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The BSA-induced Ca^{2+} influx during sperm capacitation is CATSPER channel-dependent

Jingsheng Xia and Dejian Ren*

Address: Department of Biology, University of Pennsylvania, 415 S University Ave, Philadelphia, Pennsylvania 19104, USA

Email: Jingsheng Xia - xiaj@sas.upenn.edu; Dejian Ren* - dren@sas.upenn.edu

* Corresponding author

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Abstract

Background: Serum albumin is a key component in mammalian sperm capacitation, a functional maturation process by which sperm become competent to fertilize oocytes. Capacitation is accompanied by several cellular and molecular changes including an increased tyrosine phosphorylation of sperm proteins and a development of hyperactivated sperm motility. Both of these processes require extracellular calcium, but how calcium enters sperm during capacitation is not well understood.

Methods: BSA-induced changes in intracellular calcium concentration were studied using Fluo-4 and Fura-2 calcium imaging with wild-type and Catsper1 knockout mouse sperm.

Results: We found that the fast phase of the BSA-induced rises in intracellular calcium concentration was absent in the Catsper1 knockout sperm and could be restored by an EGFP-CATSPER1 fusion protein. The calcium concentration increases were independent of G-proteins and phospholipase C but could be partially inhibited when intracellular pH was clamped. The changes started in the principal piece and propagated toward the sperm head.

Conclusion: We conclude that the initial phase of the increases in intracellular calcium concentration induced by BSA requires the CATSPER channel, but not the voltage-gated calcium channel. Our findings identify the molecular conduit responsible for the calcium entry required for the sperm motility changes that occur during capacitation.

Background

During mammalian fertilization, freshly ejaculated sperm do not have the ability to fertilize oocytes until after they undergo capacitation, a functionally defined, but poorly understood maturation process by which sperm become capable of fertilizing eggs [1-3]. Sperm become capacitated *in vivo*, by interacting with environmental stimuli in the female reproductive tract before encountering eggs. This process can also be mimicked *in vitro* by incubating

sperm in defined capacitation media. Several commonly used components are essential for successful *in vitro* capacitation in sperm from many mammalian species. Among them are bovine serum albumin (BSA), Ca^{2+} and bicarbonate (HCO_3^-) [3]. Capacitation leads to several cellular and behavioral changes, including an increase in tyrosine phosphorylation of sperm proteins, rises in intracellular pH (pH_i) and Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), membrane hyperpolarization, and hyperactivated motility [4-6].

Increases in $[Ca^{2+}]_i$ and intracellular $[pH]_i$ are believed to play central roles in both sperm capacitation and the acrosome reaction (AR) [3,7,8]. The capacitating agent BSA induces Ca^{2+} influx in sperm, but the molecular mechanisms underlying such an influx are not well understood. Multiple Ca^{2+} -permeable ion channels have been detected in mammalian sperm, including voltage-gated Ca^{2+} channels (Ca_v s), transient receptor potential (TRP) channels, cyclic nucleic gated (CNG) channels and CATSPER channels [9-12]. Among these ion channel proteins, only the four mammalian CATSPER members (CATSPER 1-4) are specifically found in sperm and spermatogenic cells [13-17]. All four *Catsper* genes are required for male fertility as mice with any of these genes disrupted are infertile [15,18-20]. Disruptions in *Catsper1* and *Catsper2* are also associated with male infertility in humans [21-23].

Using Ca^{2+} -sensitive fluorescent probes, we and others have shown that CATSPERs are required for the Ca^{2+} entry induced by stimuli such as cyclic nucleotides, alkaline depolarizing medium and egg coat proteins [24-26]. Ca^{2+} entering the channel in sperm tail can trigger Ca^{2+} propagation toward the head [25,26]. CATSPER's roles in the migration of sperm toward the oocyte and in penetrating the egg coat have been clearly established by studies showing that *Catsper* mutant sperm cannot migrate to the egg *in vivo* [27] and that, in *in vitro* fertilization (IVF), they cannot penetrate coat-intact eggs but can fertilize those without the zona pellucida [18]. In contrast, CATSPER's function in sperm capacitation is less clear. *Catsper* mutant sperm do not develop hyperactive motility after incubation in capacitation medium, as do normal sperm. The mutant sperm also have a progressive decrease of motility under certain incubation conditions [14,15,19,24,28,29]. This finding suggests that CATSPER has a role in the motility aspect of sperm capacitation. On the other hand, wild-type and *Catsper* mutant sperm do not differ in their patterns of protein tyrosine phosphorylation after sperm capacitation [19,24] or in their capacitation and AR efficiency, as examined with the chlortetracycline (CTC) assay [25,26]. In this study, we investigated CATSPER's potential role in sperm capacitation by studying the Ca^{2+} influx induced by BSA.

