Shedding light on the control of CatSper Ca^{2+} channels by cAMP and chemicals used to probe cAMP signaling

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Abstract: The sperm-specific CatSper channel controls the influx of Ca^{2+} into the flagellum and, thereby, the swimming behavior of sperm. A hallmark of human CatSper is its polymodal activation by membrane voltage, intracellular pH, and oviductal hormones. Whether CatSper is also activated by signaling pathways involving an increase of cAMP and ensuing activation of protein kinase A (PKA) is, however, a matter of controversy. Here, using kinetic ion-sensitive fluorimetry and patch-clamp recordings, we study transmembrane Ca^{2+} flux and membrane currents in human sperm from healthy donors and from patients that lack functional CatSper channels. We show that human CatSper is neither activated by intracellular cAMP directly nor indirectly by the cAMP/PKA-signaling pathway. Moreover, we demonstrate that non-physiological concentrations of cAMP and membrane-permeable cAMP analogs used to mimic the action of intracellular cAMP activate human CatSper from the outside via a previously unknown extracellular cyclic nucleotide-binding site. Finally, we demonstrate that the effects of common PKA inhibitors on human CatSper rest on off-target drug actions on CatSper itself

rather than on inhibition of PKA. We conclude that the concept of an intracellular cAMP/PKA-activation of CatSper is primarily based on unspecific effects of chemical probes used to interfere with cAMP signaling. Altogether, our findings solve several controversial issues, which has important bearings on future studies of cAMP and Ca^{2+} signaling and the ligand-control of CatSper in sperm.

Introduction

The CatSper channel (cation channel of sperm) represents the principal pathway for Ca^{2+} entry into the flagellum of sperm from many species (Kirichok et al., 2006; Lishko et al., 2011; Loux et al., 2013; Ren et al., 2001; Seifert et al., 2015; Strunker et al., 2011; Sumigama et al., 2015). The activity of CatSper is controlled by both the membrane potential (V_m) and intracellular pH (pH_i) (Hwang et al., 2019; Kirichok et al., 2006; Lishko et al., 2010; Lishko et al., 2011; Seifert et al., 2015; Strunker et al., 2011), and, in human sperm, also by oviductal steroids and prostaglandins (Brenker et al., 2018b; Lishko et al., 2011; Luo et al., 2019; Schiffer et al., 2020; Smith et al., 2013; Strunker et al., 2011; Williams et al., 2015). Thereby, CatSper translates changes of the chemical microenvironment into changes of $[Ca^{2+}]_i$ and swimming behavior, which enables sperm to reach the site of fertilization, to overcome the egg´s protective vestments, and, ultimately, to fertilize the egg (Achikanu et al., 2018; Alasmari et al., 2013; Oren-Benaroya et al., 2008; Ren et al., 2001; Rennhack et al., 2018; Schiffer et al., 2020; Tamburrino et al., 2014; Tamburrino et al., 2015). CatSper is, hence, absolutely required for fertilization in mice and humans (Avenarius et al., 2009; Avidan et al., 2003; Luo et al., 2019; Qi et al., 2007; Ren et al., 2001; Schiffer et al., 2020; Williams et al., 2015; Zhang et al., 2007).

Not only the control of $[Ca^{2+}]_i$ by CatSper, but also the flagellar cAMP dynamics is key for the function of sperm (Akbari et al., 2019; Balbach et al., 2018; Buffone et al., 2014; Esposito et al.,

2004; Visconti et al., 1995). In mammalian sperm, cAMP is predominately synthesized by the soluble adenylyl cyclase (sAC) that is controlled by bicarbonate (Brenker et al., 2012; Buffone et al., 2014; Hess et al., 2005; Kleinboelting et al., 2014; Wennemuth et al., 2003b; Xie et al., 2006). Bicarbonate-induced synthesis of cAMP by sAC activates protein kinase A (PKA) (Buffone et al., 2014; Moseley et al., 2005); and the cAMP/PKA-signaling pathway controls the flagellar beat frequency and capacitation (Esposito et al., 2004; Hess et al., 2005; Morgan et al., 2008; Xie et al., 2006), a maturation process that primes sperm to fertilize the egg (Yanagimachi, 1994).

The interplay of Ca^{2+} and cAMP in sperm is only ill-defined. In particular, it has remained controversial whether intracellular cAMP and/or activation of PKA stimulate Ca^{2+} influx via CatSper. It is unequivocal that membrane-permeable analogues of cAMP (e.g. 8-Br-cAMP) that are used to mimic the action of intracellular cAMP activate CatSper in both mouse and human sperm (Brenker et al., 2012; Orta et al., 2018; Ren et al., 2001; Xia et al., 2007). However, in a series of studies by independent groups, elevation of intracellular cAMP levels in mouse and human sperm by bicarbonate or other measures, including adenosine, synthetic adenosine- or catecholamine-receptor agonists, photorelease of cAMP from caged cAMP, or control of cAMP by optogenetics, failed to stimulate Ca^{2+} influx via CatSper (Brenker et al., 2012; Carlson et al., 2007; Carlson et al., 2003; Jansen et al., 2015; Nolan et al., 2004; Schuh et al., 2006; Strunker et al., 2011; Wennemuth et al., 2003a). These results indicate that mouse and human CatSper are neither activated by cAMP directly nor indirectly via activation of PKA, and that activation of CatSper by membrane-permeable cAMP derivatives might represent a non-specific action of these compounds in sperm. The latter is supported by the finding that membrane-permeable derivatives of cGMP (e.g. 8-Br-cGMP) activate human CatSper only from the outside (Brenker

et al., 2012). However, there are studies that contradict this concept and rather suggest a cAMP/PKA-activation of CatSper: in a study on human sperm, sizable bicarbonate-evoked Ca^{2+} signals were recorded (Spehr et al., 2004). Moreover, in a recent study by Orta et al (Orta et al., 2018), both 8-Br-cAMP and bicarbonate evoked Ca^{2+} influx via CatSper in mouse sperm. The Ca^{2+} influx by 8-Br-cAMP and bicarbonate was suppressed by inhibitors of PKA. Additionally, in patch-clamp recordings, CatSper-mediated membrane currents were enhanced by superfusion with bicarbonate and by cAMP in the pipette solution; inhibition of PKA suppressed the action of bicarbonate and intracellular cAMP. Based on these results, it was proposed that an increase of intracellular cAMP activates CatSper via activation of PKA (Orta et al., 2018).

Here, to solve this controversy, we studied the action of cAMP, membrane-permeable cAMP analogs, cAMP/PKA signaling, and PKA inhibitors in human sperm from healthy donors and patients that suffer from the deafness-infertility syndrome (DIS). In DIS patients, the *CATSPER2* gene is deleted (Avidan et al., 2003; Schiffer et al., 2020; Zhang et al., 2007), resulting in the loss of CatSper function (Brenker et al., 2018b; Schiffer et al., 2020; Smith et al., 2013). We demonstrate that human CatSper is neither activated by intracellular cAMP directly nor indirectly via activation of the cAMP/PKA-signaling pathway. In fact, membrane-permeable cAMP analogs and cAMP itself activate CatSper only from the outside via a so far unknown binding site that is distinct from that employed by steroids and prostaglandins. Furthermore, we found that several commonly used PKA inhibitors affect the activity of CatSper. The action of these drugs on CatSper does, however, not rest on the inhibition of PKA. Finally, we show that bicarbonate is prone to evoke artefactual alkaline-induced Ca^{2+} influx via CatSper that might be misinterpreted as cAMP/PKA-activation of the channel. Altogether, we conclude that the concept of a cAMP/PKA-activation of CatSper is rather based on chemical probes that are

unspecific and, therefore, ill-suited to study the interplay of Ca^{2+}- and cAMP-signaling pathways in sperm.

Results

CatSper is not activated by intracellular cAMP or cAMP/PKA signaling

To scrutinize whether CatSper is activated by intracellular cAMP or cAMP/PKA signaling, we studied the action of bicarbonate, IBMX, and adenosine in human sperm. Bicarbonate-activation of sAC rapidly increases cAMP (Fig. 1A) (Brenker et al., 2012) and activates PKA, i.e. cAMP levels and PKA activity peak within ≤ 60 s upon stimulation of sperm with bicarbonate (Battistone et al., 2013; Brenker et al., 2012). In sperm bathed in low concentrations of bicarbonate (e.g. 4 mM), isobutylmethylxanthine (IBMX) and adenosine evoke an increase of cAMP levels (Fig. 1A) (Brenker et al., 2012; Nolan et al., 2004; Schuh et al., 2006); IBMX and presumably also adenosine inhibit cAMP breakdown by phosphodiesterases (PDEs). However, neither bicarbonate nor IBMX or adenosine increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of human sperm, whereas CatSper activation by progesterone as a control evoked a biphasic Ca^{2+} response (Fig. 1B, C). Next, we recorded CatSper currents from human sperm by whole-cell patch clamping. In extracellular solution containing Ca^{2+} and Mg^{2+}, we recorded only miniscule currents (Fig. 1D, E; HS); in Na^+-based divalent-free solution, the prototypical monovalent CatSper currents were recorded (Fig. 1D, E; NaDVF) (Lishko et al., 2011; Strunker et al., 2011). The current amplitudes were similar in the absence and presence of cAMP in the pipette solution (Fig. 1D, E): mean CatSper inward currents at -80 mV were -20.7 ± 8.4 pA (n = 50) and -23.6 ± 11.9 pA (n = 16); mean outward currents at +80 mV were 53.3 ± 16.2 pA (n = 50) and 58.7 ± 21.4 pA (n = 16), respectively. Together, these results demonstrate that human CatSper is neither directly activated by intracellular cAMP nor indirectly by activation of the

cAMP/PKA-signaling pathway (Brenker et al., 2012; Strunker et al., 2011). Of note, the intracellular cAMP level and activation of the cAMP/PKA-signaling pathways do not modulate stimulus-induced gating of human CatSper either: bicarbonate and IBMX did not affect CatSper activation by progesterone and simultaneous alkalization and depolarization (K8.6), respectively (Fig. 1H, I).

Bicarbonate is prone to evoke alkaline-induced Ca^{2+} influx via CatSper

In some studies on mouse and human sperm, sizeable bicarbonate-evoked Ca^{2+} signals were recorded (Orta et al., 2018; Spehr et al., 2004). We entertained several eventualities that might explain these seemingly contradictory findings. We identified the pH of bicarbonate-containing buffers as a potential source of artifacts: the pH of buffers containing bicarbonate is tied to the partial pressure of CO_2 (Kohn and Dunlap, 1998) and, therefore, unstable at ambient conditions. To illustrate this issue, we monitored the pH of HTF exposed to ambient air in the absence and presence of bicarbonate, using a fluorescent pH indicator. In the absence of bicarbonate, the pH remained stable at pH 7.35 (Fig. 2A). In the presence of bicarbonate, due to continuous degassing of CO_2, the pH alkalized to pH 7.9 with a time constant (τ) of about 40 min (Fig. 2A). In sperm loaded with a fluorescent pH indicator, air-exposed (> 60 min) bicarbonate-HTF evoked an alkalization of the intracellular pH (pH_i) that was similar to the pH_i alkalization evoked by alkaline (pH 7.8) HTF lacking bicarbonate (HTF7.8) or by the weak base ammonium chloride (NH_4Cl) (Fig. 2B); stimulation of sperm with pH-controlled bicarbonate-HTF rather acidified pH_i (Fig. 2B). It is well-established that an intracellular alkalization by HTF7.8 or NH_4Cl evokes Ca^{2+} influx via CatSper (Fig. 2C, D) (Rennhack et al., 2018; Schiffer et al., 2020; Strunker et al., 2011). Therefore, it is not surprising that air-exposed bicarbonate-HTF also evokes a sizeable $[Ca^{2+}]_i$ increase in human sperm (Fig. 2C, D). Thus, bicarbonate-evoked

145 $[Ca^{2+}]_i$ increases observed in mouse and human sperm might reflect alkaline- rather than
146 cAMP/PKA-induced CatSper activation, caused by unintended alkalization of bicarbonate
147 buffers.

