Identical Phenotypes of CatSper1 and CatSper2 Null Sperm

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Among several candidate Ca\textsuperscript{2+} entry channels in sperm, only CatSper1 and CatSper2 are known to have required roles in male fertility. Past work with CatSper1 null sperm indicates that a critical lesion in hyperactivated motility underlies the infertility phenotype and is associated with an absence of depolarization-evoked Ca\textsuperscript{2+} entry. Here we show that failure of hyperactivation of CatSper2 null sperm similarly correlates with an absence of depolarization-evoked Ca\textsuperscript{2+} entry. Additional shared aspects of the phenotypes of CatSper1 and -2 null sperm include unperturbed regional distributions of conventional voltage-gated Ca\textsuperscript{2+} channel proteins and robust acceleration of the flagellar beat by bicarbonate. Further study reveals that treatment of both wild-type and CatSper2 null sperm with procaine increases beat asymmetry, a characteristic of the flagellar waveform of hyperactivation. This partial rescue of the loss-of-hyperactivation phenotype suggests that an absence of CatSper2 precludes hyperactivation by preventing delivery of needed Ca\textsuperscript{2+} messenger rather than by preventing flagellar responses to Ca\textsuperscript{2+}. CatSper2 null sperm also have an increased basal cAMP content and beat frequency. Protein kinase A inhibitor H89 lowers beat frequency to that of wild-type sperm, suggesting that CatSper2 is required for protein kinase A-mediated, tonic control of resting cAMP content. Relative to wild-type testis, CatSper1 and -2 null testes contain normal amounts of CatSper2 and -1 transcripts, respectively. However, CatSper1 null sperm lack CatSper2 protein and CatSper2 null sperm lack CatSper1 protein. Hence, stable expression of CatSper1 protein requires CatSper2 and vice versa. This co-dependent expression dictates identical loss-of-function sperm phenotypes for CatSper1 and -2 null mutants.

The four members of the CatSper family are cation-channel-like proteins found exclusively in sperm and spermatogenic cells (1–3). The pore-lining residues and overall sequences of CatSper1 and CatSper2 most resemble those of a single repeat from a conventional four-repeat voltage-gated Ca\textsuperscript{2+} channel (1, 2). Thus, CatSper1 and CatSper2 are proposed to form all or part of a novel, hetero- or homotetrameric, Ca\textsuperscript{2+}-selective channel. Several of the conventional four-repeat voltage-gated Ca\textsuperscript{2+} channel proteins that also are detected immunologically in sperm (4–6) are not required for male fertility as revealed by targeted gene disruption. These include Ca\textsubscript{v}1.3 (7), Ca\textsubscript{v}2.2 (8), Ca\textsubscript{v}2.3 (9), Ca\textsubscript{v}3.1 (10), and Ca\textsubscript{v}3.2 (11). Mice carrying null mutations for CNGA3 (12), TRPC2 (13), and TRPC3 (14), which also are candidates for sperm Ca\textsuperscript{2+} entry channels, likewise have no reported fertility deficits. Null mutants for Ca\textsubscript{v}1.2 (15) and Ca\textsubscript{v}2.1 (16) are embryonic lethal and thus unsuitable for fertility studies. Only CatSper1 (2) and CatSper2 (17) are required for male fertility as determined by targeted gene disruption.