Methods

Reagents

Fluo-4 AM, Fura-2 AM and pluronic F-127 were purchased from Molecular Probes (Invitrogen, Eugene, OR). Pertussis toxin (PTX) and ionomycin were from CalBiochem (Gibbstown, NJ) and Cell-Tak was from BD Biosciences (Bedford, MA). BSA (fraction V, fatty acid-depleted, Sigma #A3059), disodium salt ATP, and other reagents were purchased from Sigma. Similar Ca^{2+} responses in sperm were also observed with fatty acid-free BSA (Sigma #A8806; not shown).

Animals

Animals were treated according to institutional regulations. This study used *Catsper1* knockout mice that were backcrossed to C57BL/6J for more than 10 generations [18]. Sperm of the *Catsper1* knockout mice lack not only CATSPER1, but also CATSPER2 [28] and the associated auxiliary proteins CATSPER β [16] and CATSPER γ [17]. To reflect this fact, we do not distinguish CATSPER1 from the other CATSPERs throughout the paper. The *EGFP-Catsper1* transgenic mice have a *Catsper1* null background but carry an EGFP-CATSPER1 fusion protein gene that rescues the male sterile phenotype of the *Catsper1* null mutant [16].

Sperm Ca^{2+} imaging

Non-capacitated caudal sperm were used for Ca^{2+} imaging, as previously described [25,26]. Briefly, sperm were released into HS medium containing (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 HEPES, 10 glucose, 10 lactic acid, and 1 pyruvic acid (pH adjusted to 7.4 with NaOH), and concentrated to $5 \times 10^6 - 1 \times 10^7$ /ml. Cells were loaded with 10 μ M Fluo-4 AM and 0.05% pluronic F-127 for 30 min at room temperature in the dark, followed by two washes in imaging medium (HS supplemented with 15 mM $NaHCO_3$), each with a 4 min spin at $300 \times g$. Washed sperm were resuspended in imaging medium and loaded into a small-volume imaging chamber (~ 1 cm diameter, ~ 90 μ l) formed with Sylgard on a Cell-Tak coated coverslip, and allowed to attach for ~ 10 min.

For imaging sperm from *EGFP-Catsper1* transgenic mice, a ratiometric measurement with Fura-2 (5 μ M for loading) was used because of the EGFP fluorescence. A monochromator (DeltaRAM V, PTI) with a 75-W Xenon lamp was used to generate the excitation at 488 nm for Fluo-4 (or 340 nm and 380 nm for Fura-2). A 60 \times objective and a 1.6 \times adaptor on an inverted microscope (IX-71, Olympus) were used for imaging. Emissions (515-565 nm) were bandpass filtered (HQ540/50, Chroma) and collected with a cooled CCD camera (CoolSNAP HQ, Roper Scientific) for 25 ms in every 0.5 s for fast recording, or 100 ms in every 6 s for slow recording. Online control, data collection, and image processing were conducted using commercial software (ImageMaster 3, PTI).

For imaging using Fluo-4, $[Ca^{2+}]_i$ changes are presented as $\Delta F/F_0$ ratios after background subtraction, where ΔF was the change in fluorescence signal intensity and F_0 was the baseline as calculated by averaging the 10 frames before stimulus application. In sperm loaded with Fura-2, $[Ca^{2+}]_i$ changes are presented as the ratio of fluorescence from excitation at 340 nm to that at 380 nm (F_{340}/F_{380}) after background subtraction. All the imaging experiments were done at room temperature. Cells with uneven dye loading were excluded from the analysis. Motile sperm ($\sim 60\%$ of the population) that had at least two points

attached to the coverslip were used for analysis. Cells with peak changes of >50% in $\Delta F/F_0$ (for Fluo-4) or >0.1 in F340/380 (for Fura-2) after application of stimuli were counted as responsive. To detect the Ca^{2+} responses at "clamped" membrane potential (V_m), K^+ ionophore valinomycin (1 μM) was added to the imaging buffer, with additional K^+ as indicated to replace an equimolar amount of Na^+ . E_K was calculated with the assumption of a 120 mM intracellular K^+ concentration [30,31]. To "clamp" intracellular pH, sperm were preincubated for 5 min with 3 μM carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) and 1 μM valinomycin [32].

Statistical methods

Data analyses were performed with ImageMaster3, Excel and Origin. Student's t-tests and ANOVA were used for statistical comparisons between different treatment groups. $P < 0.05$ was considered statistically significant.

Results

CATSPER channels are required for the BSA-induced $[Ca^{2+}]_i$ rise in mouse sperm

When applied to non-capacitated sperm lightly immobilized onto coverslips, BSA elevated $[Ca^{2+}]_i$ in the sperm head (Figure 1A) at concentrations as low as 0.1 mg/ml (Figure 1B). The responses were dependent on the presence of extracellular Ca^{2+} (Figure 1A), suggesting a role for Ca^{2+} entry. These properties of BSA-induced changes under our conditions are comparable with other studies in mouse and human sperm [33-35].

How BSA induces $[Ca^{2+}]_i$ changes in sperm is not well understood, but one possibility is through Ca_v s [34-36].