148 ***Membrane-permeable cAMP analogs activate CatSper only from the outside***

149 Next, considering that human CatSper is not activated by intracellular cAMP, we set out to
150 elucidate the mechanism underlying the Ca^{2+} influx evoked by membrane-permeable cAMP
151 analogs. To this end we studied the action of the most common cAMP analogs used to mimic the
152 action of intracellular cAMP (8-CPT-cAMP, 8-Br-cAMP, Db-cAMP) or to activate PKA (6-
153 Bnz-cAMP) or EPAC (8-pCPT-2-O-Me-cAMP-AM, i.e. 007-AM) (Schmidt et al., 2013). Any of
154 these cAMP analogs evoked a biphasic $[Ca^{2+}]_i$ increase, and the amplitude of the Ca^{2+} response
155 rose in a dose-dependent fashion (Fig. 3A, B; Supplementary Fig. 1A). The potency of the
156 cAMP analogs varied: 8-pCPT-2-O-Me-cAMP-AM and 8-CPT-cAMP increased $[Ca^{2+}]_i$ already
157 at micromolar concentrations, whereas the action of 8-Br-cAMP, Db-cAMP, and 6-Bnz-cAMP
158 commenced only at $\geq$ 1 mM (Fig. 3B; Supplementary Fig. 1A). Because we used the Na^+ salts of
159 the cAMP analogs, we studied the action of NaCl as a surrogate for the vehicle. Only at > 10
160 mM, the vehicle evoked a small Ca^{2+} response on its own (Fig. 3B). To scrutinize whether the
161 $[Ca^{2+}]_i$ increase evoked by cAMP analogs is mediated by CatSper, we studied their action in
162 CatSper-deficient sperm from infertile patients that lack the *CATSPER2* gene (Brenker et al.,
163 2018b; Schiffer et al., 2020). In CatSper-deficient sperm, the Ca^{2+} response evoked by 8-CPT-
164 cAMP, 8-Br-cAMP, 6-Bnz-cAMP, and 8-pCPT-2-O-Me-cAMP-AM was abolished, and Db-
165 cAMP evoked only a small residual Ca^{2+} signal (Fig. 3A; Supplementary Fig. 1A). Furthermore,
166 in whole-cell patch-clamp recordings, superfusion of sperm with 8-CPT-cAMP enhanced
167 monovalent currents at -80 mV and +80 mV by 2.47 ± 0.8-fold and by 1.22 ± 0.16-fold (n = 8),

respectively, confirming that 8-CPT-cAMP activates CatSper. The vehicle had only a minuscule, if any, action on the amplitude of CatSper currents (Supplementary Fig. 1B-D); and in CatSper-deficient sperm, 8-CPT-cAMP did not affect residual monovalent currents. (Supplementary Fig. 2A, B). These results demonstrate that CatSper is promiscuously activated by structurally and functionally diverse membrane-permeable analogs of cAMP. Of note, CatSper is not only activated by cAMP analogs, but also by analogs of cGMP such as 8-CPT-cGMP or 8-Br-cGMP (Supplementary Fig. 3A, B) (Brenker et al., 2012), which activate human CatSper only via an extracellular binding site (Brenker et al., 2012). We examined whether cAMP analogs activate CatSper by a similar mechanism. To this end, we first tested by Ca^{2+} fluorimetry whether CatSper might also be activated by extracellular application of cAMP itself, which hardly permeates the cell membrane. Indeed, not only its membrane-permeable analogs, but also cAMP (at $\geq$ 1 mM), evoked a $[Ca^{2+}]_i$ increase in human sperm (Fig. 4A, B). The cAMP-evoked Ca^{2+} response was abolished in sperm that lack functional CatSper (Fig. 4A). Next, we examined the kinetics of the Ca^{2+} signals evoked by 8-CPT-cAMP in a stopped-flow apparatus. Upon rapid mixing of sperm with 8-CPT-cAMP, $[Ca^{2+}]_i$ rose with no measurable latency within the time resolution of the system (36 ms) (Fig. 4C, D). Finally, in patch-clamp recordings, superfusion of sperm with cAMP enhanced monovalent CatSper currents at -80 mV and +80 mV by 1.58 ± 0.17-fold and by 1.36 ± 0.4-fold (n = 4) (Fig. 5A-C), respectively. The presence of cAMP or 8-CPT-cAMP in the pipette solution did not suppress activation of CatSper by superfusion with 8-CPT-cAMP (Fig. 5D-I): 8-CPT-cAMP enhanced inward and outward currents by 1.88 ± 0.45-fold (-80 mV) and 1.03 ± 0.13-fold (+80 mV) (n = 8) in the presence of cAMP (n = 8); and by 1.86 ± 0.2-fold (-80 mV) and 1.04 ± 0.08-fold (+80 mV) in the presence of 8-CPT-cAMP (n = 4). Altogether, the activation of CatSper by extracellular cAMP, the failure of intracellular

cAMP or 8-CPT-cAMP to suppress CatSper activation by superfusion with 8-CPT-cAMP, and the virtually instantaneous onset of 8-CPT-cAMP-evoked Ca^{2+} responses indicate that cAMP and membrane-permeable cAMP analogs activate CatSper only from the extracellular space.

Human CatSper is activated by cyclic nucleotides via a so far unknown binding site

We wondered whether cyclic nucleotides compete with steroids and/or prostaglandins to activate CatSper. Ca^{2+}-fluorimetric cross-desensitization experiments revealed that steroids and prostaglandins employ different binding sites to activate human CatSper (Brenker et al., 2018b; McBrinn et al., 2019; Schaefer et al., 1998; Strunker et al., 2011). We used this approach to study the mechanism of cyclic nucleotide-induced CatSper activation. Validating our experimental conditions, pre-incubation of sperm with a saturating concentration of progesterone abolished the Ca^{2+} response evoked by 17-OH-progesterone, but not that evoked by PGE_1 (Fig. 6A, B); and pre-incubation with a saturating concentration of PGE_1 abolished the Ca^{2+} response evoked by PGE_2, but not that evoked by progesterone (Fig. 6C, D). Pre-incubation with saturating concentration of progesterone or PGE_1, however, did not abolish Ca^{2+} responses evoked by cAMP, cAMP analogs, or cGMP analogs (Fig. 6A-D); vice versa, pre-incubation of sperm with 8-CPT-cAMP or 8-CPT-cGMP did not abolish progesterone- or PGE_1-evoked Ca^{2+} responses (Fig. 6E-H). The lack of cross-desensitization demonstrates that cyclic nucleotides do not compete with progesterone or prostaglandins to activate CatSper. However, pre-incubation with 8-CPT-cAMP or 8-CPT-cGMP abolished the responses evoked by cAMP as well as by other analogs of cAMP or cGMP (Fig. 6E-G), indicating that cAMP, cAMP analogs, and cGMP analogs compete with each other to activate CatSper. Thus, cyclic nucleotides, steroids, and prostaglandins activate human CatSper via three distinct binding sites.

The action of PKA inhibitors on CatSper does not rest on inhibition of PKA

Given that CatSper is not activated by cAMP/PKA signaling, how can inhibitors of PKA, i.e. H89 and PKI 14-22, suppress CatSper-mediated Ca^{2+} influx in human and mouse sperm (Baron et al., 2016; Orta et al., 2018). To solve this conundrum, we studied the action of the five most common pharmacological probes (i.e. PKI 5-24, Rp-cAMPS, KT 5720, H89, and PK 14-22) in human sperm. The action of these drugs was heterogeneous: PKI 5-24 and Rp-cAMPS affected neither resting Ca^{2+} levels nor CatSper-mediated Ca^{2+} influx evoked by 8-CPT-cAMP, progesterone, PGE_1, or alkalization (NH_4Cl) (Fig. 7A-D). Similarly, KT 5720 did not suppress ligand- or alkaline-induced Ca^{2+} influx via CatSper (Fig. 7A-D). However, on its own, KT 5720 evoked a small, transient $[Ca^{2+}]_i$ increase that was abolished in CatSper-deficient human sperm (Fig. 7A, B; Supplementary Fig. 4A). In patch-clamp recordings from human sperm, KT 5720 increased the amplitude of monovalent CatSper inward (-80 mV) and outward (+80 mV) currents by 1.48 ± 0.47-fold and 1.33 ± 0.11-fold, respectively (Fig. 7E, F, n = 5). The action of H89 was particularly complex: H89 on its own evoked a transient Ca^{2+} increase, followed by a $[Ca^{2+}]_i$ decrease below resting levels (Fig. 8A, B). The dose-response relation for the Ca^{2+} transient was bell shaped, i.e. the signal amplitude grew with increasing concentrations, saturated at about 10 µM, and decreased again at >10 µM (Fig. 8B). The H89-evoked Ca^{2+} response was abolished in CatSper-deficient human sperm, indicating that H89 acts via CatSper (Supplementary Fig. 4A). Of note, in the presence of H89, both ligand- and alkaline-evoked Ca^{2+} influx via CatSper was strongly attenuated (Fig. 8C, D); and in patch-clamp recordings, H89 reversibly inhibited monovalent CatSper currents (Fig. 8E, F; Supplementary Fig. 4B). Finally, PKI 14-22 evoked a small decrease of $[Ca^{2+}]_i$ on its own, which was abolished in CatSper-deficient sperm (Fig. 8A; Supplementary Fig. 4A). PKI 14-22 strongly attenuated alkaline-evoked Ca^{2+} influx via CatSper,

whereas ligand-evoked Ca^{2+} influx was only slightly suppressed (Fig. 8C, D). In patch-clamp recordings, PKI 14-22 inhibited monovalent CatSper currents (Supplementary Fig. 4C).

In summary, H89 and PKI 14-22 indeed inhibit CatSper. This action rests, however, on a direct inhibition of CatSper itself rather than on inhibition of PKA: in patch-clamp recordings, both H89 and PKI 14-22 suppress basal CatSper currents recorded under conditions that do not foster PKA signaling. Moreover, H89 and PKI 14-22 suppress alkaline-evoked Ca^{2+} influx via CatSper, which does not involve activation of PKA. Thus, H89 and PKI 14-22 are not suited to study the interconnection of cAMP, PKA, and Ca^{2+} signaling in human sperm. In contrast to H89 and PKI 14-22, KT 5720 rather activates the channel, which renders also KT 5720 ill-suited for studies of PKA and/or CatSper function in sperm. Of note, the nonspecific action of the PKA inhibitors on CatSper does not come as a surprise. In fact, these drugs are well known to be rather nonspecific (Murray, 2008).

Discussion

Our results reinforce the conclusion that human CatSper is not activated by intracellular cAMP or cAMP/PKA signaling (Brenker et al., 2012). In fact, the concept of a cAMP/PKA-activation of CatSper rests on pharmacological tools that are, across the board, prone to unspecific effects. First, if not properly controlled for pH, bicarbonate solutions can activate CatSper via an increase of pH_i. Second, the common PKA inhibitors H89 and PKI 14-22 directly block CatSper, whereas KT 5720 activates the channel. Finally, membrane-permeable analogs of cyclic nucleotides activate CatSper only via an extracellular binding site. These findings enlarge the list of drugs that are frequently used to interfere with sperm signaling, but directly affect CatSper. For example, the popular PLC and PDE inhibitors U73122 and trequensin, respectively, as well as a diverse array of synthetic chemicals evoke Ca^{2+} influx via CatSper (Brenker et al., 2012; Diao et

al., 2017; McBrinn et al., 2019; Schiffer et al., 2014; Tavares et al., 2013). By contrast, MDL12330A, an inhibitor of transmembrane ACs, and RU1968, a sigma-receptor agonist, both block CatSper (Brenker et al., 2012; Rennhack et al., 2018; Schiffer et al., 2014). Moreover, also inhibitors of EPAC, another cAMP effector that reportedly controls $[Ca^{2+}]_i$ in sperm (Itzhakov et al., 2019; Lucchesi et al., 2016), directly act on CatSper. The EPAC inhibitors CE3F4, ESI 05, and HJC 0350 activate human CatSper, whereas ESI 09 blocks the channel (Supplementary Fig. 5). Thus, the most common EPAC inhibitors are also not suited to investigate the role of EPAC in Ca^{2+}-signaling of sperm. Altogether, these results caution against rash interpretation in mechanistic terms of results derived from experiments with pharmacological tools that seemingly control $[Ca^{2+}]_i$ and/or CatSper. Because of the miniscule flagellar volume, even minute changes in CatSper activity strongly affect $[Ca^{2+}]_i$ and, thereby, downstream signaling events. Therefore, quite general, using pharmacological tools to study signaling in sperm requires careful assessment of potential off-target actions on CatSper.

We did not examine whether the off-target action of membrane-permeable cAMP/cGMP analogs and PKA inhibitors on CatSper are similar in human and mouse sperm. Examples of species-specific ligand actions on CatSper are steroids and prostaglandins that activate CatSper in human (Brenker et al., 2012; Lishko et al., 2011; Strunker et al., 2011), but not in mouse sperm (Tamburrino et al., 2015). Therefore, we cannot exclude that there are differences concerning the cAMP/PKA-activation of CatSper among species. However, H89 suppressed alkaline-induced membrane currents also in mouse sperm (Orta et al., 2018), suggesting that H89 also directly blocks mouse CatSper. Moreover, a large number of independent studies by different groups consistently provided no evidence that a rise of cAMP and ensuing PKA activation activates mouse CatSper (Carlson et al., 2007; Carlson et al., 2003; Jansen et al., 2015; Nolan et al., 2004;

Schuh et al., 2006; Wennemuth et al., 2003a). Together, this comprehensive body of evidence strongly suggests that also mouse CatSper is not directly activated by intracellular cAMP/PKA signaling.

Of note, the activity of CatSper is remodeled during the capacitation process: the voltage dependence of CatSper shifts to more negative potentials (Lishko et al., 2011), and human CatSper becomes more sensitive to progesterone and prostaglandins (Bedu-Addo et al., 2005; Strunker et al., 2011). The underlying mechanism(s) are unknown, but might involve capacitation-associated remodeling of the membrane-lipid environment (Kawai et al., 2019), intracellular pH (Puga Molina et al., 2018), and/or V_m (Puga Molina et al., 2018) rather than cAMP signaling.

