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The Control of Male Fertility by Spermatozoan Ion Channels

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Abstract

Ion channels control the sperm ability to fertilize the egg by regulating sperm maturation in the female reproductive tract and by triggering key sperm physiological responses required for successful fertilization such as hyperactivated motility, chemotaxis, and the acrosome reaction. CatSper, a pH-regulated, calcium-selective ion channel, and KSper (Slo3) are core regulators of sperm tail calcium entry and sperm hyperactivated motility. Many other channels had been proposed as regulating sperm activity without direct measurements. With the development of the sperm patch-clamp technique, CatSper and KSper have been confirmed as the primary spermatozoan ion channels. In addition, the voltage-gated proton channel Hv1 has been identified in human sperm tail, and the P2X2 ion channel has been identified in the midpiece of mouse sperm. Mutations and deletions in sperm-specific ion channels affect male fertility in both mice and humans without affecting other physiological functions. The uniqueness of sperm ion channels makes them ideal pharmaceutical targets for contraception. In this review we discuss how ion channels regulate sperm physiology.

Keywords

sperm ion channels; intracellular pH; capacitation; patch clamp; hyperactivation; chemotaxis; male fertility; CatSper; Hv1; KSper; acrosome reaction

INTRODUCTION

In sexual reproduction two haploid gametes (spermatozoon and egg) fuse and restore the original number of chromosomes, resulting in the zygote and the development of a new organism. In many aquatic organisms, mature sperm cells sense the egg and swim toward it in an almost infinite unregulated environment. In contrast, spermatozoa of terrestrial animals are delivered directly into the confined, strictly regulated environment of the female

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reproductive tract, where they must undergo final maturation before fertilization can occur. Thus, the female reproductive tract has the capacity to select and orient sperm, making it an active recipient of male gametes.

Ion channels control sperm membrane potential, cytoplasmic Ca^{2+}, and intracellular pH (pH_i), which in turn regulate motility, the acrosome reaction, and other diverse physiological processes essential for successful fertilization (1–3). The dramatic improvement in our understanding of the sperm ion channels was triggered by the discovery of CatSper (*cat*ionic channel of *sper*m), a novel and complex ion channel that mediates Ca^{2+} entry in sperm flagellum and is required for sperm hyperactivation and male fertility (4). The interest in functional characterization of the CatSper channel and the mechanisms of its regulation led to the first successful application of the whole-cell patch-clamp technique to mice and then to human spermatozoa (5, 6). The scope of this review is to discuss the role of plasma membrane ion channels in normal sperm physiology, to provide an update of recent important developments in sperm ion channel research, and to discuss sperm channelopathies that cause male infertility.

SPERM MORPHOLOGY

Spermatozoa are terminally differentiated motile cells with a clear cell polarity determined by the two main structural elements: the head, which contains tightly packed DNA, and the motile flagellum, which delivers the genetic material of the sperm head into the egg (Figure 1*a*). Structurally similar flagella are present in all spermatozoa across the animal and plant kingdoms (Figure 1*b*). The sperm head consists of the nucleus, the tiny residual nuclear envelope vestiges, and the acrosome (a Golgi-derived vesicle that helps spermatozoa to penetrate egg's protective vestments). The mammalian flagellum has a central axoneme surrounded by specialized structural components and is composed of three parts: the midpiece, which contains mitochondria wrapped in a spiral pattern around the axoneme; the principal piece, which is primarily responsible for motility; and the endpiece, which contains few structural elements (Figure 1*a*,*c*). The midpiece and the principal piece are separated by a ringed septin structure termed the annulus, which prevents diffusion of plasma membrane proteins between these two flagellar domains (Figure 1*d*) (7).

The sperm plasma membrane is tightly attached to the underlying cellular structures along the whole sperm body to provide stiffness. Many membrane proteins of the sperm principal piece appear to be anchored to the underlying fibrous sheath to ensure their strict compartmentalization. The fibrous sheath functions as a scaffold for proteins in signaling pathways that regulate sperm maturation, motility, capacitation, hyperactivation, and/or the acrosome reaction. Interestingly, the fibrous sheath also anchors enzymes of the sperm-specific glycolytic pathway that provide ATP for motility (8). The cytoplasmic droplet, the remnant of the precursor cell's cytoplasm, is the only region of the plasma membrane loosely attached to the intracellular structures (Figure 1*d*). The cytoplasmic droplet is likely to serve as a reservoir for adaptation to osmotic changes occurring at ejaculation (9) but in many species is shed from spermatozoa after ejaculation.

The sperm axoneme is composed of microtubules: Nine outer doublet microtubules surround a central pair of singlet microtubules (a 9+2 arrangement) and the associated proteins such as the molecular motor dynein. Axonemal bending is produced by sliding between pairs of outer doublet microtubules (10), and this sliding is powered by ATP hydrolysis by dynein's heavy chains. This active sliding of microtubules is a linear phenomenon, but the bending and propagation of the wave of motion down the flagellum are not well understood. One theory for the generation of flagellar motion is the geometric clutch hypothesis, whereby dynein engagement alternates between sides of the axoneme as the flagellum bends (11).

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Sperm axoneme bending is sensitive to intracellular alkalinization (12) and intracellular Ca^{2+} ($[Ca^{2+}]_i$) (13) so that increasing pH_i above 7 stimulates dynein activity and promotes flagellar beating, whereas increasing intracellular Ca^{2+} enhances asymmetrical flagellum bending (14, 15). As spermatozoa travel through the environment of changing pH, osmolarity and sense extracellular cues such as progesterone and chemoattractants, sperm ion channels, and transporters regulate ion concentrations within the sperm's cytoplasm to control motility and to trigger physiological responses such as hyperactivation of motility (hyperactivation) and the acrosome reaction.

ACTIVATION OF MOTILITY, CAPACITATION, AND HYPERACTIVATION

Mammalian spermatozoa from all portions of the epididymis have an acidic pH_i (~6.8) and are essentially quiescent (16). When spermatozoa are mixed with seminal plasma (pH > 7.0) upon ejaculation, the sperm cytoplasm is alkalinized (17), and sperm become motile. Despite being motile, freshly ejaculated mammalian spermatozoa are unable or poorly able to fertilize the oocyte. To become competent to fertilize the egg, they must undergo capacitation: a phenomenon reported in 1951 by Austin (18) and Chang (19).

Capacitation results in the removal of noncovalently attached glycoproteins acquired in the epididymis, in the removal of adherent seminal plasma proteins, and in the depletion of the membrane cholesterol and other sterols (20, 21). Moreover, during capacitation, intracellular Ca^{2+}, pH, and cyclic adenosine monophosphate (cAMP) increase, and sperm membrane proteins are phosphorylated on tyrosines (22–24). The first steps of capacitation may begin anywhere extracellular pH (pH_o) is elevated, such as at the cervical mucus, but the ampulla of Fallopian tubes is critical for the completion of the process. Motility is hyperactivated during capacitation. Hyperactivation is defined by an increase in the angle of the flagellar bend, which results in more asymmetrical (whip-like) movements and more powerful swimming force (14, 15, 25). The second major change is that sperm acquire the ability to undergo the acrosome reaction (18, 19, 26). Capacitation increases the fluidity of the plasma membrane and sensitizes sperm to fertilization cues.

When capacitated spermatazoa encounter the cumulus oophorus (27) or bind the glycoproteins of the egg's zona pellucida (ZP), there are additional steep increases in sperm pH_i and Ca^{2+}, resulting in the acrosome reaction (28–32). The sperm plasma membrane contains specific ion channels and transporters that initiate changes in these ions in the sperm cytoplasm (4–6, 33–39). During the acrosome reaction, hydrolytic enzymes are expelled from the sperm acrosome to facilitate penetration through the egg's protective vestments (29).

