subsequently interpolated to cAMP concentrations using a cAMP standard curve from the cAMP calibrator provided with the assay kit. Measurements of changes in intracellular pH (pH_i) were measured in 384-multiwell plates in a fluorescence plate reader (FLUOstar Omega, BMG Labtech). Sperm were loaded with the fluorescent pH indicator BCECF (10 µM) for 15 minutes at 37 °C. Fluorescence was excited at 440 and 480 nm (dual excitation) and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after the addition of 25 µL (1:3 dilution) of the treatments, negative control, and positive control (30 mM NH_4Cl). Changes in the ratio of BCECF fluorescence between the 440 and 480nm excitation are shown as $\Delta R/R_0$ indicating the change in the ratio of fluorescence between the 2 modes of excitation (ΔR) to the mean basal ratio of fluorescence between the 2 modes of excitation (R_0) before the addition of treatments.

Acrosome reaction

Swim-up–recovered spermatozoa were capacitated using HTF with 25-mM $NaHCO_3$ with 10-mg/mL human serum albumin and NaCl of 97.8 mM for at least 3 hours at 37 °C in a 10% CO_2 atmosphere. Spermatozoa were divided into equal aliquots and mixed with 5 µg/mL fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA) (Sigma-Aldrich No. L0770), 0.5 µg/mL propidium iodide (PI) (ChemoMetec, No. 910-3016), and 10 µg/mL Hoechst-33342 (No. 910–3015) in 25mM HTF with or without selected treatments. Ionomycin (10 µM) and progesterone (1 µM) were used as positive controls, while 0.2% dimethyl sulfoxide was used as control. Samples were mixed and thereafter incubated with gentle rotation for 30 minutes at 37 °C. A 50 µL sample was then mixed with 100 µL immobilizing solution containing 0.6 M $NaHCO_3$ and 0.37% formaldehyde. The solution was mixed and loaded in an A2 slide (ChemoMetec, No. 942-0001) and assessed in a NucleoCounter NC-3000 image cytometer. The following protocol was applied: 2-color flexicyte with Hoechst defining the spermatozoa to be analyzed: Ex475-Em560/35: exposure time 3000 ms, Ex530-Em675/75: exposure time 500 ms: with a minimum of 5000 analyzed cells (positive for Hoechst). PI intensity as a function of FITC-PSA intensity was plotted on biexponential scales and 3 specific gates were used to distinguish the PI-positive cells (nonviable), PI-negative, and FITC-PSA–positive cells (acrosome-reacted viable), and the PI-negative and FITC-PSA–negative cells (acrosome-intact viable) (20). The definitions of the 3 gates were made individually for each donor according to the ionomycin reaction.

Biochemical analysis

A representative sample of 9 healthy semen donors was compared with patients with FHH1 and ADH1. Fasting blood samples were collected between 8 AM and 10 AM. Serum was analyzed immediately, and all hormones were determined. The ionized calcium concentrations in the seminal fluid were measured using the Konelab 30i (Thermo Scientific) with a coefficient of variation of less than 2%, and total calcium and PTH levels were measured using the Cobas 8000 (Roche) with a coefficient of variation of 4%. Ionized calcium in the seminal fluid was analyzed immediately. We measured 5 seminal fluid samples, but the pH was too high in 3 of the samples. In one of the remaining samples, 1 µL HCl was added to adjust the pH for the Konelab 30i instrument. The remaining analyses on total calcium and magnesium were conducted on thawed samples.

RNA and quantitative reverse-transcriptase–polymerase chain reaction

RNA was isolated from tissue using NucleoSpin RNA (Macherey-Nagel, No. 740952), and the RNA concentration was determined using NanoDrop ND-1000 (Thermo Scientific). Complementary DNA synthesis was conducted using dT20 primer and random hexamers in 4:1 (0.5 µg/µL). qRT-PCR was conducted in triplicates with SYBR Green QPCR Master Mix (Agilent, No. 600828). Relative gene expression levels were normalized to *B2M*, *RPLP0*, or *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) using the ΔΔCT method. Primer sequences are listed in Table S2 (21).

Immunohistochemistry, immunofluorescence, and Western blot

Immunohistochemistry was conducted as described previously (22). Primary antibodies, dilutions, and retrieval buffers are listed in Supplementary Table S3 (21). After incubation, the sections were incubated with secondary antibody (ImmPRESS, MP-7402 or HistostainR Plus [859043, Life Technologies]). Sections with buffer instead of primary antibody were used as negative controls. None of the control slides showed any staining. Counterstaining was conducted with Mayer's hematoxylin. A slightly different protocol was used for the Abcam antibody (ab19347) on human testis: pressure cooker treatment for 30 minutes. Immunostaining with ab19347 and sc-47741 on cytospins with human spermatozoa was performed by antigen retrieval in a microwave oven for 1 minute at 750 W and 15 minutes at 350 W. All sections were scanned on a NanoZoomer 2.0 HT (Hamamatsu Photonics) and analyzed using the software NDPview version 2.6.13 (Hamamatsu Photonics). For staining of spermatozoa, the ejaculate was diluted in Tris-buffered saline (TBS) depending on the concentration of spermatozoa before centrifugation (5 minutes, 300*g*) onto a Superfrost+ slide (Thermo Scientific, No. J1800AMNT) and dried. The slides were fixed in 4% formaldehyde. Immunofluorescence was performed with TBS washes (3 × 5 minutes) between each step except between blockade for unspecific binding and primary antibody treatment. Paraffin sections were deparaffinized and rehydrated using standard procedures, followed by antigen retrieval (pressure cooker) in 0.01-M citrate buffer (pH 6) and peroxidase block in 1% (v/v) H_2O_2 in methanol for 30 minutes. Next, the sections were blocked in normal horse serum (Vector laboratories, No. MP-7402) diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin (BSA) (normal chicken serum [NCS]/TBS/BSA), followed by incubation with the first primary antibody (CaSR, N-term 2, Supplementary Table S3 [21]) diluted 1:50 in NCS/TBS/BSA overnight at 4 °C. Sections were incubated with peroxidase-conjugated secondary antibody (Santa Cruz, No. SC2962), diluted 1:200 in NCS/TBS/BSA for 30 minutes at RT and followed by incubation with Tyr-Cy3 (PerkinElmer, No. NEL744B001KT). Sections were subjected to antigen retrieval again and blocking in NCS/TBS/BSA, followed by overnight incubation at 4 °C with the second primary antibody; androgen receptor for human (No. MS-443-P, diluted 1:75) and SOX9 for mouse (No. AB5535, diluted 1:3000) in NCS/TBS/BSA. On the third day, slides were incubated with peroxidase-conjugated chicken antirabbit secondary antibody (Santa Cruz, No. 2963) diluted 1:200 in NCS/TBS/BSA for 30 minutes at RT, followed by incubation with Tyr-Fluorescein (PerkinElmer, No. NEL741E001KT) according to the manufacturer's instructions. Sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole; Sigma, No. D9542) diluted 1:500 in TBS for 10 minutes. Finally, slides were mounted with PermaFluor (Thermo Scientific, No. P36930) and fluorescent images captured using an Olympus BX61 microscope. For Western blot, samples were homogenized in radioimmunoprecipitation assay lysis buffer with 1:250 Protease Inhibitor Cocktail Set III (Calbiochem). After centrifugation, the supernatant was collected and diluted 1:2 in SDS Sample Buffer and heated to 95 °C for 5 minutes. Proteins were separated on 8% to 16% gradient protein gels (Bio-Rad). Membranes were blocked for 1 hour with 5% BSA in TBST and incubated overnight with primary antibodies. After washing in TBST, membranes were further incubated for 1 hour with secondary rabbit antimouse antibody (Dako, No. P0260). Western blot was conducted on the kidney from wild-type, *Casr* heterozygous, and *Casr* knockout mice (P3) in addition to semen or spermatozoa from 3 donors.