Ca\textsuperscript{2+} is an important messenger in capacitation, the processes that prepare sperm for fertilization while they reside in the female reproductive tract after mating (18). Past work indicates that capacitation includes obligatory changes in sperm swimming behavior that are mediated by elevation of [Ca\textsuperscript{2+}]. (19–23). The requirement of CatSper1 and CatSper2 for male fertility suggests that these putative channels open to allow Ca\textsuperscript{2+} entry to generate such instructive Ca\textsuperscript{2+} signals. Localization of CatSper1 and -2 to the membrane of the principal piece of the flagellum (2, 17) indicates a role in the control of flagellar function rather than in acrosomal exocytosis, another essential Ca\textsuperscript{2+}-dependent component of capacitation. Indeed, a defect in sperm hyperactivation is prominent in the phenotypes of CatSper1 and -2 null sperm (17, 24, 25). Although engagement of the protein tyrosine phosphorylation cascade, the zona pellucida-induced acrosome reaction, and several other landmarks in capacitation do not require CatSper1 or CatSper2 (1, 2, 17, 25), the extent of identity in the phenotypes of CatSper1 and -2 null sperm has remained unclear. Here, we further examine the phenotype of CatSper2 null sperm and document additional similarities to those of sperm of the CatSper1 null mutant. We propose that these nearly or completely identical phenotypes result from a co-dependent expression of CatSper1 and CatSper2 proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2 acetoxymethyl (AM) and Pluronic F127 were from Molecular Probes, Inc. (Eugene, OR), and H89 from Calbiochem. Antibodies to CatSper1 and α-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and InnoGenex (San Ramon, CA), respectively. The CatSper2 polyclonal antibody used here was directed against the carboxyl-terminal epitope, as described previously (1). Unless noted, other chemicals were from Sigma.

**Sperm Preparation and Incubation**—Sperm were prepared as in prior work (6, 26, 27). Briefly, cauda epididymides and vasa deferentia were excised from male mice that were euthanized by CO\textsubscript{2} asphyxiation. After rinsing with medium Na\textsubscript{7.4} (135 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgSO\textsubscript{4}, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, 1 mM pyruvic acid, adjusted to pH 7.4 with NaOH), the tissue was transferred to 1 ml of a "swimout/capacitation" medium (medium Na\textsubscript{7.4} with 5 mM of bovine serum albumin/ml and 15 mM NaHCO\textsubscript{3}). Semen was allowed to elute (15 min at 37 °C, 5% CO\textsubscript{2}) from several small incisions.

The abbreviations used are: AM, acetoxymethyl; PKA, protein kinase A; TAPS, 3-(N-tris(hydroxymethyl)aminomethyl)-1-propanesulfonic acid.
All subsequent operations were at room temperature (22–25°C) in medium Na7.4, unless noted otherwise. Sperm were washed twice and then dispersed and stored at 1–2 × 10⁷ cells ml⁻¹. Potassium-evoked responses were produced with medium K8.6 (135 mM KCl, 5 mM NaCl, 2 mM CaCl₂, 1 mM MgSO₄, 30 mM TAPS, 10 glucose, 1 lactic acid, 1 pyruvic acid), adjusted to pH 8.6 with NaOH. For in vitro capacitation, sperm were transferred to the “swimout/capacitation” medium and incubated for 90 min at 37°C in a 95% air, 5% CO₂ atmosphere. Sperm were then washed twice in medium Na7.4 and examined at room temperature.

Dye Loading and Photometry—Fura-2 AM was dispensed from 2 mM stocks in Me₂SO, dispersed in 10–15% Pluronic F127, diluted to 20 μM in 0.25 ml medium Na7.4, and then immediately mixed with an equal volume of the sperm suspension. After 15–20 min, medium Na7.4 (0.5 ml) was added, and the cells were sedimented. After resuspension in 0.25 ml of fresh medium, incubation continued for 45 min before use. Ten microliters of cell suspension was added to the surface of an uncoated glass coverslip resting in a glass-bottomed incubation chamber. Ten microliters of Na7.4 containing a small cluster (3–5 cells) of loosely tethered sperm, each pivotting about a single point of attachment at the base of the head. The raw photometric signals were corrected for cell-free background, collected prior to each series of measurements. The ratio of the corrected signals was calibrated (5) with the constants Rₘₐₙ (0.380), Rₘₐₓ (1.795), and Kₜₐ (1228 nm) obtained from cells equilibrated in solutions fortified with ionomycin (10 μM) and containing 20 mM EGTA, 15 mM CaCl₂, or 20 mM EGTA with 15 mM CaCl₂ (calculated free Ca²⁺ concentration of 226 nM). The calibrated signal reports spatially averaged internal [Ca²⁺] from the head and proximal flagellum of several sperm. Further analyses were performed in Igor (Wavemetrics, Lake Oswego, OR). Statistical analyses were performed in Excel (Microsoft, Redmond, WA). All results are presented as mean ± S.E., except where noted.

