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Ca^{2+} Signals Generated by CatSper and Ca^{2+} Stores Regulate Different Behaviors in Human Sperm*[S]

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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_i 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 μM 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_i 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 NNC55-039, 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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(2, 10). CatSper is pH-activated and weakly voltage-dependent, but in human sperm it has been shown to be sensitive also to progesterone, prostaglandin E_1, and other ligands (11). It is thus a polymodal Ca^{2+} signaling "node," which can integrate the actions of diverse stimuli on sperm motility. Mammalian spermatogenic cells and mature sperm also contain Ca^{2+} storage organelles and express inositol trisphosphate receptors and ryanodine receptors (12–19). Studies on sperm from rodents, bulls, and humans have shown that mobilization of Ca^{2+} from a store at the sperm neck region stimulates flagellar activity and can even support hyperactivation in CatSper-null sperm (12, 16, 18, 20, 21).

Stimulation of human sperm with progesterone activates CatSper in the flagellum, but the consequent $[Ca^{2+}]_i$ elevation is amplified and propagated forward, apparently by Ca^{2+}-induced Ca^{2+} release (CICR)[5] at the sperm neck (16, 22, 23, 24). A subset of progesterone-stimulated sperm also generate $[Ca^{2+}]_i$ oscillations by cyclically mobilizing this store (18, 25, 26), each $[Ca^{2+}]_i$ transient being accompanied by synchronous flexure of the sperm neck and a burst of increased flagellar excursion (18, 27). Removal of extracellular Ca^{2+} abolishes these $[Ca^{2+}]_i$ oscillations, but reduction of $[Ca^{2+}]_o$ to low micromolar concentrations has no inhibitory effect. If progesterone is withdrawn or CatSper is partially blocked, oscillations arrest but may restart after a brief interval (18, 24, 26). Thus, only a minimal level of $[Ca^{2+}]_i$ influx is required for their maintenance, apparently supporting both CICR and store refilling. CatSper channels and release of stored Ca^{2+} at the sperm neck thus provide two separate but interlinked Ca^{2+} signaling mechanisms for regulation of motility in human sperm.

CatSper integrates diverse signals and cues into a common Ca^{2+} message at the flagellum (11), yet the sperm must employ and switch between functionally different behaviors in response to different environments and stimuli. Release of stored Ca^{2+} at the sperm neck could provide a mechanism for generating diversity in sperm $[Ca^{2+}]_i$ signals, adding temporal and spatial complexity and allowing for selection between functionally different types of motility. Consistent with this hypothesis, the direction of the primary flagellar bend (closest to the head) in mouse sperm differs according to the source of Ca^{2+} (28). We have investigated the effects of CatSper activation and Ca^{2+} store mobilization on $[Ca^{2+}]_i$ signaling, motility, and functional competence of human sperm. We demonstrate that these two components of the sperm $[Ca^{2+}]_i$ signaling apparatus have strikingly different effects on sperm motility. This allows the cell to generate functionally different behaviors according to the source of mobilized Ca^{2+}. We propose that modulation of CICR at the sperm neck Ca^{2+} store, by capacitation and in response to signals from the female tract, may regulate the behavioral output in response to activation of CatSper.

[5] The abbreviations used are: CICR, Ca^{2+}-induced Ca^{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-side movement of the sperm head; NNC, NNC55-0396.