Statistics

Data were analyzed using GraphPad Prism, version 8 (GraphPad Software). Gaussian distribution of all numerical variables was evaluated by QQ-plots to secure the validity of the statistical tests used. As a result, the following variables were transformed with natural logarithm: ionized calcium concentrations in seminal and follicular fluid, amplitude $\Delta F/F_0$ (%) comparison between control men and a patient with FHH1 on 3.7- and 1.23-mM $CaCl_2$, and cAMP on $CaCl_2$ or $MgSO_4$ treatment in 4- and 50-mM HCO_3^-. The *t* test was used between ionized calcium concentrations in seminal and follicular fluid, amplitude comparisons between control men and the patient with FHH1 on $CaCl_2$ and $MgSO_4$, cAMP measurements in 4 or 50mM HCO_3^- on 2.04-mM $MgSO_4$, ABC motility of spermatozoa exposed to 2 µM cinacalcet, and acrosome-reacted spermatozoa. If the data did not have a gaussian distribution even after transformation, a nonparametric test was used. As a result, the Mann-Whitney test was used for the following variables: total calcium and magnesium measurements in seminal and follicular fluid. One-way analysis of variance (followed by a Dunnett multiple comparisons test) was used between cAMP measurements on $CaCl_2$ stimulation in 4- or 50-mM HCO_3^- and ABC motility (%) of spermatozoa stratified according to calcium concentration in seminal fluid (followed by a Tukey multiple comparisons test). Statistical significance was determined at the following levels: not significant (NS) *P* greater than .05, **P* less than .05, ***P* less than .01, *** *P* less than or equal to .001, and *****P* less than .0001.

Sequence analysis of calcium-sensing receptor gene

Genomic DNA was extracted from blood. Next-generation sequencing (Illumina) analyses of all coding regions and splice sites (± 50 bp) and copy number variation/multiplex ligation-dependent probe amplification analyses of *CASR* were conducted. Variants are detected by being present in more than 20% of sequence reads with coverage greater than 50×.

Results

Calcium and magnesium in reproductive fluids and calcium-sensing receptor expression in human male germ cells

Total calcium was 3-fold higher in seminal fluid compared with follicular fluid (5.13 ± 2.50 mM vs 2.11 ± 0.05 mM, respectively, $P = .001$) (Fig. 1A). Only 3% to 4% of calcium was available in the ionized form (0.19 mM ± 0.03) in seminal fluid and thus significantly lower than in the follicular fluid, in which 50% (1.30 ± 0.21 mM) was available as ionized calcium ($P < .001$) (see Fig. 1A). The concentration of total magnesium was also higher in seminal fluid compared with follicular fluid (3.44 ± 1.75 mM vs 0.69 ± 0.08 mM, respectively, $P = .001$) (see Fig. 1A). *CASR* was detected at the messenger RNA (mRNA) level in the testis and the expression level ranged from 6% to 22% of the expression in human kidney specimens (Fig. 1B; Supplementary Fig. S1A [21]). In accordance, CaSR expression was found in the cytoplasm/membrane of spermatogonia and spermatocytes within the seminiferous tubules and thus clearly distinct from the Sertoli-specific androgen receptor (Fig. 1C). In mature spermatozoa, CaSR was expressed in the neck and midpiece (Fig. 1D). Western blot of CaSR on human semen from 3 donors showed bands at the predicted size for the CaSR monomer (140-160 kDa) and dimer (280-300 kDa), including in spermatozoa after swim-up using 2 CaSR antibodies (Fig. 1E, one blot shown) that were validated on *Casr* knockout mice and human kidney specimens (Supplementary Fig. S1B [21]; data not shown).

Calcium-sensing receptor agonists induce increases in intracellular calcium in human spermatozoa, and responses are changed in patients with mutations in *CASR*

Changes in extracellular calcium induced a concentration-dependent increase in the $[Ca^{2+}]_i$ in human spermatozoa (Fig. 2A). High concentrations of extracellular Ca^{2+} evoked a rapid and sustained elevation of $[Ca^{2+}]_i$, whereas low concentrations induced a rapid increase in $[Ca^{2+}]_i$ followed by complete or partial restoration back to baseline (see Fig. 2A). Increased extracellular Ca^{2+} was achieved by the addition of $CaCl_2$ and virtually all the added calcium was available as ionized calcium (Ca^{2+}) (Supplementary Fig. S2A) (21). The dependence of CaSR for the observed $[Ca^{2+}]_i$ response was tested in spermatozoa from 3 patients with a heterozygous dominant loss-of-function mutation in *CASR* causing FHH1 and in spermatozoa from one patient with a heterozygous dominant activating mutation in *CASR* causing ADH1. The patients with loss-of-function mutations had 3 different mutations: Ile81Thr or Tyr63Cys in exon 3 or Trp818Stop in exon 7 identified by next-generation sequencing (Illumina). The patient with ADH1 had an Arg955Stop mutation. All patients with FHH1 had as expected elevated ionized serum calcium and the 2 men with inactivating mutations in exon 3 had normal semen quality, whereas the patient with a stop-codon in exon 7 had low sperm concentration and very low sperm motility and morphology (Table 1). As expected, the patient with ADH1 had low ionized serum calcium without inappropriately normal PTH (see Table 1). Interestingly, spermatozoa from both FHH1 patients with substitutions of exon-3 residues (Ile81Thr and Tyr63Cys mutations) elicited a much lower response to extracellular Ca^{2+} compared with control men, whereas the ADH1 patient elicited a higher response (Fig. 2A-2D). The patient with a stop-codon in exon 7 was completely unresponsive but data are not presented because it may be due to low semen quality rather than due to the *CASR* mutation. In response to Ca^{2+} (0.4-100 mM) spermatozoa from control men had a rapid elevation of $[Ca^{2+}]_i$ that returned promptly to baseline, whereas the patient with FHH1 Ile81Thr had a sustained elevated $[Ca^{2+}]_i$ after the initial peak (Fig. 2A and 2B). Concentration-response curves based on the peaks for Ca^{2+}-induced $[Ca^{2+}]_i$-influx were right-shifted in all patients with FHH1 compared with control men and left-shifted in the patient with ADH1 (Fig. 2E). Furthermore, the positive allosteric CaSR modulator cinacalcet left-shifted the concentration-response curve in spermatozoa from normal men but not when applied to spermatozoa from the FHH1 patient with the Ile81Thr mutation (Fig. 2F). As for Ca^{2+}, spermatozoa from both FHH1 patients with Ile81Thr and Tyr63Cys mutations elicited a lower response to extracellular Mg^{2+} compared to control men, whereas the ADH1 patient elicited a higher response (Fig. 3A-3D). Concentration-response curves induced by $MgSO_4$ were also right-shifted in the patients with FHH1 compared with control men and left-shifted in the patient with ADH1 (Fig. 3E). $MgCl_2$ induced a marked calcium response but with a higher median effective concentration

