Ca\(^{2+}\) Signals Generated by CatSper and Ca\(^{2+}\) Stores Regulate Different Behaviors in Human Sperm*§

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Background: Ca\(^{2+}\) signals, elicited by cues from the oocyte and female tract, regulate human sperm behavior.

Results: CatSper channel activation (flagellum) and Ca\(^{2+}\) store mobilization (neck) caused similar [Ca\(^{2+}\)]\(_i\) elevation but induced functionally different behaviors.

Conclusion: Sperm motility pattern is determined by the site of Ca\(^{2+}\) mobilization.

Significance: Selection of Ca\(^{2+}\) signaling components and/or regulation of their availability for activation controls human sperm behavior.

[Ca\(^{2+}\)]\(_i\) signaling regulates sperm motility, enabling switching between functionally different behaviors that the sperm must employ as it ascends the female tract and fertilizes the oocyte. We report that different behaviors in human sperm are recruited according to the Ca\(^{2+}\) signaling pathway used. Activation of CatSper (by raising pH, or stimulating with progesterone) caused sustained [Ca\(^{2+}\)]\(_i\), elevation but did not induce hyperactivation, the whiplash-like behavior required for progression along the oviduct and penetration of the zona pellucida. In contrast, penetration into methylcellulose (mimicking penetration into cervical mucus or cumulus matrix) was enhanced by activation of CatSper. NNC55-0396, which abolishes CatSper currents in human sperm, inhibited this effect. Treatment with 5 \(\mu\)M thimerosal to mobilize stored Ca\(^{2+}\) caused sustained [Ca\(^{2+}\)]\(_i\), elevation and induced strong, sustained hyperactivation that was completely insensitive to NNC55-0396. Thimerosal had no effect on penetration into methylcellulose. 4-Aminopyridine, a powerful modulator of sperm motility, both raised pH, and mobilized Ca\(^{2+}\) stored in sperm (and from microsomal membrane preparations). 4-Aminopyridine-induced hyperactivation even in cells suspended in Ca\(^{2+}\)-depleted medium and also potentiated penetration into methylcellulose. The latter effect was sensitive to NNC55-0393, but induction of hyperactivation was not. We conclude that these two components of the [Ca\(^{2+}\)]\(_i\) signaling apparatus have strikingly different effects on sperm motility. Furthermore, since stored Ca\(^{2+}\) at the sperm neck can be mobilized by Ca\(^{2+}\)-induced Ca\(^{2+}\) release, we propose that CatSper activation can elicit functionally different behaviors according to the sensitivity of the Ca\(^{2+}\) store, which may be regulated by capacitation and NO from the cumulus.

In order to fertilize an oocyte, a mammalian sperm must be able to enter and progress through viscous and visco-elastic substances, bind to and then successfully detach from the oviductal epithelium, and penetrate both the gelatinous cumulus matrix and the fibrous zona pellucida. In all of these instances, adoption of an appropriate flagellar beat pattern, which generates a characteristic behavior, is crucial (1–3). The ability of mammalian spermatozoa to change their pattern of motility was first described by Yanagimachi (4). He observed that incubation of hamster sperm under conditions supporting capacitation (the maturation process that occurs in the female tract by which sperm gain competence to fertilize) induced a change in flagellar beat from high frequency, low amplitude, symmetrical bending to a pattern characterized by asymmetry, deep flagellar bends, increased flexure of the midpiece/proximal flagellum, and exaggerated lateral movement of the head. This more vigorous type of motility, known as hyperactivation, varies between species and may even take more than one form within the same population of cells (5, 6), suggesting that a number of functionally different sperm “behaviors” occur.

[Ca\(^{2+}\)]\(_i\) signaling is the primary regulator of sperm flagellar beat (7) and CatSper, a Ca\(^{2+}\)-permeable channel that is expressed only in the membrane of the sperm flagellum (8), is central to this process. Sperm from CatSper-null mice are motile but sterile because they fail to hyperactivate and cannot fully ascend the female tract (9) or penetrate the zona pellucida.
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Experimental Procedures

Salines/Media

Supplemented Earle’s balanced salt solution (sEBSS) contained 1.0167 mM Na\textsubscript{2}PO\textsubscript{4}, 5.4 mM KCl, 0.811 mM MgSO\textsubscript{4}, 7H\textsubscript{2}O, 5.5 mM C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}, 2.5 mM C\textsubscript{2}H\textsubscript{3}NaO\textsubscript{3}, 19.0 mM CH\textsubscript{3}COONa, 1.8 mM CaCl\textsubscript{2}, 2H\textsubscript{2}O, 25.0 mM NaHCO\textsubscript{3}, 118.4 mM NaCl, and 15 mM HEPES (pH 7.35, 285–295 mosM), supplemented with 0.3% (w/v) fatty acid-free BSA. EGTA-buffered medium contained 5 mM Ca\textsuperscript{2+}, 6 mM EGTA (<100 nM at pH 8.4). Osmotic strength was maintained by adjusting NaCl.

Synthetic tubal fluid (STF) (based on Ref. 29) consisted of 4.7 mM KCl, 3 mM CaCl\textsubscript{2}, 1 MgSO\textsubscript{4}, 7H\textsubscript{2}O, 106 mM NaCl, 5.6 mM D-glucose, 1.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 1.0 mM sodium pyruvate, 41.8 mM sodium lactate, 25 mM NaHCO\textsubscript{3}, 1.33 mM glycine, 0.68 mM glutamine, 0.07 mM taurine, non-essential amino acids (1:100 dilution in STF) and 3% (w/v) BSA. HEPES-buffered non-capacitating medium adapted from STF contained 5.4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 0.8 mM MgSO\textsubscript{4}, 7H\textsubscript{2}O, 116.4 mM NaCl, 5.6 mM D-glucose, 1.0 mM NaH\textsubscript{2}PO\textsubscript{4}, 2.7 mM sodium pyruvate, 41.8 mM sodium lactate, and 25 mM HEPES. Both media were adjusted to 290–320 mosM, pH 7.4.