Ester Loading of cAMP—The cAMP-AM was dispensed from a 2 mM stock in Me₂SO, dispersed in 10–15% Pluronic F127, diluted to 20 μM in 0.25 ml medium Na7.4, and then immediately mixed with 0.15 ml of sperm suspension for a final concentration of 60 μM. After 30 min, an aliquot was added to the sample chamber containing medium Na7.4 for imaging. Data were collected within 5 min to preclude loss of signal due to declining cAMP content following dilution of external cAMP-AM.

Waveform Analysis—The flagellar waveform was analyzed as described (27). Briefly, stop-motion digital images were collected at 30 Hz from a 128 × 128-pixel region of the camera chip (larger regions were used for asymmetry measurements), under the direction of Metamorph (Universal Imaging, West Chester PA). Images were stored in TIFF format for subsequent semiautomated tracing of the flagellum. Additional software routines analyzed flagellar images to (i) determine the flagellar beat frequency; (ii) tabulate the distance along the flagellum (arc length), the angular deviation (tangent angle) along the flagellum, and the time-averaged tangent angle; (iii) present the time-averaged tangent angle versus arc length data (shear curves) as a measure of flagellar beat asymmetry; (iv) determine the flagellar beat envelope and the beat amplitude at regular intervals along the beat axis; and (v) calculate the maximal curvature of the 20-μm midpiece in both the pro-hook and anti-hook directions (28).

Immunocytochemistry and Immunoblotting—As in prior work (5, 6, 25), sperm were probed with the affinity-purified antibodies CNB1, CNC1, or CNE2, directed respectively against the pore-forming α sub-units of the Ca₂⁺.2, Ca₂⁺.1.2, and Ca₂⁺.2.3 channels that specify N-, L-, and R-type currents in somatic cells, using methods for confocal immunomicroscopy as described (5, 6). Briefly, fixed and permeabilized sperm were washed, blocked, and rinsed before incubation with diluted (1:15) antibody. Samples were rinsed again, treated with biotinylated anti-rabbit IgG (1:300), rinsed, treated with avidin D fluorescein (1:300), rinsed, and mounted for examination by confocal microscopy. For immunoblotting, spermatozoa were boiled in Laemmli sample buffer (1 × 10⁶ cells/20 μl), separated by SDS-PAGE, transferred to nitrocellulose, probed with the indicated antibodies diluted in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 2.5% nonfat milk, and treated with enhanced chemiluminescence reagents (Pierce).

Quantitative Real-time PCR—Real-time PCR analysis was performed as described using previously validated primers for CatSper1 and CatSper2 (29). Testicular total RNA was treated with TURBO DNA-free reagent (Ambion) and reverse transcribed (2 μg) with random primers and Superscript III (Invitrogen) according to the manufacturer’s protocol. The resultant cDNA samples were diluted 10-fold, and 3 μl was used as a template for amplification with the SYBR Green PCR Master Mix (Applied Biosystems). All samples were normalized to the 18S ribosomal RNA signal for determination of relative expression levels, which were calculated according to the Applied Biosystems Comparative Ct method, assuming that a 2-fold difference in concentration changes Ct by ±1.

Cyclic AMP Measurements—Epididymal sperm were harvested at 37°C in a “swimout/capacitation” medium that lacked NaHCO₃ and Ca²⁺. After washing in the same modified medium, sperm were diluted 12-fold into medium Na7.4 with 5 mg of bovine serum albumin/ml containing or lacking Ca²⁺, NaHCO₃, or both. After the indicated times at 37°C, aliquots were diluted into an equal volume of cold 1 N perchloric acid, mixed, and frozen in liquid nitrogen. For the t = 0 samples, sperm were diluted into the various media already mixed with cold 1 N perchloric acid. After disruption by five freeze/thaw cycles (dry ice/ethanol bath and then 37°C), cAMP was isolated and determined by radioimmunoassay as described (30).