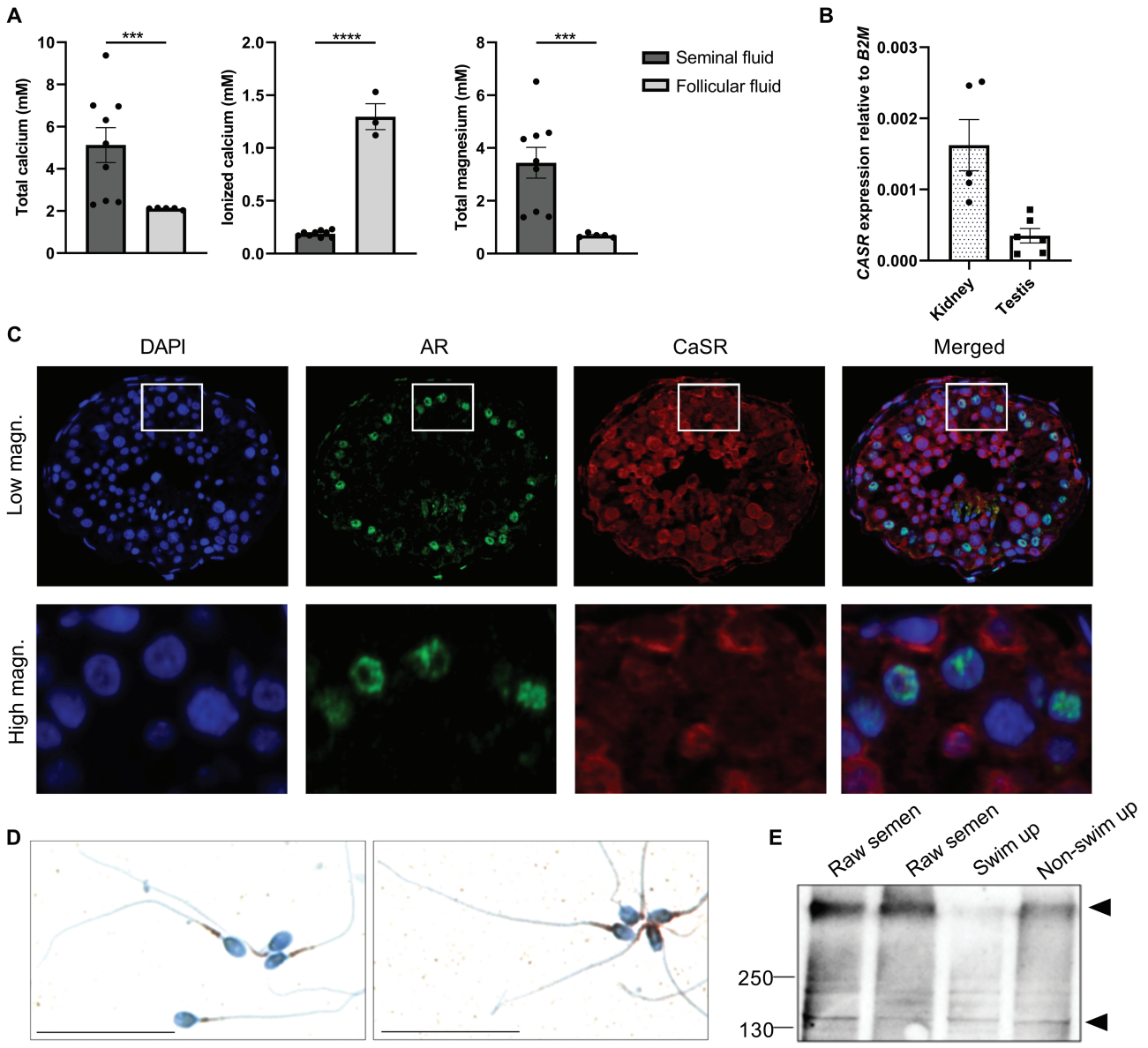

Figure 1. Seminal fluid concentration of calcium and magnesium and expression of calcium-sensing receptor (CaSR) in human testis and spermatozoa. A, Total calcium, ionized calcium, and total magnesium. n = 5-9 (total calcium, magnesium), n = 3 (ionized calcium). B, Gene expression of *CASR* in human testis and kidney. The expression of *CASR* is normalized to the expression of β-2-microglobulin (*B2M*). n = 5-6. C, Triple immunofluorescence staining of human testis using antibodies against the androgen receptor (AR) used as a Sertoli cell marker (green), the CaSR (red), and DAPI (4′,6-diamidino-2-phenylindole) as a nuclear marker (blue). White inserts mark area in high magnification. D, Immunostaining of CaSR in human spermatozoa with N-term 4 (left) and N-term 1 (right) antibodies (Supplementary Table S1 [21]). Counterstaining with Mayer's hematoxylin. The scale bar corresponds to 25 µm. E, Western blot of CaSR on human semen, spermatozoa from swim-up, and the non–swim-up fraction with N-term 1 antibody (Supplementary Table S1 and Supplementary Fig. S1B [21]). Arrows indicate CaSR monomer (~ 140 kDa) and dimer (~ 280-300 kDa). Data are presented individually and as mean ± SEM. ***P less than or equal to .001; ****P less than .0001.

(EC_{50}) and lower maximum amplitude compared with the $MgSO_4$-induced calcium response (Supplementary Fig. S3A and S3B [21]). Measurements of magnesium and calcium showed no significant differences between FHH1 patients and healthy controls, although the FHH1 patient with impaired semen quality had a lower seminal fluid concentration of both cations (see Table 1). The high seminal fluid Ca^{2+} concentration in the FHH1 patient with the Ile81Thr mutation may be partly explained by low pH and vitamin D insufficiency or CaSR may be involved in calcium transport in the male reproductive tract. The endogenous CaSR agonist spermine and the exogenous allosteric modulator cinacalcet also evoked elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3F and 3G). The CatSper calcium ion channel can be activated by increased pH_i, but none of the tested CaSR ligands, except spermine, increased pH_i level (Supplementary Fig. S2B [21]). Moreover, L-tryptophan lowered the EC_{50} for the

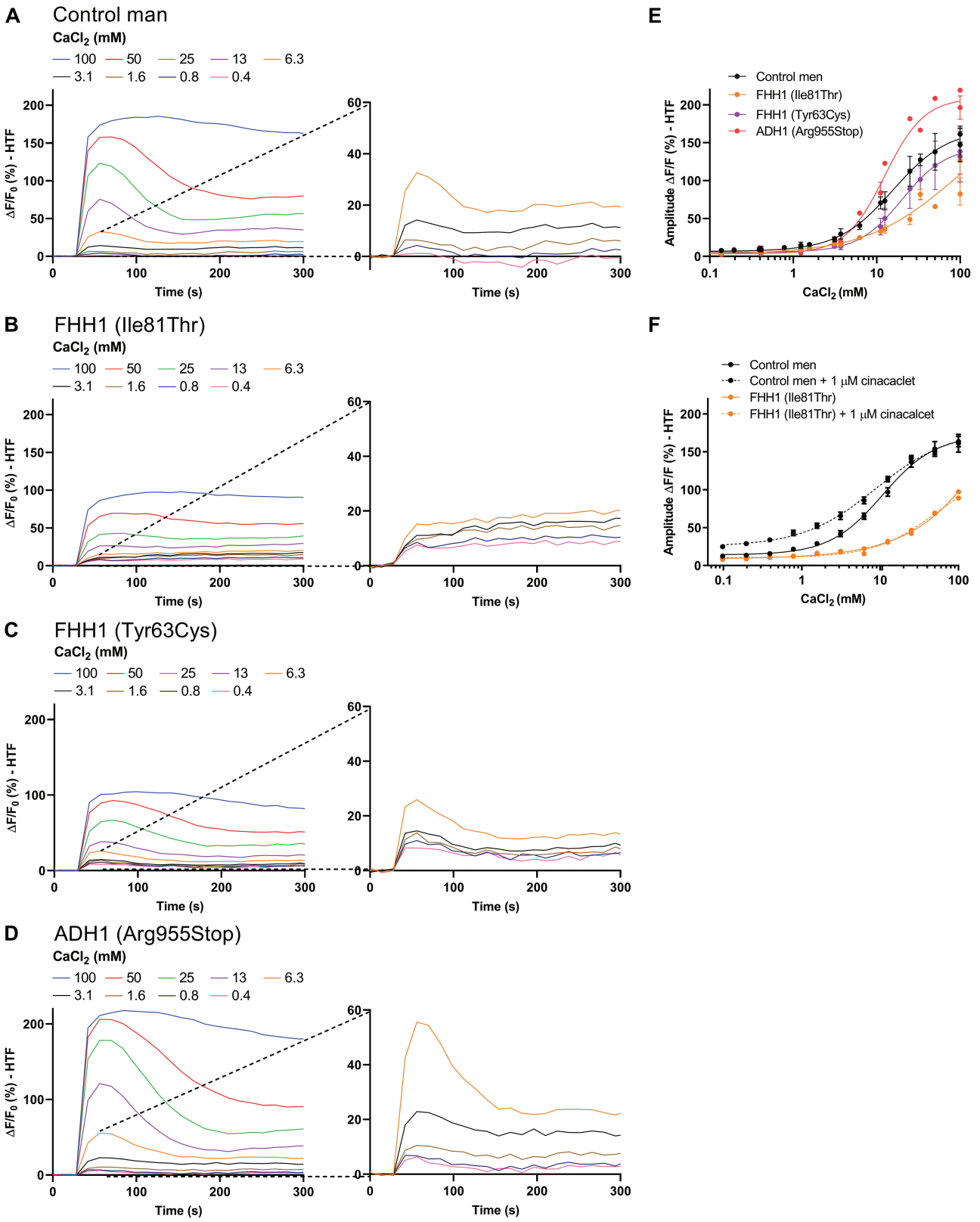

Figure 2. Free ionized calcium (Ca^{2+})-induced Ca^{2+}-signals in human sperm from healthy donors and patients with mutations in *CASR*. Intracellular Ca^{2+} signals induced by $CaCl_2$ in A, a representative control donor; B and C, 2 patients with loss-of-function mutations (Ile81Thr or Tyr63Cys) in *CASR* resulting in a familial hypocalciuric hypercalcemia type 1 (FHH1) phenotype; and D, in a patient with a gain-of-function mutation in *CASR* (Arg955Stop) resulting in autosomal dominant hypocalcemia type 1 (ADH1). $\Delta F/F_0$ (%)–human tubal fluid (HTF) indicates the percentages change in fluorescence with respect to the mean basal fluorescence before the application of $CaCl_2$ and HTF subtracted (medium without ligand). Dotted

$MgSO_4$ response from 6.3 to 3.7 mM, which implies that the high content of amino acids in the seminal fluid may influence the potencies of Ca^{2+} and Mg^{2+} (Supplementary Fig. S4A and S4B [21]).