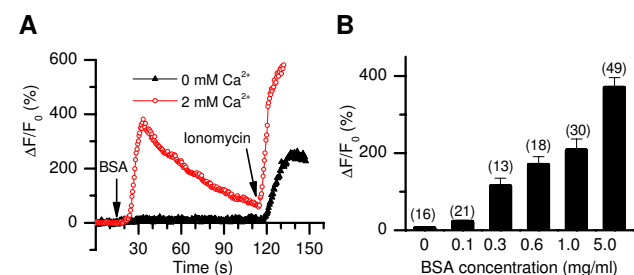


Figure 1
BSA-induced $[Ca^{2+}]_i$ changes in the sperm head of wild-type mice monitored with single cell imaging. (A) Representative recordings of $[Ca^{2+}]_i$ changes (represented as normalized Fluo-4 fluorescence changes) in response to application of BSA (5 mg/ml; indicated by vertical arrow) to a bath containing 2 mM Ca^{2+} or no Ca^{2+} (with 5 mM EGTA). Some cells also had a second phase of $[Ca^{2+}]_i$ rise (see Figure 2A). Ionomycin (5 μM , used as a control) caused a $[Ca^{2+}]_i$ rise in the absence of extracellular Ca^{2+} by releasing intracellular Ca^{2+} . (B) Dose-dependence of peak amplitude of the BSA-induced Ca^{2+} responses.

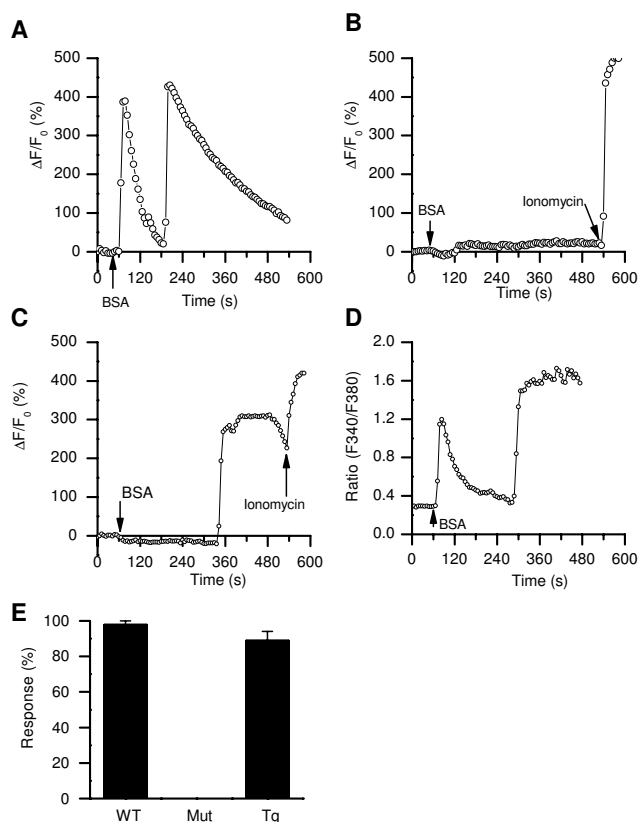


Figure 2
BSA-induced $[Ca^{2+}]_i$ rises in sperm head depend on CATSPER. (A) Example of a wild-type sperm with two phases of $[Ca^{2+}]_i$ changes. (B, C) Recordings from *Catsper1* null sperm. None had the fast (1st phase) response within 2 min, but one (C) had a 2nd phase response. The Ca^{2+} ionophore ionomycin (5 μM) was applied as a control stimulus. (D) A representative recording from a transgenic sperm expressing an EGFP-CATSPER fusion protein in the *Catsper1* null background. (E) Percentage of sperm responsive to BSA application in wild-type (WT; n = 18 imaging runs; 9 mice, 109 cells), *Catsper1* null (Mut; n = 7; 3 mice, 30 cells), and a *Catsper1* mutant rescued with the EGFP-CATSPER transgene (Tg; n = 12; 5 mice, 57 cells). Cells from Tg mice were imaged with Fura-2 and the others were imaged with Fluo-4. BSA was used at a concentration of 5 mg/ml.

Our recent studies, however, suggest that mature sperm do not have detectable functional Ca_v channels [25]. To determine whether the BSA-induced $[Ca^{2+}]_i$ rises are dependent on CATSPERs, we compared the $[Ca^{2+}]_i$ changes in wild-type mouse sperm with those in the *Catsper1* null mutants. Upon bath application of BSA (5 mg/ml), 98% of wild-type sperm (107/109) showed initial responses in the head within 20 s and 17% (10 of 58) had a 2nd response more than 2 min later (Figure 2A, E). In contrast, the initial $[Ca^{2+}]_i$ changes were absent in CATSPER1-deficient sperm within 2 min of BSA stimula-

tion (Figure 2B, E), although the delayed responses were present in some sperm (23%, Figure 2C). The BSA-induced $[Ca^{2+}]_i$ rises were restored by a transgene encoding an EGFP-CATSPER1 fusion protein in the Catsper1 null background (Figure 2D, E). These results indicate that CATSPER1 is required for the initial $[Ca^{2+}]_i$ responses.