Preparation and Capacitation of Spermatozoa

Healthy donors were recruited in accordance with the Human Fertilisation and Embryology Authority Code of Practice (University of Birmingham Life and Health Sciences ERC 07-009 and ERN-12-0570; Tayside Committee of Medical Research Ethics B 08/S1402/6). Cells from >40 donors were used over the duration of the study. Sperm collected by masturbation after 2–3 days of sexual abstinence was allowed to liquefy for ~30 min (37 °C, 6% CO\textsubscript{2}). Cells were prepared either by swim-up or density gradient centrifugation.

Swim-up—Cells were harvested by direct swim-up as described previously (27) and adjusted to 6 × 10\textsuperscript{6} cells/ml. Aliquots of 200 μl or 2 ml (according to experimental protocol) were left to capacitate (37 °C, 6% CO\textsubscript{2}) for 5–6 h (30).

Density Gradient Centrifugation—1.5 ml of semen was gently added to the top layer of the density gradient (1 ml of 80% non-capacitating medium-buffered PureSperm™ overlaid with 1 ml of 40% PureSperm™) and centrifuged at 300 × g for 20 min. The pellet was washed in non-capacitating medium (500 × g, 10 min) and then resuspended in STF (8–20 × 10\textsuperscript{6} cells/ml) and incubated for ~2 h (37 °C, 6% CO\textsubscript{2}). Further incubation did not enhance spontaneous hyperactivation or agonist-stimulated [Ca\textsuperscript{2+}]<sub>i</sub> responses.

Assessment of pH<sub>i</sub>

2-ml aliquots (6 × 10\textsuperscript{6} cells·ml\textsuperscript{-1}) were labeled with 1 μM 2′,7′-bis-(2-carboxyethyl)-5-((and-6)-carboxyfluorescein ace-toxymethyl ester (30 min at 37 °C, 6% CO\textsubscript{2}) and then centrifuged (300 × g, 5 min) and resuspended in sEBSS. Emission (535 nm) in response to 12.5-Hz alternating 440/495-nm excitation (slit width = 15 nm) was used to calculate a ratio. After the experiment, pH<sub>i</sub> was calibrated by permeabilizing with Triton X-100 (final concentration 0.12% (v/v)) and then making sequential additions of HCl. At each step, pH was determined.

The abbreviations used are: CICR, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release; sEBSS, supplemented Earle’s balanced salt solution; STF, synthetic tubal fluid; OGB, Oregon Green BAPTA 1; CASA, computer-assisted semen analysis; TMA, trimethylamine hydrochloride; 4-AP, 4-aminopyridine; ALH, amplitude of side-to-side movement of the sperm head; NNC, NNC55-0396.
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with a conventional pH electrode to calibrate the emission ratio (31).

**Microsomal Ca\textsuperscript{2+} Release**

Microsomes were suspended in 2 ml of 40 mM Tris, 100 mM KCl (pH 7.2) in a stirred methylacrylate cuvette at 37 °C. Rabbit skeletal muscle sarcoplasmic reticulum (2 μg) or porcine brain vesicles (40–50 μg) were added in the presence of 250 nm fluo-3 (free acid), 10 μg/ml creatine kinase, and 10 mM phosphocreatine. The addition of Mg-ATP initiated Ca\textsuperscript{2+} uptake. 4-Aminopyridine (4-AP)-induced Ca\textsuperscript{2+} release was measured in the presence of thapsigargin (1–1.5 μM). Fluorescence changes were monitored using excitation/emission of 506/526 nm. [Ca\textsuperscript{2+}] was estimated using [Ca\textsuperscript{2+}] = \( K_d \times (F - F_{\text{min}})/(F_{\text{max}} - F) \), where \( K_d \) is the dissociation constant for Ca\textsuperscript{2+} binding to fluo-3 (900 nm at 37 °C, pH 7.2, in 40 mM Tris and 100 mM KCl), \( F \) is fluorescence intensity of the sample, and \( F_{\text{min}} \) and \( F_{\text{max}} \) are fluorescence intensities with 1.25 mM EGTA and 2 mM CaCl\textsubscript{2}, respectively (32). %Ca\textsuperscript{2+} release was determined by comparison with release observed with 25 μM A23187.

**Single Cell [Ca\textsuperscript{2+}], Imaging**

Loading with Oregon Green BAPTA 1 (OGB) and time lapse fluorescence imaging was as described previously (33). Fluorescence of OGB shows negligible pH sensitivity over the range pH 6–9, making it suitable for the experiments in which pH is varied.

All experiments were performed at 25 ± 0.5 °C (unless stated otherwise) in a continuous flow of medium. Images were normally captured at 0.1 Hz using a 40× oil objective and Q Imaging Rolera-XR cooled CCD or Andor Ixon 897 EMCCD camera controlled by iQ software (Andor Technology, Belfast, UK). Fast (9–60-Hz) imaging was as above, but a ×60 oil objective was used with the Andor camera. Fluorescence from the sperm posterior head/neck was background-corrected and normalized. All experiments, cells were centrifuged (300 × g, 5 min) and resuspended in 1 ml of EGTA-buffered saline ±3 min before stimulation. Saline (control) or agonist was added, and cells were introduced into a prewarmed 20-μm chamber (Microcell, Conception Technologies Ltd.) on an HTM IVOS system (Hamilton Thorn Biosciences, Beverly, MA) (37 °C). Recording (60 Hz) commenced <2 min after the addition of agonist. 2) 1 μl of agonist was added to 99 μl of sperm suspension prepared by density gradient centrifugation, already containing any pretreatment. 4 μl was loaded into a prewarmed 20-μm chamber (Hamilton-Thorn 2X-Cel) and placed immediately onto the heated stage of an Olympus CX41 microscope connected to a Hamilton Thorn CEROS CASA system. Slides were maintained at 37 °C for ~2 min prior to the start of data acquisition. For responses to NH\textsubscript{4}Cl, total time between agonist addition and motility assessment was sometimes reduced to 1 min. Motion characteristics were assessed at 60 Hz.