In the past decade, a picture has emerged that human CatSper serves as a polymodal chemosensor that translates the chemical code of the oviductal environment to changes in $[Ca^{2+}]_i$ and motility (Alasmari et al., 2013; Brenker et al., 2012; Brenker et al., 2018b; Diao et al., 2014; Diao et al., 2017; Lishko et al., 2011; Strunker et al., 2011; Williams et al., 2015). Oviductal steroids and prostaglandins activate CatSper in a highly synergistic fashion (Brenker et al., 2018a) via two distinct binging sites (Lishko et al., 2011; McBrinn et al., 2019; Schaefer et al., 1998; Strunker et al., 2011). Progesterone has been proposed to activate human CatSper via the receptor alpha/beta hydrolase domain-containing protein 2 (ABHD2) (Miller et al., 2016). By contrast, the mechanism of CatSper activation by prostaglandins is elusive, except that it does not involve ABHD2, classical G protein-coupled receptors, and second messengers (Brenker et al., 2012; Brenker et al., 2018c; Lishko et al., 2011; McBrinn et al., 2019; Miller et al., 2016; Schaefer et al., 1998; Strunker et al., 2011). Our results demonstrate that CatSper is also controlled by an extracellular binding site that accommodates neither steroids nor prostaglandins,

but cyclic nucleotides. Yet, extracellular cAMP activates CatSper only at concentrations ≥ 1 mM, which exceeds physiological extracellular cAMP concentrations by several orders of magnitude. This indicates that the true physiological ligand of the cyclic nucleotide-binding site is to be deorphanized. Moreover, future studies are required to elucidate the molecular identity of this novel binding site, and it needs to be examined whether activation of CatSper via this third binding site evokes motility responses that are similar or distinct to that evoked by steroids or prostaglandins.

Materials and Methods

Reagents. cAMP, cGMP, and their derivatives (sodium salts) were obtained from BIOLOG Life Science Institute (Bremen, Germany). 8-pCPT-2-O-Me-cAMP-AM, H89 dihydrochloride, PKI 14-22 amide myristoylated, ESI 09, CE3F4, HJC 0350, and ESI 05 were from Tocris (Minnesota, USA). Prostaglandin E_1 (PGE_1), Prostaglandin E_2 (PGE_2), and PKI 5-24 were obtained from Cayman (Hamburg, Germany). Fluo-4-AM, pHrodo Red-AM, and BCECF were obtained from Invitrogen (California, USA). Human serum albumin (HSA) was obtained from Irvine Scientific (Santa Ana, USA). All other chemicals were from Sigma-Aldrich.

Human sperm preparation. Semen samples were obtained from volunteers and DIS patients with prior written consent, under approval from the ethical committees of the medical association Westfalen-Lippe and the medical faculty of the University of Münster (4INie). Semen samples were produced by masturbation and ejaculated into plastic containers. The samples were allowed to liquefy for 15~30 min at 37°C and motile sperm were purified by a swim-up procedure: liquefied semen (0.5–1 ml) was directly layered in a 50 ml falcon tube below 4 ml of human tubal fluid (HTF) medium, containing (in mM): 97.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 Na-pyruvate, 21.4 lactic acid, 4 $NaHCO_3$, 2.78 glucose, and 21 HEPES, pH

7.35 (adjusted with NaOH). Alternatively, the liquefied semen was diluted 1:10 with HTF, and sperm, somatic cells, and cell debris were pelleted by centrifugation at 700g for 20 min (37°C). The pellet was resuspended in the same volume HTF, 50 ml falcon tubes were filled with 5 ml of the suspension, and cells were pelleted by centrifugation at 700 g for 5 min (37°C). In both cases, motile sperm were allowed to swim up into HTF for 60–90 min at 37 °C. Sperm were washed two times (700g, 20 min, 37°C) and re-suspended in HTF containing 3 mg/ml HSA at a density of 1×10^7 sperm/ml for measurement of changes in intracellular Ca^{2+}, pH, or cAMP as well as for patch-clamp recordings. To study the action of bicarbonate, sperm were purified by swim-up in HTF medium lacking $NaHCO_3$, which was substituted with NaCl. Sperm were washed two times (700g, 20 min, 37°C) and re-suspended in bicarbonate-free HTF containing 3 mg/ml HSA.

Measurement of changes in $[Ca^{2+}]_i$ and pH. Changes in $[Ca^{2+}]_i$ and pH_i were measured in sperm loaded with the fluorescent Ca^{2+} and pH indicator, Fluo-4-AM and pHrodo Red-AM, respectively, at 30°C in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany) (Strunker et al., 2011). Sperm were loaded with Fluo-4-AM (5 µM, 20 min) and pHrodo Red-AM (5 µM, 30 min) at 37°C in the presence of Pluronic F-127 (0.05% w/v). After incubation, excess dye was removed by centrifugation (700g, 5 min, room temperature). Sperm were resuspended in HTF at a concentration of 5×10^6/ml. The wells were filled with 50 µl of the sperm suspension and the fluorescence was excited at 480 nm (Fluo-4, pHrodo Red) and fluorescence emission was recorded at 520 nm. Changes in Fluo-4 and pHrodo Red fluorescence are depicted as $\Delta F/F_0$ (%), that is, the change in fluorescence (ΔF) relative to the mean basal fluorescence (F_0) before application of buffer or stimuli (25 µl), to correct for intra- and inter-experimental variations in basal fluorescence among individual wells.

To study the action of bicarbonate, wells were filled with 40 µl of the sperm suspension in bicarbonate-free HTF and 40 µl of HTF containing 50 mM bicarbonate were injected. Of note, the bicarbonate-HTF was stored in air-tight tubes filled to capacity to avoid alkalization due to exposure to room air. Moreover, right prior to the experiments, the pH was checked again and, if required, (re)adjusted. To measure Ca^{2+} signals evoked by simultaneous alkalization/depolarization, wells were filled with 40 µl of the sperm suspension and 40 µl of K8.6 solution, containing (in mM): 98.5 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 21.4 lactic acid, 4 $KHCO_3$, 2.78 glucose, and 21 HEPES, pH 9.3 (adjusted with KOH), was injected; the final pH in the well was 8.6. To measure the pH change of HTF containing 50 mM $NaHCO_3$ exposed to room air, 5 µM BCECF was used. The wells were filled with 50 µl HTF and BCECF was exited at 440 and 480 nm (dual excitation, BCECF) and the emission was recorded at 520 nm over 2 hours. Changes in BCECF-fluorescence ratio (R, 480/440 nm) are depicted as ΔR/R (%), that is, the change in ratio (ΔR) relative to the mean ratio (R0) measured within the first minute of the recording.

Stopped-flow experiments were performed as described before (Brenker et al., 2012; Strunker et al., 2011) with some modifications (see also (Hamzeh et al., 2019)). Briefly, In a SFM-400 stopped-flow device (Bio-Logic, France), a suspension of Fluo-4-loaded sperm (1×10^7/ml) in HTF was rapidly mixed (1:1; flow rate = 1 ml/s) with HTF containing 10 mM 8-CPT-cAMP. Fluorescence was excited with a blue light-emitting diode (LED; M470D2, Thorlabs, Germany; powered with a custom-made power supply) that was modulated at 10 kHz using a function generator (4060MV, PeakTech, Germany). The light was passed through a 475/28 nm excitation filter (Semrock, Buffalo NY, USA). Emission was passed through a 536/40 nm filter (Semrock) and recorded with a photomultiplier (H9656-20; Hamamatsu Photonics, Hamamatsu, Japan).

Signals were amplified with a lock-in amplifier (MFLI, Signal Zürich Instruments, Switzerland) and recorded with a data acquisition pad (PCI-6221; National Instruments, Germany) and BioKine software v. 4.49 (Bio-Logic).

Electrophysiology. We recorded CatSper currents from human sperm in the whole-cell configuration, as described before (Strunker et al., 2011). Seals between pipette and sperm were formed either at the cytoplasmic droplet or in the neck region in solution (HS) containing (in mM): 135 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 5 glucose, 1 Na-pyruvate, 10 lactic acid, 20 HEPES, adjusted to pH 7.4 with NaOH. Monovalent CatSper currents were recorded in a sodium-based divalent-free solution (NaDVF) containing (in mM): 140 NaCl, 40 HEPES, and 1 EGTA, adjusted to pH 7.4 with NaOH. The osmolarity of HS and NaDVF solution was approximately 320 mOsm. The pipette (10–15 MΩ) solution contained (in mM): 130 Cs-aspartate, 50 HEPES, 5 EGTA, 5 CsCl, adjusted to pH 7.3 with CsOH. The pipette solution was approximately 325 mOsm. To examine the effect of intracellular cAMP on CatSper, cAMP was dissolved in the pipette solution. The action of extracellular application of H89 (50 mM stock in DMSO), KT 5720 (20 mM stock in DMSO), PKI 14-22 (800 µM stock in water) was examined by diluting the stocks with NaDVF; 8-CPT-cAMP was directly dissolved at 5 mM in NaDVF. All experiments were performed at room temperature (21-25°C). Data were not corrected for liquid junction potentials.

Measurement of intracellular cAMP content. To examine the action of adenosine and IBMX, 308 µl of sperm in HTF at a density of 2×10^7 cells/ml (6×10^6 cells in total) were mixed with 32 µl of HTF containing adenosine or IBMX to reach a final concentration of (in mM): 0.1 adenosine and 0.5 IBMX. To examine the action of bicarbonate, 154 µl of sperm in bicarbonate-free HTF at a density of 4×10^7 cells/ml (6×10^6 cells in total) were mixed with 154 µl HTF

containing 46 mM $NaHCO_3$ and 32 µl of HTF containing 25 mM $NaHCO_3$. After mixing with the respective stimulus, the samples were incubated for 30 min at 37°C, followed by the addition of 18 µl of 5 M HCl (0.25 M final concentration) to quench the biochemical reactions. After incubation for 30 min at room temperature, cell debris was sedimented by centrifugation at 3,000 g for 5 min at room temperature. The cAMP concentration in the supernatant was determined by a competitive enzyme immunoassay according to the product manual (Catalog #: ADI-900-066, Enzo Life Sciences), including acetylation of cAMP. Calibration curves were obtained by serial dilutions of cAMP standards (acetylated format).

Data analysis. All data are given as mean ± SD.

References and Notes:

Achikanu, C., Pendekanti, V., Teague, R., and Publicover, S. (2018). Effects of pH manipulation, CatSper stimulation and Ca2+-store mobilization on [Ca2+]i and behaviour of human sperm. Hum Reprod *33*, 1802-1811.

Akbari, A., Pipitone, G.B., Anvar, Z., Jaafarinia, M., Ferrari, M., Carrera, P., and Totonchi, M. (2019). ADCY10 frameshift variant leading to severe recessive asthenozoospermia and segregating with absorptive hypercalciuria. Hum Reprod *34*, 1155-1164.

Alasmari, W., Costello, S., Correia, J., Oxenham, S.K., Morris, J., Fernandes, L., Ramalho-Santos, J., Kirkman-Brown, J., Michelangeli, F., Publicover, S., *et al.* (2013). Ca2+ signals generated by CatSper and Ca2+ stores regulate different behaviors in human sperm. J Biol Chem *288*, 6248-6258.

Avenarius, M.R., Hildebrand, M.S., Zhang, Y., Meyer, N.C., Smith, L.L., Kahrizi, K., Najmabadi, H., and Smith, R.J. (2009). Human male infertility caused by mutations in the CATSPER1 channel protein. Am J Hum Genet *84*, 505-510.

Avidan, N., Tamary, H., Dgany, O., Cattan, D., Pariente, A., Thulliez, M., Borot, N., Moati, L., Barthelme, A., Shalmon, L., *et al.* (2003). CATSPER2, a human autosomal nonsyndromic male infertility gene. Eur J Hum Genet *11*, 497-502.

Balbach, M., Beckert, V., Hansen, J.N., and Wachten, D. (2018). Shedding light on the role of cAMP in mammalian sperm physiology. Mol Cell Endocrinol *468*, 111-120.

Baron, L., Fara, K., Zapata-Carmona, H., Zuniga, L., Kong, M., Signorelli, J., Diaz, E.S., and Morales, P. (2016). Participation of protein kinases and phosphatases in the progesterone-induced acrosome reaction and calcium influx in human spermatozoa. Andrology *4*, 1073-1083.

Battistone, M.A., Da Ros, V.G., Salicioni, A.M., Navarrete, F.A., Krapf, D., Visconti, P.E., and Cuasnicu, P.S. (2013). Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. Mol Hum Reprod *19*, 570-580.

Bedu-Addo, K., Lefievre, L., Moseley, F.L., Barratt, C.L., and Publicover, S.J. (2005). Bicarbonate and bovine serum albumin reversibly 'switch' capacitation-induced events in human spermatozoa. Mol Hum Reprod *11*, 683-691.

Brenker, C., Goodwin, N., Weyand, I., Kashikar, N.D., Naruse, M., Krahling, M., Muller, A., Kaupp, U.B., and Strunker, T. (2012). The CatSper channel: a polymodal chemosensor in human sperm. The EMBO journal *31*, 1654-1665.