Elevations in BSA -induced $[Ca^{2+}]_i$ start in the sperm tail and propagate to the head

The finding that the BSA-induced increase in $[Ca^{2+}]_i$ in the sperm head was dependent on CATSPER1 was intriguing because CATSPER proteins and the current through the channels are strictly localized in sperm principal piece, which is 20 mm away from the head [18,37]. To test whether the BSA-induced $[Ca^{2+}]_i$ changes in the sperm head were a result of Ca^{2+} entry through CATSPER in the tail, we analyzed the spatial-temporal kinetics of $[Ca^{2+}]_i$ changes along the entire length of the sperm. After BSA application, $[Ca^{2+}]_i$ rises started in the principal piece, and then were seen in the mid-piece and head of the sperm (Figure 3A). The differences in the response onsets between the principal piece and head were 2.46 ± 0.52 s ($n = 10$) with 1 mg/ml BSA (Figure 3B) and 2.76 ± 0.25 s ($n = 10$) when induced with a higher concentration (5 mg/ml, Figure 3C). In contrast to BSA, the Ca^{2+} ionophore ionomycin (5 μ M) increased $[Ca^{2+}]_i$ simultaneously in the principal piece, mid-piece and sperm head (Figure 3A).

The BSA-induced increases in $[Ca^{2+}]_i$ partially depend on pH_i changes

How does BSA induce a CATSPER-dependent Ca^{2+} entry? Stimuli such as egg coat proteins (e.g. zona pellucida) can lead to CATSPER-dependent Ca^{2+} influx likely via pertussis toxin (PTX)-sensitive G proteins and a phospholipase C-dependent signaling pathway that can be inhibited by neomycin [10,25,38]. To test whether the CATSPER-dependent Ca^{2+} influx activated by BSA uses a similar pathway, we recorded BSA-induced $[Ca^{2+}]_i$ changes in the absence and presence of PTX (100 ng/ml, Figure 4B) or neomycin (1 mM, Figure 4C). Neither PTX or neomycin inhibited the BSA-induced $[Ca^{2+}]_i$ rises (Figure 4D).

BSA also changes the membrane lipid composition by facilitating cholesterol efflux, and such a change likely contributes to capacitation-associated events such as tyrosine phosphorylation [39-42]. The ability of BSA to remove lipids, however, does not seem to be required for its induction of CATSPER-dependent Ca^{2+} influx since pre-incubating sperm with cholesterol sulfate to presumably saturate BSA and reduce its ability to remove cholesterol [36,41] did not inhibit the BSA-induced Ca^{2+} influx (Figure 5).

Depolarization-activated voltage-gated channels such as the T-type Ca^{2+} channel have also been proposed to medi-

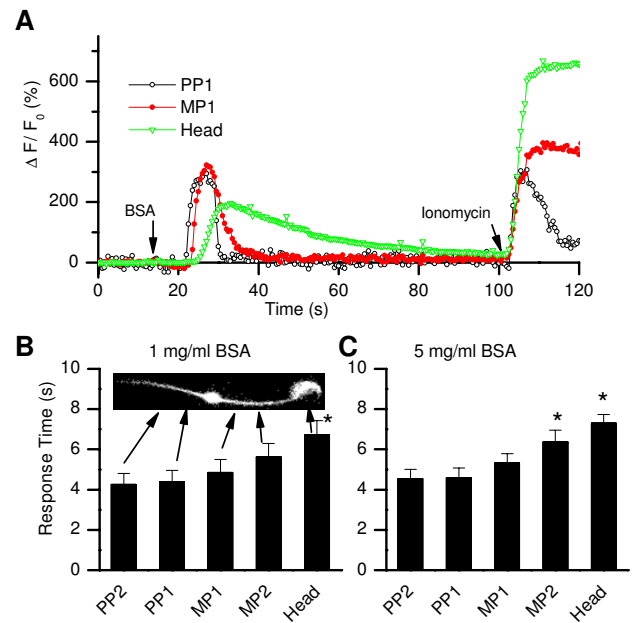


Figure 3
The BSA -induced $[Ca^{2+}]_i$ rises start in sperm tail and propagate toward the head. (A) Representative time courses of the $[Ca^{2+}]_i$ changes in the principal piece (PP1), midpiece (MP1) and head of the sperm. Increases in $[Ca^{2+}]_i$ start in the principal piece and propagate to the head. The Ca^{2+} ionophore ionomycin (5 μ M) increased $[Ca^{2+}]_i$ increases simultaneously in all subregions. (B-C) Time differences between the application of BSA (B, 1 mg/ml; C, 5 mg/ml) and the onset of fluorescence changes (defined as the time point when $\Delta F/F_0$ started to have steep rise) in different regions within the principal piece (PP1, PP2), midpiece (MP1, MP2) and head as shown in the inset. PP1 and MP1 are both 5 μ m from the annulus. There is a 5 μ m distance from PP1 to PP2, and from MP1 to MP2. * indicates statistically significant ($P < 0.05$).