EXPERIMENTAL PROCEDURES

Salines/Media

Supplemented Earle's balanced salt solution (sEBSS) contained 1.0167 mM NaH_2PO_4, 5.4 mM KCl, 0.811 mM $MgSO_4 \cdot 7H_2O$, 5.5 mM $C_6H_{12}O_6$, 2.5 mM $C_3H_3NaO_3$, 19.0 mM $CH_3CH(OH)COONa$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 25.0 mM $NaHCO_3$, 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^{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 $CaCl_2$, 1 $MgSO_4 \cdot 7H_2O$, 106 mM NaCl, 5.6 mM D-glucose, 1.5 mM NaH_2PO_4, 1 mM sodium pyruvate, 41.8 mM sodium lactate, 25 mM $NaHCO_3$, 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 $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 116.4 mM NaCl, 5.6 mM D-glucose, 1.0 mM NaH_2PO_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. Semen collected by masturbation after 2–3 days of sexual abstinence was allowed to liquefy for ~30 min (37 °C, 6% CO_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 6×10^6 cells/ml. Aliquots of 200 μl or 2 ml (according to experimental protocol) were left to capacitate (37 °C, 6% CO_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 ($8–20 \times 10^6$ cells/ml) and incubated for ~2 h (37 °C, 6% CO_2). Further incubation did not enhance spontaneous hyperactivation or agonist-stimulated $[Ca^{2+}]_i$ responses.

Assessment of pH_i

2-ml aliquots (6×10^{-6} cells·ml^{-1}) were labeled with 1 μM 2′-7′-bis-(2-carboxyethyl)-5-(and-6-)-carboxyfluorescein acetoxymethyl ester (30 min at 37 °C, 6% CO_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_i 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

with a conventional pH electrode to calibrate the emission ratio (31).

Microsomal Ca^{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 sacroplasmic 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^{2+} uptake. 4-Aminopyridine (4-AP)-induced Ca^{2+} release was measured in the presence of thapsigargin (1–1.5 μM). Fluorescence changes were monitored using excitation/emission of 506/526 nm. $[Ca^{2+}]$ was estimated using $[Ca^{2+}] = K_d \times ((F - F_{min})/(F_{max} - F))$, where K_d is the dissociation constant for Ca^{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_{min} and F_{max} are fluorescence intensities with 1.25 mM EGTA and 2 mM $CaCl_2$, respectively (32). $\%Ca^{2+}$ release was determined by comparison with release observed with 25 μM A23187.

Single Cell $[Ca^{2+}]_i$ 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_i 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 as described previously (33) using $\Delta F = ((F - F_{rest})/F_{rest}) \times 100\%$, where ΔF is percentage change in intensity, F is fluorescence intensity at time t, and F_{rest} is the mean of ≥10 determinations of F during the control period. Mean ΔF of all cells in the experiment (ΔF_{mean}) was used to compare responses between experiments, and n values represent the number of experiments.

Assessment of Hyperactivation

Two computer-assisted semen analysis (CASA) protocols were used. 1) 200-μl aliquots of spermatozoa (6×10^{-6} cells·ml^{-1}) were prepared by swim-up into sEBSS and capacitated for 5–6 h as described above. For Ca^{2+}-buffered 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_4Cl, 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. Percentage 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, *E–H*), cells were prepared as described in method 1, diluted to ~0.5×10^6 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™. Sperm were prepared by density gradient centrifugation, adjusted to ~30×10^6/ml, and incubated for 2 h (37 °C, 6% CO_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_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_2O) in STF, adjusted (with the same medium) to ~25×10^6 cells/ml and incubated for ~2 h (37 °C in a 6% CO_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-D-lysine (BD Biosciences), PureSperm™ (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.