RESULTS

CatSper2 Null Sperm Lack Evoked Ca²⁺ Entry—Ca²⁺ entry in sperm is evoked by treatment with a high potassium, high pH medium (31), and the rate of depolarization-evoked Ca²⁺ entry, reported by CatSper2 photometry, indicates the relative number of open, voltage-gated Ca²⁺ channels (27). In Fig. 1, fura-2 monitored spatially averaged [Ca²⁺] from small clusters of 3–7 motile sperm loosely adhered to a coverslip. The cells were perfused with medium Na7.4 alone or with 15 mM NaHCO₃ except during 30-s depolarizing stimuli with medium K8.6. For wild-type sperm (Fig. 1A), [Ca²⁺]i, rose abruptly during each stimulus and then returned slowly toward the initial resting level. As in past work (5, 25, 27, 32), channel opening was facilitated by incubation with the bicarbonate anion. The K8.6-evoked rate of rise was 21 ± 3 nM s⁻¹ before and 33 ± 6 nM s⁻¹ after conditioning with HCO₃⁻ (Fig. 1C). The modest facilitation observed here presumably results from channel inactivation during the initial 30-s stimulus. For CatSper2 null sperm, depolarization evoked little or no increase in Ca²⁺ before or after conditioning with HCO₃⁻ (Fig. 1B). Rates of rise were <1 nM s⁻¹ under both conditions. As for CatSper1 (25), CatSper2 is required for depolarization-evoked Ca²⁺ entry in sperm.

Unaffected Regional Distributions of Caᵥ Channels in CatSper2 Null Sperm—A requirement of CatSper2 for evoked Ca²⁺ entry suggests, but does not demonstrate, that the CatSper2 protein functions as a Ca²⁺ entry channel. Therefore, we considered the alternate hypothesis that
the CatSper2 protein instead is required for membrane targeting and functional expression of conventional voltage-gated Ca\textsuperscript{2+} channels, which also are candidates for the route of depolarization-evoked Ca\textsuperscript{2+} entry in sperm (5, 6). However, we find indistinguishable regional localizations of Ca\textsubscript{V}1.2, Ca\textsubscript{V}2.2, and Ca\textsubscript{V}2.3 immunoreactivity in wild-type and CatSper2 null sperm (Fig. 2). The loss of Ca\textsuperscript{2+} entry channel function in CatSper2 null sperm apparently does not result from disrupted regional distributions of Ca\textsubscript{V} channel proteins.

No Hyperactivated Motility for CatSper2 Null Sperm—We also examined whether CatSper2 is required for the highly asymmetrical flagellar waveform that is a hallmark of sperm hyperactivation. In Fig. 3 the time-averaged distribution of bending along the flagellum provides a quantitative measure of asymmetry. For wild-type sperm bathed in Na\textsubscript{7.4}, asymmetry was low initially but became greater after incubation under capacitating conditions (Fig. 3A). The mean value for asymmetry at 40 \( \mu \)m along the flagellum increased from <0.3 to >1.0 radians. In contrast, the asymmetry of CatSper2 null sperm changed little after capacitating incubations (Fig. 3B). The mean value for asymmetry at 40 \( \mu \)m along the flagellum remained <0.2 radians.

The hyperactivated waveform also has a larger beat amplitude, here measured by the maximal excursion from the flagellar beat axis for each point along the flagellum (Fig. 3, C and D). The amplitude at 30 \( \mu \)m for wild-type sperm in Na\textsubscript{7.4} increased from 28 \( \pm \) 2 to 35 \( \pm \) 2 \( \mu \)m after capacitating incubations. The beat amplitude of CatSper2 null sperm decreased slightly from 25 \( \pm \) 1 to 23 \( \pm \) 2 \( \mu \)m after capacitating incubations.