Activation of soluble adenylyl cyclase and intracellular calcium concentration influx by HCO_3^- is influenced by extracellular cations and calcium-sensing receptor

The concentration of HCO_3^- is high in the female reproductive tract, and paired experiments with spermatozoa from 5 control men showed an augmented amplitude of the increase in $[Ca^{2+}]_i$ on $CaCl_2$ addition in a high vs low HCO_3^- environment (Fig. 4A). The stimulation was not caused by pH changes alone because adjusting pH to 7.35 only slightly changed the potency of Ca^{2+} (Supplementary Fig. S5 (21)). Cinacalcet further increased the sensitivity to external Ca^{2+} both in low and high HCO_3^- conditions in sperm from normal men (Fig. 4A). High HCO_3^- also resulted in augmented $[Ca^{2+}]_i$ in the FHH1 patient with the *CASR* Ile82Thr mutation but only when stimulated with supraphysiological concentrations of Ca^{2+} (Fig. 4A and 4B). The addition of 1.56-mM $CaCl_2$ to the media resulted in a marked response in the presence of cinacalcet and HCO_3^- in healthy donors, which differed from the impaired response in the patient with FHH1. cAMP was not generated in a low Ca^{2+} and HCO_3^- environment, and 12.5-mM Ca^{2+} was needed to significantly induce cAMP. The situation differed in a high bicarbonate environment where all concentrations of Ca^{2+} (≥ 3.13 mM) increased cAMP, which suggests a fully saturated response already at 3.13-mM Ca^{2+} (Fig. 4C). However, incubation with 1 µM cinacalcet did not further induce cAMP levels in either 4- or 50-mM HCO_3^- (Supplementary Fig. S6 [21]). CatSper does not allow permeability of Mg^{2+}, so to exclude a direct CatSper effect we tested and showed that HCO_3^- induced a 15-fold increase in cAMP when 2.04-mM $MgSO_4$ was added to a nominal calcium-free medium. This suggests that it is the presence of external Mg^{2+} or Ca^{2+} and not calcium influx that potentiates the activity of sAC, which thereby may be merely dependent on subsequent intracellular signals or calcium-release from intracellular stores than exclusively on CatSper activation (Fig. 4D).

Calcium and magnesium in the activation of CatSper and soluble adenylyl cyclase

Cross-desensitization studies using Ca^{2+} and Mg^{2+} showed that $CaCl_2$ concentration-dependently increased EC_{50} for $MgSO_4$-induced increases in $[Ca^{2+}]_i$. Moreover, Mg^{2+} cannot pass CatSper and was unable at any concentration to induce an increase in $[Ca^{2+}]_i$ in nominal Ca^{2+}-free medium (Fig. 5A). The CatSper inhibitor RU1968 changed the kinetics of the cinacalcet response (2.5 µM) but did not abrogate the increase in $[Ca^{2+}]_i$. The $G_{q/11}$ inhibitor YM-254890 did not affect the cinacalcet response, while KH7 that inhibits sAC changed the kinetics but did not abrogate the response to cinacalcet (Fig. 5B). RU1968 had strong inhibition of the progesterone, Ca^{2+}, Mg^{2+}, and cinacalcet response. The negative allosteric CaSR modulator NPS 2143 (5 µM) lowered all investigated treatments by 40% to 50% but inhibited the cinacalcet (2.5 µM) response more than 95% (Fig. 5C). However, NPS 2143 also induced a Ca^{2+} influx on its own (Supplementary Fig. S7). On cinacalcet treatment, changes in $[Ca^{2+}]_i$ occurred simultaneously in the head and midpiece of spermatozoa in accordance with the subcellular location of CaSR (Fig. 5D and 5E).

Calcium-sensing receptor influences the progesterone response and acrosome reaction

The progesterone responses in spermatozoa from patients with FHH1 were greatly reduced compared with the signal in spermatozoa from control men (Fig. 6A). Comparing the maximal changes in the Fluo-4 fluorescence signal can be challenging because it depends on the gain, background fluorescence, internal dye concentration, and the number of sperm in the well and their resting $[Ca^{2+}]_i$. Therefore, to correct for these variations the maximal response levels were determined in the individual experiments with ionomycin or 100-mM Ca^{2+} to show the progesterone response relative to the maximal response (Fig. 6B), which confirmed a lower response in the FHH1 patients. Calcium (5 and 10 mM) induced an increase in acrosome-reacted spermatozoa in control men, whereas cinacalcet treatment induced a borderline significant increase ($P = .08$) (data not shown). Interestingly, cinacalcet or Ca^{2+} did not affect acrosome reaction in the patient with FHH1 Ile81Thr but did increase acrosome-reacted sperm cells from FHH1 Tyr63Cys and the ADH1 patient (Fig. 6C). Treatment with

Figure 2: continued

lines mark the magnified areas comprising physiological relevant levels of calcium in follicular fluid or seminal fluid. A to D, Representative blots are shown. E, Concentration-dependent relationship of the signal amplitudes induced by $CaCl_2$ in control men and patients with FHH1. n = 7 different healthy donors, n = 4 from 4 different days (FHH1 Ile81Thr), n = 3 from 3 different days (FHH1 Tyr63Cys), n = 2 from 2 different days (ADH1 Arg955Stop). F, Concentration-dependent relationship of the signal amplitudes induced by $CaCl_2$ in control men and the FHH1 patient with *CASR* Ile81Thr mutation with or without preincubation of 1 µM cinacalcet. n = 5 (same healthy donor, 5 different days), n = 1 (FHH1 Ile81Thr). E to F, Data are presented as mean with ± SEM.

Table 1. Mineral homeostasis in serum and seminal fluid from patients with calcium-sensing receptor mutations and healthy controls

Serum variables	Reference level	FHH1 (Ile81Thr)	FHH1 (Tyr63Cys)	FHH1 (Trp818Stop)	ADH1 (Arg955Stop)	
25-OHD_{2+3}, nmol/L	> 50	38.8 (± 14.2)	66.7 (± 6.94)	51.3 (± 5.31)	65	
S-Calcium, mmol/L	2.15-2.51	2.50 (± 0.02)	2.7 (± 0.15)	2.70 (± 0.03)	2.24	
S-Ca^{2+}, mmol/L	1.18-1.32	1.34 (± 0.04)	1.42 (± 0.03)	1.42 (± 0.01)	1.16	
S-Magnesium, mmol/L	0.71-0.94	0.89 (± 0.04)	0.89 (± 0.01)	0.87 (± 0.03)	0.69	
PTH, pmol/L	1.6-7.1	4.28 (± 0.62)	4.15 (± 0.63)	3.2 (± 0.10)	2.2	
Semen variables	WHO lower reference	FHH1 (Ile81Thr)	FHH1 (Tyr63Cys)	FHH1 (Trp818Stop)	ADH1 (Arg955Stop)	Controls (± SD)
Ejaculate volume, mL	1.5	3.5	1.8	2.5	NA	–
Concentration, 10^6/mL	15	45	133	9	NA	–
Motility, %	32	73	75	18	NA	–
Morphology, %	4	5	7	0	NA	–
pH	–	7.00	7.26	7.26	NA	7.34 (± 0.16)
Total calcium	–	8.19	5.08	3.59	NA	5.12 (± 2.50)
Ionized calcium	–	0.29	0.15	0.16	NA	0.18 (± 0.03)
Ionized calcium pH 7.4	–	0.23	0.14	0.15	NA	0.17 (± 0.03)
Total magnesium	–	3.45	2.88	2.37	NA	2.05 (± 1.00)

Data are presented as measured value in patients with FHH1 or ADH1, whereas mean ± SD is presented for healthy controls and ranging from n = 3 to n = 9. Seminal fluid ionized calcium at pH 7.4 = $Ca^{2+} \times (1\text{-}0.53 \times [7.4 - pH])$.