As in all cells, sperm Na^+/K^+-ATPases establish the high K^+ and low Na^+ concentration of the sperm cytoplasm (38). As in serum, the extracellular fluids surrounding sperm cells contain 1–2 mM $[Ca^{2+}]$. Cells maintain a remarkable 20,000-fold gradient from outside the cell to inside the cell, with resting cytosolic Ca^{2+} concentrations ranging from ~50–100 nM $[Ca^{2+}]$. In somatic cells, these gradients are maintained by the export of cytoplasmic Ca^{2+} across the plasma membrane and by the import of Ca^{2+} into the endoplasmic reticulum and mitochondria. In sperm, these gradients are maintained primarily by a plasma membrane Ca^{2+}-ATPase pump (PMCA4) that extrudes Ca^{2+} (40–42). Male mice deficient in PMCA4 have impaired sperm motility and are infertile (40, 41). Unlike other cells, spermatozoa do not contain significant amounts of endoplasmic reticulum. How much Ca^{2+} is stored in sperm mitochondria remains unexplored.

The proton gradient across the sperm plasma membrane is the inverse of the Ca^{2+} gradient. Serum $[H^+]$ (40 nM, pH ~ 7.4) is fourfold lower than the intracellular $[H^+]$ of ejaculated

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spermatozoa (160 nM, pH ~ 6.8) (17, 43, 44). In epididymis, the extracellular fluid (pH 5.5 to 6.8; $[H^+]$ from 3160 to 160 nM) is even more acidic (16). Epididymal sperm pH_i drops below 6.0 due to the activity of different exchangers, including Na^+/H^+ and bicarbonate exchangers (45–48). Thus, there is always a concentration gradient in protons between cytoplasm and extracellular fluid. Low epididymal pH (and thus low pH_i) appears to be a major factor in rendering spermatozoa quiescent before ejaculation by inhibiting axonemal dynein activity (16, 17, 49). Also, the high viscosity of the cauda epididymal fluid (50, 51) and proteins such as semenogelin (52) inhibits sperm motility. Upon ejaculation, sperm cells are mixed with seminal plasma of much higher pH (~7.4; $[H^+]$ = 40 nM), and as sperm pH_i rises to ~6.5 ($[H^+]$ = 316 nM), sperm become motile for the first time (1, 44, 47). Lactobacilli and other vaginal flora acidify the vagina (pH ~ 4; $[H^+]$ = 100 μM); seminal plasma transiently increases female vaginal pH from 4.3 to 7.2 after intercourse (53), alkalinizing the environment and thus enabling spermatozoa to begin swimming. During subsequent transit through the female reproductive tract, pH_i increases further but still lags behind pH_o. Interestingly, at the peak of fertility in the middle of the menstrual cycle, cervical mucus becomes less viscous, and its pH can reach 9.0, making it less of a barrier to sperm (54). The pH of follicular fluid varies between 7 and 8, depending on the species and the phase of the menstrual cycle (55).

Upon ejaculation, sperm intracellular cAMP is elevated due to HCO_3^- activation of sperm soluble adenylyl cyclase (sAC) in a pH-independent manner (56). HCO_3^- concentration is higher in the seminal plasma/female reproductive tract than in the epididymal fluid (57), and HCO_3^- transporters deliver HCO_3^- into sperm cells. Moreover, the female oviduct is enriched in CO_2, which is converted into HCO_3^- by sperm extracellular glycosyl phosphatidylinositol–anchored carbonic anhydrase IV (58). Intracellular cAMP induces phosphorylation of axonemal dynein by protein kinase A (PKA) (59–61) to increase flagellar beating and sperm motility (62). sAC is also activated by Ca^{2+} (63, 64), and extracellular Ca^{2+} is required for the sAC-dependent increase in the frequency of flagellar beat triggered by HCO_3^- (65). Not surprisingly, male sAC and PKA knockout mice have impaired sperm motility and are infertile (66–68). PKA and sAC do not seem to be required for initiation of sperm motility but rather increase the frequency of sperm tail beating and improve progressive motility (61, 68, 69).

As mentioned above, the high proton and low Ca^{2+} concentrations in the sperm cytoplasm suppress sperm motility. Activation of spermatazoa requires alkalinization of sperm cytoplasm by the extrusion of protons and the elevation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. However, because transporters pump ions much more slowly than do channels, changes in intracellular ion concentrations are relatively slow compared with the rapid changes elicited by ion channels. As such, ion channels are primarily responsible for rapid signaling events (70). A more rapid change in sperm motility is achieved by fast diffusion of K^+, protons, and Ca^{2+} down their K^+, H^+, and Ca^{2+} concentration gradients across the sperm plasma membrane through selective ion channels. The opening of such channels is controlled by specific cues in the female reproductive tract that regulate the activity of the sperm cells (Figure 2). This regulation is both spatial and temporal in accordance with female anatomy and the phase of the menstrual cycle. Known cues for human spermatozoa are H^+ concentration, progesterone, and anandamide released by the cumulus oophorus; glycoproteins of the ZP; and proteins of the oviductal fluid such as serum albumin (31, 32, 71–75). However, there are probably more yet-to-be-discovered factors in the female reproductive tract that directly or indirectly control activity of sperm ion channels that synchronize arrival of the egg and the sperm at the fertilization site.

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SPERM ION CHANNELS

Calcium Channels

Ca^{2+} is critical to the initiation of cellular motion of all kinds (76). In spermatozoa, however, normal swimming behavior does not require the elevation of Ca^{2+}, a fact that at first surprised physiologists who focus on muscle and nerve. Sperm can swim over a range of $[Ca^{2+}]_i$, even in the absence of a plasma membrane, because they are essentially ciliary dynein ATPase motors. Just as in muscle and nerve, changing Ca^{2+} triggers changes in the behavior of motor proteins. Nonetheless, the elevation of intracellular Ca^{2+} is essential for changes in flagellar function that are manifested by capacitation, chemotaxis, and hyperactivated motility. Moreover, Ca^{2+} is required for initiation of the acrosome reaction.

Increases in $[Ca^{2+}]_i$ regulate the sperm's flagellar waveform and promote its asymmetrical bending (77). Before 2001, the channel responsible for sperm Ca^{2+} elevation was believed to be a voltage-gated Ca^{2+} channel (Ca_v; VGCC) and was perceived as the principal Ca^{2+} conductance of sperm (78–80). This notion was supported by electrophysiological identification of Ca_v channels in testicular spermatocytes (immature spermatogenic cells) using the patch-clamp technique (81–83) and by observation of a putative voltage-gated Ca^{2+} influx into mature sperm cells in response to the application of a high-K^+/high-pH extracellular medium (84). Yet, male mice deficient in $Ca_v2.2$, $Ca_v2.3$, and $Ca_v3.1$ were fertile, indicating that these VGCC channels were not essential for sperm physiology or functioned redundantly (85–87). Knockouts of $Ca_v1.2$ and $Ca_v2.1$ were lethal either at embryonic stages or soon after birth, thus precluding assessment of Ca^{2+} elevation in sperm (88, 89).

In 2001, the first member of a completely new family of Ca^{2+}-selective ion channel subunits was discovered. Termed CatSper1, it was found to be only in sperm cells and to be required for male fertility (4). Since then, seven CatSper subunits composing the heteromeric CatSper channel have been identified, and at least five of them—CatSper1–4 and CatSperδ—have been shown to be indispensible for proper channel formation and function (Figure 3) (Table 1) (4, 90–96). CatSper's pore is formed by four α subunits, the products of four distinct genes: *Catsper1*, *Catsper2*, *Catsper3*, and *Catsper4*. The channel contains three auxiliary subunits—CatSperβ, CatSperγ, and CatSperδ—of unknown stoichiometry (32, 93, 95, 96). All CatSper subunits are sperm-specific proteins and are located in the principal piece of the sperm flagellum. CatSperβ is predicted to have two transmembrane helices connected by a large extracellular loop. CatSperγ and CatSperδ have single predicted transmembrane helices and large extracellular domains. The function of the auxiliary subunits after assembly of the CatSper channel complex is not known.