Procaine Partially Rescues Flagellar Asymmetry in CatSper2 Null Sperm—We asked whether CatSper2 null sperm lack an asymmetrical waveform after capacitating incubations due to a defective flagellar motor or due to defects in the mechanisms that control it. As a test, we...
applied an alternative, pharmacological method to induce flagellar asymmetry. Past work reports that local anesthetics such as procaine evoke hyperactivation in sperm (33–35). Procaine action requires external Ca\(^{2+}\) but may be independent of cAMP and tyrosine phosphorylation (36). We find that with a 5-min exposure to 10 mM procaine, both wild-type and CatSper2 null sperm display a highly asymmetrical flagellar beat (Fig. 4A). Thus, the lesion that prevents capacitating incubations from producing waveform asymmetry is not in the flagellar axoneme of the CatSper2 null sperm but instead in the signals that control it.

The waveform of procaine-treated sperm was similar but not identical to that of the hyperactivated waveform of wild-type sperm. Whereas capacitating incubations decreased the beat frequency of wild-type sperm, procaine treatment did not (Fig. 4B). For CatSper2 null sperm, capacitating incubations marginally increased and procaine marginally decreased beat frequency. In addition, procaine treatment decreased beat amplitude of both wild-type and CatSper2 null sperm (Fig. 4C), whereas hyperactivating conditions increased only the beat amplitude of wild-type sperm (Fig. 3C). We further characterized the procaine-induced waveform by measuring the maximal curvature of the flagellar midpiece in both the pro-hook and anti-hook bend directions (i.e. in the same or opposite direction as the hook of the head (28)). After capacitating incubations, wild-type sperm displayed increased pro-hook curvature (indicated by a positive value) of the midpiece. Procaine treatment did not increase pro-hook curvature but decreased anti-hook curvature (indicated by a positive value) of both wild-type and CatSper2 null sperm (Fig. 4D). Thus, the similar asymmetry of the waveform of hyperactivation produced by incubation under capacitating conditions and the waveform produced by procaine (cf. Figs. 3A and 4A) are produced by different distributions of bending in pro- and anti-hook directions.

Activation of CatSper2 Null Sperm by Bicarbonate—One of the earliest stages of capacitation is the acceleration of sperm motility that occurs shortly after mating, a process termed activation. Activation is probably signaled by the high HCO\(_3^-\) concentrations in male and female reproductive fluids. In vitro, the flagellar beat frequency increases severalfold within seconds of exposing sperm to HCO\(_3^-\) (25, 27). This Ca\(^{2+}\)-dependent action of HCO\(_3^-\) (25) occurs by stimulation of the atypical adenylyl cyclase of sperm (37), and by cAMP-mediated activation of protein kinase A (PKA) (32). Past work found that bicarbonate-evoked activation of motility is not impaired in CatSper1 null sperm (25). We now find that, like wild-type sperm, the CatSper2 null sperm also increase their beat frequency nearly 3-fold upon exposure to 15 mM NaHCO\(_3\) or upon incubation with the membrane-permeant cAMP-AM ester (Fig. 5). Thus, CatSper2 is not required for signaling events downstream of cAMP in the bicarbonate-evoked acceleration of the flagellar beat.

Increased Basal Beat Frequency of CatSper2 Null Sperm Due to Elevated cAMP—Fig. 5 also shows that, as for CatSper1 null sperm (25), the CatSper2 null sperm cells have an elevated basal (before exposure to HCO\(_3^-\)) beat frequency (3.8 ± 0.1 Hz versus 2.8 ± 0.2 Hz for wild-type sperm). Examining the cause of the accelerated beat, we measured the cAMP content of CatSper2 null and wild-type sperm before and during treatment with HCO\(_3^-\) (Fig. 6A). The CatSper2 null sperm had an elevated basal cAMP content (inset). In Ca\(^{2+}\)-deficient medium, HCO\(_3^-\) had little or no effect on cAMP content. In the presence of external Ca\(^{2+}\), HCO\(_3^-\) altered the cAMP content of both wild-type and CatSper2 null sperm with similar but not identical time courses. For sperm of both types, cAMP content rapidly increased 5-fold within the first 1 min of exposure, declined to ∼3-fold of the basal value by 5 min, and then again rose to ∼5-fold the initial value by 30 min. However, the secondary rise was more rapid for CatSper2 null sperm, whose contents at 15 and 30 min were similarly elevated.