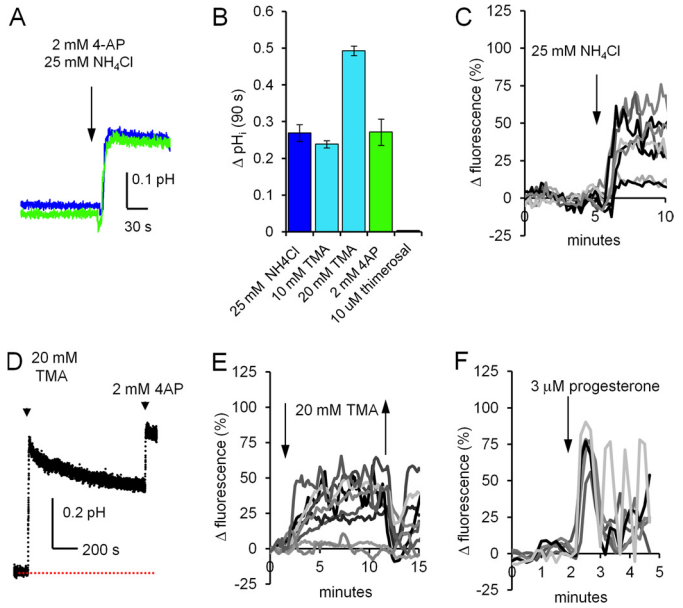

FIGURE 1. **Alkalinization raises $[Ca^{2+}]_i$ in human sperm.** *A*, 2 mM 4-AP (*green trace*) and 25 mM NH_4Cl (*dark blue trace*) cause similar changes in pH_i of human sperm populations. The additions are marked by an *arrow*. Both aliquots were from the same ejaculate. *B*, amplitude of pH_i increment imposed by 25 mM NH_4Cl (*dark blue*), 10 and 20 mM TMA (*light blue*), 2 mM 4-AP (*green*), and 10 mM thimerosal (*red*). Each *bar* shows the mean of 4–10 experiments ± S.E. (*error bars*). *C*, 25 mM NH_4Cl (added at the *arrow*) increases $[Ca^{2+}]_o$ (OGB fluorescent intensity) in human sperm. Shown are the responses of eight cells in the same experiment. *D*, 20 mM TMA (*first arrow*) induces a large prolonged increment in pH_i of human sperm. 2 mM 4-AP was added at the *second arrow*. *E*, 20 mM TMA (added at the *first arrow*) increases $[Ca^{2+}]_o$ (OGB fluorescent intensity) in human sperm. The *upward arrow* shows TMA washout. Shown are the responses of eight cells in the same experiment. *F*, 3 μM progesterone (added at the *arrow*) causes a biphasic increase in $[Ca^{2+}]_o$ (OGB fluorescent intensity) in human sperm. Shown are the responses of six cells in the same experiment, one of which generates $[Ca^{2+}]_i$ oscillations after the initial transient.

Statistical Analysis

Values in are stated as mean ± S.E. *n* represents the number of independent experiments. Microsoft Excel was used to perform paired or unpaired Student's *t* tests, χ^2 tests, and correlations as appropriate. Statistical significance was set at $p < 0.05$. Hyperactivation percentage data were arcsine-transformed before testing for significance.

RESULTS

CatSper Channel Agonists Do Not Induce Robust Hyperactivation in Human Sperm—To activate CatSper channels, we increased pH_i. Resting pH_i of human sperm capacitated in sEBSS was 6.99 ± 0.06 ($n = 23$). 25 mM NH_4Cl raised pH_i by 0.27 ± 0.02 units in <1 min ($p < 10^{-4}$; $n = 6$; Fig. 1, *A* and *B*). $[Ca^{2+}]_i$ increased more slowly in response to NH_4Cl, the mean change in fluorescence of OGB (ΔF_{mean}) stabilizing at 22.4 ± 4.1% above control levels within 5 min ($n = 4$; Fig. 1*C*; $p < 0.02$). 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 NH_4Cl recruited only a further 4.1 ± 1.3% of cells into the hyperactivated class ($p < 0.01$; paired *t* test; $n = 21$; Fig. 2*A*). 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