437 Brenker, C., Rehfeld, A., Schiffer, C., Kierzek, M., Kaupp, U.B., Skakkebaek, N.E., and Strunker, T. (2018a).
438 Synergistic activation of CatSper Ca2+ channels in human sperm by oviductal ligands and endocrine disrupting
439 chemicals. Hum Reprod *33*, 1915-1923.
440 Brenker, C., Schiffer, C., Wagner, I.V., Tuttelmann, F., Ropke, A., Rennhack, A., Kaupp, U.B., and Strunker, T.
441 (2018b). Action of steroids and plant triterpenoids on CatSper Ca2+ channels in human sperm. P Natl Acad Sci
442 USA *115*, E344-E346.
443 Brenker, C., Schiffer, C., Wagner, I.V., Tuttelmann, F., Ropke, A., Rennhack, A., Kaupp, U.B., and Strunker, T.
444 (2018c). Action of steroids and plant triterpenoids on CatSper Ca(2+) channels in human sperm. Proceedings of
445 the National Academy of Sciences of the United States of America *115*, E344-E346.
446 Buffone, M.G., Wertheimer, E.V., Visconti, P.E., and Krapf, D. (2014). Central role of soluble adenylyl cyclase and
447 cAMP in sperm physiology. Bba-Mol Basis Dis *1842*, 2610-2620.
448 Carlson, A.E., Hille, B., and Babcock, D.F. (2007). External Ca2+ acts upstream of adenylyl cyclase SACY in the
449 bicarbonate signaled activation of sperm motility. Developmental Biology *312*, 183-192.
450 Carlson, A.E., Westenbroek, R.E., Quill, T., Ren, D., Clapham, D.E., Hille, B., Garbers, D.L., and Babcock, D.F.
451 (2003). CatSper1 required for evoked Ca2+ entry and control of flagellar function in sperm. Proc Natl Acad Sci
452 U S A *100*, 14864-14868.
453 Diao, R., Fok, K.L., Chen, H., Yu, M.K., Duan, Y., Chung, C.M., Li, Z., Wu, H., Li, Z., Zhang, H., *et al.* (2014).
454 Deficient human beta-defensin 1 underlies male infertility associated with poor sperm motility and genital tract
455 infection. Sci Transl Med *6*, 249ra108.
456 Diao, R., Wang, T., Fok, K.L., Li, X., Ruan, Y., Yu, M.K., Cheng, Y., Chen, Y., Chen, H., Mou, L., *et al.* (2017).
457 CCR6 is required for ligand-induced CatSper activation in human sperm. Oncotarget *8*, 91445-91458.
458 Esposito, G., Jaiswal, B.S., Xie, F., Krajnc-Franken, M.A., Robben, T.J., Strik, A.M., Kuil, C., Philipsen, R.L., van
459 Duin, M., Conti, M., *et al.* (2004). Mice deficient for soluble adenylyl cyclase are infertile because of a severe
460 sperm-motility defect. Proc Natl Acad Sci U S A *101*, 2993-2998.
461 Hamzeh, H., Alvarez, L., Strunker, T., Kierzek, M., Brenker, C., Deal, P.E., Miller, E.W., Seifert, R., and Kaupp,
462 U.B. (2019). Kinetic and photonic techniques to study chemotactic signaling in sea urchin sperm. Methods Cell
463 Biol *151*, 487-517.
464 Hess, K.C., Jones, B.H., Marquez, B., Chen, Y., Ord, T.S., Kamenetsky, M., Miyamoto, C., Zippin, J.H., Kopf,
465 G.S., Suarez, S.S., *et al.* (2005). The "soluble" adenylyl cyclase in sperm mediates multiple signaling events
466 required for fertilization. Dev Cell *9*, 249-259.
467 Hwang, J.Y., Mannowetz, N., Zhang, Y., Everley, R.A., Gygi, S.P., Bewersdorf, J., Lishko, P.V., and Chung, J.J.
468 (2019). Dual Sensing of Physiologic pH and Calcium by EFCAB9 Regulates Sperm Motility. Cell *177*, 1480-
469 1494 e1419.
470 Itzhakov, D., Nitzan, Y., and Breitbart, H. (2019). Protein kinase A inhibition induces EPAC-dependent acrosomal
471 exocytosis in human sperm. Asian J Androl *21*, 337-344.
472 Jansen, V., Alvarez, L., Balbach, M., Strunker, T., Hegemann, P., Kaupp, U.B., and Wachten, D. (2015).
473 Controlling fertilization and cAMP signaling in sperm by optogenetics. eLife *4*.
474 Kawai, T., Miyata, H., Nakanishi, H., Sakata, S., Morioka, S., Sasaki, J., Watanabe, M., Sakimura, K., Fujimoto, T.,
475 Sasaki, T., *et al.* (2019). Polarized PtdIns(4,5)P2 distribution mediated by a voltage-sensing phosphatase (VSP)
476 regulates sperm motility. Proc Natl Acad Sci U S A *116*, 26020-26028.
477 Kirichok, Y., Navarro, B., and Clapham, D.E. (2006). Whole-cell patch-clamp measurements of spermatozoa reveal
478 an alkaline-activated Ca2+ channel. Nature *439*, 737-740.
479 Kleinboelting, S., van den Heuvel, J., and Steegborn, C. (2014). Structural analysis of human soluble adenylyl
480 cyclase and crystal structures of its nucleotide complexes-implications for cyclase catalysis and evolution. FEBS
481 J *281*, 4151-4164.
482 Kohn, R.A., and Dunlap, T.F. (1998). Calculation of the buffering capacity of bicarbonate in the rumen and in vitro.
483 J Anim Sci *76*, 1702-1709.
484 Lishko, P.V., Botchkina, I.L., Fedorenko, A., and Kirichok, Y. (2010). Acid extrusion from human spermatozoa is
485 mediated by flagellar voltage-gated proton channel. Cell *140*, 327-337.
486 Lishko, P.V., Botchkina, I.L., and Kirichok, Y. (2011). Progesterone activates the principal Ca2+ channel of human
487 sperm. Nature *471*, 387-391.
488 Loux, S.C., Crawford, K.R., Ing, N.H., Gonzalez-Fernandez, L., Macias-Garcia, B., Love, C.C., Varner, D.D.,
489 Velez, I.C., Choi, Y.H., and Hinrichs, K. (2013). CatSper and the relationship of hyperactivated motility to
490 intracellular calcium and pH kinetics in equine sperm. Biol Reprod *89*, 123.

Lucchesi, O., Ruete, M.C., Bustos, M.A., Quevedo, M.F., and Tomes, C.N. (2016). The signaling module cAMP/Epac/Rap1/PLCepsilon/IP3 mobilizes acrosomal calcium during sperm exocytosis. Biochim Biophys Acta *1863*, 544-561.

Luo, T., Chen, H.Y., Zou, Q.X., Wang, T., Cheng, Y.M., Wang, H.F., Wang, F., Jin, Z.L., Chen, Y., Weng, S.Q., *et al.* (2019). A novel copy number variation in CATSPER2 causes idiopathic male infertility with normal semen parameters. Hum Reprod *34*, 414-423.

McBrinn, R.C., Fraser, J., Hope, A.G., Gray, D.W., Barratt, C.L.R., Martins da Silva, S.J., and Brown, S.G. (2019). Novel pharmacological actions of trequinsin hydrochloride improve human sperm cell motility and function. Br J Pharmacol *176*, 4521-4536.

Miller, M.R., Mannowetz, N., Iavarone, A.T., Safavi, R., Gracheva, E.O., Smith, J.F., Hill, R.Z., Bautista, D.M., Kirichok, Y., and Lishko, P.V. (2016). Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone. Science *352*, 555-559.

Morgan, D.J., Weisenhaus, M., Shum, S., Su, T., Zheng, R., Zhang, C., Shokat, K.M., Hille, B., Babcock, D.F., and McKnight, G.S. (2008). Tissue-specific PKA inhibition using a chemical genetic approach and its application to studies on sperm capacitation. Proc Natl Acad Sci U S A *105*, 20740-20745.

Moseley, F.L., Jha, K.N., Bjorndahl, L., Brewis, I.A., Publicover, S.J., Barratt, C.L., and Lefievre, L. (2005). Protein tyrosine phosphorylation, hyperactivation and progesterone-induced acrosome reaction are enhanced in IVF media: an effect that is not associated with an increase in protein kinase A activation. Mol Hum Reprod *11*, 523-529.

Murray, A.J. (2008). Pharmacological PKA inhibition: all may not be what it seems. Sci Signal *1*, re4.

Nolan, M.A., Babcock, D.F., Wennemuth, G., Brown, W., Burton, K.A., and McKnight, G.S. (2004). Sperm-specific protein kinase A catalytic subunit Calpha2 orchestrates cAMP signaling for male fertility. Proc Natl Acad Sci U S A *101*, 13483-13488.

Oren-Benaroya, R., Orvieto, R., Gakamsky, A., Pinchasov, M., and Eisenbach, M. (2008). The sperm chemoattractant secreted from human cumulus cells is progesterone. Hum Reprod *23*, 2339-2345.

Orta, G., de la Vega-Beltran, J.L., Martin-Hidalgo, D., Santi, C.M., Visconti, P.E., and Darszon, A. (2018). CatSper channels are regulated by protein kinase A. J Biol Chem *293*, 16830-16841.

Puga Molina, L.C., Luque, G.M., Balestrini, P.A., Marin-Briggiler, C.I., Romarowski, A., and Buffone, M.G. (2018). Molecular Basis of Human Sperm Capacitation. Front Cell Dev Biol *6*, 72.

Qi, H., Moran, M.M., Navarro, B., Chong, J.A., Krapivinsky, G., Krapivinsky, L., Kirichok, Y., Ramsey, I.S., Quill, T.A., and Clapham, D.E. (2007). All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci U S A *104*, 1219-1223.

Ren, D., Navarro, B., Perez, G., Jackson, A.C., Hsu, S., Shi, Q., Tilly, J.L., and Clapham, D.E. (2001). A sperm ion channel required for sperm motility and male fertility. Nature *413*, 603-609.

Rennhack, A., Schiffer, C., Brenker, C., Fridman, D., Nitao, E.T., Cheng, Y.M., Tamburrino, L., Balbach, M., Stolting, G., Berger, T.K., *et al.* (2018). A novel cross-species inhibitor to study the function of CatSper Ca(2+) channels in sperm. Br J Pharmacol *175*, 3144-3161.

Schaefer, M., Hofmann, T., Schultz, G., and Gudermann, T. (1998). A new prostaglandin E receptor mediates calcium influx and acrosome reaction in human spermatozoa. Proc Natl Acad Sci U S A *95*, 3008-3013.

Schiffer, C., Muller, A., Egeberg, D.L., Alvarez, L., Brenker, C., Rehfeld, A., Frederiksen, H., Waschle, B., Kaupp, U.B., Balbach, M., *et al.* (2014). Direct action of endocrine disrupting chemicals on human sperm. EMBO Rep *15*, 758-765.

Schiffer, C., Rieger, S., Brenker, C., Young, S., Hamzeh, H., Wachten, D., Tuttelmann, F., Ropke, A., Kaupp, U.B., Wang, T., *et al.* (2020). Rotational motion and rheotaxis of human sperm do not require functional CatSper channels and transmembrane Ca(2+) signaling. The EMBO journal, e102363.

Schmidt, M., Dekker, F.J., and Maarsingh, H. (2013). Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev *65*, 670-709.

Schuh, S.M., Carlson, A.E., McKnight, G.S., Conti, M., Hille, B., and Babcock, D.F. (2006). Signaling pathways for modulation of mouse sperm motility by adenosine and catecholamine agonists. Biol Reprod *74*, 492-500.

Seifert, R., Flick, M., Bonigk, W., Alvarez, L., Trotschel, C., Poetsch, A., Muller, A., Goodwin, N., Pelzer, P., Kashikar, N.D., *et al.* (2015). The CatSper channel controls chemosensation in sea urchin sperm. The EMBO journal *34*, 379-392.

Smith, J.F., Syritsyna, O., Fellous, M., Serres, C., Mannowetz, N., Kirichok, Y., and Lishko, P.V. (2013). Disruption of the principal, progesterone-activated sperm Ca2+ channel in a CatSper2-deficient infertile patient. Proc Natl Acad Sci U S A *110*, 6823-6828.

Spehr, M., Schwane, K., Riffell, J.A., Barbour, J., Zimmer, R.K., Neuhaus, E.M., and Hatt, H. (2004). Particulate adenylate cyclase plays a key role in human sperm olfactory receptor-mediated chemotaxis. J Biol Chem *279*, 40194-40203.

Strunker, T., Goodwin, N., Brenker, C., Kashikar, N.D., Weyand, I., Seifert, R., and Kaupp, U.B. (2011). The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature *471*, 382-386.

Sumigama, S., Mansell, S., Miller, M., Lishko, P.V., Cherr, G.N., Meyers, S.A., and Tollner, T. (2015). Progesterone Accelerates the Completion of Sperm Capacitation and Activates CatSper Channel in Spermatozoa from the Rhesus Macaque. Biol Reprod *93*, 130.

Tamburrino, L., Marchiani, S., Minetti, F., Forti, G., Muratori, M., and Baldi, E. (2014). The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. Hum Reprod *29*, 418-428.

Tamburrino, L., Marchiani, S., Vicini, E., Muciaccia, B., Cambi, M., Pellegrini, S., Forti, G., Muratori, M., and Baldi, E. (2015). Quantification of CatSper1 expression in human spermatozoa and relation to functional parameters. Hum Reprod *30*, 1532-1544.

Tavares, R.S., Mansell, S., Barratt, C.L., Wilson, S.M., Publicover, S.J., and Ramalho-Santos, J. (2013). p,p'-DDE activates CatSper and compromises human sperm function at environmentally relevant concentrations. Hum Reprod *28*, 3167-3177.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D.Y., Oldsclarke, P., and Kopf, G.S. (1995). Capacitation of Mouse Spermatozoa .2. Protein-Tyrosine Phosphorylation and Capacitation Are Regulated by a Camp-Dependent Pathway. Development *121*, 1139-1150.