To examine whether the elevated basal CAMP increases the basal flagellar beat frequency of CatSper2 null sperm by a PKA-mediated pathway, we applied pharmacological blockade with PKA inhibitor H89, shown previously (27) to block HCO\(_3^-\)-evoked acceleration of the beat of wild-type sperm. Fig. 6B compares the beat frequency before and during exposure to a 30 μM concentration of the PKA inhibitor H89. The H89 had little or no effect on the 2.5 ± 0.2 Hz basal beat of wild-type sperm but decreased the beat of CatSper2 null sperm from 4.1 ± 0.2 to 2.5 ± 0.2 Hz, the same value as the basal beat of wild-type sperm (Fig. 6B). We conclude that the increased basal CAMP content of CatSper2 null sperm raises the resting beat frequency by PKA-mediated protein phosphorylation.
Co-dependent Expression of CatSper1 and CatSper2 Proteins—The numerous similarities in the phenotypes of CatSper1 null and CatSper2 null sperm indicate that the two proteins are required components in the same pathway(s) that controls Ca2+ entry and flagellar waveform asymmetry. In the simplest interpretation, the sperm of both null mutants lack functional channels for an entry of Ca2+ that is required to produce the flagellar asymmetry of hyperactivation. Such functional co-dependence could have molecular explanation at several levels: formation of a functional membrane channel; trafficking of the putative channel proteins to the membrane; translation and post-translational modification of the channel proteins, or production and processing of the mRNA that encode them.

Previous studies of testicular gene expression by microarray analyses indicate that the transcription of CatSper2 begins several days before that of CatSper1 in the developing mouse testis (29, 38). To determine whether CatSper2 transcription is required for subsequent production of CatSper1 mRNA, we examined adult CatSper1 and -2 null testes by quantitative real-time PCR. Compared with wild-type testes, neither the relative content of CatSper1 mRNA in the CatSper2 null testes (1.65 ± 1.64-fold; n = 3) nor the CatSper2 mRNA content of CatSper1 null testes (1.01 ± 1.12-fold; n = 3) was significantly altered. Next, we considered whether CatSper1 and -2 proteins stabilize each other through an interaction at the protein level. Fig. 7 shows immunoblots of wild-type sperm and CatSper1 and -2 null sperm probed with antibodies directed against the CatSper1 (Fig. 7 A) and CatSper2 proteins (Fig. 7B). The wild-type sperm show prominent immunoreactive ~82 kDa CatSper1 and ~72 kDa CatSper2 bands. Neither of these bands was detected in either the CatSper1 or -2 null sperm examined at similar protein loading as determined with an antibody for α-tubulin (Fig. 7C).

DISCUSSION

The database-mining strategy that led to the discovery of CatSper1 (2) and CatSper2 (1) was followed (3) by the identification of two other members of this new family of putative cation channels. Although demonstration of channel function by heterologous expression has remained a difficult and unmet challenge, interest in the CatSperms has remained high due to (i) their unique expression in sperm (1, 2), (ii) their requirement for male fertility (2, 17) and possible role(s) in heritable male infertility (39, 40), and (iii) the unique and informative loss-of-function sperm phenotype produced by their targeted disruption (2, 17, 25). Here, we have further characterized and compared the phenotypes of CatSper1 and -2 null sperm. Our results reveal new aspects of the CatSper2 null phenotype and show that several previously described characteristics of CatSper1 null sperm are shared by the CatSper2 null mutants. These findings lead to the proposal that the phenotypes in fact are identical and to a demonstration that such identity of phenotype is a consequence of co-dependent expression of the two proteins.