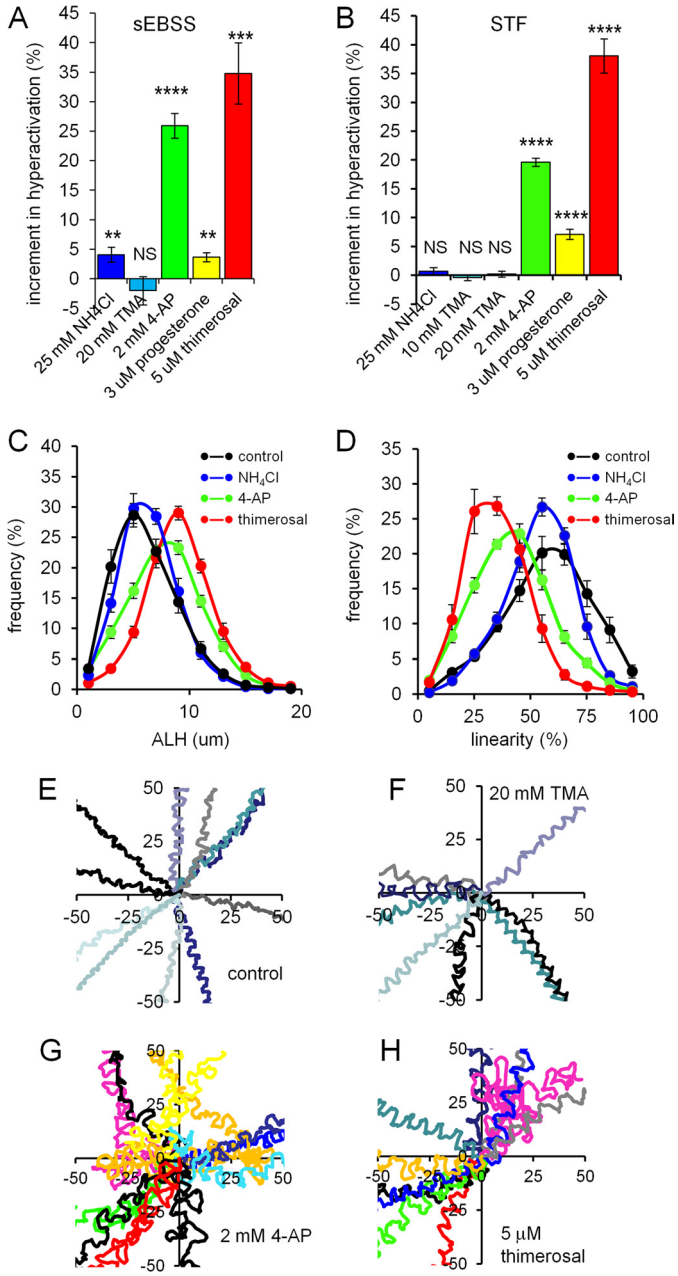

FIGURE 2. **Stored Ca^{2+} but not activation of CatSper induces hyperactivation.** *A* and *B*, increment in percentage of hyperactivated cells induced by 25 mM NH_4Cl (*dark blue*), 20 and 10 mM TMA (*light blue*), 2 mM 4-AP (*green*), 3 μM progesterone (*yellow*), and 5 μM thimerosal (*red*). *A*, cells prepared by swim-up into sEBSS; *B*, cells prepared by density gradient centrifugation into STF. Each *bar* shows mean ± S.E. (*error bars*) of 20–60 experiments except for thimerosal swim-up ($n = 8$), TMA ($n = 4$–5). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ compared with control. *C* and *D*, frequency distributions of ALH (*C*) and linearity (*D*) under control conditions (*black*) and after stimulation with 25 mM NH_4Cl (*dark blue*), 2 mM 4-AP (*green*), and 5 μM thimerosal (*red*). Each plot shows mean ± S.E. of 19 (control, 4-AP, progesterone), 9 (NH_4Cl), and 10 (thimerosal) STF samples. *E–H*, example tracks of control (*E*) and cells exposed to 20 mM TMA (*F*), 2 mM 4-AP (*G*), and 5 μM thimerosal (*H*). All traces start at the origin (0, 0), and *scales* show distance (μm). Sample rate = 100 Hz.

motility (recorded in the absence of stimulation) was increased >3-fold (12.2 ± 1.0%; $n = 73$; $p < 10^{-11}$), but the effects of NH_4Cl on hyperactivated motility were negligible (Fig. 2*B*). Frequency distributions of two key kinematic parameters measured by CASA, amplitude of side-side movement of the sperm

head (ALH) and linear distance from first to last track point/total track length (linearity) confirmed this observation. Activation of CatSper by alkalinization 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*).