For both methods, hyperactivation was defined as those cells with curvilinear velocity ≥150 μm/s, linearity <50%, and lateral head displacement ≥7 μm (34). ≥13 track points were required for inclusion of data. At least 20 fields were scored across each slide. Percent motility (control = 85.9 ± 1.5%) was assessed in parallel with assessment of hyperactivation (2–3 min after application of stimulus) in all experiments and was not inhibited compared with control by any treatment unless specifically stated.

For obtaining detailed tracks (e.g. Fig. 2, F–H), cells were prepared as described in method 1, diluted to ~0.5 × 10\textsuperscript{6} cells/ml, and viewed in a 20-μm depth chamber using a Hamamatsu Photonics C9300 CCD camera at 100 Hz. The stage was maintained at 37 °C (LINKAM C0102 stage heater).

**Penetration of Artificial Viscous Medium**

Penetration into methylcellulose (1%, w/v) was assessed as described previously (35). Methylcellulose was made up using non-capacitating medium supplemented with 0.3% BSA was introduced into 5-cm flattened capillary tubes (1.2 × 4.8-mm section, 0.4-mm inner depth; Camlab Ltd., Cambridge, UK). One end was then sealed with Plasticine\textsuperscript{TM}. Sperm were prepared by density gradient centrifugation, adjusted to ~30 × 10\textsuperscript{6}/ml, and incubated for 2 h (37 °C, 6% CO\textsubscript{2}). 1 μl of agonist and 1 μl of NNC55-0396 (when employed) were added to a 99-μl sperm suspension. Open ends of the capillary tubes were inserted into the samples and incubated (37 °C, 6% CO\textsubscript{2}). After 1 h, tubes were removed, wiped, and viewed (final magnification ×200). At 1 and 2 cm from the base of the tube, three fields in each of four planes were counted, and average cells/field was calculated. Cell densities were normalized to values from parallel, untreated controls.

To assess motility in methylcellulose, spermatozoa were harvested by direct swim-up into 1% (w/v) methylcellulose (4000 centipoise at 2% in H\textsubscript{2}O) in STF, adjusted (with the same medium) to ~25 × 10\textsuperscript{6} cells/ml and incubated for ~2 h (37 °C in a 6% CO\textsubscript{2}). 1 μl of agonist (or saline in controls) was added to 99 μl of sperm suspension. After ~1–5 min at 25 °C, 4 μl of sperm suspension was loaded into a prewarmed 20-μm chamber, and motion characteristics were assessed as described above.

**Materials**

Sources for materials were as follows: fatty acid-free BSA (SAFC Biosciences, Lenexa, KS), OGB and fura-2/AM (Invitrogen), poly-n-lysine (BD Biosciences), PureSperm\textsuperscript{TM} (Nidacon, Molndal, Sweden), methylcellulose (Aldrich). All other chemicals used in preparation of media, 4-AP, FITC-PSA, trimethylamine hydrochloride, DMSO, NNC55-0396, and Pluronic F-127 were from Sigma-Aldrich. Hydromount was from BDH Merck. All other chemicals were from Calbiochem.
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**RESULTS**

**CatSper Channel Agonists Do Not Induce Robust Hyperactivation in Human Sperm**—To activate CatSper channels, we increased pH. Resting pH of human sperm capacitated in sEBSS was 6.99 ± 0.06 (n = 23). 25 mM NH₄Cl raised pH by 0.27 ± 0.02 units in <1 min (p < 10⁻⁴; n = 6; Fig. 1, A and B). [Ca²⁺], increased more slowly in response to NH₄Cl, the mean change in fluorescence of OGB (∆F) stabilizing at 22.4 ± 4.1% above control levels within 5 min (n = 4; Fig. 1C; p < 0.02).

In sperm incubated in sEBSS, the proportion of cells showing hyperactivated motility, as assessed by CASA (34), was 3.6 ± 0.4% (n = 60). Stimulation with 25 mM NH₄Cl recruited only a further 4.1 ± 1.3% of cells into the hyperactivated class (p < 0.01; paired t test; n = 21; Fig. 2A). These experiments were repeated using sperm incubated in STF, a medium that promotes rapid and potent sperm capacitation. In STF-incubated cells, the proportion showing “spontaneous” hyperactivated motility (recorded in the absence of stimulation) was increased >3-fold (12.2 ± 1.0%; n = 73; p < 10⁻¹¹), but the effects of NH₄Cl on hyperactivated motility were negligible (Fig. 2B). Frequency distributions of two key kinematic parameters measured by CASA, amplitude of side-side movement of the sperm
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head (ALH) and linear distance from first to last track point/total track length (linearity) confirmed this observation. Activation of CatSper by alkalization reduced the proportion of cells showing very low ALH and very highly linear behavior (≥75%), but there was no increase in the proportion of cells showing ALH ≥9 μm or linearity ≤35% (Fig. 2, C and D).