ate the BSA-induced $[Ca^{2+}]_i$ increases [34-36]. In addition, CATSPER channels are not primarily voltage-activated, but do have some weak voltage-sensitivity [37]. To test whether voltage changes are required for the BSA-induced Ca^{2+} entry into mature sperm, we compared $[Ca^{2+}]_i$ changes at various membrane potentials "clamped" with a K^+ ionophore valinomycin. Although the amplitudes of the $[Ca^{2+}]_i$ change varied with the clamped membrane potentials, BSA still raised $[Ca^{2+}]_i$ even when the voltage of the membrane was held at -40 (Figure 6B, D) or -20 mV (Figure 6C, D), conditions that are expected to completely inactivate the T-type Ca^{2+} channel [25]. These data suggest that voltage changes nor T-type Ca^{2+} channels are required for the BSA-induced elevations in $[Ca^{2+}]_i$.

Another effect of BSA in particular, and capacitation in general, is an intracellular alkalinization by 0.4 pH_i units

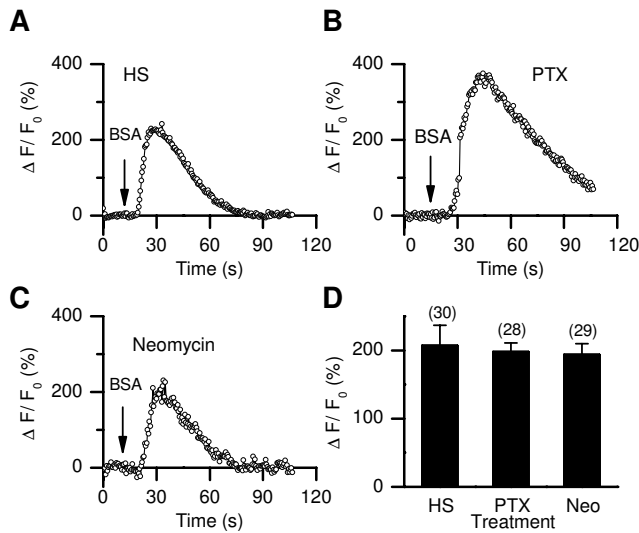


Figure 4
PTX and neomycin do not inhibit the BSA-induced $[Ca^{2+}]_i$ change. (A-C) Representative recordings of the $[Ca^{2+}]_i$ changes ($\Delta F/F_0$) in the sperm head induced by BSA (1 mg/ml) from cells pre-incubated with control (A), PTX (100 ng/ml, 30 min pre-incubation) or neomycin (C) (1 mM, 5 min pre-incubation). (D) Averaged peak $\Delta F/F_0$ changes. The numbers of total sperm (from two to three mice) are indicated.

[33,43]. When pH_i was clamped with a H^+ ionophore FCCP and K^+ ionophore valinomycin [32], the amplitude of the BSA-induced $[Ca^{2+}]_i$ rise was decreased and the percentage of responsive cells was reduced (Figure 6E, F), suggesting that BSA induces $[Ca^{2+}]_i$ increases, at least partially, through pH_i changes.

Consistent with a role of pH_i changes in the BSA-induced $[Ca^{2+}]_i$ increases, the CATSPER channel can be activated by intracellular alkalization [37] and alkaline medium with an elevated concentration of K^+ (the K8.6 medium) can lead to a CATSPER-dependent rise in $[Ca^{2+}]_i$ [24]. Thus, we investigated whether intracellular alkalization alone can induce CATSPER-dependent $[Ca^{2+}]_i$ rises and the extent to which CATSPER channels contribute to the increases. To do this, we compared the $[Ca^{2+}]_i$ responses to bath application of 20 mM NH_4Cl , which leads to intracellular alkalization and induces a slow $[Ca^{2+}]_i$ rises in sperm [44-47], in the wild-type and *Catsper1* mutant sperm. NH_4Cl application evoked $[Ca^{2+}]_i$ rises in wild-type, but not in *Catsper1* null sperm (Figure 7). The NH_4Cl -induced $[Ca^{2+}]_i$ changes were restored by an EGFP-CATSPER1 fusion protein in the *Catsper1* null background (Figure 7C, E). These results suggest that the CATSPER channel is necessary for the increase in $[Ca^{2+}]_i$ induced by pH_i change.