NH_4Cl-induced elevation of pH_i is temporary, alkalinization of mouse sperm decaying in minutes (36). To address the possibility that elevation of pH_i decayed too rapidly for effects on motility to be assessed, we used trimethylamine hydrochloride (TMA), a larger base that causes prolonged, dose-dependent alkalinization (37). 10 and 20 mM TMA raised pH_i 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 $[Ca^{2+}]_i$, ΔF_{mean} 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. 1*E*). 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 $[Ca^{2+}]_i$ elevation (Fig. 1*F*). During the initial, large $[Ca^{2+}]_i$ 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 capacitating medium STF ($p < 0.05$; Fig. 2, *A* and *B*). Reduction of the interval between progesterone application and CASA recording did not reveal any stronger hyperactivating response.

Induction of Hyperactivation by 4-AP Is Not by Cytoplasmic Alkalinization or Depolarization—4-Aminopyridine strongly hyperactivates mammalian sperm (28, 41), an effect that is correlated with fertilization rate in *in vitro* fertilization (42). Application of 2 mM 4-AP to cells bathed in sEBSS rapidly raised $[Ca^{2+}]_i$ ($\Delta F_{mean} = 32 \pm 4\%$; $n = 7$ experiments; $p < 0.0001$; Fig. 3*A*) and recruited 26 ± 2% of cells into the hyperactivated population ($n = 48$; $p < 10^{-19}$; Fig. 2, *A* and *G*). In cells suspended in STF, a similar hyperactivating effect of 4-AP was seen ($n = 63$; $p < 10^{-33}$; Fig. 2, *B–D*). 4-AP is a weak base and in mouse sperm causes hyperpolarization of membrane potential (E_m) (43) due to activation of a 4-AP-insensitive, pH-regulated K^+ channel (44, 45). Hyperactivation might thus reflect alkalinization-induced activation of CatSper (28, 43). 2 mM 4-AP increased pH_i by 0.27 ± 0.04 units, $n = 7$, an effect similar to that seen with 25 mM NH_4Cl ($p > 0.9$) and only half that seen with 20 mM TMA ($p < 0.003$), yet stimulation of hyperactivation by 4-AP was strikingly more potent than either NH_4Cl or TMA (in sEBSS cells, *cf.* NH_4Cl ($p < 10^{-7}$) and 20 mM TMA ($p < 10^{-4}$); in STF cells, *cf.* NH_4Cl ($p < 10^{-14}$) and 20 mM TMA ($p < 0.002$)). 4-AP also raised pH_o (~1 unit), which was left uncorrected in order to enhance cytoplasmic entry of the drug (46, 47). Control experiments showed that elevation of pH_o to 8.5 raised pH_i by 0.13 ± 0.05 units ($p < 0.005$; $n = 13$, paired *t* 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 alkalinization ($p > 0.5$; $n = 6$). 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