NH4Cl-induced elevation of pHi is temporary, alkalization of mouse sperm decaying in minutes (36). To address the possibility that elevation of pHi decayed too rapidly for effects on motility to be assessed, we used trimethylamine hydrochloride (TMA), a larger base that causes prolonged, dose-dependent alkalization (37). 10 and 20 mM TMA raised pHi by 0.24 ± 0.01 (n = 3) and 0.49 ± 0.13 (n = 4) pH units (p < 0.005 and p < 0.0001, respectively) this effect persisting for >15 min with only limited decay (Fig. 1, B and D). At both concentrations, we observed a persistent, reversible increase in [Ca2+]i, ΔFmean increasing by 20.6 ± 5.5% (p < 0.002; n = 4) and 25.1 ± 2.4% (p < 0.05; n = 4) with 10 and 20 mM TMA, respectively (Fig. 1E). Neither concentration of TMA increased the proportion of hyperactivated cells (Fig. 2, A, B, and F). Examination of individual components of motility confirmed the lack of effect of this stimulus (data not shown). Progesterone activates CatSper channels (38, 39). Treatment with 3 μM progesterone, a saturating dose for CatSper activation, induces a biphasic [Ca2+]i elevation (Fig. 1F). During the initial, large [Ca2+]i transient, which lasts 1–2 min and includes a component restricted to the sperm neck region (24), some cells show effects on motility resembling hyperactivation (40). When we assessed motility 2–3 min after progesterone application, we recorded only a small increment in the proportion of hyperactivated cells, this effect being greater when cells were prepared in the potently channel blocker, but the sperm K+ channel Slo3 shows very low sensitivity to the drug (44), and it does not depolarize human sperm,6 and the effects of 4-AP test) but did not significantly increase spontaneous hyperactivation (p = 0.09; n = 20). 4-AP-induced hyperactivation (p = 0.17; n = 20), or 4-AP-induced alkalization (p > 0.5; n = 6). 4-AP is commonly used as a K+ channel blocker, but the sperm K+ channel Slo3 shows very low sensitivity to the drug (44), and it does not depolarize human sperm,6 and the effects of 4-AP

6 S. Mansell, S. Publicover, C. Barratt, and M. Wilson, manuscript in preparation.
were not inhibited by clamping \( E_m \) to \( E_K \) with 1 \( \mu M \) valinomycin (data not shown). We conclude that the potent hyperactivating effect of 4-AP on human sperm is not exerted through alkalization or depolarization of the cell.

4-AP Mobilizes Stored Ca\(^{2+}\) — 4-AP is reported to mobilize stored Ca\(^{2+}\) in several types of cells (48–50). We confirmed this effect of 4-AP using rabbit skeletal muscle heavy sarcoplasmic reticulum, which expresses predominantly RyR1 (ryanodine receptor 1) (51). Ca\(^{2+}\) uptake upon the addition of 1.5 mM Mg-ATP was not inhibited by the prior addition of 2 mM 4-AP. Application of 0.1–3.0 mM 4-AP to Ca\(^{2+}\)-loaded microsomes stimulated release of up to 10% of stored (A23187-releasable) Ca\(^{2+}\) (Fig. 3B). To confirm that 4-AP mobilizes stored Ca\(^{2+}\) in human sperm, we tested the effect of the drug on cells super fused with EGTA-buffered saline ([Ca\(^{2+}\)]\(_i\) < 100 \( nM \)). Upon application of EGTA-buffered saline, there was an immediate fall in [Ca\(^{2+}\)]\(_i\), which stabilized at a lower level within 5–10 min. Application of 4-AP within 5 min of EGTA exposure induced a transient [Ca\(^{2+}\)]\(_i\) increase that was superimposed on the EGTA-induced fall (24 ± 8% of cells; \( n = 7 \) experiments; Fig. 3C). Transient duration was 5–10 min, and amplitude ranged from 2.5 to 55% of fluorescence intensity prior to application of 4-AP (mean = 8.7 ± 1.4%; 121 cells). Exposure to EGTA for ≥10 min occluded the effect of 4-AP. Using a high frame rate (60 Hz), we investigated the spatial characteristics of 4-AP-induced [Ca\(^{2+}\)]\(_i\) elevation. 21 of 32 cells (from eight experiments) where head and flagellum remained in focus showed a 4-AP-induced rise in [Ca\(^{2+}\)]\(_i\). 17 of these 21 cells clearly responded first in the head. In similar experiments using the CatSper agonist progesterone (38, 39), 31 of 35 cells responded first in the flagellum (\( p = 2 \times 10^{-7} \); \( \chi^2 \)). It was rarely possible to identify a point of origin of the Ca\(^{2+}\) signal within the head, but when such a focus was visible, it occurred at the head-flagellum junction (sperm neck), the signal spreading into the head, midpiece, and principal piece (supplemental Movie 1). We often observed a bend (typically 10–20º) at the midpiece/neck region during [Ca\(^{2+}\)]\(_i\), elevation. Similarly to the reversibility of bending in the neck region observed during [Ca\(^{2+}\)]\(_i\), oscillations induced by progesterone or NO` (16, 18), the fall in [Ca\(^{2+}\)]\(_i\), upon 4-AP washout caused the flagellum to relax to its original position, and a second application repeated the effect (supplemental Movie 2). During bending at the neck, the principal piece of the flagellum continued to beat but was displaced laterally.

**Mobilization of Stored Ca\(^{2+}\) Induces Hyperactivation** — To test whether the ability of 4-AP to induce hyperactivation was due to its action on stored Ca\(^{2+}\), we investigated effects on motility when [Ca\(^{2+}\)]\(_o\) was buffered with EGTA. When cells capacitated in sEBSS were resuspended in EGTA-buffered medium shortly before the addition of 4-AP, the hyperactivating effect of the drug recorded 1 min after stimulation was similar to that in parallel controls maintained in sEBSS (34.4 ± 6.6% (\( n = 4 \)) compared with 37.3 ± 8.2% (\( n = 3 \)); Fig. 3D; not significant). This effect of 4-AP decayed completely within 12 min, although motility of the cells in EGTA-buffered medium persisted for 20–30 min. In contrast, hyperactivation in parallel control experiments on sperm maintained in sEBSS persisted for >45 min (32.0 ± 4.1% at 45 min, \( n = 3 \); Fig. 3D). These data show that mobilization of stored Ca\(^{2+}\), and probably consequent store-operated Ca\(^{2+}\) influx, is important in the induction and maintenance of hyperactivation by 4-AP.