Although interaction of CatSperβ and CatSperγ with the CatSper complex was clearly demonstrated biochemically (93, 95), whether they are required for the functional CatSper channel assembly is not clear. In contrast, CatSperδ not only interacts with the CatSper complex but is essential for functional CatSper (96). Like mice lacking any of the four CatSper α subunits (94), *CatSperδ*$^{-/-}$ mice have no measurable CatSper current ($I_{CatSper}$) and have the identical phenotype of male infertility due to loss of hyperactivated motility (96). Interestingly, CatSperβ, CatSperγ, CatSperδ, CatSper2, CatSper3, and CatSper4 are all undetectable on CatSper1 knockout sperm plasma membranes (92, 93, 95, 96), suggesting that all CatSper subunits are required for proper channel assembly; the absence of a single subunit may lead to degradation of remaining CatSper proteins. Humans with mutations or deletions in *CatSper1* and *CatSper2* are infertile (97–100). We suspect that loss-of-function mutations in any of the seven known CatSper subunits result in male infertility.

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Direct electrophysiological characterization of CatSper1 was achieved with the whole-cell patch-clamp technique applied to mouse spermatozoa (6). Comparison of ion currents recorded from wild-type and *CatSper1*-deficient spermatozoa confirmed that CatSper1 is required for a highly selective Ca^{2+} current. Recording from fragments of mouse spermatozoa established that $I_{CatSper}$ originated from the principal piece of the sperm flagellum, corresponding to antibody localization of the CatSper1 protein. $I_{CatSper}$ is weakly voltage dependent (the slope factor of the voltage activation curve $k = 30$) in comparison to strongly voltage-activated channels ($k = 4$) (6). Interestingly, the S4 transmembrane helix of CatSper1 contains six positively charged lysine/arginine residues aligned in the same manner as in strongly voltage-sensitive channels. However, CatSper2 has only four such residues, and only two are preserved in CatSper3 and -4. Because the pore of this heteromeric channel is formed by all four CatSpers, the voltage sensitivity of the complete channel is weak (34, 94).

The mouse CatSper channel is gated by changes in pH_i: The current is increased approximately sevenfold when pH_i is increased from 6.0 to 7.0 (6), corresponding to a (leftward) shift in the G-V curve of −70 mV. The abundance of histidines in the mouse CatSper1 N-terminal domain (51 His in the 250-residue N terminus) is one possible mechanism for this pH sensitivity (4, 34). Intracellular alkalinization by extracellular application of NH_4Cl not only causes $[Ca^{2+}]_i$ elevation by activating the CatSper channel but also triggers sperm hyperactivation (101).

Another hallmark of capacitation is reduction of sperm membrane cholesterol. Albumin, the main protein of the tubular fluid and an important component of in vitro capacitation media, also causes CatSper-dependent Ca^{2+} influx into mouse spermatozoa (74), perhaps affecting CatSper gating by modification of the lipid composition of the sperm plasma membrane. Finally, Ca^{2+} influx into mouse spermatozoa induced by the glycoproteins of the egg's ZP requires the CatSper channel (31), a property formerly assigned to the putative sperm Ca_v channels (78, 79). In this regard, the Ca_v current present in spermatocytes is not detected in mature spermatozoa—all channel-mediated Ca^{2+} entry in mature spermatozoa is via CatSper.

The subunits of CatSper channel are present in all mammalian genomes and some invertebrate species, such as the sea urchin and the freshwater mold *Allomyces macrogynus* (102, 103), but not in genomes of birds, amphibians, insects, and worms. The rapid disappearance of CatSper from some intermediate species over millions of years reflects the strong evolutionary pressure on gamete genes (102).

The CatSper channel is present in human sperm (5, 104) and, like mouse CatSper, is weakly voltage dependent but potently activated by intracellular alkalinization. The voltage dependency of human CatSper is slightly steeper ($k = 20$ compared with $k = 30$ in mice) than in mouse CatSper. Importantly, the $V_{1/2}$ (the voltage at which half of the channels are activated) of human CatSper is +85 mV versus +11 mV of mouse CatSper at the same pH_i ($pH_i = 7.5$) (6, 104), leading to the question of how human CatSper might be activated at such high membrane potentials.

Progesterone, a major steroid hormone released by the ovaries and the cumulus cells surrounding the egg, induces robust Ca^{2+} influx into human sperm cells (105, 106), triggers sperm hyperactivation, and initiates the acrosome reaction. These rapid effects are not via the nuclear progesterone receptor (107, 108). Progesterone exerts its effect on *Xenopus laevis* oocyte maturation and affects neural function without binding nuclear DNA or regulating gene expression. For example, *X. laevis* oocytes that are arrested in the G2 phase undergo maturation after the addition of extracellular progesterone. This phenomenon can

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occur even in enucleated cells. Also, progesterone can modulate γ-aminobutyric acid–mediated, glycine-mediated, and 5-hydroxytryptamine-mediated currents in neurons. However, the elusive progesterone receptor associated with humanspermatozoa is probably the best-known example of a nongenomic progesterone receptor (107, 108).

The mystery of progesterone's short-term responses on sperm was recently solved. Progesterone activates human CatSper at low concentrations [$EC_{50} \approx 7.7$ nM (104)] by shifting the voltage dependency of the human CatSper channel into the physiological range (104). The action of progesterone is rapid (latency <36 ms) and does not depend on intracellular ATP, GDP, cyclic nucleotides, Ca^{2+}, or other soluble intracellular messengers (104, 109). The simplest explanation of these results is that the progesterone-binding site may be located on one of the CatSper subunits or on a currently unidentified protein associated with the CatSper complex. The binding site associated with the CatSper channel for this hormone has not been identified but appears to be accessible from the extracellular space (104).

Prostaglandins are abundant in the seminal plasma (110) and are secreted by the oviduct and cumulus cells surrounding the oocyte (111). Nanomolar concentrations of select prostaglandins, including PGE_1, evoke intracellular Ca^{2+} transients similar in amplitude and waveform to those induced by progesterone (112–114). The relative potency of the human CatSper activators is as follows: progesterone > PGF_1 ≥PGE_1 > PGA_1 > PGE_2 > PGD_2 (104). Prostaglandin effects are additive to those of progesterone and thus may be mediated through a different receptor (104, 109). High levels of Zn^{2+} in seminal plasma (115) are likely to block the CatSper channel and to prevent its activation in the seminal plasma, but once spermatozoa are in the female-dominant environment, Zn^{2+} should be diluted or chelated (116–118).

In conclusion, the CatSper complex is encoded by at least seven genes, making it the most biochemically complex of all ion channels. This complexity may be required for its assembly, trafficking, and localization to the flagella and for its sensitivity to pH_i, progesterone, prostaglandins, and perhaps other proteins. Because orthologs of CatSper subunits present in different species have low identity (50% or less) (32, 93, 102), regulation of the CatSper channel may differ significantly between species. The CatSper channel of murine epididymal sperm cells, for example, is not sensitive to the activators of human CatSper such as progesterone and prostaglandins (104). This difference in CatSper channel regulation and even the absence of *CatSper* genes in some species highlight the common trend in evolutionary pressure on gamete genes, which applies also to critical genes in sex determination pathways such as *SRY* and *DAX1*.

KSper (Slo3): The Principal K^+ Channel of Spermatozoa

Incapacitated murine sperm hyperpolarize to approximately −60 mV during capacitation (119), an effect attributed to an increase in K^+ permeability. In a series of experiments in which voltage and intracellular and extracellular solutions were controlled, Navarro et al. (120) determined that pH_i sets the sperm membrane potential primarily by modifying the K^+ conductance. Under direct voltage clamp of mouse epididymal spermatozoa, resting membrane potential hyperpolarized to −45 mV within a few seconds after alkalinization. This hyperpolarization was due to a weakly outwardly rectifying K^+ current (I_{KSper}). I_{KSper} exhibited minimal time and voltage dependence, was relatively K^+ selective, and originated from the principal piece of the sperm flagellum. Intracellular alkalinization strongly potentiated I_{KSper} independent of extracellular [K^+]. I_{KSper} was not affected by 2 mM membrane-permeant cAMP and cGMP analogs, by increasing extracellular [Ca^{2+}], or by changes in bath osmolarity. Barium, quinine, clofilium, EIPA [a Na^+/H^+ exchanger (NHE) antagonist], and mibefradil reversibly inhibited I_{KSper}. Thus, I_{KSper} is the only detectable

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hyperpolarizing current in spermatozoa and largely sets its resting membrane potential. These authors suggested that I_{KSper} is encoded by Slo3 on the basis of its tissue localization and other properties (Table 1). Like I_{KSper}, *Xenopus* oocyte–expressed Slo3 is also weakly voltage sensitive (~16 mV/*e*-fold), has relaxed K^+ selectivity, and is insensitive to $[Ca^{2+}]_i$ and to external tetraethyl ammonium chloride (36, 121–123). This prediction was confirmed by recordings from mice lacking the *Slo3* gene (124, 125). However, heterologously expressed mSlo3 had different pH sensitivity, which suggests that Slo3 in spermatozoa may be regulated by other subunits or mechanisms that are absent from heterologous expression systems.