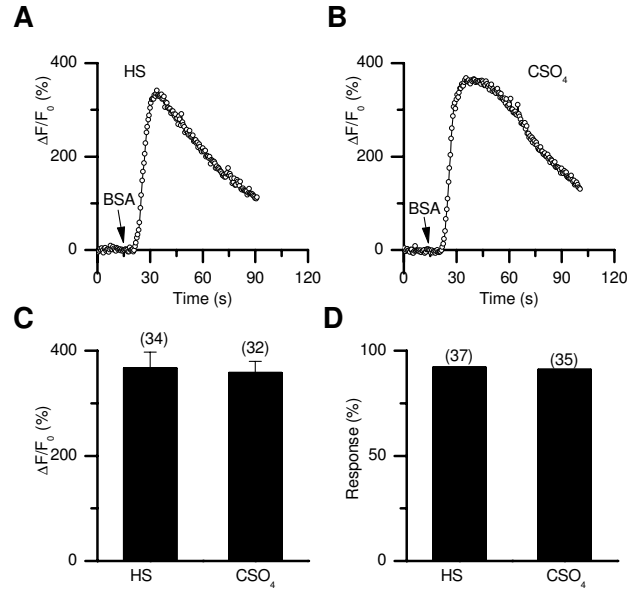


Figure 5
BSA-induced $[Ca^{2+}]_i$ changes in the presence and absence of cholesterol. (A, B) Representative $[Ca^{2+}]_i$ rises induced by BSA (5 mg/ml) in the absence (A) and presence (B) of CSO_4 (preincubated with 750 μM CSO_4 for 30 min). (C, D) Averaged peak $\Delta F/F_0$ changes (C) and percentages of cells responsive to BSA in the absence and presence of CSO_4 .

The ATP-induced $[Ca^{2+}]_i$ rises in mouse sperm are independent of CATSPER channel

The findings that *Catsper1* mutant sperm lack the initial $[Ca^{2+}]_i$ increased induced by several stimuli tested (8-Br-cGMP, 8-Br-cAMP, alkaline depolarizing medium, zona pellucida, pH_i change (NH_4Cl) and BSA [24-26]) raises a concern that a deficiency in the CATSPER channel leads to a non-specific defect in sperm Ca^{2+} entry. To test this idea, we compared the *Catsper1* mutant and wild-type's sperm $[Ca^{2+}]_i$ responses to bath application of ATP, which was reported to increase $[Ca^{2+}]_i$ in bovine and mouse sperm by activating a P2 purinergic receptor[48,49]. Similar to other observations, ATP induced rises in $[Ca^{2+}]_i$ in the wild-type sperm head in a concentration-dependent manner (Figure 8A). Unlike the BSA-induced $[Ca^{2+}]_i$ increases, those induced by ATP did not begin in the tail, but instead, started simultaneously along the whole length of the sperm (Figure 8C, D). The ATP-induced $[Ca^{2+}]_i$ changes were intact in the *Catsper* null sperm (Figure 8B), indicating that the CATSPER channel does not contribute to the ATP-induced elevation of $[Ca^{2+}]_i$.

Discussion

The major finding of our study is that CATSPER channels are required for the BSA induced $[Ca^{2+}]_i$ increases in mouse sperm. Stimuli known to induce a CATSPER-

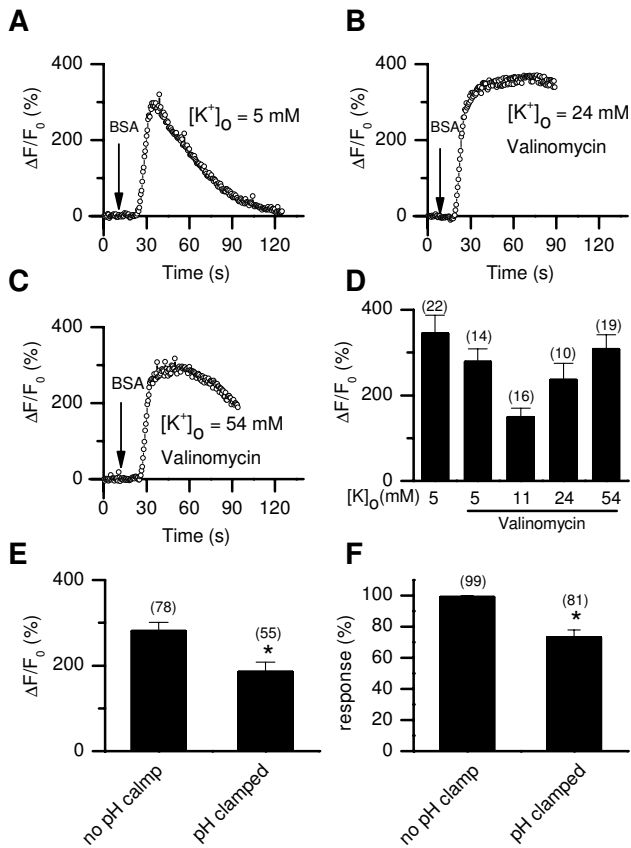


Figure 6
BSA-induced [Ca²⁺]_i rises under clamped membrane potential (V_m) or intracellular pH (pH_i). (A-C) Representative recordings in different [K⁺]_o as indicated, without (A) or with (B and C) 1 μM valinomycin in the bath to "clamp" the membrane potentials to -40 mV ([K⁺]_o = 24 mM) or -20 mV ([K⁺]_o = 54 mM). (D) Averaged peak ΔF/F₀ in responsive cells under various [K⁺]_o, with or without valinomycin. (E, F) Peak [Ca²⁺]_i rise amplitude (E) and response percentages (F) induced by 5 mg/ml BSA without pH_i clamp or under pH_i clamp. Numbers of tested cells are indicated within the bars. *, P < 0.05 compared to the no pH_i clamp condition.