Thimerosal, at low micromolar concentrations, sensitizes intracellular Ca\(^{2+}\) release (52). At 25 °C, the effect of 5 \( \mu M \) thimerosal was inconsistent, but at 30 °C, the drug reliably induced sustained [Ca\(^{2+}\)]\(_i\) elevation (15.8 ± 1.1% after 7 min; \( n = 6; p < 0.0001; \) Fig. 3E). This temperature sensitivity is consistent with previous observations on the activation of stored Ca\(^{2+}\) release by thimerosal (53). 5 \( \mu M \) thimerosal potently and persistently hyperactivated human sperm (Figs. 2 (A and B, read bars) and 3F), tracks showing the pronounced side-side movements of the sperm head characteristic of hyperactivated motility (Fig. 2H). Analysis of two key kinematic parameters, ALH and linearity, showed striking differences from cells treated with agonists of CatSper. In the presence of thimerosal, the majority of cells showed ALH ≥9 \( \mu M \) and linearity of ≤35% (Fig. 2, C and D). At higher doses, thimerosal was similarly effective in raising [Ca\(^{2+}\)]\(_i\) and inducing hyperactivation, but this effect then decayed (Fig. 3F), accompanied ultimately by a loss of motility as described in mouse sperm (28).

**Penetration of Viscous Medium Is Enhanced by Activation of CatSper but Not by Mobilization of Stored Ca\(^{2+}\)** — A crucial aspect of sperm motility is the ability to penetrate viscous medium. To assess the significance of CatSper and store-mediated [Ca\(^{2+}\)]\(_i\) signals in regulating this aspect of sperm behavior, we assessed penetration of STF-prepared sperm into methylcellulose. In this functional test, efficacy of the various stimuli was reversed compared with their ability to induce hyperactivated motility. 25 mM NH\(_4\)Cl and 10 mM TMA; 2 mM 4-AP, and 3 \( \mu M \) progesterone all enhanced sperm penetration into methylcellulose medium, cell numbers at 1 and 2 cm being significantly increased over those in parallel controls (Fig. 4, A and B). 20 mM TMA was less effective (\( p > 0.5; n = 3 \)). In contrast, 5 \( \mu M \) thimerosal, the most powerful inducer of hyperactivated motility (see above), caused negligible enhancement of penetration into methylcellulose (Fig. 4, A and B). To distinguish between effects on penetration into and progression through viscous medium, we assessed kinematics of cells swimming in methylcellulose. Similarly to rodent sperm (54), methylcellulose greatly reduced speed and lateral head movement. Inclusion of 5 \( \mu M \) thimerosal in the methylcellulose significantly enhanced ALH and decreased beat frequency (\( p < 0.05; n = 6 \)), but this effect was small. None of the stimuli caused a significant change in speed of progression through methylcellulose (data not shown), showing that stimulation of CatSper activity, either directly (progesterone) or via pH, elevation, enhances entry of sperm into methylcellulose rather than their progress through it.

**NCC55-0396 Inhibits Effects of CatSper Manipulation and Reduces Spontaneous Hyperactivation** — NCC55-0396 (NNC) is the most effective blocker of CatSper channels currently available, abolishing CatSper currents of human sperm at low micromolar concentrations (38, 39). When 10 \( \mu M \) NNC was applied to cells incubated in STF, penetration of unstimulated cells into methylcellulose was inhibited by ~20% (\( p < 0.001 \)), and the stimulatory effects of CatSper agonists were significantly inhibited (2 mM 4-AP, \( p < 0.05; 3 \mu M \) progesterone, \( p < 0.01; \) and 25 mM NH\(_4\)Cl, \( p < 0.01; \) Fig. 4C).
The significantly increased level of spontaneous hyperactivation that was seen in cells incubated in STF suggests that this potent capacitating medium leads to spontaneous mobilization of stored Ca\(^{2+}\) and hyperactivation, perhaps reflecting the increased incidence of [Ca\(^{2+}\)]\(_i\) oscillations in highly capacitated cells (25, 26). Because oscillations are generated by CICR, downstream of Ca\(^{2+}\) influx at the plasma membrane, CatSper channels may play a key role in this effect of capacitation. Consistent with this idea, treatment of cells prepared in STF with 10 \(\mu\)M NNC significantly reduced the level of spontaneous hyperactivation compared with parallel controls (from 16.7 \(\pm\) 2.2 to 11.3 \(\pm\) 1.9%; \(p < 0.0005; n = 20\)). Examination of individual experiments showed that the size of this inhibitory effect was correlated with the level of spontaneous hyperactivated motility (Fig. 4D; \(r = 0.75\)). The small hyperactivating effect of progesterone was also sensitive to NNC (Fig. 4E; \(p < 0.001\)), but the powerful hyperactivating actions of 4-AP and thimerosal, which directly mobilize stored Ca\(^{2+}\), were not inhibited (Fig. 4E; \(p > 0.2\)).

**DISCUSSION**

The findings described here confirm the important role of CatSper in regulation of motility but show that, at least in human sperm, its role is not directly to support hyperactivation. In fact, whereas mobilization of stored Ca\(^{2+}\) was a potent inducer of hyperactivated motility, the main functional effect of activating CatSper, by alkalization or by stimulation with progesterone, was to enhance penetration into viscous medium. 4-AP, which activates CatSper (28, 43) and also mobilizes stored Ca\(^{2+}\) (48–50) (Fig. 3), enhanced both aspects of motility.