In a carefully done study, genetic deletion of *Slo3* abolished all pH-dependent K^+ current at physiological membrane potentials in mouse corpus epididymal sperm (125). *Slo3*$^{-/-}$ mice are infertile and do not exhibit capacitation-dependent membrane hyperpolarization, and Slo3-deficient sperm morphological abnormalities are accentuated by hypotonic challenge. Solutions of lower osmolality (230–310 mOsm kg^{-1}) resulted in an increase in bent and hairpin shapes, whereas spermatozoa kept in a hyperosmolar solution were protected against these changes. Incapacitated *Slo3*$^{-/-}$ sperm also have modest defects in motility, which may be related to a requirement for osmolar adaptation during spermatogenesis and sperm maturation (125). Only 10% of *Slo3*$^{-/-}$ sperm were able to fertilize oocytes during in vitro fertilization experiments. In summary, mSlo3 accounts for KSper, the dominant, if not the only, K^+-selective channel in mouse epididymal spermatozoa.

The protein responsible for K^+ current in human spermatozoa has not been identified but is likely to be the human homolog of Slo3, KCNU1. However, in contrast to the situation in murine sperm cells, human KSper seems to be independent of intracellular alkalinization (P. Lishko & Y. Kirichok, unpublished observation). Murine Slo3 and human Slo3 proteins are 65% identical; mouse Slo3 is more enriched in histidines in the cytoplasmic C terminus. The difference between human and murine sperm K^+ current represents another discrepancy in physiology between human and mouse spermatozoa.

The Voltage-Gated Proton Channel Hv1: A Fast Regulator of Intracellular pH in Human Sperm

Intracellular alkalinization is essential for the initiation of motility, capacitation, hyperactivation, and the acrosome reaction. On the basis of experiments with pH_i-sensitive fluorescent probes that detected changes in pH_i, the NHE (45, 126, 127) and a Na^+-dependent Cl^-/HCO_3^- exchanger (46, 47) were proposed to participate in sperm alkalinization. Upon ejaculation, mammalian spermatozoa are exposed to 100–150 mM $[Na^+]$ in seminal plasma, a much higher Na^+ concentration than the 30 mM $[Na^+]$ found in the cauda epididymis. In the female reproductive tract, Na^+ levels are similar to those in sera (140–150 mM) (128, 129). Thus, in the exchange of Na^+ for H^+, spermatozoan pH_i should increase. Sperm-specific molecules homologous to known Na^+/H^+ exchangers (sNHE) (130) are found in the principal piece of sperm flagellum. *sNHE* knockout mouse spermatozoa have impaired motility, and these males were completely infertile (130). Unfortunately, it has been difficult to demonstrate that sNHE actually functions as an NHE, as no significant difference in pH was found between wild-type and sNHE$^{-/-}$ spermatozoa (130). Complicating matters are the findings that sAC expression levels are significantly reduced in sNHE-deficient spermatozoa and that the sperm motility defect could be rescued by the addition of membrane-permeable cAMP analogs (130, 131).

The proton-selective, voltage-gated ion channel Hv1 (HVCN1) was cloned in 2006. This unusual channel is composed of a voltage sensor domain homologous to the voltage sensor of voltage-gated cation channels (132, 133). In contrast to the conventional ion channel, Hv1 lacks a classical pore region. The permeation pathway seems to be formed by an internal

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water wire completed by a movement of the charged S4 helix (134). Hv1 molecules dimerize, but each Hv1 subunit can function independently as a voltage-gated proton channel (135–137). The primary function of Hv1 in phagocytes is to allow intracellular protons to flow down their electrochemical gradient as electrons are extruded from cells via NADPH oxidase (NOX); block of Hv1 inhibits the innate immunity function of NOX (138, 139). Hv1 is characterized by strong voltage dependence, activation by high intracellular [H^+], unidirectional proton extrusion (Hv1 is physiologically unidirectional), and inhibition by low micromolar concentrations of zinc and potentiation by fatty acids (140).

A voltage-gated proton channel was recorded in human spermatozoa (Table 1) (5). Although it has the electrophysiological and pharmacological properties of Hv1 (5, 141), its function in sperm may not be simply to support NOX. Hv1 is abundantly expressed in human sperm cells within the principal piece of the sperm flagellum, making it ideally positioned to activate pH-dependent proteins of the axoneme and thus to control sperm motility (5, 25, 141). The normal to alkaline pH of the upper female reproductive tract (~7.4) may rapidly alkalinize the acidic intracellular compartments of sperm as they leave the acidic environment of the cauda epididymis and vagina. However, these changes need not be rapid and may easily be accomplished by exchangers, leaving one to wonder about the need for fast H^+ adaptation.

Human sperm flagella are long (40 μm), thin (<2 μm), and filled with axonemal structures. Because diffusion is inversely proportional to the area through which a substance diffuses, molecules take many seconds to travel within the extremely narrow flagellum from the mid-piece to the endpiece. Thus, ATP, generated in the mitochondria of the midpiece, is slow to reach the end of the flagellum. Therefore, flagellar movement, especially at the distal parts of the sperm tail, is powered mainly by glycolysis (142–144), which results in cytoplasm acidification. Moreover, axonemal dynein hydrolyzes ATP to produce ADP, P_i, and H^+, all of which also contribute to intracellular acidification. The prompt removal of protons is thus vital to dynein function (Figure 4). Sperm Hv1 conducts protons much more rapidly and efficiently than do exchangers or transporters and conducts them unidirectionally to the extracellular space.

Another possible role assigned to Hv1 is the regulation of intracellular Ca^{2+} homeostasis. Ca^{2+} delivered through CatSper is pumped out by a flagellar Ca^{2+}-ATPase that exports a cytoplasmic Ca^{2+} ion and imports extracellular protons. Its functioning results in decreasing flagellar pH_i, potentially inhibiting the CatSper channel. To prevent this scenario and to return the system to the status quo, Hv1 may balance pH_i by proton extrusion (Figure 3).

Hv1 is activated by the combination of the pH gradient and membrane depolarization (5, 132, 133, 140). However, because there is always a H^+ gradient out of the spermatozoa, the sperm's membrane potential is an important unknown and changes during sperm travel through the female reproductive tract. Membrane potential is set by Na^+/K^+-ATPases, which distribute ions over long durations, but is rapidly changed by the opening of ion channels.

Sperm Hv1 can be activated by the removal of extracellular zinc (5, 141). Zinc in humans is highest in seminal plasma (total 2.2 ± 1.1 mM compared with 14 ± 3 μM in serum) (115). Seminal zinc should inhibit Hv1, but as sperm travel through the female reproductive tract, any bound zinc is released through dilution, absorption by the uterine epithelium, and chelation by albumin and other molecules (116–118). Upon arrival at the Fallopian tube, spermatozoa should be essentially free from zinc inhibition. In addition, low micromolar concentrations of the endogenous cannabinoid anandamide strongly potentiate sperm Hv1 (5). The effect of anandamide is not mediated by CB1 or CB2 cannabinoid receptors and is likely due to a direct interaction of anandamide with Hv1 (5). Bulk concentrations of

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anandamide in the fluids of the male and female reproductive tracts are in the nanomolar range (145). However, because cumulus cells also synthesize and release anandamide, spermatozoa may experience much higher anandamide concentrations during the sperm's penetration of the cumulus oophorus (75). Finally, Hv1 is activated during in vitro capacitation (5), a time when tyrosine phosphorylation is very active. The mechanism of this potentiation remains unknown, but one hypothesis is that Hv1 is phosphorylated, especially because phosphorylation is the primary mechanism of Hv1 regulation in other tissues (146, 147). Moreover, intracellular alkalinization is considered to be a key factor during capacitation (15), and the coincidence of capacitation and the enhancement of Hv1 activity suggest a strong connection between these two events.