Abbreviations: ADH1, autosomal dominant hypocalcemia type 1; Ca^{2+}, free ionized calcium; FHH1, familial hypocalciuric hypercalcemia type 1; NA, not available; PTH, parathyroid hormone; WHO, World Health Organization.

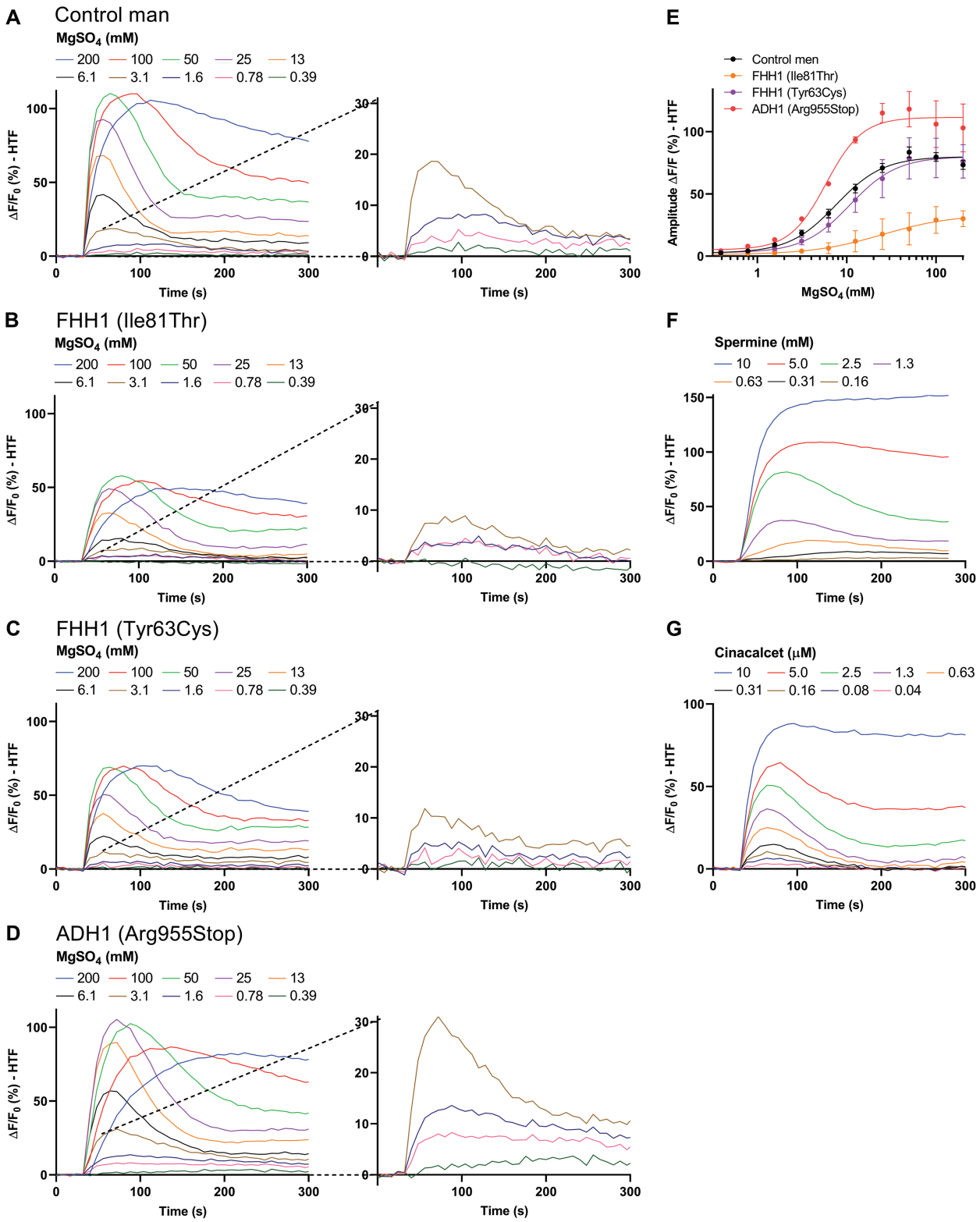

Figure 3. Magnesium induced free ionized calcium (Ca^{2+})-signals in human sperm from healthy donors and patients with mutations in *CASR*. Intracellular Ca^{2+} signals induced by $MgSO_4$ in A, a healthy donor; B and C, 2 patients with *CASR* loss-of-function mutations (Ile81Thr or Tyr63Cys) resulting in an familial hypocalciuric hypercalcemia type 1 (FHH1) phenotype; D, and an autosomal dominant hypocalcemia type 1 (ADH1) patient with a gain-of-function mutation in *CASR* (Arg955Stop). Dotted lines mark the magnified areas on the curves that contain the physiological relevant levels of calcium in follicular fluid or seminal fluid. A to D, Representative blots are shown. E, Concentration-dependent relationship of the signal amplitudes induced by $MgSO_4$ in control men and patients with FHH1. n = 8 different control men, n = 3 from 4 different days (FHH1 Ile81Thr), n = 3 from 3 different days (FHH1 Tyr63Cys), n = 2 from 2 different days (ADH1 Arg955Stop). Data are presented as mean with ± SEM. F and G, Intracellular Ca^{2+} signals induced by spermine or cinacalcet in a healthy donor. Representative blots are shown.