**FIGURE 4.** CatSper activity enhances penetration into viscous medium and contributes to spontaneous hyperactivation. A and B, increment in cell density (percentage of control) 1 cm (A) and 2 cm (B) into methylcellulose. Cells were stimulated with 25 mM NH\(_4\)Cl (dark blue), 10 mM TMA (light blue), 2 mM 4-AP (green), 3 \(\mu\)M progesterone (yellow), and 5 \(\mu\)M thimerosal (red). Bars show the mean \(\pm\) S.E. (error bars) of 10–20 experiments except for TMA (4). C, increment in cell density (percentage of control) 2 cm into methylcellulose of cells stimulated with 2 mM 4-AP (green), 3 \(\mu\)M progesterone (yellow), and 25 mM NH\(_4\)Cl (dark blue) and in parallel incubations pretreated with 10 \(\mu\)M NNC (black). Bars, mean \(\pm\) S.E. of 8–20 experiments except for TMA (4). D, inhibition of spontaneous hyperactivation upon exposure of STF-capacitated cells to NNC (difference between control and NNC-treated cells; \(\mu\)M NNC) is dependent upon the level of spontaneous hyperactivation prior to application of the drug (\(r = 0.75, n = 19\)). E, increment in hyperactivation (percentage of cells) in response to 2 mM 4-AP (green), 3 \(\mu\)M progesterone (yellow), 25 mM NH\(_4\)Cl (dark blue), and 5 \(\mu\)M thimerosal (red). Black bars, responses in parallel 10 \(\mu\)M NNC-pretreated experiments. Each bar shows mean \(\pm\) S.E. of 9–20 experiments. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\); ****, \(p < 0.0001\) compared with control (A and B) or NNC alone (C and E). NS, not significant.
but only entry into viscous medium was sensitive to the CatSper antagonist NNC. We conclude that regulation of motility in human sperm through [Ca^{2+}], signaling is flexible, different behaviors being recruited according to the source of mobilized Ca^{2+}.

**Effects of CatSper Activation and Ca^{2+} Store Mobilization on Motility**—Changes in motility attributable specifically to CatSper activation, although functionally significant, were subtle. Stimulation with NH_{4}Cl and TMA reduced the proportion of cells with very low lateral head displacement and high track linearity, but there was no increase in cells with extravagant lateral head displacement or low linearity (Fig. 2, C and D). Photolysis of caged progesterone, which will activate CatSper, induced changes in curvature primarily in the distal flagellum (55). CASA, which measures only movement of the sperm head, may be poorly suited to identify subtle changes in flagellar beat that promote entry into viscous medium, and further investigation by three-dimensional tracking (56) and high speed video microscopy of the flagellum in free-swimming cells (57) may be required. Stimulation of hyperactivated motility did not enhance entry into viscous medium, and, as has been described previously for mouse sperm (54), manipulations that induce such movement did not enhance progression within methylcellulose. Instead, hyperactivation is believed to be essential for successful interaction with the oviduct wall and for penetration of the zona (3, 5, 58). [Ca^{2+}], signals mediated by stored Ca^{2+} and by alkalization will differ in their point of origin but were similar in amplitude and kinetics. The mechanism by which these different signals induce different “behaviors” clearly demands further investigation. The bending seen at the sperm neck associated with store mobilization (16) (see supplemental Movies 1 and 2) may contribute to the asymmetric nature of hyperactivated flagellar beating, but this resembles more the “lever” bends that have been observed during zona penetration (5). Significantly, both [Ca^{2+}], elevation and hyperactivation of human sperm induced by 4-AP are positively correlated with fertilization rate in *in vitro* fertilization (42).

**Signaling Pathways Regulating Functional Sperm Behavior**—By selective activation of CatSper and store mobilization, we were able to demonstrate their functionally different effects in regulating motility. How are these two components of Ca^{2+} signaling (and their effects on sperm behavior) recruited in the female tract? CatSper channels are activated by a range of small organic molecules, including progesterone and prostaglandin E_1, and are also sensitive to depolarization, cytoplasmic alkalinization, and probably other aspects of capacitation (38, 39).

CatSper thus acts as a polymodal signaling “node” on which many stimuli converge (11). Mobilization of stored Ca^{2+} at the sperm neck, generating [Ca^{2+}], oscillations, occurs secondarily to influx of Ca^{2+} induced pharmacologically or by progesterone-induced activation of CatSper (18, 25) (Fig. 1F). Progesterone does not directly mobilize stored Ca^{2+} (39), but release at the sperm neck, where both ryanodine receptors (18, 19) and inositol trisphosphate receptors (13) have been described in human sperm, can occur by CICR. The proportion of cells in which oscillations are observed is enhanced by nitric oxide (NO; a product of the oviduct epithelium and cumulus cells surrounding the oocyte) and by capacitation (25, 26). NO’S-nitrosoylates ryanodine receptors in human sperm (59), an action that sensitizes CICR (60). An important component of capacitation may be oxidative stress, which has similar effects (61). NO sensitizes the release of stored Ca^{2+} in human sperm by low doses of progesterone and enhances the effect of progesterone on flagellar excursion (62, 63). In addition, release of stored Ca^{2+} may be modulated by exchange proteins activated directly by cyclic AMP (EPACs). These proteins are known to facilitate release of stored Ca^{2+} and have been detected at the acrosome and neck region in human sperm (64–66). A potential mechanism for mobilization of stored Ca^{2+} at the sperm neck is thus through CatSper-mediated Ca^{2+} influx followed by CICR, where CICR is a “gatekeeper,” determining the availability of each cell for recruitment into the hyperactivated population. Sensitization/desensitization through S-nitrosoylation and signaling events related to capacitation will regulate this “available” population (5). If store mobilization through CICR is supported by CatSper, high levels of spontaneous HA induced by potent capacitating media should be sensitive to blockade of CatSper, and this was the case, the effect of NNC being most marked in cells where the rate of spontaneous hyperactivation was greatest.