Wennemuth, G., Carlson, A.E., Harper, A.J., and Babcock, D.F. (2003a). Bicarbonate actions on flagellar and Ca2+-channel responses: initial events in sperm activation. Development *130*, 1317-1326.

Wennemuth, G., Carlson, A.E., Harper, A.J., and Babcock, D.F. (2003b). Bicarbonate actions on flagellar and Ca2+-channel responses: initial events in sperm activation. Development *130*, 1317-1326.

Williams, H.L., Mansell, S., Alasmari, W., Brown, S.G., Wilson, S.M., Sutton, K.A., Miller, M.R., Lishko, P.V., Barratt, C.L., Publicover, S.J., *et al.* (2015). Specific loss of CatSper function is sufficient to compromise fertilizing capacity of human spermatozoa. Hum Reprod *30*, 2737-2746.

Xia, J., Reigada, D., Mitchell, C.H., and Ren, D. (2007). CATSPER channel-mediated Ca2+ entry into mouse sperm triggers a tail-to-head propagation. Biol Reprod *77*, 551-559.

Xie, F., Garcia, M.A., Carlson, A.E., Schuh, S.M., Babcock, D.F., Jaiswal, B.S., Gossen, J.A., Esposito, G., van Duin, M., and Conti, M. (2006). Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. Dev Biol *296*, 353-362.

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. Zygote *2*, 371-372.

Zhang, Y., Malekpour, M., Al-Madani, N., Kahrizi, K., Zanganeh, M., Lohr, N.J., Mohseni, M., Mojahedi, F., Daneshi, A., Najmabadi, H., *et al.* (2007). Sensorineural deafness and male infertility: a contiguous gene deletion syndrome. J Med Genet *44*, 233-240.

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Data and materials availability: All data are present in the main text, figures, and the supplementary material.

Figures

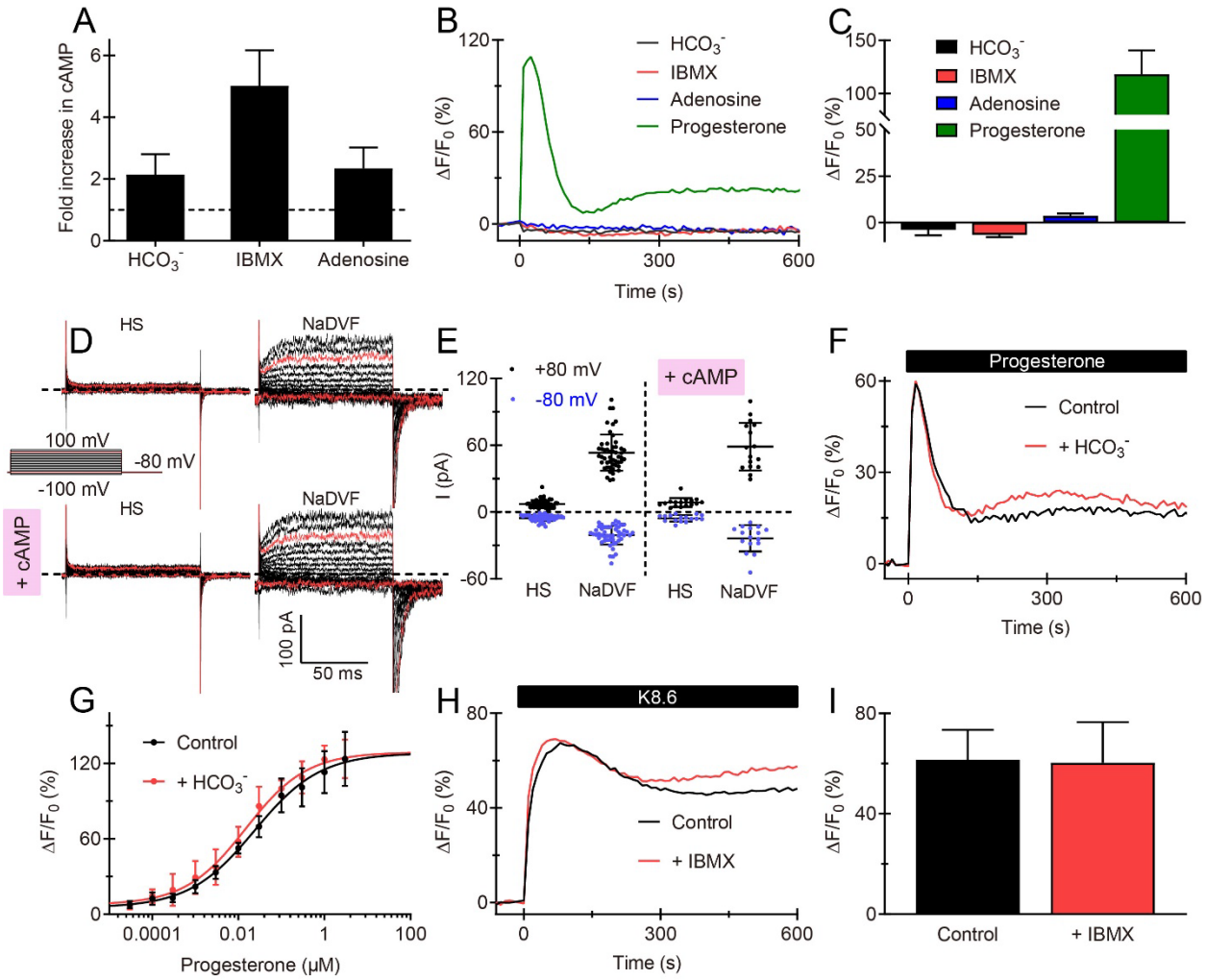


Figure 1 Human CatSper is not activated by intracellular cAMP. (A) Changes in total cAMP content in human sperm upon increasing bicarbonate from 0 mM to 25 mM HCO_3^-, or by IBMX (0.5 mM) or adenosine (0.1 mM) in the presence of 4 mM HCO_3^-. Dotted black line = 1 (control) (n = 3). **(B)** Representative Ca^{2+} signals evoked by increasing HCO_3^- from 0 to 25 mM, or by application of IBMX (0.5 mM), adenosine (0.1 mM), or progesterone (10 µM) in the presence of 4 mM bicarbonate. $[Ca^{2+}]_i$ was monitored using a fluorescence plate reader (Strunker et al., 2011). Sperm were loaded with the fluorescent Ca^{2+} indicator Fluo-4. $\Delta F/F_0$ (%) indicates the percent change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before application of stimulants at t = 0. **(C)** Mean amplitude of Ca^{2+} signals from B ($n \geq 3$). **(D)** Representative whole-cell currents recorded from a human sperm cell at pH_i 7.3 in extracellular solution containing Mg^{2+} and Ca^{2+} (HS) and during perfusion with Na^+-based divalent-free bath solution (NaDVF). Currents were evoked by stepping the membrane voltage from -100 to +100 mV (step 10 mV) from a holding potential of -80 mV in the absence (upper panel) or presence

(lower panel) of cAMP (1 mM) in the pipette. Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV. **(E)** Steady-state current amplitudes at +80 mV and -80 mV in HS and NaDVF in the absence (n = 50, left) and presence of cAMP (1 mM) in the pipette (n = 16; right). **(F)** Ca^{2+} signals evoked by progesterone (10 nM) in sperm bathed in 0 (control) or 25 mM HCO_3^-. **(G)** Dose-response relationship for progesterone in the absence (control; EC_{50} = 23 ± 4 nM) and presence of HCO_3^- (EC_{50} = 19 ± 19 nM) (n = 4). **(H)** Ca^{2+} signals in sperm bathed in 4 mM HCO_3^- evoked by a simultaneous alkalization and depolarization (K8.6) in the absence (control) and presence of IBMX (0.5 mM). **(I)** Mean amplitude of Ca^{2+} signals from (H) (n = 4).

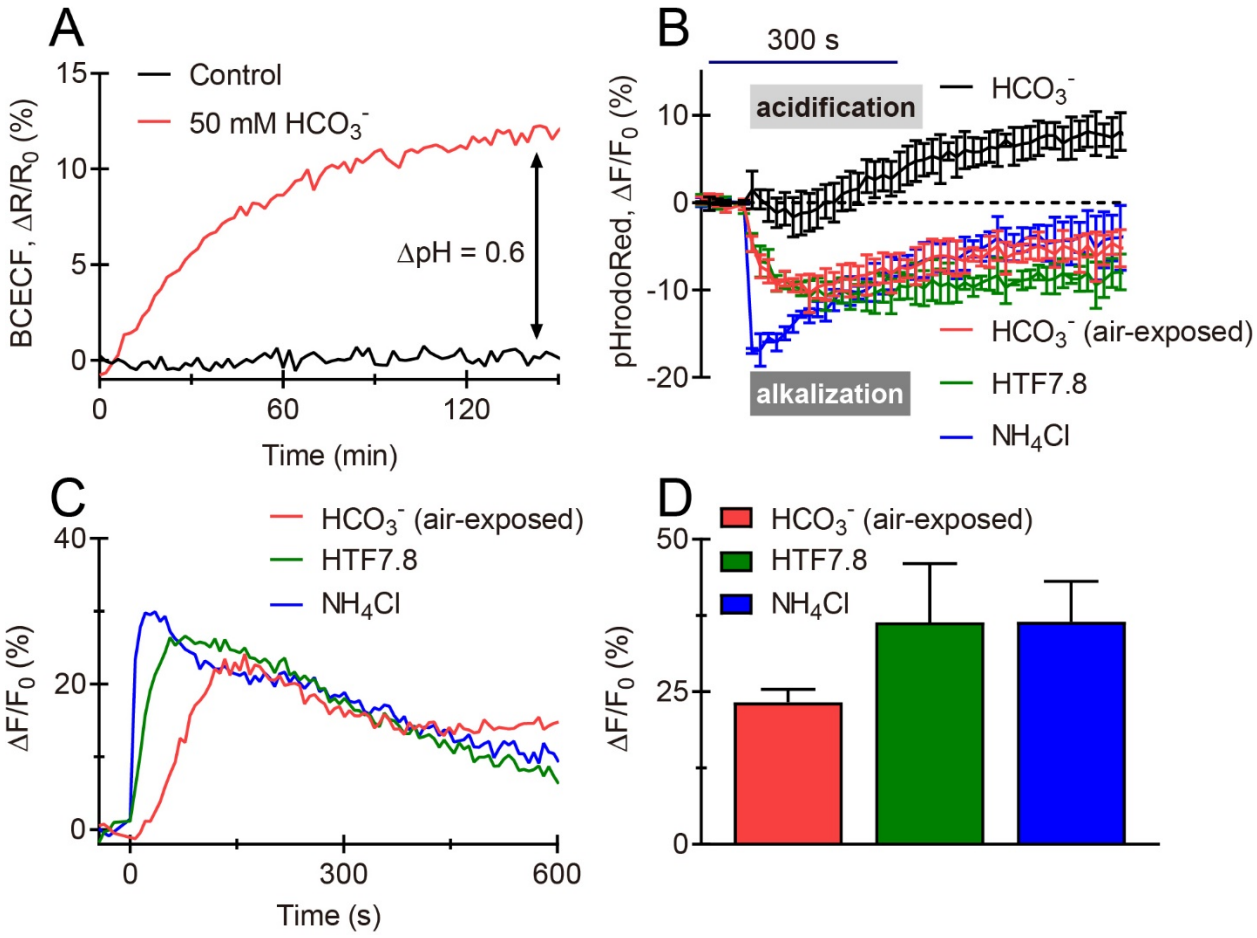


Figure 2 Bicarbonate is prone to evoke alkaline-induced Ca^{2+} influx via CatSper (A) Changes in the pH of HTF buffer in the absence (control) and presence of HCO_3^- (50 mM) at ambient air, measured with the fluorescent pH-indicator BCECF. **(B)** pH_i changes evoked by pH-controlled HTF containing HCO_3^- (25 mM), air-exposed, alkaline HTF containing HCO_3^- (25 mM), alkaline HCO_3^--free HTF (pH 7.8, HTF7.8), and NH_4Cl (5 mM) in human sperm loaded with the fluorescent pH indicator pHrodoRed. Dotted black line indicates Y = 0 (n = 3). **(C)** Ca^{2+} signals evoked by air-exposed, alkaline HTF containing HCO_3^- (25 mM), HTF7.8, and NH_4Cl (5 mM). **(D)** Mean amplitude of Ca^{2+} signals from (C) (n = 3).