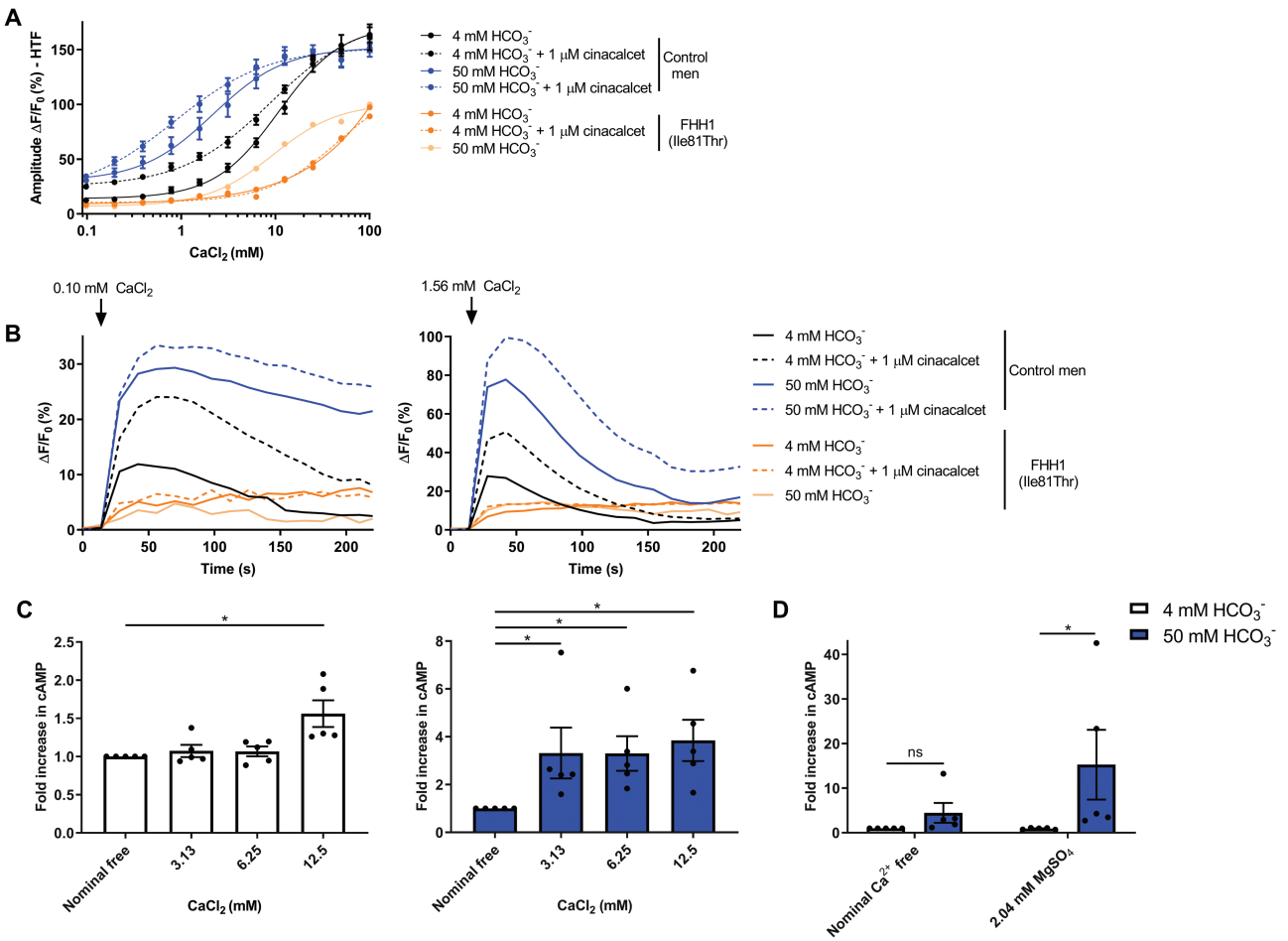

Figure 4. Bicarbonate and the free ionized calcium (Ca^{2+})-induced Ca^{2+}-influx and 3′,5′-cyclic adenosine 5′-monophosphate (cAMP) generation. A, Concentration-response relationship for $CaCl_2$ in control men and the familial hypocalciuric hypercalcemia type 1 (FHH1) patient with *CASR* Ile81Thr mutation in human tubal fluid (HTF) medium containing 4 or 50 mM HCO_3^- with or without preincubation of 1 μM cinacalcet. n = 5 (same healthy donor, 5 different days), n = 1 (FHH1 Ile81Thr). B, Ca^{2+} signals induced by 0.10- or 1.56-mM $CaCl_2$ in HTF medium containing 4 or 50 mM HCO_3^- with or without preincubation of 1 μM cinacalcet in control men and a patient with FHH1. n = 5 (average from the same donor, 5 different days), n = 1 (FHH1 Ile81Thr). C, Changes in cAMP concentration in sperm evoked by increasing $CaCl_2$ concentrations in 4 or 50 mM HCO_3^-. n = 5 different healthy donors. D, Changes in cAMP concentration in human sperm evoked by 4 or 50 mM HCO_3^- with or without the presence of $MgSO_4$. n = 5 men. Data are presented individually and as mean with or without ± SEM. **P* less than .05.

ionomycin increased the fraction of dead cells, which was not affected by other treatments (Fig. 6D). The patients with FHH1 Ille81Thr had a higher fraction of spontaneous acrosome-reacted sperm, more dead cells after almost all the treatments, and a lower increase in acrosome-reacted sperm in response to ionomycin (see Fig. 6C and 6D). The proposed CaSR-signaling pathways in human spermatozoa are illustrated in Fig. 7.

Discussion

This study demonstrates that CaSR in human spermatozoa is essential for normal sensitivity to Ca^{2+}, Mg^{2+}, and HCO_3^-. The endogenous agonists of CaSR—Ca^{2+}, Mg^{2+}, spermine, and the exogenous positive allosteric modulator cinacalcet—all induced significant $[Ca^{2+}]_i$ signals, which were dependent on CatSper-mediated calcium influx from the extracellular compartment. An essential role of CaSR for mediating the effect of cations in the extracellular environment was shown by the suppressed response in patients with loss-of-function mutations in *CASR* and by an increased response in a patient with a gain-of-function mutation in *CASR*. Loss-of-function mutation in amino acid 81 has been shown to reduce CaSR responses by 50% to 80% (23), and to the best of our knowledge, the Tyr63Cys, Trp818Stop, or Arg955Stop mutations have not been characterized before in vitro. All men with loss-of-function mutations had, in addition to the described *CASR* mutations, a characteristic clinical FHH phenotype with hypocalciuria, hypercalcemia, normal serum phosphate, and PTH. Our findings are the first indication that a patient's own cells can be used to prove the FHH1

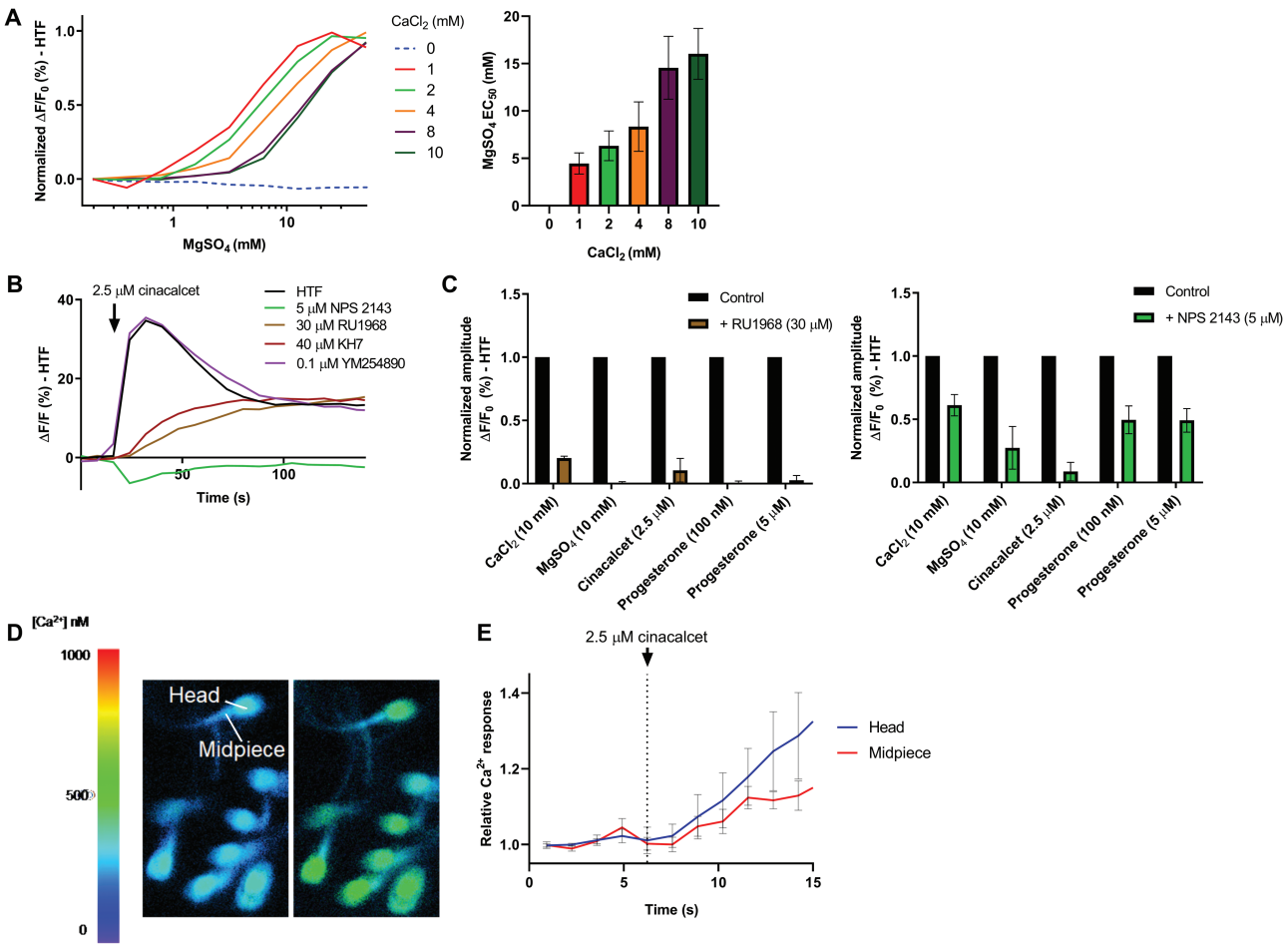

Figure 5. Free ionized calcium (Ca^{2+}) and Mg^{2+} compete for CatSper activation and soluble adenylyl cyclase (sAC) activity. A, Concentration-response relationship of $MgSO_4$ in the absence or presence of increasing $CaCl_2$ concentrations (left). Median effective concentration (EC_{50}) values of $MgSO_4$ in the absence or presence of increasing $CaCl_2$ concentrations (right). Average from 3 men. B, Ca^{2+} signals induced by 2.5 μM cinacalcet in the absence or presence of an inhibitor of CatSper (RU1968), calcium-sensing receptor (CaSR) (NPS 2143), sAC (KH7), or $G_{q/11}$-mediated signaling (YM254890). Average from 3 control men. C, The amplitude of Ca^{2+} signals induced by cinacalcet, $CaCl_2$, $MgSO_4$, or progesterone in the absence or presence of the RU1968 or NPS 2143. n = 3-5 men. D, Fura-2–loaded sperm before (left) and after (right) addition of 2.5 μM cinacalcet. Color indicates $[Ca^{2+}]_i$. E, Relative increase in $[Ca^{2+}]$ after addition of 2.5 μM cinacalcet. n = 5 different sperm. Data are presented as mean with or without ± SEM.

phenotype (in males) rather than expressing the receptor in HEK293 cells to study the effects (24).