To date, patch-clamp experiments with mouse epididymal spermatozoa have not detected proton currents (5), and Hv1-deficient mice do not exhibit fertility defects (138, 139). Unfortunately, the NHE is electroneutral, and its activity cannot be recorded with patch-clamp techniques. Thus, the identification of all components of H^+ exchange in spermatozoa in mammals is an area for future detailed exploration.

In conclusion, sperm Hv1 may play an important role in the regulation of human sperm pH_i. By doing so, it could potentially influence almost every aspect of sperm behavior in the female reproductive tract, including initiation of motility, capacitation, hyperactivation, and the acrosome reaction. However, the physiological function of sperm Hv1 remains to be established. To date, the only correlation between human infertility and Hv1 is low levels of sperm HVCN1 mRNA in some infertility patients (148). Studies of genetic infertility in humans may thus help us understand the exact role of Hv1 in male fertility.

The ATP-Gated P2X2 Channel of Mammalian Sperm

To date, transmitter-mediated currents have not been reported in mouse spermatozoa. After screening a number of neurotransmitters and other biological molecules for their ability to induce ion channel currents in the whole spermatazoon, Navarro et al. (37) found a cation-nonselective, Ca^{2+}-permeable current originating from the midpiece of mouse epididymal spermatozoa that is activated by external ATP (I_{ATP}) (Table 1). Various plasma membrane purinergic receptors for ATP (purinergic receptors) were found in sperm by immunocytochemical studies (149), and ATP was reported to mediate an increase in intracellular Ca^{2+} (74). Navarro et al. (37) show that the behavior of this slowly desensitizing and strongly inwardly rectifying ATP-gated current has biophysical and pharmacological properties that mimic those of the heterologously expressed P2X2 oligomeric cation channel. Moreover, I_{ATP} is absent in spermatozoa of mice lacking the *P2rx2* gene. Despite the loss of I_{ATP}, *P2rx2*-deficient mice are fertile and have normal sperm morphology, sperm count, motility, and percent of sperm undergoing the acrosome reaction. However, the fertility of $P2rx2^{-/-}$ males declines with frequent mating over days, suggesting that the P2X2 receptor may confer a selection advantage under these conditions, perhaps through energizing mitochondria in the midpiece. ATP reportedly triggers the acrosome reaction in ejaculated bovine and human spermatozoa, reportedly via an uncharacterized sperm ATP-gated Na^+ channel (150).

Other Spermatozoan Ion Channels

In addition to the four sperm ion channels reviewed above, less evidence exists for other functional ion channels. Before 2001, the VGCCs were perceived as the principal Ca^{2+} conductance of sperm (78–80), but patch-clamp recording from mature sperm did not reveal any functional VGCCs and established that the CatSper channel is the principal sperm Ca^{2+} channel (6). In addition, several stimuli (e.g., increase in pH_i, depolarization, bovine serum albumin, and egg coat proteins) that trigger sperm Ca^{2+} influx previously assigned to

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VGCCs were later found to do so via the CatSper channel (31, 32). Finally, as discussed above, male mice deficient in $Ca_v2.2$, $Ca_v2.3$, and $Ca_v3.1$, three proteins detected only by antibodies in sperm, are fertile (85–87), indicating that these channels are not essential for sperm physiology or function redundantly (85–87). Our opinion is that VGCCs do not function in mature spermatozoa and do not have a significant role in mature sperm.

Cyclic nucleotide–gated (CNG) channels have also been proposed as mediating sperm Ca^{2+} influx. Both cAMP and cGMP elicit increases in $[Ca^{2+}]_i$ in sperm, as demonstrated in assays in which cell-permeable cAMP or cGMP is applied or when caged cGMP is uncaged (151). Thus, similar to photoreceptors and olfactory neurons, CNG channels may be responsible for the cyclic nucleotide–induced Ca^{2+} influx in sperm (151). A more recent variation of this model proposes that cyclic nucleotides activate the hyperpolarization-activated and cyclic nucleotide–gated (HCN) channels, resulting in the depolarization and subsequent opening of Ca^{2+} channels (79, 152). Although CNG and HCN channels may be present in sea urchin spermatozoa, mice and humans deficient in the CNG and HCN channels are fertile and have not been shown to exhibit defects in sperm function despite deficiencies in vision and cardiac function. Furthermore, no CNG or HCN currents in mouse or human sperm have been detected to date. Finally, because CatSper is responsible for the cAMP/cGMP-induced Ca^{2+} influx into sperm (32), cyclic nucleotides may activate CatSper indirectly, possibly via a PKA-dependent mechanism.

Several of the 28 members of the transient receptor potential (TRP) ion channels were recently proposed to function in mature spermatozoa. These include TRPM8, TRPV1, TRPC2, and others (153–155). For example, the TRPC2 protein was detected in sperm, and an anti-TRPC2 antibody reduced the sustained Ca^{2+} response elicited by egg coat proteins in mouse sperm and the ZP-induced acrosome reaction (154). However, mice deficient in *TRPC2* (as well as in the genes encoding TRPC1–7, TRPV1–4, TRPA1, TRPM1–4, and TRPM8) have no obvious defects in sperm physiology or male fertility. Indeed, in humans, *TRPC2* is a pseudogene. Therefore, the contribution of TRPC2, TRPM8, and TRPV1 in sperm physiology, if any, remains to be clarified.

SPERM CHEMOTAXIS

In the search for the egg, spermatozoa of many species are aided by chemotactic factors (73, 152, 156). Chemotaxis was first discovered in invertebrate marine animals such as the sea urchin, starfish, and sea squirt (157–159). Most marine animals produce and release sperm cells and eggs into seawater. To reach the egg in time, sperm cells must navigate a gradient of the chemoattractant(s) released by the egg and swim toward it.

The first putative chemoattractant of sea urchin, *Strongylocentrotus purpuratus* spermatozoa, a small peptide speract, was isolated from egg coat in 1981 by Hansbrough & Garbers (159). Later, picomolar concentrations of speract were found to activate a K^+ channel of *S. purpuratus* sperm (160). Interestingly, speract binding to the sea urchin spermatozoa also resulted in an increase in pH_i (161). Resact, a peptide from the egg coat of another species, Arbacia punctulata, was discovered in 1985 and was clearly shown to attract spermatozoa (162). The current model for sea urchin chemotaxis is built around the actions of resact on spermatozoa from *A. punctulata*, which suggested that resact activates flagellar guanylyl cyclase (GC) and triggers a signal transduction pathway leading to Ca^{2+} influx into the sperm flagellum. In short, the sea urchin's sperm flagellum contains a high density of membrane GC that produces cGMP from GTP in response to resact binding. cGMP is proposed to open K^+-selective cyclic nucleotide–gated (KCNG) channels; such opening briefly hyperpolarizes the sperm membrane (163). As a result, HCN channels open and allow Na^+ entry into the sperm flagellum. The resulting depolarization opens VGCCs,

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which conduct Ca^{2+} into flagellum and change the beating pattern. This sequence of events will require direct confirmation by recording under voltage clamp. Because all the CatSper α genes are present in the sea urchin (102, 103), we suspect that the story of sea urchin chemotaxis is not yet complete.