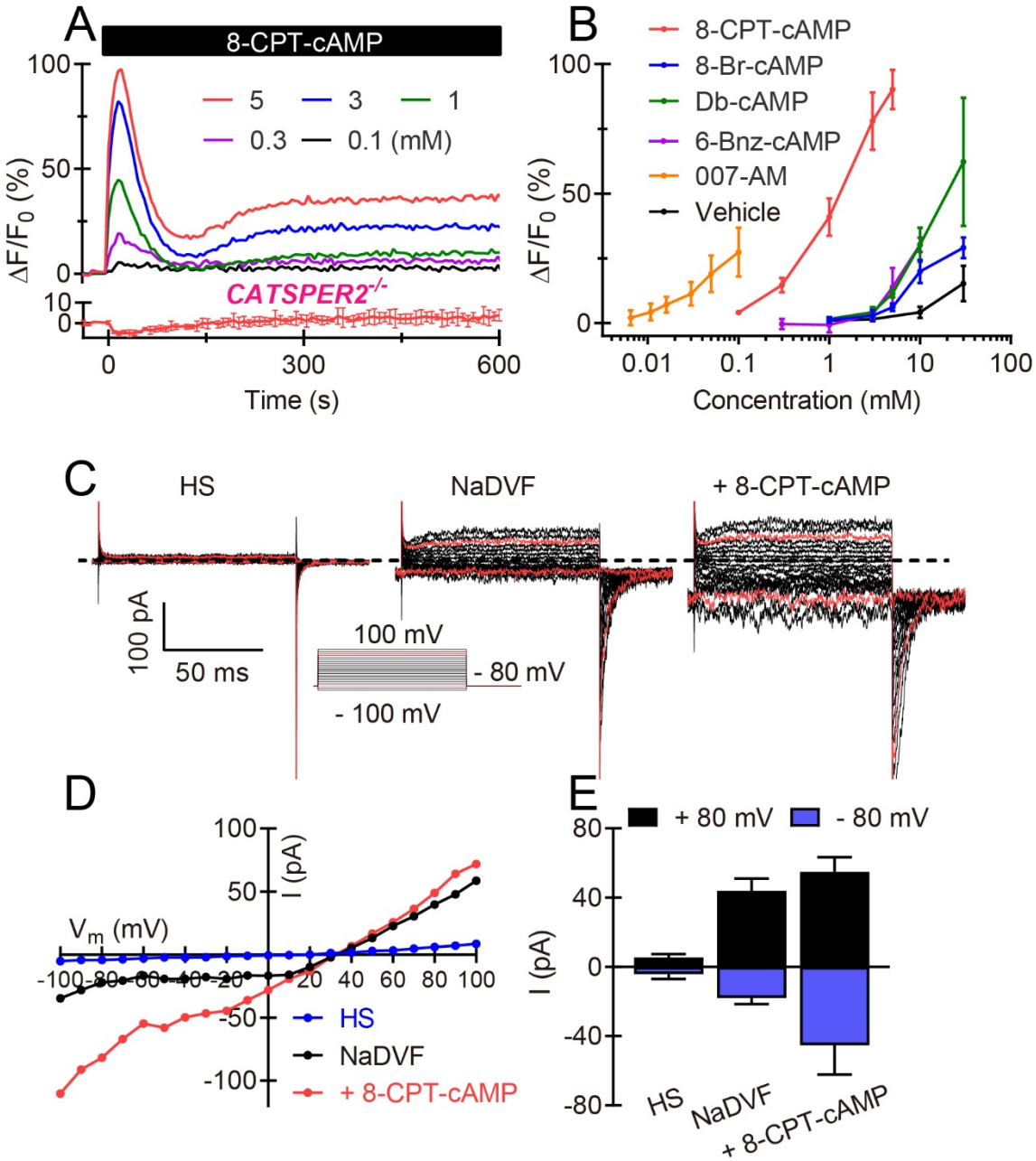


Figure 3 Membrane-permeable cAMP analogs activate CatSper in human sperm. **(A)** Representative Ca^{2+} signals evoked by 8-CPT-cAMP in sperm from a healthy donor (upper panel), and averaged Ca^{2+} signal (lower panel, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(B)** Mean amplitudes of Ca^{2+} signals evoked by membrane-permeable cAMP analogs and by the vehicle (NaCl) (n = 3). **(C)** Representative whole-cell currents recorded from human sperm at pH_i 7.3 in extracellular solution containing Ca^{2+} and Mg^{2+} (HS), in divalent-free Na^{+}-based bath solution (NaDVF), and during perfusion with NaDVF containing 8-CPT-cAMP (5 mM). Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV. **(D)** Steady-state current-voltage relationship from (C). **(E)** Mean current amplitudes at +80 mV and -80 mV in HS, in NaDVF, and in NaDVF containing 5 mM 8-CPT-cAMP (n = 8).

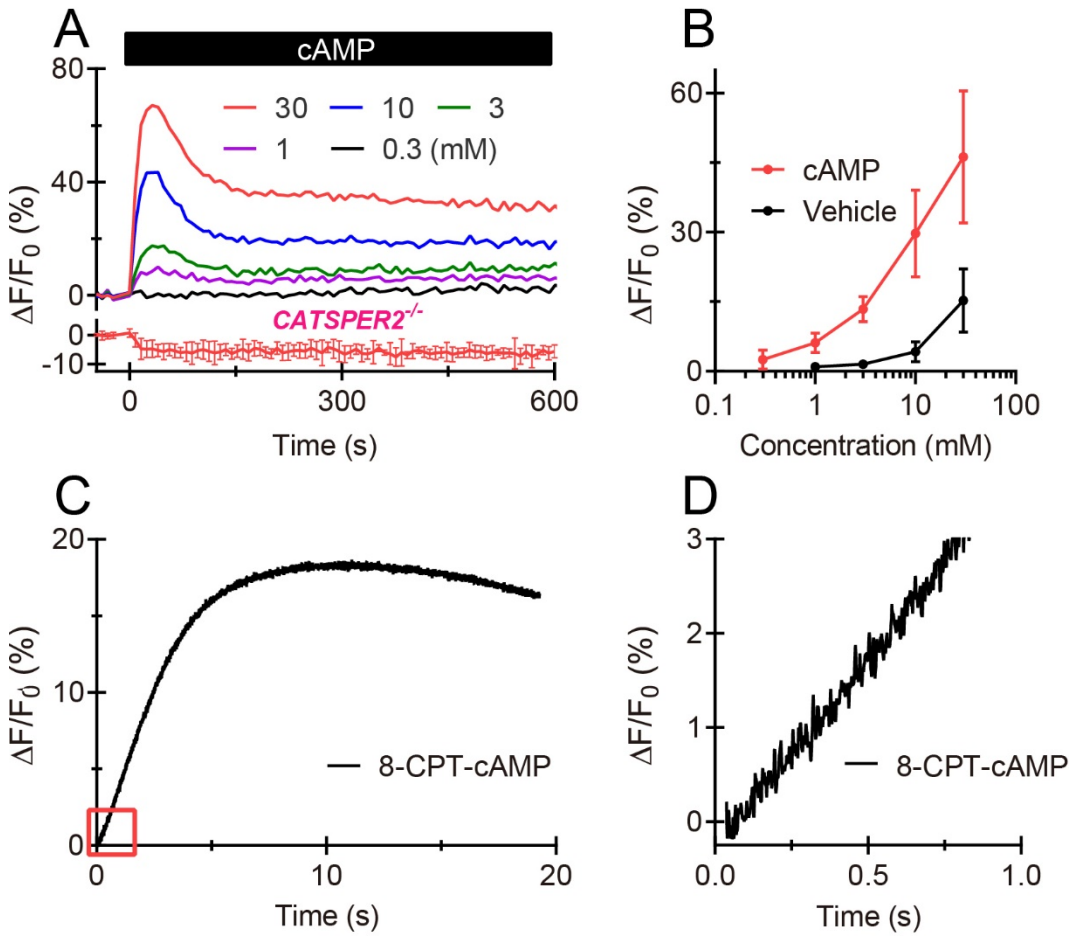
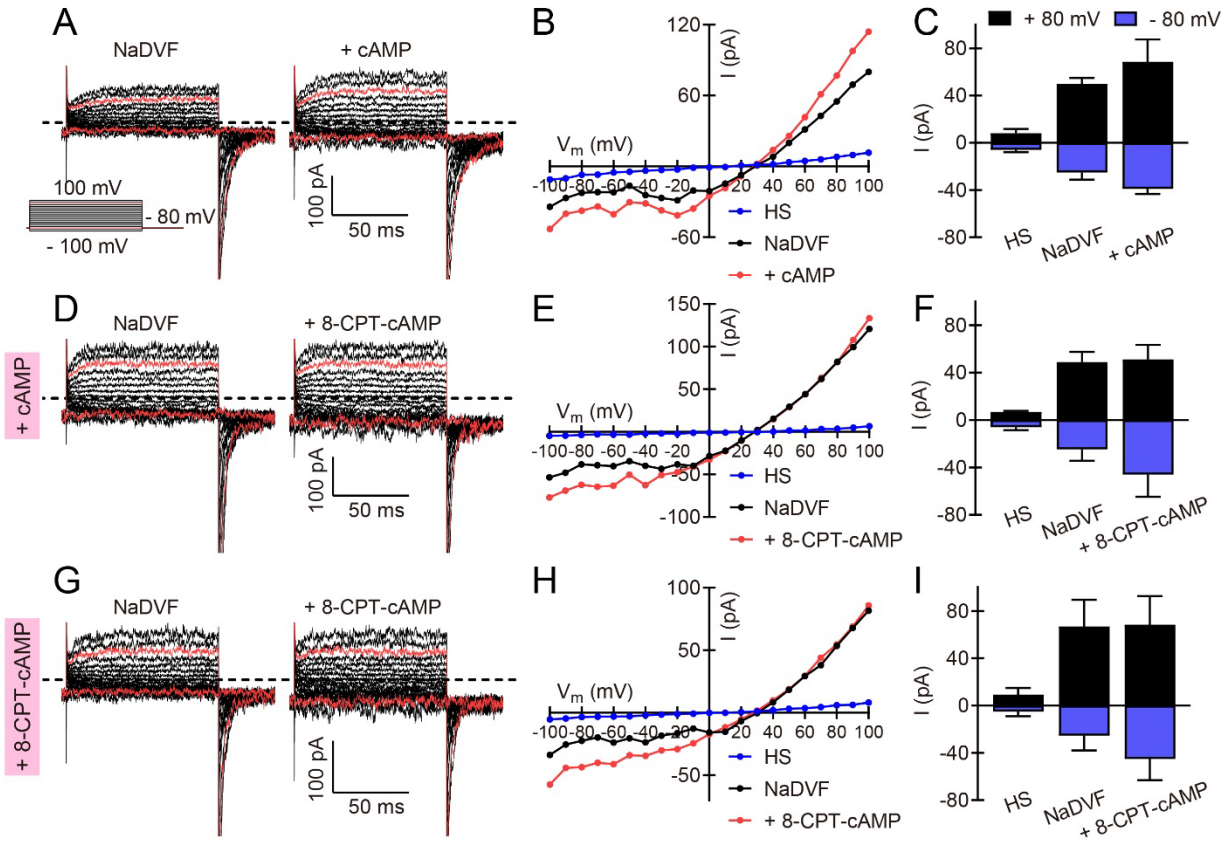


Figure 4 Activation of CatSper by extracellular cAMP and kinetics of the 8-CPT-cAMP-evoked Ca^{2+} response in human sperm. (A) Representative Ca^{2+} signals evoked by cAMP in sperm from a healthy donor (upper panel), and averaged Ca^{2+} signal (lower panel, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(B)** Mean amplitudes of Ca^{2+} signals evoked by cAMP (n = 4) and the vehicle (NaCl) (n = 3). **(C)** Ca^{2+} signal evoked by rapid mixing of sperm with 8-CPT-cAMP (5 mM) in a stopped-flow apparatus. **(D)** Onset of the 8-CPT-cAMP-evoked Ca^{2+} signal from (C) shown on an extended time scale.



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655 **Figure 5 cAMP and 8-CPT-cAMP activate human CatSper from the outside. (A, D, and G)**
656 Representative whole-cell currents at pH_i 7.3 in divalent-free Na^+-based bath solution (NaDVF)
657 and in NaDVF containing 10 mM cAMP (A) or 5 mM 8-CPT-cAMP (D and G). Currents were
658 evoked in the absence (A) or in the presence of 1 mM cAMP (D) or 5 mM 8-CPT-cAMP (G) in
659 the pipette. Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV. **(B,**
660 **E, and H)** Steady-state current-voltage relationships from (A), (D), and (G), respectively. **(C, F,**
661 **and I)** Mean current amplitudes at +80 mV and -80 mV in HS, in NaDVF, and in NaVDF
662 containing 10 mM cAMP (C, n = 4) or 5 mM 8-CPT-cAMP (F, n = 8; I, n = 4). Currents were
663 evoked in the absence (C) or presence of 1 mM cAMP (F) or 5 mM 8-CPT-cAMP (I) in the
664 pipette.