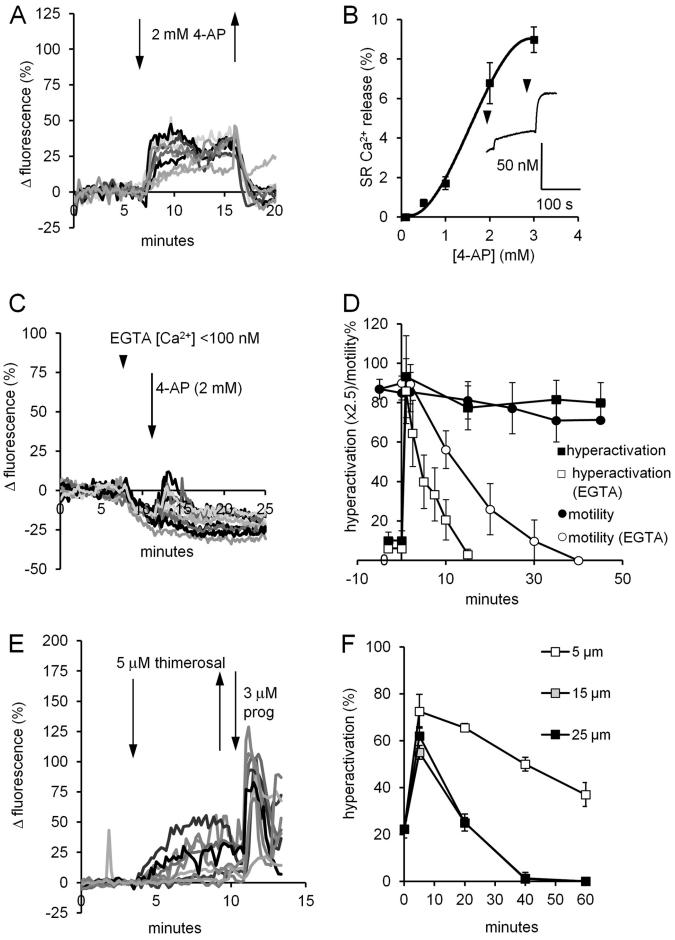

FIGURE 3. **4-AP causes hyperactivation by mobilizing stored Ca^{2+}.** *A*, sustained increase in $[Ca^{2+}]_i$ (OGB fluorescent intensity) in human sperm exposed to 2 mM 4-AP (*first arrow*). 4-AP was washed off at the *second arrow*. Shown are responses of eight cells in same experiment. *B*, release of Ca^{2+} from sacroplasmic reticulum microsomes by 4-AP. The graph shows 4-AP-induced Ca^{2+} release (percentage of A23187-releasable Ca^{2+}); each point represents the mean ± S.E. (*error bars*) of 4–7 experiments. *Inset*, example trace. 2 mM 4-AP was added at the *first arrow*, and remaining Ca^{2+} was released by 25 μM A23187 (*second arrow*). Calibration shows time and $[Ca^{2+}]$. *C*, effect of 2 mM 4-AP in Ca^{2+}-depleted medium. Sperm were superfused with EGTA-buffered medium (*first arrow*), which caused a rapid fall in $[Ca^{2+}]_i$. Subsequent application of 4-AP caused a $[Ca^{2+}]_i$ transient in a subset of cells. Shown are responses of 11 cells in the same experiment. *D*, time course of hyperactivation (percentage of cells; *squares*) induced by 2 mM 4-AP in cells suspended in sEBSS (*filled symbols*) and in parallel experiments where cells were resuspended in EGTA-buffered sEBSS immediately before use (*open symbols*). *Circles* show the percentage of motile cells. Each point shows mean ± S.E. of four experiments. *E*, sustained increase in $[Ca^{2+}]_i$ (OGB fluorescent intensity) in human sperm exposed to 5 μM thimerosal (*first arrow*). Thimerosal was washed off at the *second arrow*, and 3 μM progesterone was applied at the *third arrow*. Shown are the responses of eight cells in the same experiment. Temperature was 30 °C. *F*, time course and concentration dependence of thimerosal-induced hyperactivation (percentage of cells). Each *line* shows mean ± S.E. of four experiments.

[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 μM valinomycin (data not shown). We conclude that the potent hyperactivating effect of 4-AP on human sperm is not exerted through alkalinization 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 sacroplasmic 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. 3*B*). To confirm that 4-AP mobilizes stored Ca^{2+} in human sperm, we tested the effect of the drug on cells superfused with EGTA-buffered saline ($[Ca^{2+}]_o < 100$ 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. 3*C*). 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 ($p = 2 \times 10^{-7}$; χ^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 10–20°) 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 $NO^{\bullet}$ (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. 3*D*; 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. 3*D*). 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 μM 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. 3*E*). This temperature sensitivity is consistent with previous observations on the activation of stored Ca^{2+} release by thimerosal (53). 5 μM thimerosal potently and persistently hyperactivated human sperm (Figs. 2 (*A* and *B*, *red bars*) and 3*F*), tracks showing the pronounced side-side movements of the sperm head characteristic of hyperactivated motility (Fig. 2*H*). 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 μm 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. 3*F*), 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 $[Ca^{2+}]_o$ 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_4Cl and 10 mM TMA, 2 mM 4-AP, and 3 μM 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 μM 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 μM 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_i elevation, enhances entry of sperm into methylcellulose rather than their progress through it.