In contrast to the well-studied chemotaxis of sea urchin spermatozoa, much less is known about the chemotaxis of mammalian sperm. Mammalian spermatozoa are not likely to be engaged in the competitive-race model. Out of millions of spermatozoa delivered into the female reproductive tract, only one of every million succeeds in entering the Fallopian tubes, and <100 are able to reach the ampulla at any given time (73). Spermatozoa may be directed to the oocyte by specific cues or by chemicals released by the cumulus oophorus. Indeed, in 1991 researchers showed that human spermatozoa tend to accumulate in the follicular fluid (164) and that there is a positive correlation between sperm accumulation in the fluid and fertilization rate. Sperm chemotaxis was proposed in frogs, mice, and rabbits (for review see Reference 73). The discovery that human and rabbit spermatozoa are sensitive to picomolar concentrations of female hormone progesterone (165) established progesterone as a potential chemoattractant for human spermatozoa. Progesterone secreted from the cumulus oophorus peaks at midcycle and is present in oviductal and follicular fluid. As mentioned above, picomolar concentrations of progesterone activate CatSper and may regulate directional movement of the spermatozoa (104, 109). Mouse epididymal sperm CatSper is insensitive to progesterone (104), but this hypothesis should also be tested in ejaculated mouse spermatozoa.

Interestingly, *Ciona intestinalis* (sea squirt) eggs release sperm-attracting and -activating factor (SAAF), a molecule structurally similar to progesterone, and SAAF is a potent chemoattractant for *Ciona* spermatozoa (166). The molecular target for SAAF is not known, but because the *Ciona* genome contains *CatSper* and *Hvcn1* genes, the mechanism may be similar to that in human spermatozoa.

CONCLUSIONS

Ion channels of the sperm plasma membrane control the sperm membrane potential, establish intracellular Ca^{2+} and proton concentrations, direct cell movement, and, most importantly, are required for male fertility. With the ability to patch-clamp sperm, more light will be shed on the molecular identities and physiological regulation of sperm ion channels, resulting in new tools to control the behavior of spermatozoa and to increase or decrease male fertility. Given the enormous evolutionary pressure on genes optimizing gamete performance, there are likely many modifications or fine-tuning of the basic framework discussed above.

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Glossary

pH_i	intracellular pH
Acrosome	a cap-like vesicle covering the anterior portion of the head of a spermatozoon. The acrosome contains hydrolytic enzymes for penetrating through the protective vestments of the oocyte

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Acrosome reaction	exocytosis of the acrosomal vesicle upon the spermatozoon's contact with egg's protective vestments. Hydrolytic enzymes released by the acrosome digest the zona pellucida and help spermatozoa to reach the egg's surface
Cationic channel of sperm (CatSper)	a pH-regulated, calcium-selective ion channel required for sperm hyperactivation
Hyperactivation	a whip-like, high-amplitude asymmetrical beat of the sperm flagellum that helps spermatozoa overcome the egg's protective vestments. Hyperactivation is different from the low-amplitude symmetrical beat observed in normal motility
Fibrous sheath	a unique cytoskeletal structure of two longitudinal columns, connected by closely arrayed semicircular ribs. Fibrous sheath surrounds the outer dense fibers and the axoneme. The fibrous sheath influences the degree of flexibility, the plane of flagellar motion, and the shape of the flagellar beat
Capacitation	spermatozoa's acquisition of fertilizing capacity upon exposure to the fluids of the female reproductive tract for several hours. Capacitation results in sperm hyperactivation and the ability to undergo the acrosome reaction
Epididymis	a part of the male reproductive tract in which spermatozoa continue maturation and are stored
cAMP	cyclic adenosine monophosphate
pH_o	extracellular pH
Cumulus oophorus	the mass of cells, derived from granulosa cells of the Graafian follicle, that surrounds the oocyte upon its release during ovulation. To fertilize the oocyte, spermatozoon must first penetrate the cumulus oophorus
Zona pellucida (ZP)	the dense extracellular matrix surrounding the developing oocyte. The ZP prevents fertilization by multiple spermatozoa (polyspermy), prevents premature implantation, and protects the embryo during its first week of development
sAC	soluble adenylyl cyclase
PKA	protein kinase A
Chemotaxis	directional migration of the spermatozoa toward higher concentrations of chemoattractant released by the egg or cumulus oophorus
VGCC	voltage-gated calcium channel
$I_{CatSper}$	CatSper current
G-V curve	plot of membrane conductance (G) versus voltage (V) that is used to show the percentage of activated ion channels in relation to membrane potential
Slo3	a K^+-permeant ion channel
sNHE	sperm Na^+/H^+ exchanger

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Hv1	the voltage-gated, proton-selective ion channel found usually in phagocytes but also present in human sperm
CNG channel	cyclic nucleotide–gated channel
HCN channel	hyperpolarization-activated and cyclic nucleotide–gated channel

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SUMMARY POINTS

1. Sperm are free-swimming gametes that must adapt to changes in local environments on their journey to the egg. Ion channels of sperm enable sperm to respond and adjust to constantly changing environments and are required for male fertility.
2. Spermatozoa are compartmentalized cells, and plasma membrane ion channels are found primarily in the flagella. CatSper, KSper, and, in humans, Hv1 are localized in the principal piece of sperm flagellum, where they regulate sperm motility.
3. Direct recordings of spermatozoan ion currents under voltage clamp are essential for the proper identification of putative ion channel proteins found in sperm by other techniques.

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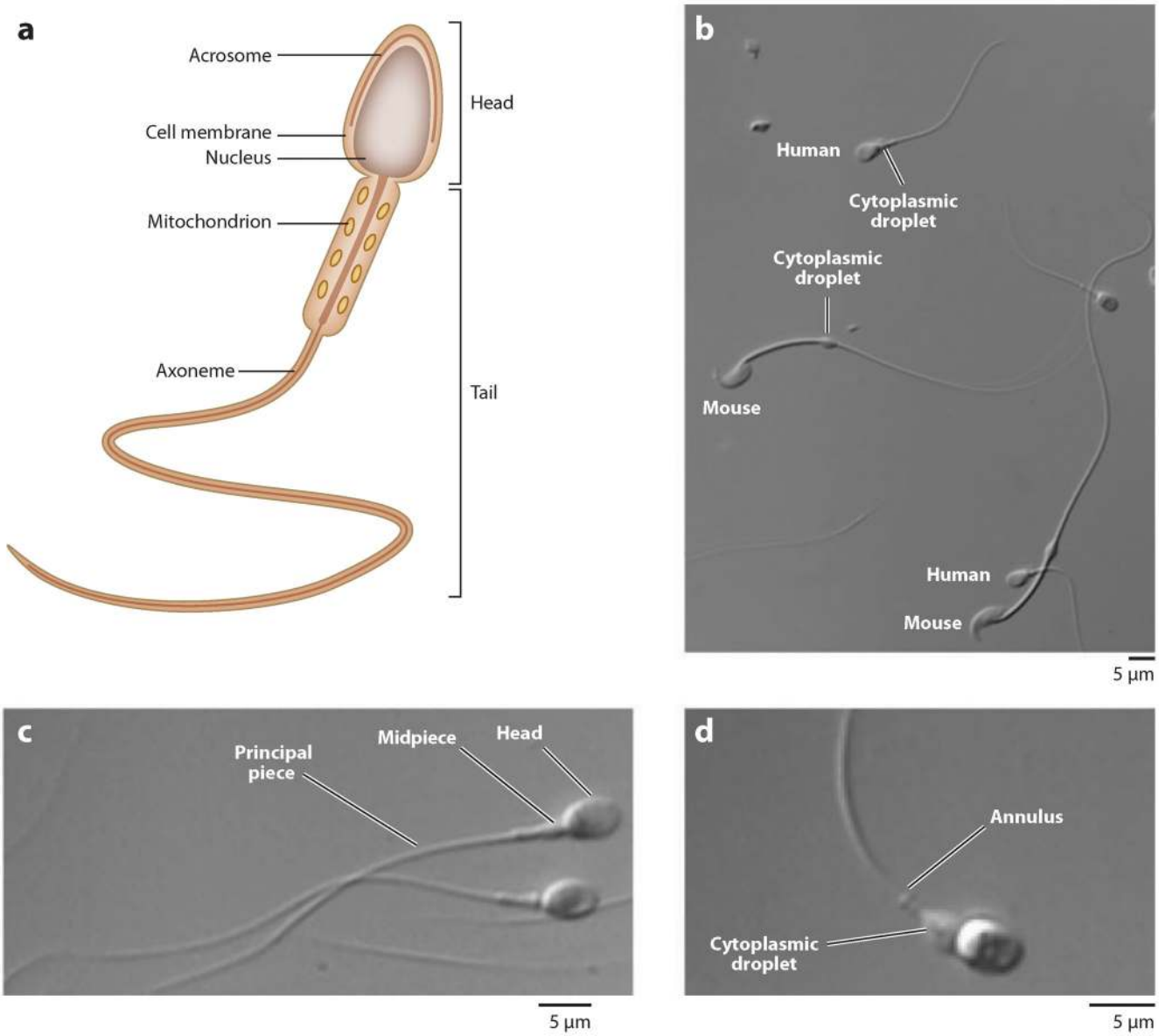