An important question here is whether the sperm neck Ca^{2+} store is available for direct activation in response to agonists, recruiting cells into the hyperactivated population without the requirement for propagation downstream of CatSper. The available evidence suggests that this does not occur with progesterone (39). However, solubilized zona pellucida stimulates generation of IP_3 in mouse sperm (67), which is believed to bind acrosomal inositol trisphosphate receptors (17) and mobilize the acrosomal Ca^{2+} store prior to acrosome reaction (68–71). Indeed, although the initial [Ca^{2+}], elevation induced by zona proteins (≤2 min) is not observed in sperm of CatSper-null mice, delayed [Ca^{2+}], responses still occur in a minority of cells, and these may be due to mobilization of stored Ca^{2+} (72).
thermore, recombinant human zona proteins cause a marked reduction in the linearity of human sperm motility (73), an effect that may reflect activation of inositol trisphosphate receptors on the store at the sperm neck (12, 13). Other agonists that may have a direct effect include vitamin D (74), but no stimuli have yet been described that have an efficacy comparable with 4-AP.

Hyperactivation of Sperm from Other Species—In humans, mating and ovulation are not synchronized. Capacitation and hyperactivation may be delayed or even reversed, and flexibility in regulation of behavior is required (75–77). In “model” species, this is not the case, and this may be reflected in regulation of motility. In vitro capacitation of mouse sperm is accompanied by development of hyperactivated motility in a much higher proportion of cells than occurs in humans. In sperm from CatSper-null mice, this hyperactivation does not occur (2, 78), but hyperactivation of these cells can be induced by release of stored Ca\(^{2+}\) (20). Failure of hyperactivation in these cells may be equivalent to the effect of CatSper blockade on spontaneous hyperactivation of human sperm (Fig. 4D), the transition in motility depending on store mobilization downstream of CatSper-mediated Ca\(^{2+}\) influx. If this is the case, the proportion of cells where CICR occurs is much greater than in human sperm. In bovine sperm, activation of CatSper by alkalinization with 25 mM NH\(_4\)Cl (as used in this study) potently stimulated hyperactivated motility (79). One explanation for this difference is that direct induction of hyperactivation by Ca\(^{2+}\) entering through CatSper channels occurs in these cells. However, store mobilization has been shown to induce hyperactivation in bovine sperm (12) and may be involved in this effect.

In summary, we have shown that activation of CatSper and mobilization of stored Ca\(^{2+}\) induce functionally different behaviors in human sperm. Although diverse cues and signals from the cumulus-oocyte complex and female tract apparently converge to activate CatSper (11), diversity in the consequent [Ca\(^{2+}\)], signal, permitting “selection” of components of sperm behavior, may be achieved by regulation of downstream mobilization of stored Ca\(^{2+}\) at the sperm neck. These findings reveal new complexity to the biology of human sperm and their interaction with the tract and oocyte during fertilization.