NNC55-0396 Inhibits Effects of CatSper Manipulation and Reduces Spontaneous Hyperactivation—NNC55-0396 (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 μM 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 μM progesterone, $p < 0.01$; and 25 mM NH_4Cl, $p < 0.01$; Fig. 4*C*).

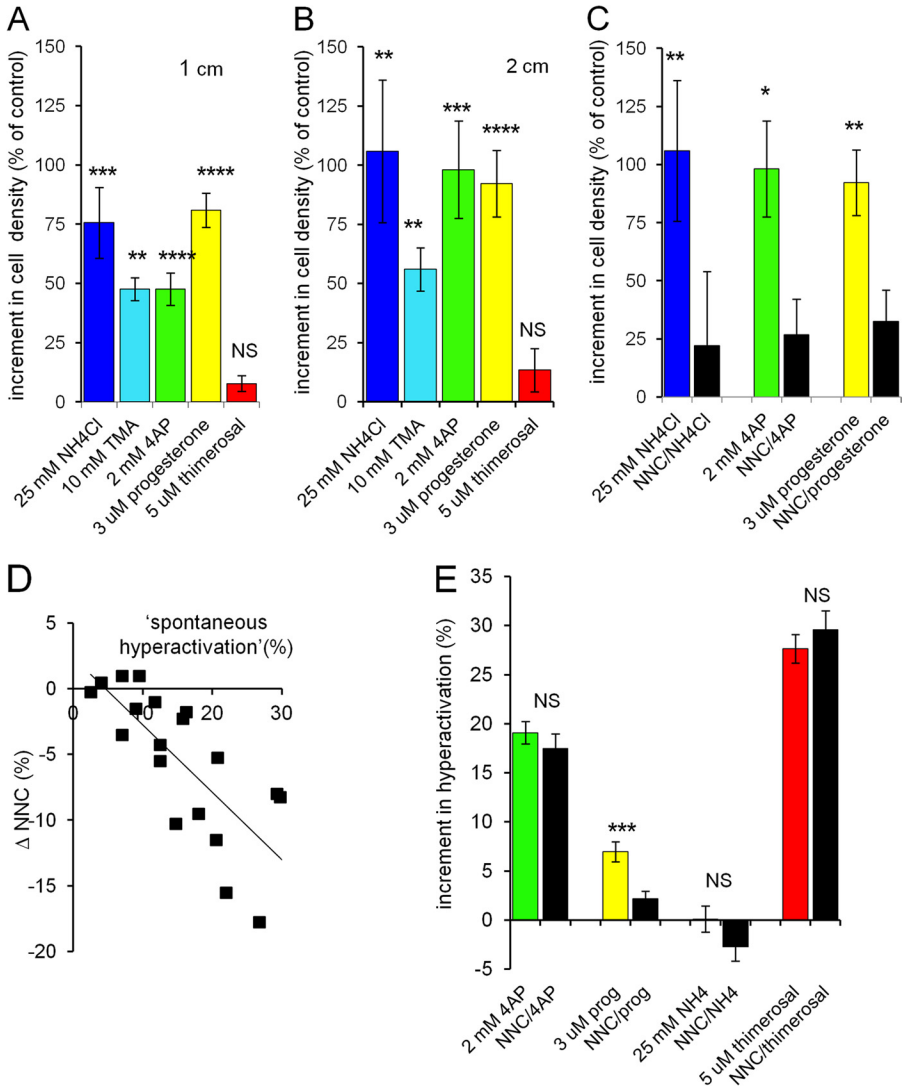

FIGURE 4. **CatSper activity enhances penetration into viscous medium and contributes to spontaneous hyperactivation.** *A* and *B*, increment in cell density (percentage of control) 1 cm (*A*) and 2 cm (*B*) into methylcellulose. Cells were stimulated with 25 mM NH_4Cl (*dark blue*), 10 mM TMA (*light blue*), 2 mM 4-AP (*green*), 3 μM progesterone (*yellow*), and 5 μM thimerosal (*red*). *Bars* show the mean ± 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 μM progesterone (*yellow*), and 25 mM NH_4Cl (*dark blue*) and in parallel incubations pretreated with 10 μM NNC (*black*). *Bars*, mean ± 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; μM 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 μM progesterone (*yellow*), 25 mM NH_4Cl (*dark blue*), and 5 μM thimerosal (*red*). *Black bars*, responses in parallel 10 μM NNC-pretreated experiments. Each *bar* shows mean ± 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.

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 μM NNC significantly reduced the level of spontaneous hyperactivation compared with parallel controls (from 16.7 ± 2.2 to 11.3 ± 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. 4*D*; $r = 0.75$). The small hyperactivating effect of progesterone was also sensitive to NNC (Fig. 4*E*; $p < 0.001$), but the powerful hyperactivating actions of 4-AP and thimerosal, which directly mobilize stored Ca^{2+}, were not inhibited (Fig. 4*E*; $p > 0.2$).

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 alkalinization 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,