Figure 1.
Mammalian spermatozoa. (*a*) Schematic representation of mammalian sperm. (*b*) Comparison between human and mouse spermatozoa. (*c*) Human spermatozoa with head, midpiece, and principal piece as indicated. (*d*) Cytoplasmic droplet and annulus are labeled.

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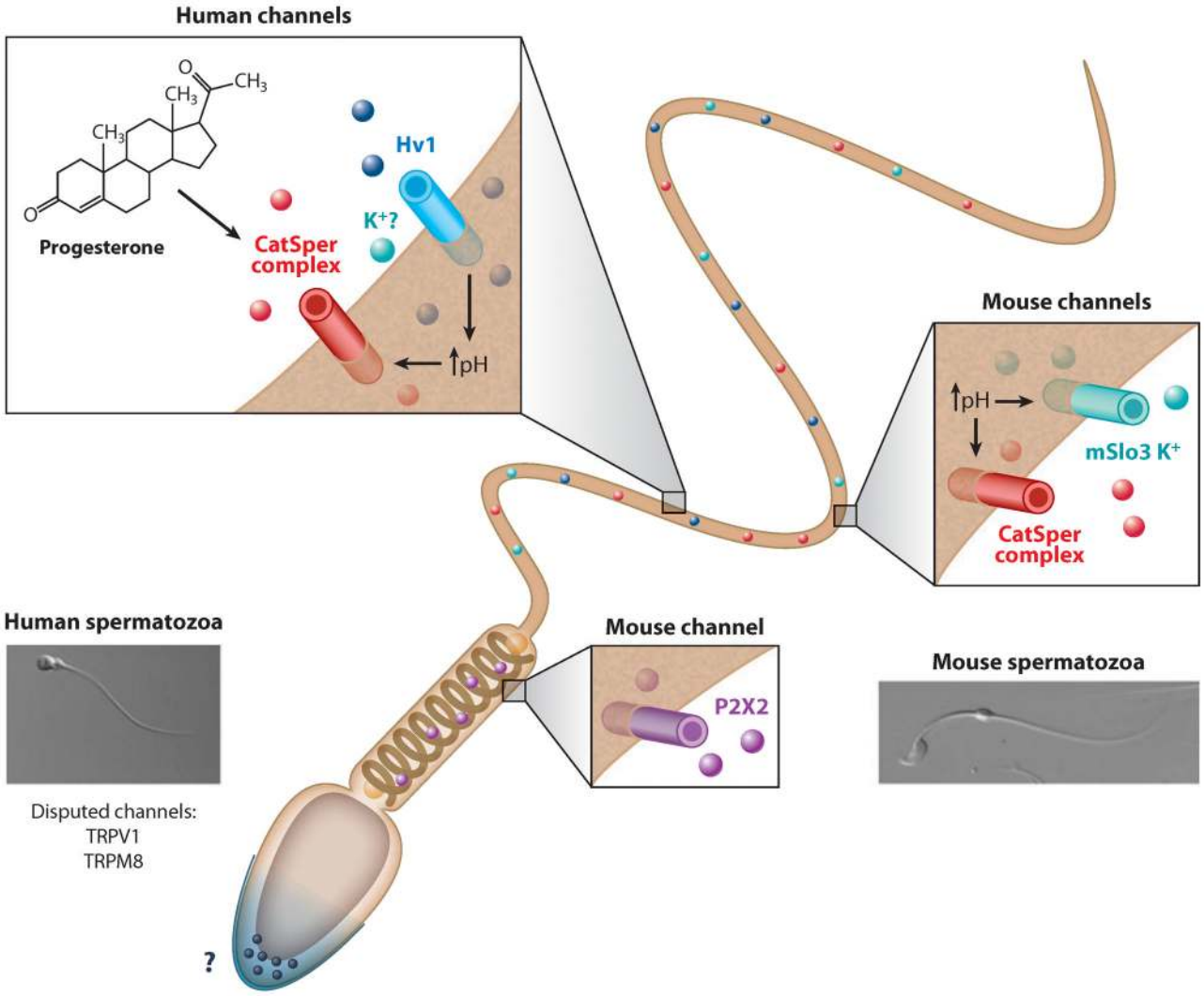

Figure 2.

Sperm ion channel localization and function. Flagellar beating is regulated by at least three ion channels: alkaline-sensitive CatSper (Ca^{2+} entry), pH-regulated Slo3 (K^+ exit), and Hv1 (H^+ exit; human sperm only). The upper half of the figure depicts ion channels and their regulation as detected in human sperm (the regulation of the CatSper complex by progesterone and Hv1), whereas the lower half of the figure shows ion channels found in mouse spermatozoa (CatSper complex, Slo3, and P2X2).

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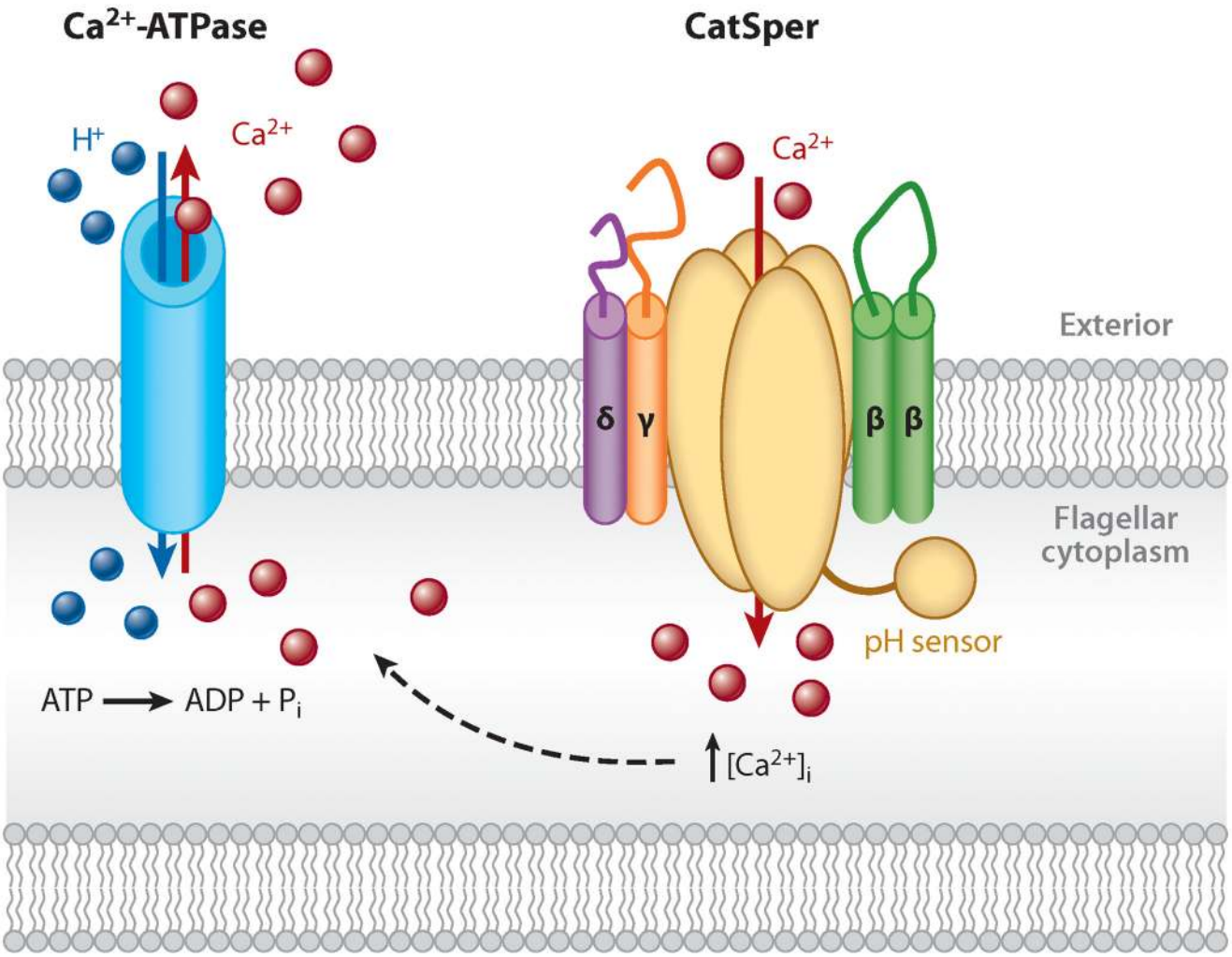