dependent Ca²⁺ entry now include some of the most important mediators in sperm physiology: cyclic nucleotides, zona pellucida and serum albumin. There are clearly also CATSPER-independent Ca²⁺ entry paths that are responsible for the [Ca²⁺]_i increases induced by stimuli such as extracellular ATP, as we showed in this study, and mechanical force (Xia and Ren, unpublished). Differences in signal transduction pathways that link various stimuli to CATSPER channels also exist. For example, the Ca²⁺ entry induced by the zona pellucida is dependent on G-proteins and phospholipase C [10,25,38] whereas the BSA-induced one is not (Figure 4).

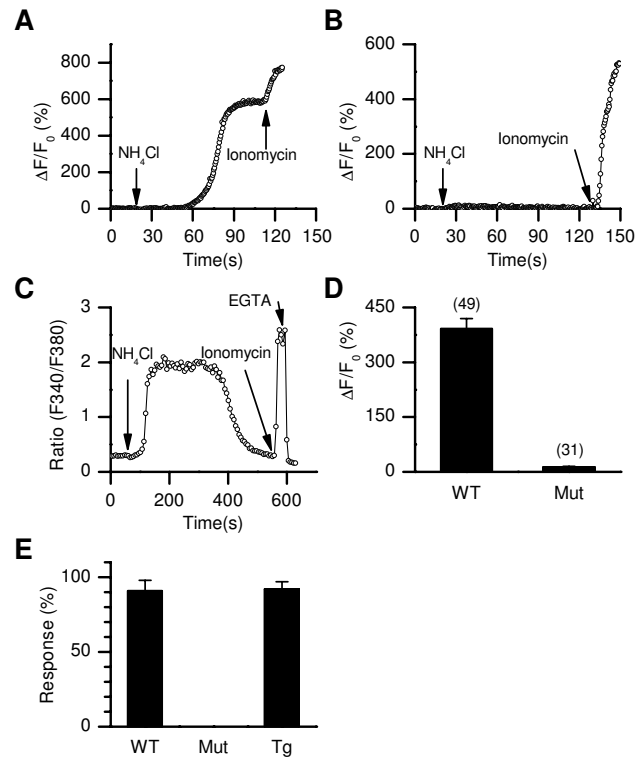


Figure 7
CATSPER1 is required for the NH₄Cl-induced [Ca²⁺]_i rise in mouse sperm. (A-C) Representative responses to 20 mM NH₄Cl in sperm head from a wild-type (A), a *Catsper1* null (B), and transgenic sperm expressing an EGFP-CATSPER1 fusion protein on the *Catsper1* null background (C). Ionomycin (10 μM, A-C) and EGTA (50 mM) were applied as control stimuli. (D) Averaged ΔF/F₀ changes induced by 20 mM NH₄Cl in wild-type (WT) and *Catsper1* null (Mut) sperm. Numbers of analyzed cells are in parentheses. (E) Percentages of sperm responsive to NH₄Cl application in WT (n = 7 imaging runs; 3 mice, 56 cells), *Catsper1* null (Mut; n = 7; 3 mice, 31 cells), and the *Catsper1* mutant rescued with the EGFP-CatSper1 transgene (Tg; n = 7; 2 mice, 31 cells). Cells from Tg mice were imaged with Fura-2 and the others were imaged with Fluo-4.

Like several other stimuli such as 8-Br-cGMP [37] and the zona pellucida [25], BSA does not directly activate CATSPER channel currents in corpus sperm under whole cell voltage clamp patch clamp recording (with intracellular and extracellular pH buffered at 7.2 and 7.4, respectively [25,37]; Xia and Ren, unpublished observation). We cannot exclude the possibility that BSA can directly activate CATSPER under more physiological conditions. During sperm capacitation with BSA-containing medium, there is an intracellular alkalization [43]. Consistent with a role of the alkalization, the BSA-induced [Ca²⁺]_i rises were reduced when pH_i was "clamped" (Figure 6). The [Ca²⁺]_i