CaSR was found to be expressed in the germ cells during all stages of spermatogenesis, and in the head and neck region of mature human spermatozoa, and Western blot using 2 antibodies validated in *Casr* knockout mice confirmed the presence of CaSR. Previous studies have found CaSR in rodent, stallion, and boar spermatozoa, in which CaSR seemed to be important for capacitation and sperm motility and the most upregulated protein in good quality bull spermatozoa (25-31). A RNA sequencing transcriptome analysis of human sperm samples reported low *CASR* mRNA levels (32). However, mRNA levels are not a good indicator of presence in the transcriptionally silent sperm, but the presence of the protein and functional biochemical evidence suggest a role for CaSR in human sperm. Interestingly, human spermatozoa are exposed to high concentrations of total calcium in the ejaculate, which appears to be in opposition to the functionally important role of low calcium in the cauda epididymis (19). However, the coexisting high citrate and phosphate concentrations in the seminal fluid provide enormous Ca^{2+} buffering capacity and as a result low extracellular ionized calcium concentrations (average 0.22 mM) corresponding to 3% to 4% of total calcium, which agrees with previous studies (17, 18).

Extracellular magnesium was 6-fold higher in the ejaculate compared with serum and able to induce a potent increase in $[Ca^{2+}]_i$. However, the high Mg^{2+} level in the seminal fluid may also be buffered by citrate, although a lower free fraction than Ca^{2+} may be available due to different citrate dissociation constants (Ca^{2+} 0.7 mM vs Mg^{2+} 0.28 mM) (33, 34). Interestingly, Mg^{2+} was unable at any concentration (0-50 mM) to induce an increase in $[Ca^{2+}]_i$ in calcium-free medium, which shows the dependence of

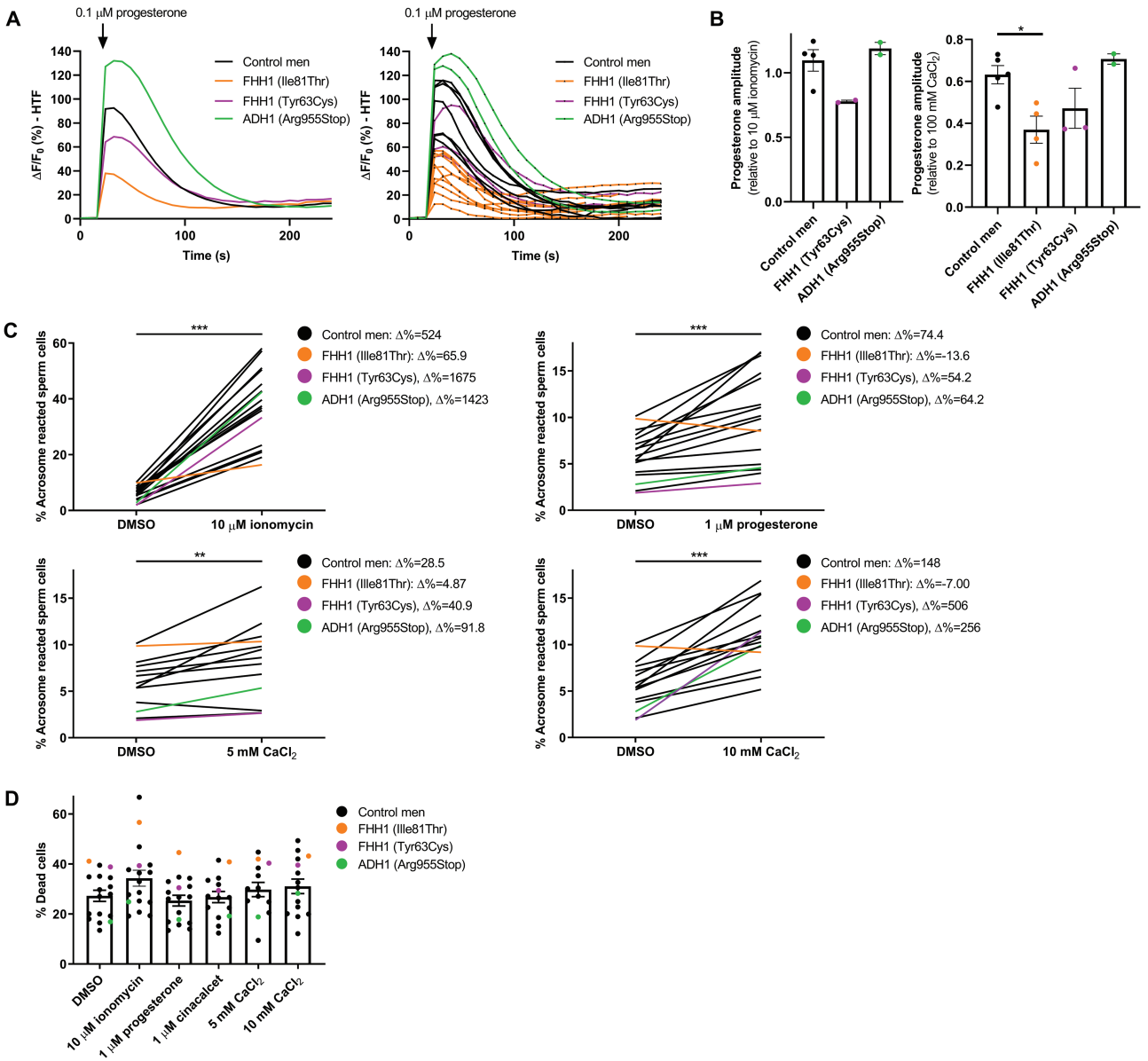

Figure 6. Progesterone response and acrosome reaction in sperm from control men and patients with familial hypocalciuric hypercalcemia type 1 (FHH1) or autosomal dominant hypocalcemia type 1 (ADH1). A, Free ionized calcium (Ca^{2+}) signals induced by progesterone in sperm from control men and patients with FHH1 or ADH1. Ca^{2+} signals are shown as average (left) or each individual Ca^{2+} signal is shown (right). n = 7 healthy donors, n = 10 from 5 different days (FHH1 Ile81Thr), n = 3 from 3 different days (FHH1 Tyr63Cys), n = 2 from 2 different days (ADH1 Arg955Stop). B, Progesterone amplitudes relative to the amplitudes of 10 μM ionomycin or 100-mM $CaCl_2$. n = 4-5 control men, n = 4 (FHH1 patient Ile81Thr), n = 2-3 (FHH1 Tyr63Cys), n = 2 (ADH1 Arg955Stop), all from different days. C, Acrosome-reacted spermatozoa after 30 minutes of treatment (percentage of total number of spermatozoa) in control men and patients with FHH1 or ADH1. n = 10-14 from 10 men (control men), n = 1 (FHH1 Ile81Thr), n = 2 average from 2 different days (FHH1Tyr63Cys), n = 1 (ADH1 Arg955Stop). D, Percentage of dead cells after treatments in the acrosome reaction assay. n = 10-14 from 10 men (control men), n = 1 (FHH1 Ile81Thr), n = 2 average from 2 different days (FHH1Tyr63Cys), n = 1 (ADH1 Arg955Stop). Data are presented individually and as mean with or without ± SEM. **P* less than .05; ***P* less than .01; ****P* less than .001.

calcium influx on the extracellular environment rather than exclusive dependence on release from intracellular stores. This implies that the high level of Mg^{2+} in the seminal fluid is unable to activate the spermatozoa because of low extracellular ionized calcium and a high concentration of the indirect CatSper-inhibitor zinc (35). The low Ca^{2+} in the cauda epididymis and ejaculate may therefore keep the spermatozoa quiescent until they reach the female reproductive tract, maybe in combination with the high phosphate concentration that can reinforce the inactive conformation of CaSR (23, 36). Spermine is available in high concentration (1-5 mM) in human seminal fluid (12) and induced a potent increase in $[Ca^{2+}]_i$. However, spermine was the only tested CaSR agonist that changed pH, which alone activates CatSper (37). Moreover, only a fraction of the spermatozoa is exposed to the fluid from the prostate and seminal