but only entry into viscous medium was sensitive to the CatSper antagonist NNC. We conclude that regulation of motility in human sperm through $[Ca^{2+}]_i$ 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_4Cl 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). $[Ca^{2+}]_i$ signals mediated by stored Ca^{2+} and by alkalinization 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ oscillations, occurs secondarily to influx of Ca^{2+} induced pharmacologically or by progesterone-induced activation of CatSper (18, 25) (Fig. 1*F*). 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 ($NO^{\bullet}$; a product of the oviduct epithelium and cumulus cells surrounding the oocyte) and by capacitation (25, 26). $NO^{\bullet}$ *S*-nitrosylates 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). $NO^{\bullet}$ facilitates 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*-nitrosylation and signaling events related to capacitation will regulate this "available" population (Fig. 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.

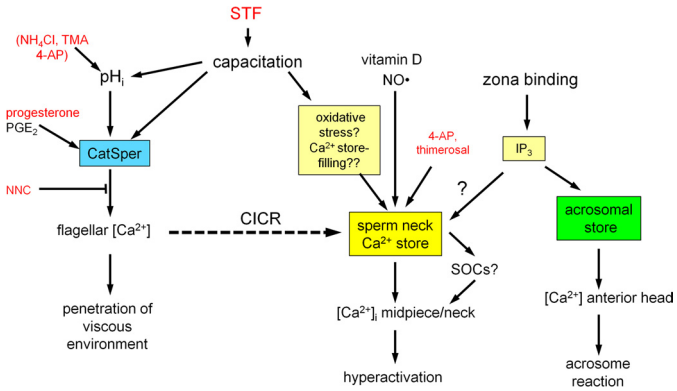

FIGURE 5. **Tentative model for the interacting effects of Ca^{2+} influx mediated by CatSper (*blue box*) and release of Ca^{2+} from the store at the sperm neck (*yellow box*) based on evidence from this and previous studies.** The acrosomal Ca^{2+} store is also shown (*green box*). CICR links these two parts of the Ca^{2+}-signaling apparatus in a subset of cells where the Ca^{2+} store is sensitized (*dashed arrow*). Agents employed in this study are shown in *red*. STF is shown acting to enhance capacitation, including sensitization of the Ca^{2+} store at the sperm neck. Other (endogenous) agents and pathways are shown in *black*. *Question marks* indicate effects that are consistent with the model or may be predicted on the basis of studies on other cell types but that have not been established in sperm. *Double question marks* denote speculation.

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 $[Ca^{2+}]_i$ elevation induced by zona proteins (≤2 min) is not observed in sperm of CatSper-null mice, delayed $[Ca^{2+}]_i$ responses still occur in a minority of cells, and these may be due to mobilization of stored Ca^{2+} (72). Fur-

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. 4*D*), 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_4Cl (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 $[Ca^{2+}]_i$ 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.

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