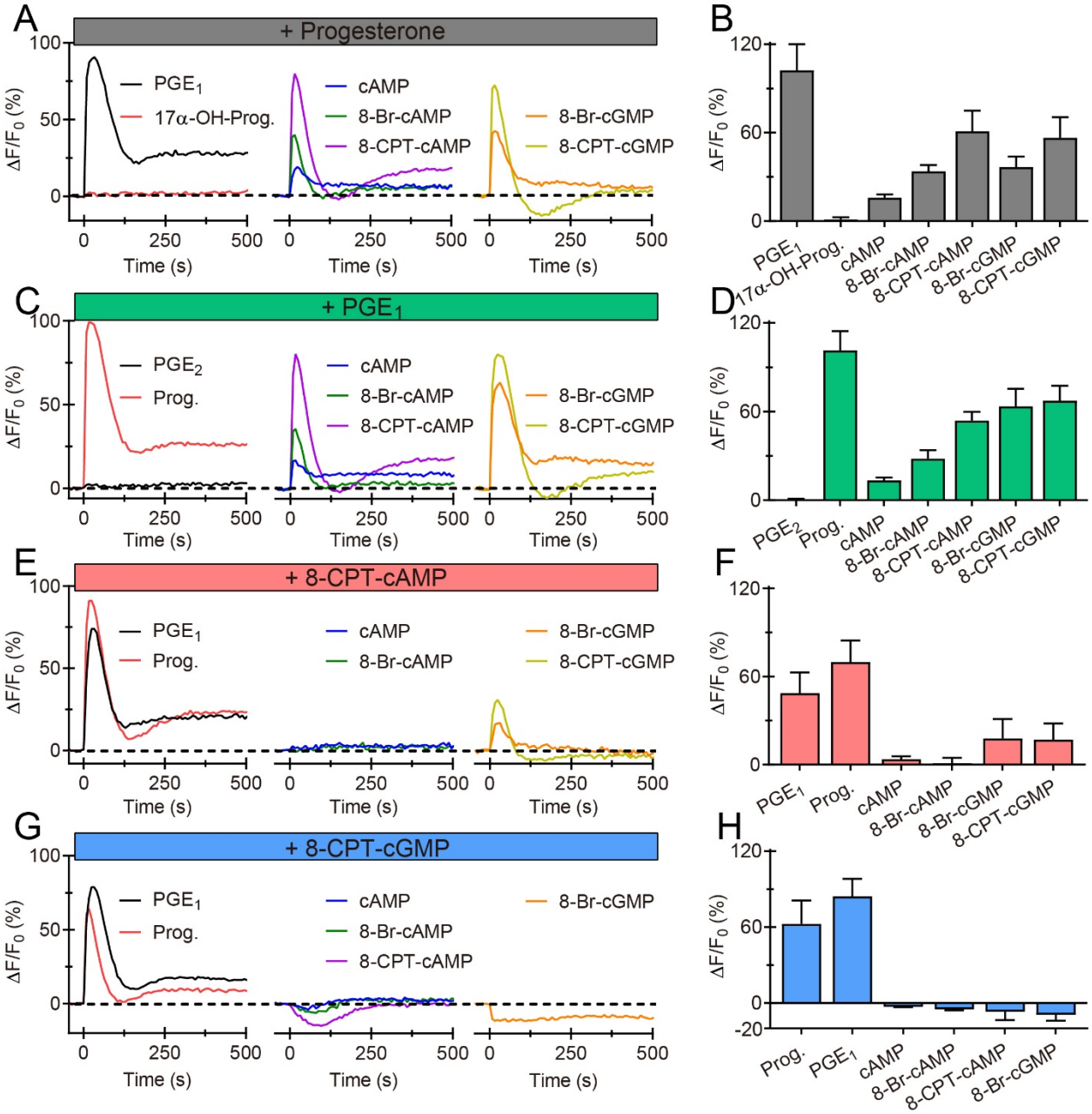


Figure 6 Cyclic nucleotides, steroids, and prostaglandins activate human CatSper via three distinct binding sites. (A, B) Representative Ca^{2+} signals (A) and mean signal amplitudes (B; n ≥ 3) evoked by 17α-OH-Progesterone (2 µM), PGE_1 (2 µM), cAMP (10 mM), 8-Br-cAMP (10 mM), 8-CPT-cAMP (5 mM), 8-Br-cGMP (10 mM), and 8-CPT-cGMP (5 mM) in sperm bathed in progesterone (10 µM). **(C, D)** Representative Ca^{2+} signals and mean signal amplitudes (n ≥ 3) evoked by PGE_2 (2 µM), progesterone (2 µM), and the indicated cyclic nucleotides in sperm bathed in PGE_1 (2 µM). **(E, F)** Representative Ca^{2+} signals and mean signal amplitudes (n ≥ 3) evoked by PGE_1, progesterone, and the indicated cyclic nucleotides in sperm bathed in 8-CPT-cAMP (5 mM), **(G, H)** Representative Ca^{2+} signals and mean signal amplitudes (n ≥ 3) evoked by PGE_1, progesterone, and the indicated cyclic nucleotides in sperm bathed in 8-CPT-cGMP (5 mM).

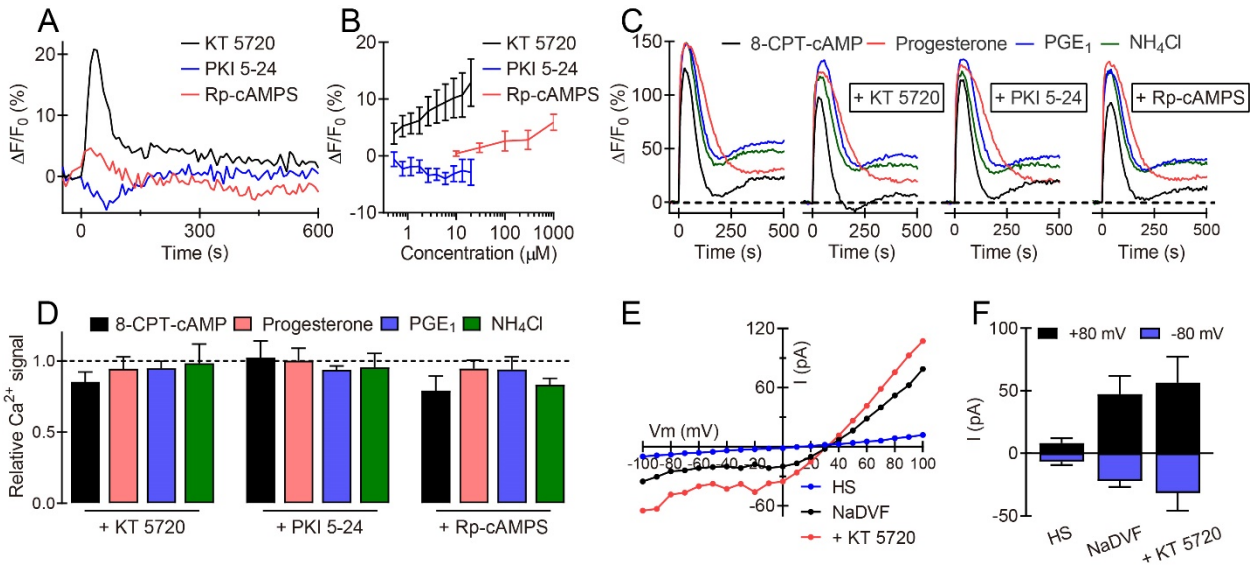


Figure 7 Action of PKA inhibitors on CatSper in human sperm. (A) Representative Ca^{2+} signals evoked by KT 5720 (20 μM), PKI 5-24 (20 μM), and Rp-cAMPS (1 mM). **(B)** Mean amplitudes of Ca^{2+} signals evoked by KT 5720, PKI 5-24, and Rp-cAMPS ($n \geq 3$). **(C)** Ca^{2+} signals evoked by 8-CPT-cAMP (5 mM), progesterone (10 μM), PGE_1 (10 μM), and NH_4Cl (10 mM), in the absence and presence of KT 5720 (20 μM), PKI 5-24 (20 μM), or Rp-cAMPS (1 mM). **(D)** Mean amplitude of Ca^{2+} signals in the presence of KT 5720 (20 μM), PKI 5-24 (20 μM), or Rp-cAMPS (1 mM) relative to the amplitude evoked in the absence of any drug ($n \geq 4$). **(E)** Representative steady-state current-voltage relationship of currents recorded at pH_i of 7.3 in extracellular solution containing Ca^{2+} and Mg^{2+} (HS), in divalent-free Na^+-based bath solution (NaDVF), and in NaDVF containing KT 5720 (20 μM). **(F)** Mean current amplitudes at +80 mV and -80 mV recorded in HS, in NaDVF, and in NaDVF containing 20 μM KT 5720 ($n = 5$).

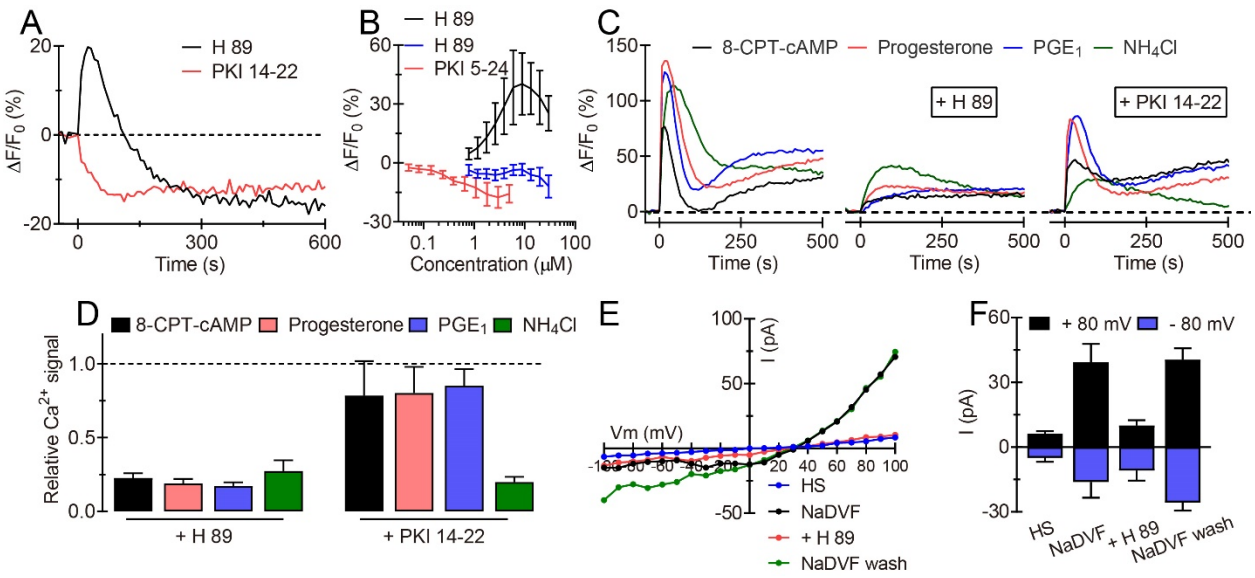
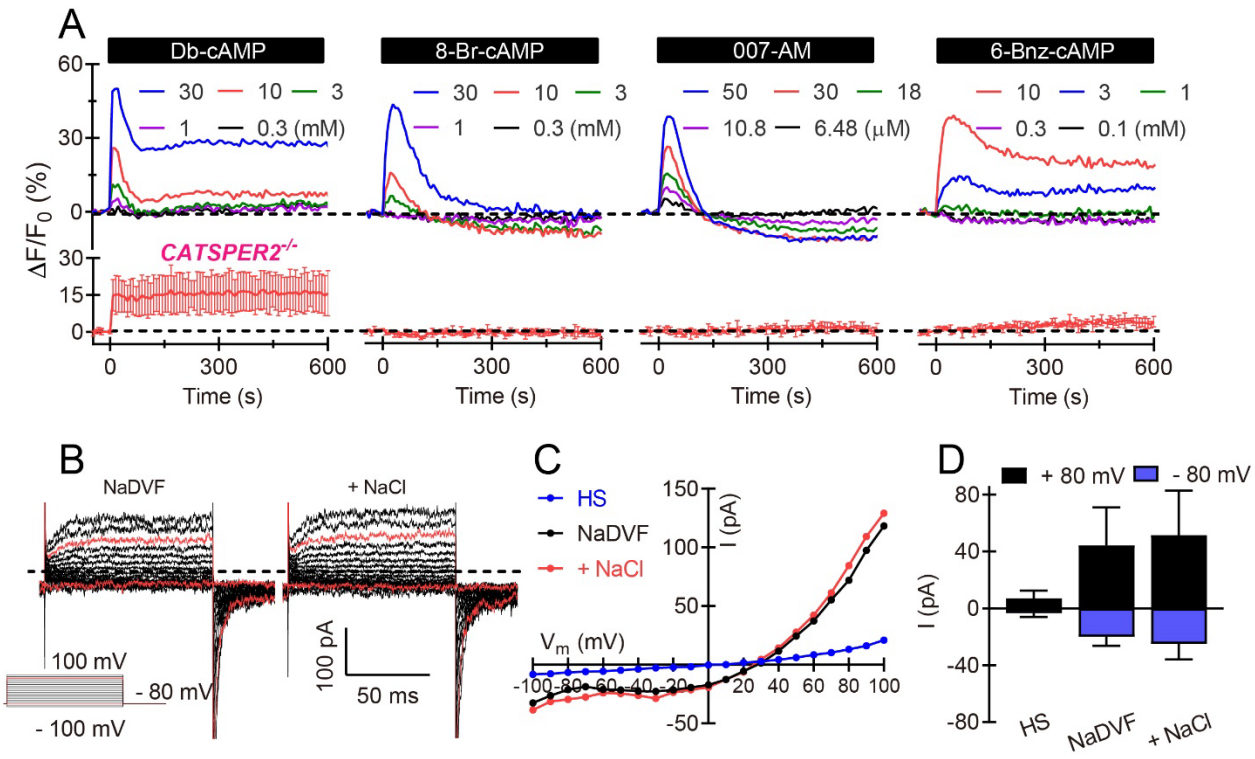
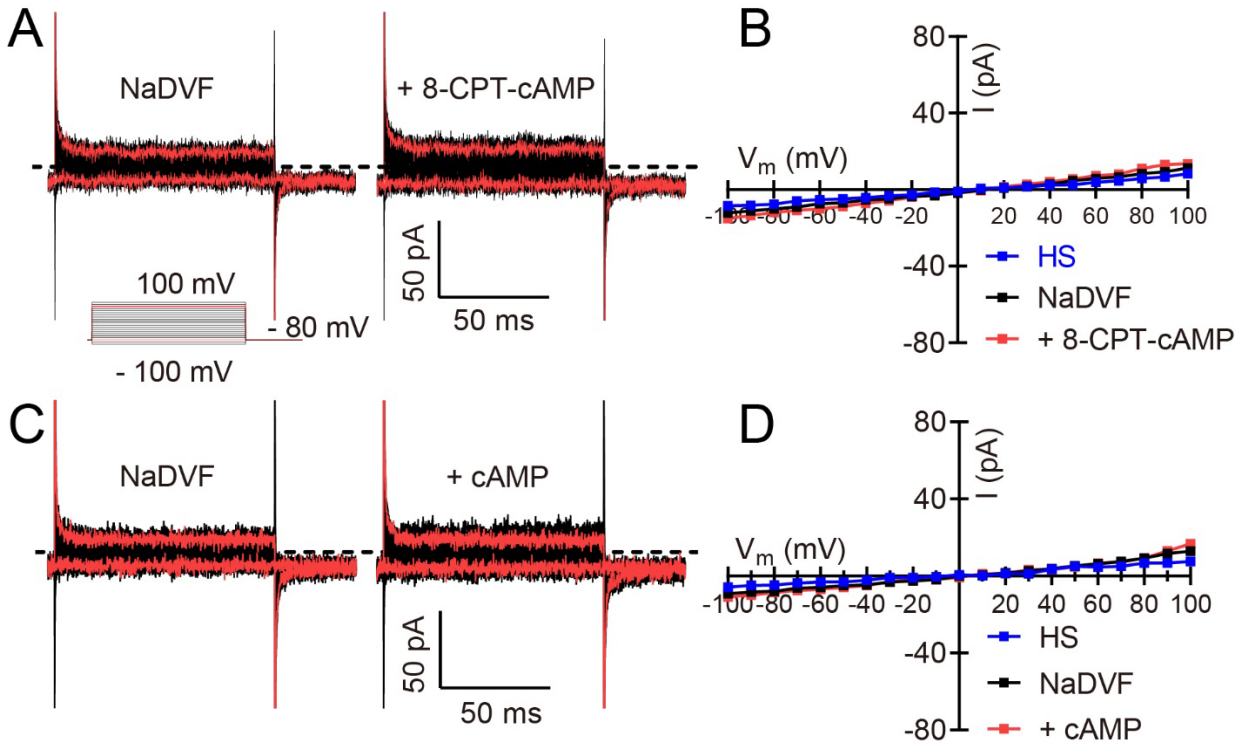