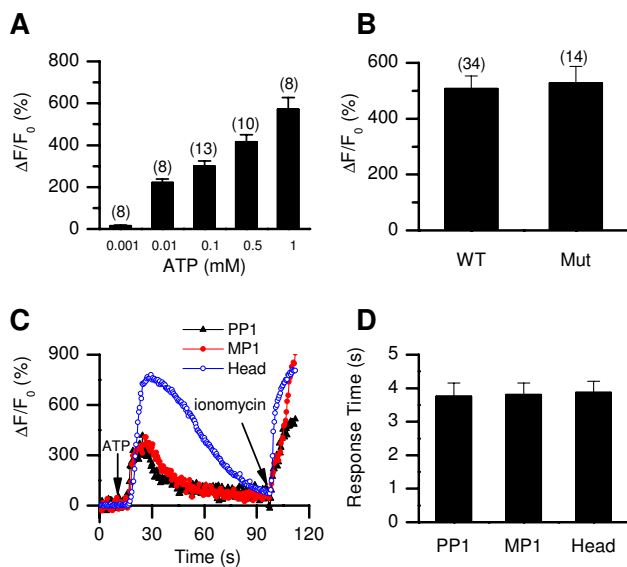


Figure 8
CATSPER channels are not required for the ATP-induced $[Ca^{2+}]_i$ rise. (A) Dose-responses of ATP-induced $[Ca^{2+}]_i$ rises in the sperm head of wild-type (WT) mouse. (B) Peak amplitudes of $\Delta F/F_0$ induced by 0.1 mM ATP in WT and *Catsper1* null (Mut) sperm. (C) Representative recordings of the time course of ATP (1 mM) -induced fluorescence changes in the principal piece (PPI), midpiece (MPI) and head, in a WT sperm. Like the ones induced by Ca^{2+} ionophore (10 μ M ionomycin), the ATP-elicited $[Ca^{2+}]_i$ increases started simultaneously in all subregions (D, n = 8). The locations of the subregions were defined as in Figure 3.

response, however, was not completely inhibited. The residual BSA-induced $[Ca^{2+}]_i$ changes under "pH_i clamp" condition are likely through pathways other than intracellular alkalization.

Although weakly voltage-dependent, whole cell CATSPER conductance can be increased by cell membrane depolarization [37]. In addition, depolarization by increased extracellular K⁺ concentrations, especially coupled with alkalization medium (K8.6), can lead to a CATSPER-dependent Ca²⁺ influx [24]. These results point to a possible role of membrane depolarization in the pathway leading to the BSA-induced Ca²⁺ entry. However, we consider this possibility unlikely for several reasons. First, we show here that the $[Ca^{2+}]_i$ rises are not dependent on a membrane voltage change since they persist when the voltage is "clamped" with K⁺ ionophore. Second, inactivating the T-type Ca_v channel, that is a proposed candidate for the Ca²⁺ entry [34-36], by clamping the membrane at -20 mV does not decrease the BSA-induced $[Ca^{2+}]_i$ rises. This result is consistent with our recent finding that mature sperm do not possess detectable functional Ca_v channels [25]. Finally, BSA does not lead to depolarization during sperm

capacitation, instead, a hyperpolarization of ~15 mV has been observed using voltage-sensitive fluorescence dyes [31]. Along these lines, we used whole cell patch clamp under current clamp mode to directly measure acute membrane potential changes in corpus sperm upon BSA application and found that BSA (4 mg/ml) produced a fast and profound membrane hyperpolarization (11 ± 2 mV, n = 6). While the exact mechanism of such a membrane potential hyperpolarization induced by BSA remains undetermined, we found no evidence for a role of membrane depolarization in the BSA-induced Ca²⁺ entry through CATSPER.

Conclusion

In summary, we determined the molecular identity of an ion channel responsible for the fast Ca²⁺ influx induced by BSA used in *in vitro* sperm capacitation. Three lines of evidence support that CATSPER is the aforementioned channel. First, the BSA-induced $[Ca^{2+}]_i$ increases are absent in *Catsper1* knockout sperm; second, such responses can be restored by an EGFP-CATSPER1 fusion protein; and finally, these $[Ca^{2+}]_i$ rises start in the principal piece of sperm where CATSPER proteins and current through CATSPER channels are localized. The mechanisms by which BSA is coupled to the CATSPER channel are largely unknown. They are unlikely through a voltage change or a cholesterol efflux. An intracellular alkalization appears to be involved (Figure 6) but there is clearly a [pH]_i change-independent component that remains to be uncovered.

Together with previous findings that the capacitation status and changes in the pattern of tyrosine phosphorylation during capacitation do not require CATSPER while the hyperactivated motility does [15,20,24,28], our data genetically separate the Ca²⁺ requirements for the two aspects of sperm capacitation: one mediating the change of motility via the CATSPER channel and another mediating the increase of tyrosine phosphorylation via a source yet to be determined. One possible source for the latter is the second phase of $[Ca^{2+}]_i$ rises that are also present in the *Catsper1* mutant sperm.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JX performed all the experiments and did data analysis. JX and DR designed the experiments and wrote the paper. Both authors read and approved the final manuscript.

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