REFERENCES
1. Strauss, C. R., Votta, T. I., and Suarez, S. S. (1995) Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. Biol. Reprod. 53, 1280–1285
2. Quill, T. A., Sugden, S. A., Rossi, K. L., Doolittle, L. K., Hammer, R. E., and Garbers, D. L. (2003) Hyperactivated sperm motility driven by CatSper2 is required for fertilization. Proc. Natl. Acad. Sci. U.S.A. 100, 14669–14674
3. Suarez, S. S. (2006) Control of hyperactivation in sperm. Hum. Reprod. Update 14, 647–657
4. Yanagimachi, R. (1970) The movement of golden hamster spermatozoa before and after capacitation. J. Reprod. Fertil. 23, 193–196
5. Drobins, E. Z., Yudin, A. I., Cherr, G. N., and Katz, D. F. (1988) Hamster sperm penetration of the zona pellucida. Kinematic analysis and mechanical implications. Dev. Biol. 130, 311–323
6. Kay, V. J., and Robertson, L. (1998) Hyperactivated motility of human spermatozoa. A review of physiological function and application in assisted reproduction. Hum. Reprod. Update 4, 776–786
7. Darszon, A., Nishigaki, T., Beltran, C., and Treviño, C. L. (2011) Calcium channels in the development, maturation, and function of spermatozoa. Physiol. Rev. 91, 1305–1355
8. Lishko, P. V., Kirichok, Y., Ren, D., Navarro, B., Chung, J. I., and Clapham, D. E. (2012) The control of male fertility by spermatozoan ion channels. Annu. Rev. Physiol. 74, 453–475
9. Ho, K., Wolff, C. A., and Suarez, S. S. (2009) CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. Reprod. Fertil. Dev. 21, 345–350
10. Ren, D., Navarro, B., Perez, G., Jackson, A. C., Hsu, S., Shi, Q., Tilly, J. L., and Clapham, D. E. (2001) A sperm ion channel required for sperm motility and male fertility. Nature 413, 603–609
11. Brenner, C., Goodwin, N., Weyand, I., Kashkar, N. D., Narse, M., Krähling, M., Müller, A., Kaupp, U. B., and Strünker, T. (2012) The CatSper channel. A polymodal chemosensor in human sperm. EMBO J. 31, 1654–1665
12. Ho, D. C., and Suarez, S. S. (2001) An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca\(^{2+}\) store is involved in regulating sperm hyperactivated motility. Biol. Reprod. 65, 1606–1615
13. Naaby-Hansen, S., Wolkowicz, M. J., Klotz, K., Bush, L. A., Westbrook, V. A., Shibahara, H., Shetty, J., Coonrod, S. A., Reddi, P. P., Shannon, J., Kinter, M., Sherman, N. F., Fox, J., Flickinger, C. J., and Herr, J. C. (2001) Co-localization of the inositol 1,4,5-trisphosphate receptor and calreticulin in the equatorial segment and in membrane bounded vesicles in the cytoplasmic droplet of human spermatozoa. Mol. Hum. Reprod. 7, 923–933
14. Treviño, C. L., Santi, C. M., Beltrán, C., Hernández-Cruz, A., Darson, A., and Lomeli, H. (1998) Localisation of inositol trisphosphate and ryanodine receptors during mouse spermatogenesis. Possible functional implications. Zygote 6, 159–172
15. Chiarrella, P., Puglisi, R., Sorrentino, V., Boitani, C., and Stefanini, M. (2004) Ryanodine receptors are expressed and functionally active in mouse spermatozoic cells and their inhibition interferes with spermatogonial differentiation. J. Cell Sci. 117, 4127–4134
16. Costello, S., Michelangeli, F., Nash, K., Lefievre, L., Morris, J., Machado-Oliveira, G., Barratt, C., Kirkman-Brown, J., and Publicover, S. (2009) Ca\(^{2+}\) stores in sperm. Their identities and functions. Reproduction 138, 425–437
17. Walensky, L. D., and Snyder, S. H. (1995) Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. J. Cell Biol. 130, 857–869
18. Harper, C. V., Barratt, C. L., and Publicover, S. I. (2004) Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca\(^{2+}\)] oscillations and cyclical transitions in flagellar beating. J. Biol. Chem. 279, 46315–46325
19. Park, K. H., Kim, B. J., Kang, J., Nam, T. S., Lim, J. M., Kim, H. T., Park, J. K., Kim, Y. G., Chae, S. W., and Kim, U. H. (2011) Ca\(^{2+}\) signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. Sci. Signal. 4, ra31
20. Marquez, B., Ignotz, G., and Suarez, S. S. (2007) Contributions of extracellular and intracellular Ca\(^{2+}\) to regulation of sperm motility. Release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. Dev. Biol. 303, 214–221
21. Bedu-Addo, K., Barratt, C. L., Kirkman-Brown, J. C., and Publicover, S. I. (2007) Patterns of [Ca\(^{2+}\)] mobilization and cell response in human spermatozoa exposed to progesterone. Dev. Biol. 302, 324–332
22. Olson, S. D., Suarez, S. S., and Fauci, L. J. (2010) A model of CatSper channel mediated calcium dynamics in mammalian spermatozoa. Bull. Math. Biol. 72, 1925–1946
23. Buffone, M. G., Ji, T. W., Cao, W., Merdiushhev, T., Aghajanian, H. K., and Gerton, G. L. (2012) Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. Mol. Reprod. Dev. 79, 4–18
24. Lefievre, L., Nash, K., Mansell, S., Costello, S., Punt, E., Correia, J., Morris, J., Kirkman-Brown, J., Wilson, S. M., Barratt, C. L., and Publicover, S. (2012) 2-APB-potentiated channels amplify CatSper-induced Ca\(^{2+}\) signals in human sperm. Biochem. J. 448, 189–200
25. Kirkman-Brown, J. C., Barratt, C. L., and Publicover, S. I. (2004) Slow calcium oscillations in human spermatozoa. Biochem. J. 378, 827–832
26. Aitken, R. J., and McLaughlin, E. A. (2007) Molecular mechanisms of sperm capacitation. Progesterone-induced secondary calcium oscillations
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68. O'Toole, C. M., Arnoult, C., Darszon, A., Steinhardt, R. A., and Florman, H. M. (2000) Ca^{2+} entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol. Biol. Cell* **11**, 1571–1584

69. Florman, H. M., Jungnickel, M. K., and Sutton, K. A. (2008) Regulating the acrosome reaction. *Int. J. Dev. Biol.* **52**, 503–510

70. Fukami, K., Yoshida, M., Inoue, T., Kurokawa, M., Fissoere, R. A., Yoshida, N., Mikoshiba, K., and Takenawa, T. (2003) Phospholipase Cδ4 is required for Ca^{2+} mobilization essential for acrosome reaction in sperm. *J. Cell Biol.* **161**, 79–88

71. Herrick, S. B., Schweisinger, D. L., Kim, S. W., Bayan, K. R., Mann, S., and Cardullo, R. A. (2005) The acrosomal vesicle of mouse sperm is a calcium store. *J. Cell Physiol.* **202**, 663–671

72. Xia, J., and Ren, D. (2009) Egg coat proteins activate calcium entry into mouse sperm via CATSPER channels. *Biol. Reprod.* **80**, 1092–1098

73. Caballero-Campo, P., Chirinos, M., Fan, X. J., González-González, M. E., Galicia-Chavarría, M., Larrea, F., and Gerton, G. L. (2006) Biological effects of recombinant human zona pellucida proteins on sperm function. *Biol. Reprod.* **74**, 760–768

74. Blomberg Jensen, M., Bjerrum, P. J., Jessen, T. E., Nielsen, J. E., Joensen, U. N., Olesen, I. A., Petersen, J. H., Juul, A., Dissing, S., and Jørgensen, N. (2011) Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa. *Hum. Reprod.* **26**, 1307–1317

75. Suarez, S. S., and Pacey, A. A. (2006) Sperm transport in the female reproductive tract. *Hum. Reprod. Update* **12**, 23–37

76. Pujianto, D. A., Curry, B. J., and Aitken, R. J. (2010) Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* **151**, 1269–1279

77. Mortimer, S. T. (1997) A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum. Reprod. Update* **3**, 403–439

78. Carlson, A. E., Westenbroek, R. E., Quill, T., Ren, D., Clapham, D. E., Hille, B., Garbers, D. L., and Babcock, D. F. (2003) CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14864–14868

79. Marquez, B., and Suarez, S. S. (2007) Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. *Biol. Reprod.* **76**, 660–665