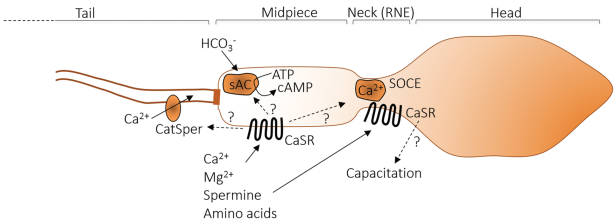

Figure 7. Proposed calcium-sensing receptor (CaSR) signaling pathways in human spermatozoa. CaSR is expressed in the neck region and midpiece and is near the intracellular calcium storage in the neck (nuclear redundant envelope) and soluble adenylyl cyclase (sAC) that may be 2 of the downstream mediators of CaSR activation. The exact downstream signaling is unclear but there seems to be dependence of CatSper activation although the complementary expression in tail and midpiece/neck indicates dependence of another signaling pathway that can bridge CaSR and CatSper. The effect of HCO_3^- on sAC activity is dependent on either free ionized magnesium (Mg^{2+}) or free ionized calcium (Ca^{2+}) in the extracellular compartment, which suggests that CaSR may be involved in mediating this activation of sAC in a high bicarbonate environment but independent of calcium influx. The amino acid concentrations are also high in the seminal fluid, and they may influence the sensitivity to the concentrations of Mg^{2+} and Ca^{2+}. The effects of CaSR seem to be most relevant in the epididymis for induction of motility and storage and in the woman possible for capacitation, hyperactivation, and survival.

vesicle, which suggests that CaSR may be of greater importance during epididymal transit and in the woman.

In the female reproductive tract, spermatozoa escape the millimolar concentrations of citrate and phosphate in seminal fluid and encounter on average 2.2 mM total and 1.23 mM Ca^{2+} in the follicular fluid, which is in line with a previous report (18). The sperm also encounter high concentrations of HCO_3^-, prostaglandins, progesterone, and Mg^{2+} that facilitate the spermatozoa to undergo capacitation, acrosome reaction, become hyperactivated, and bind to the zona pellucida of the oocyte. Ca^{2+} induced the acrosome reaction, but this induction was lost in the FHH1 patient with the Ille81Thr mutation. Interestingly, one of the patients with FHH1 (Ile81Thr) had more spontaneous acrosome-reacted spermatozoa and more dead cells compared with spermatozoa from control men. This supports a role for CaSR in sperm survival, which may be augmented in the patient with a loss-of-function of *CASR* and impaired semen quality. In accordance, sperm from the patient with FHH1 due to the Ile81Thr mutation had a sustained increase in $[Ca^{2+}]_i$ after calcium administration, whereas sperm from control men exhibited $[Ca^{2+}]_i$ levels that normalized shortly after, thus highlighting different kinetics in the responses. Possibly, CaSR plays a role in maintaining an optimal $[Ca^{2+}]_i$, and dysfunction could lead to impaired sperm survival and an untimely acrosome reaction, which correlates with the number of dead spermatozoa (38). It has previously been shown that HCO_3^- and Ca^{2+} are key activators of sAC—the most important adenylyl cyclase in spermatozoa (39). We show in line with previous reports a combined activation of sAC by HCO_3^- and Ca^{2+} that can be abrogated by the removal of external Ca^{2+} (40, 41). However, we also show that high HCO_3^- potentiates Ca^{2+} influx on calcium administration. Moreover, we show that Mg^{2+}, which is unable to enter through CatSper, can exert a similar potentiation of the HCO_3^- response in a low-extracellular Ca^{2+} environment. Moreover, increasing the concentration of Ca^{2+} increased the EC_{50} for Mg^{2+}-induced $[Ca^{2+}]_i$-signals. Combined, this suggests that Ca^{2+} and Mg^{2+} may compete for binding sites and activation possible through sAC or another downstream signaling target. Interestingly, releasing Ca^{2+} from intracellular stores does not rescue the lack of response to HCO_3^- in a low-Ca^{2+} environment (41), which highlights the dependence on extracellular Mg^{2+} or Ca^{2+}. We propose that external Ca^{2+} and Mg^{2+} through CaSR may potentiate the effects of high bicarbonate on sAC and cAMP generation. This may be different in a low-bicarbonate environment (male site), in which minor effects were observed at high concentrations of Ca^{2+} (13 mM), although this at least to some degree may be compensated by high levels of amino acids that may increase CaSR sensitivity (42-44). In a high-bicarbonate situation, the potentiation of the response is fully saturated at physiological female calcium levels. Interestingly, spermatozoa from a patient with a loss-of-function mutation in *CASR* (Ile81Thr) were responsive to high bicarbonate although with less efficacy compared with control men, and treatment with high HCO_3^- at physiological and lower supraphysiological Ca^{2+} concentrations (up to 3.5 mM) were unable to induce any response in the patient with Ile81Thr.

The CatSper inhibitor RU1968 abrogated or greatly reduced the increases in $[Ca^{2+}]_i$ induced by Ca^{2+}, Mg^{2+}, and cinacalcet, which indicates that the CaSR response depends on CatSper activation (45). CaSR is dependent on $G_{q/11}$ in other tissues, but the specific inhibitor YM-254890 (46) did not affect the sperm response to cinacalcet. Interestingly, pretreatment with the sAC inhibitor KH7 inhibited the cinacalcet responses, which suggests that sAC may be downstream of CaSR activation. The negative allosteric CaSR modulator NPS 2143 suppressed both high and low concentration effects of progesterone and calcium by 50% to 60%, whereas NPS 2143 completely abrogated the effect of cinacalcet. NPS 2143 and cinacalcet compete for an overlapping binding site in the transmembrane domain (47), whereas Ca^{2+} binds at other sites on the receptor (23, 48). Another possibility is that CaSR is directly coupled to an ion channel, for instance, CatSper as proposed in other tissues (49, 50) or to other second messenger systems or that cinacalcet or NPS 2143 can bind directly to CatSper. One may argue that calcium

influx is required for the observed bicarbonate effect, but Carlson et al. (51) showed that CatSper is not required for the action of bicarbonate in mice, and we show here that Mg^{2+} can augment the effect in a low-calcium environment in humans by increasing cAMP, thus questioning the exclusive dependence of calcium influx. The progesterone response was diminished in FHH1 patients (see Fig. 6A and 6B), indicating that the CaSR influences the sensitivity to progesterone and thereby influences fertility (2). Global *Casr* knockout mice die at P10 (52) and cannot be used to assess the role of CaSR in male fertility. Heterozygous carriers of a *CASR* loss-of-function mutation can pass on the mutation (10), but their ion channels and receptors are synthesized by their diploid progenitors during spermatogenesis, which makes the question difficult to answer fully in humans. Still, the ability to conceive naturally in humans with heterozygous loss-of-function mutations suggests that CaSR impairment in most cases can be compensated for and fertility preserved. However, FHH1 patients are typically heterozygotes, which implies that the premeiotic spermatogonia transcribe, translate, and express CaSR so the wild-type and mutant CaSR are coexpressed in postmeiotic germ cells as the protein originates from processing prior to meiosis. This could explain why FHH1 patients with a heterozygous loss-of-function mutation in *CASR* have a mild phenotype as both wild-type and mutant CaSR isoforms coexist in the same spermatozoa unlike homozygous patients that may be infertile. Interestingly, the patient with the Trp818 stop mutation had the most severe reproductive phenotype, possibly because the TMD6 truncation led to a nonfunctional protein also in the heterozygote state (53). As shown previously, Trp818Leu results in ADH1 despite theoretically 25% wt-wt CaSR on the cell surface (54).

In conclusion, this study shows that CaSR is important for sensing Ca^{2+}, Mg^{2+}, and HCO_3^- in human sperm, which may be of biological relevance and ultimately provide a new platform for supporting the FHH1 diagnosis.

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Additional Information

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Disclosures: The authors have nothing to declare.

Data Availability: Data sharing is not applicable to this article because no data sets were generated or analyzed during the present study.

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