Past work found immunological evidence that the pore-forming α subunits of several conventional voltage-gated (CaV) channels are regionally distributed in distinctive patterns in both the sperm head and flagellum (4–6) and that depolarization-evoked entry of Ca2+ has a pharmacological sensitivity profile consistent with involvement of CaV2.2 and CaV2.3 channels (5). Unexpectedly, CatSper1 null sperm lacked the evoked entry of Ca2+ (25), indicating that CatSper1 is required directly or indirectly for functional channels. Regional distributions of CaV1.2, CaV2.2, and CaV2.3 channel proteins in CatSper1 null sperm were not distinguishable from those of wild-type sperm, suggesting that the absence of CatSper1 does not affect expression and trafficking of CaV channel proteins. Here we find that CatSper2 null sperm also lack evoked Ca2+ entry (Fig. 1) and have similarly unperturbed distributions of CaV channel protein (Fig. 2). In the simplest interpretation, the CatSper1 and -2 proteins present in the membrane of the principal piece (1, 2) form voltage-gated CaV2+ channels that are the major route for depolarization-evoked entry of Ca2+ into the flagellum. The photometric methods used here and in past studies of evoked entry of Ca2+ in wild-type sperm (5, 25–27) report changes in spatially averaged [Ca2+] from the heads and proximal flagella of several cells. Evoked Ca2+ entry also occurs when the detection window is limited to the flagella of clus-
ters of wild-type sperm and when Ca$^{2+}$ is monitored by imaging of individual immobilized sperm (41). We also note that the flagellum must possess at least one other route of Ca$^{2+}$ entry, necessary to explain the preserved Ca$^{2+}$ dependence of the stimulatory action of bicarbonate on the flagellar beat of CatSper1 null sperm (25) and of the cAMP accumulation by CatSper2 null sperm (Fig. 6). Perhaps these Ca$^{2+}$-dependent, CatSper-independent actions of bicarbonate are mediated by a direct action of Ca$^{2+}$ on the sperm adenyl cyclase (37).

Prior analysis of the flagellar waveform (25) and the swimming behavior (2, 17) of CatSper1 (2, 25) and CatSper2 (17) null sperm indicated that CatSper1 and -2 are each required for sperm hyperactivation as variously assessed by flagellar beat asymmetry, by the profile of path parameters from automated analysis of swimming tracks (2), and by penetration of viscous media (17) and of zona-free but not zona-intact eggs during fertilization in vitro (2, 17). Here we show (Fig. 3) that CatSper2, like CatSper1 (25), is required for the asymmetrical waveform that underlies the swimming behavior of hyperactivated sperm. Several other sperm characteristics were found not to require CatSper1 (25) or CatSper2 (Figs. 2, 4, 5, and 6A). All available evidence is consistent with the hypothesis that the hyperactivation deficit is the critical lesion in the infertility of CatSper1 and -2 sperm.

In permeabilized sperm preparations, Ca$^{2+}$ mediates transition from a symmetrical to an asymmetric flagellar waveform (35, 42), which is similar to that observed for intact sperm hyperactivated during Ca$^{2+}$-dependent capacitation in vitro. In the simplest explanation, CatSper1 and -2 form functional channels that open to allow an entry of Ca$^{2+}$, which is required to initiate or sustain hyperactivation. In a more complicated explanation, CatSper1 and -2 might function to maintain or repolarize the asymmetric waveform. Tests to distinguish between these hypotheses have not been made.

In yet another possible explanation for the hyperactivation deficit of CatSper1 and -2 null sperm, these proteins could be required during interactions of CatSper1 and -2 and of other sperm components that include sNHE, the novel putative Na$^{+}$/H$^{+}$ exchanger of sperm (46), atypical adenylyl cyclase of sperm (37), and unique PKA C$_{3z}$ (32). Continued study of these and other yet uncharacterized sperm-specific signaling proteins (29) holds significant promise for ultimate applications to control male fertility.

Acknowledgments—We thank Drs. David L. Garbers and Stanley McKinney for critically reviewing the manuscript. T. A. Q. also thanks David L. Garbers for providing both space and resources for carrying out some of the work reported here.

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J. Biol. Chem. 2005, 280:32238-32244.
doi: 10.1074/jbc.M501430200 originally published online July 21, 2005

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