Figure 3.
Regulation of flagellar Ca^{2+}. Ca^{2+} enters the sperm flagellum via the alkaline-activated CatSper channel and is extruded from the flagellum by a plasma membrane Ca^{2+}-ATPase (42). Ca^{2+}-ATPase pumps hydrolyze ATP to export a cytoplasmic Ca^{2+} ion and to import extracellular protons. The resulting acidification of flagellar cytoplasm must be prevented by proton extrusion via channels or transporters.

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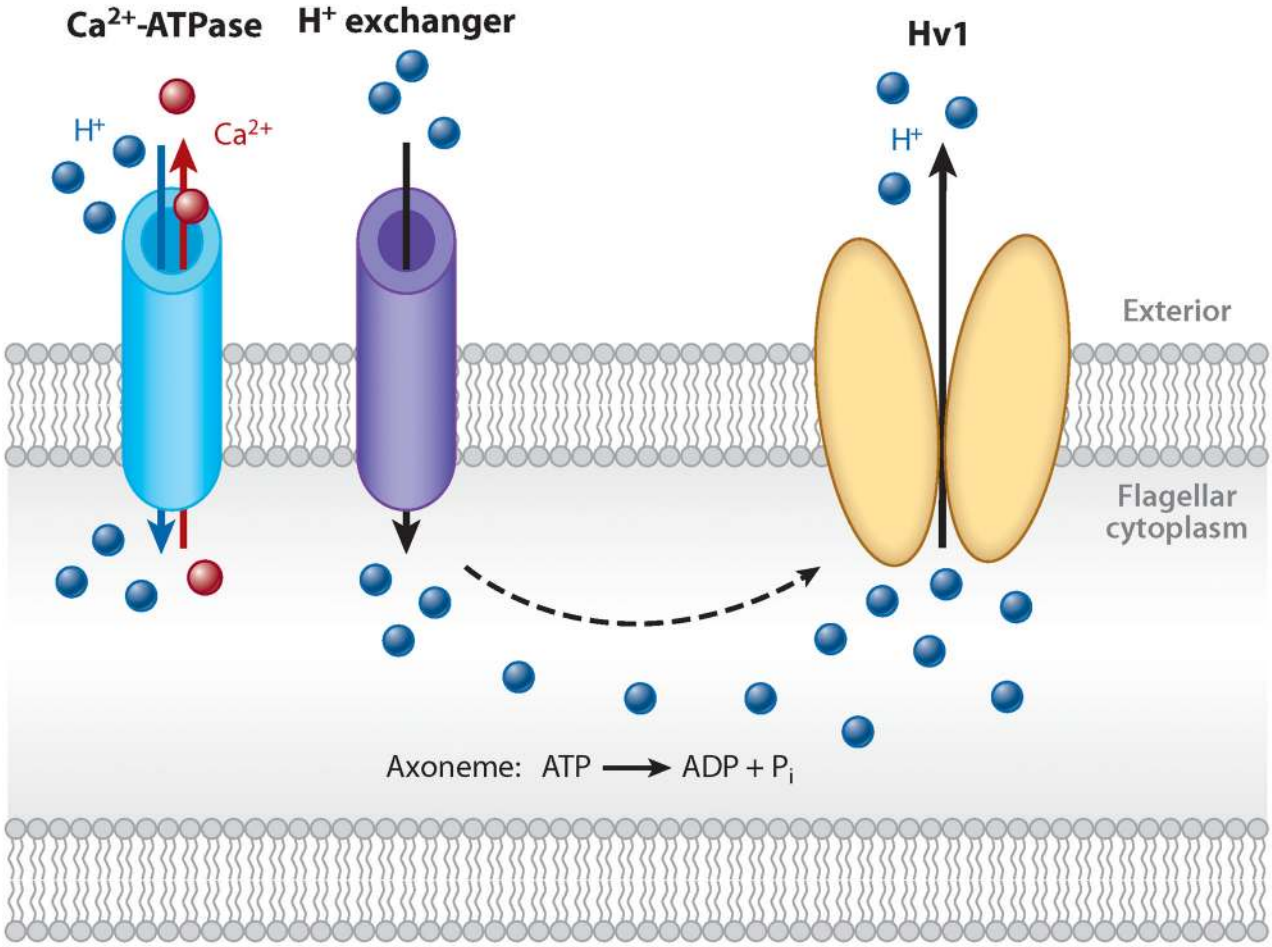

Figure 4.
Regulation of flagellar pH. Protons may accumulate in the sperm flagellum via proton exchange, ATP hydrolysis by axonemal dynein, and active glycolysis. Rapid proton extrusion from the human sperm flagella may be carried out by Hv1 proteins, which form a proton-selective, voltage-gated ion channel restricted to the sperm's principal piece.

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Table 1

Ion channels of mammalian spermatozoa

Channel name	Gene name and chromosomal location	Ion selectivity	Subunit(s)/composition	Localization in sperm	Role in sperm physiology, including sperm specificity	Endogenous regulators	Knockout phenotype
CatSper	*CatSper1* (11q13.1) *CatSper2* (15q15.3), (15q15.3- pseudogene) *CatSper3* (5q31.1) *CatSper4* (1p36.11) *CatSperβ* (14q32.11) *CatSperγ* (19p13.2) *CatSperδ* (19p13.3); locations shown are for human CatSper	Ca^{2+}	Seven subunits total: heteromeric assembly of CatSper1–4 (pore subunits), CatSperβ, -γ, -δ(auxiliary subunits); stoichiometry is unknown	Principal piece	Ca^{2+} influx into flagella, hyperactivated motility, sperm specific	Progesterone (human sperm only), pH_i, egg coat proteins, albumin	Male sterility; sperm from $CatSper^{-/-}$ mice are unable to hyperactivate
KSper	Mouse: *Slo3* (8A3); human: *Slo3* (8p11.23)	K^+	Probable tetramer	Principal piece	Hyperpolarizes sperm membrane, regulates membrane potential; sperm specific	pH_i	Male sterility–increased bent hairpin morphology of spermatozoa
Hv1	Human: *Hvcn1* (12q24.11)	H^+	Probable homomeric dimer	Principal piece	Extrudes protons from flagellum, alkalinizes cytoplasm; primarily in phagocytic cells (e.g., leukocytes), alveolar type II cells, and spermatozoa (human)	pH_i, membrane voltage, removal of zinc, anandamide	I_{Hv1} is not present in murine epididymal sperm; $Hvcn1^{-/-}$ mice are fertile
I_{ATP}	Human: *P2RX2* (12q24.33); mouse: *P2rx2* (5)	Na^+, K^+, Ca^{2+}	Probable homotrimer	Midpiece	Widespread		$P2rx2^{-/-}$ mice are fertile

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