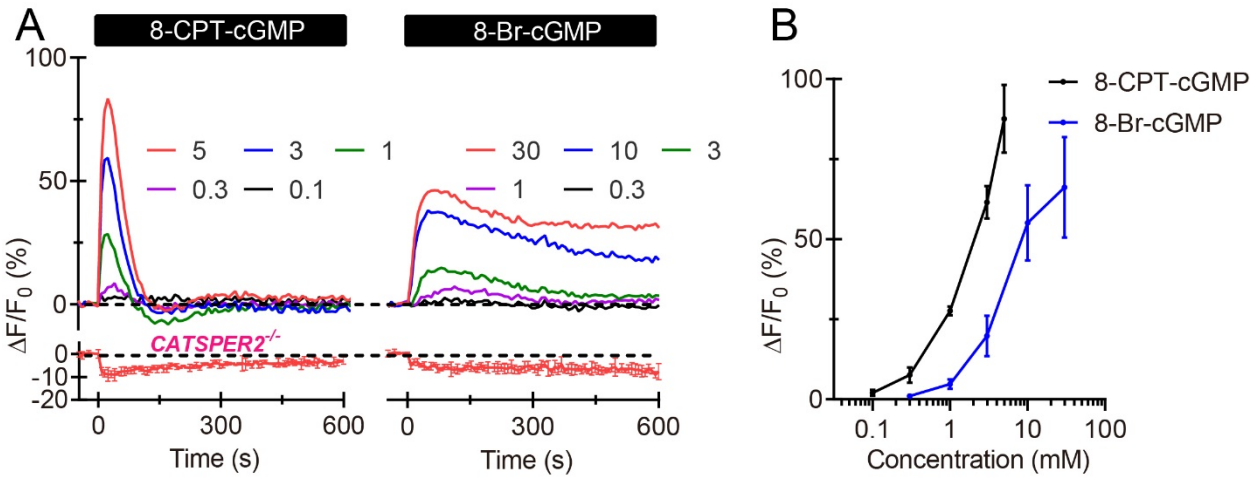
Figure 8 The PKA inhibitors H89 and PKI 14-22 inhibit human CatSper. (A) Representative Ca^{2+} signals evoked by H89 (30 μM) and PKI 14-22 (5 μM). **(B)** Mean amplitude of Ca^{2+} signals evoked by H89 (black, peak phase; blue, sustained phase) and PKI 14-22 ($n \geq 6$). **(C)** Ca^{2+} signals evoked by 8-CPT-cAMP (5 mM), progesterone (10 μM), PGE_1 (10 μM), and NH_4Cl (10 mM) in the absence and presence of H89 (30 μM) or PKI 14-22 (5 μM). **(D)** Mean amplitude of Ca^{2+} signals in the presence of H89 or PKI 14-22 relative to the amplitude evoked in the absence of any drug ($n \geq 4$). **(E)** Representative steady-state current-voltage relationship of currents recorded at pH_i 7.3 in extracellular solution containing Ca^{2+} and Mg^{2+} (HS), in divalent-free Na^+-based bath solution (NaDVF), and in NaDVF containing H89 (30 μM). **(F)** Mean current amplitudes at +80 mV and -80 mV in HS, in NaDVF, and in NaDVF containing H89 ($n \geq 3$).



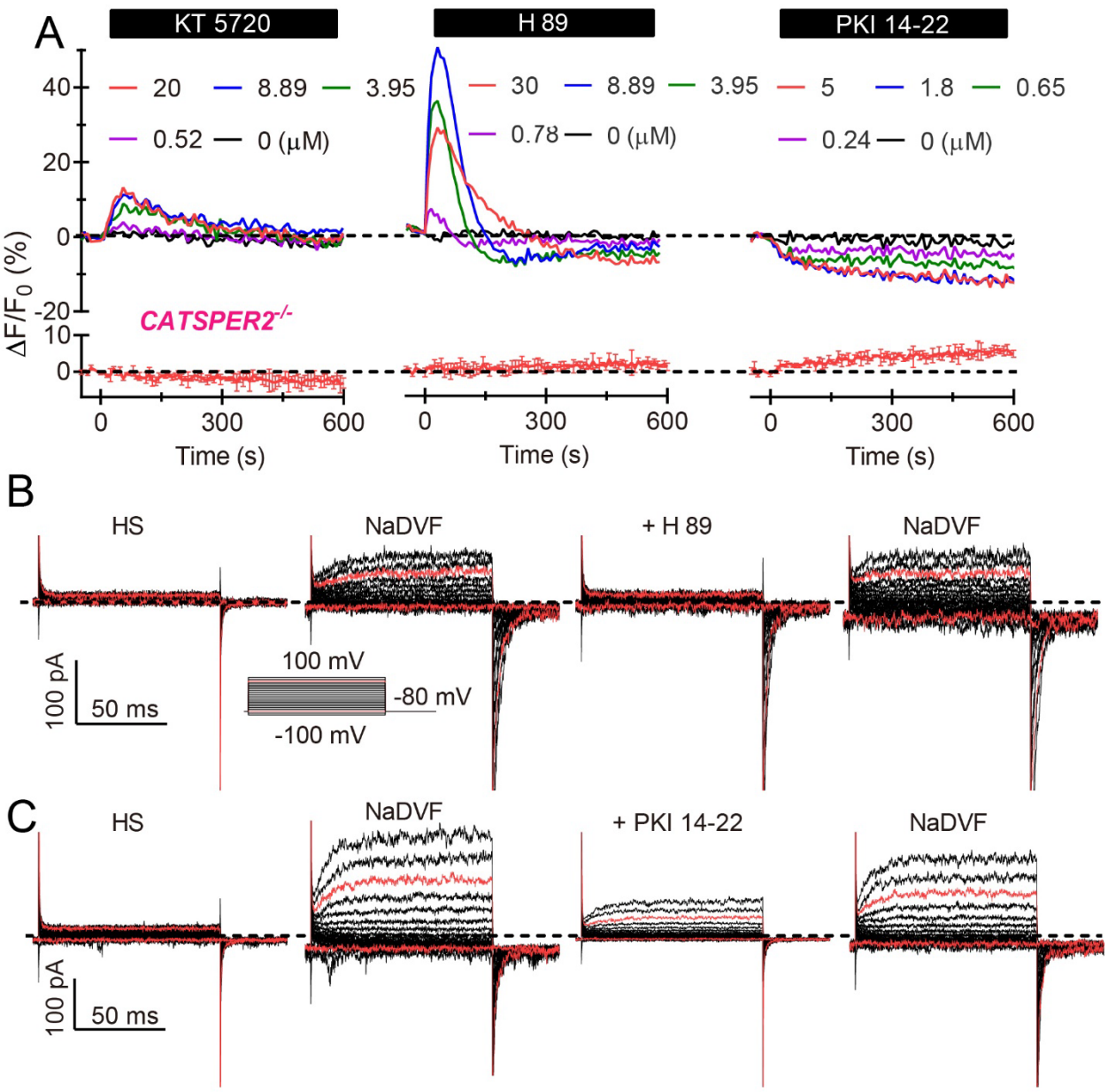
Supplementary Figure 1 cAMP analogs evoke Ca^{2+} signals in human sperm. (A) Representative Ca^{2+} signals evoked by Db-cAMP, 8-Br-cAMP, 8-pCPT-2-O-Me-cAMP (e.g. 007-AM), and 6-Bnz-cAMP in sperm from healthy donors (upper panel), and averaged Ca^{2+} signal (lower panels, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(B)** Representative whole-cell currents at pH_i 7.3 in divalent-free Na^+-based bath solution (NaDVF) and in NaDVF containing additional 5 mM NaCl. Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV. **(C)** Steady-state current-voltage relationship from (B) and in extracellular solution containing Ca^{2+} and Mg^{2+} (HS) indicated as a control. **(D)** Mean current amplitudes at +80 mV and -80 mV in HS, in NaDVF, and in NaDVF containing additional 5 mM NaCl (n = 3).



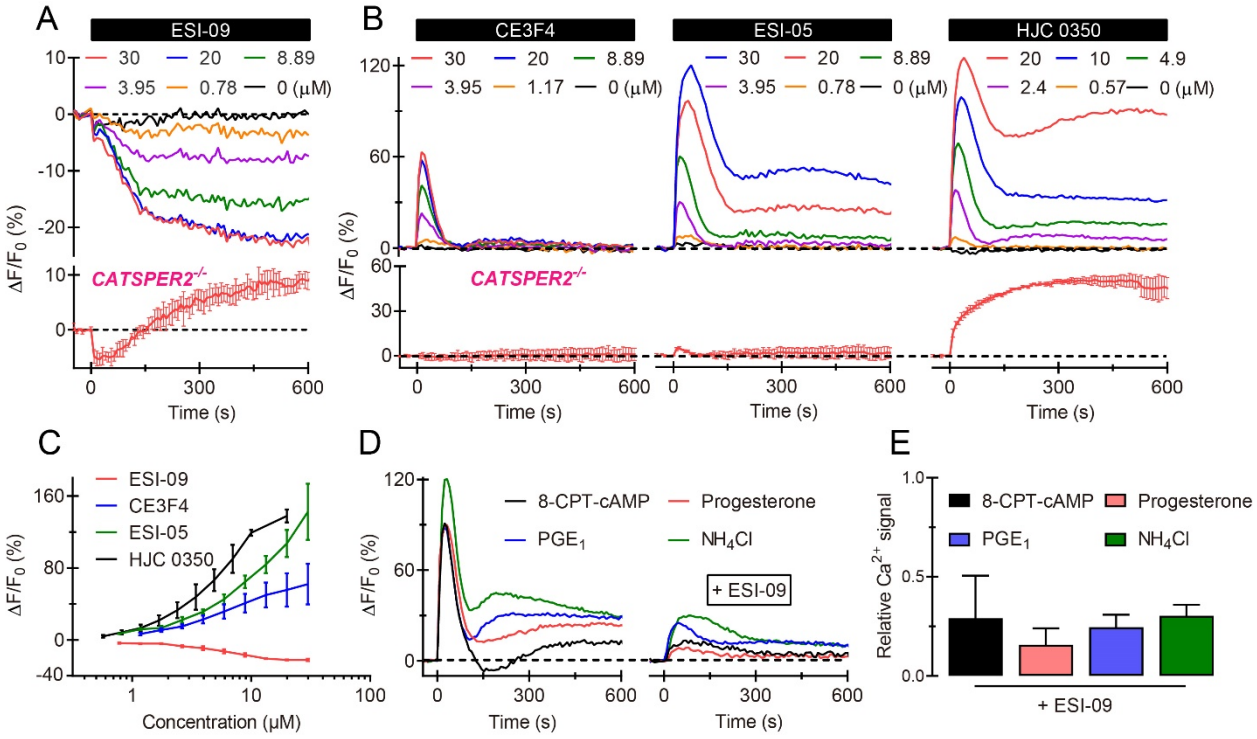
Supplementary Figure 2 Action of 8-CPT-cAMP and cAMP in CatSper-deficient human sperm. (A, C) Representative whole-cell currents at pH_i 7.3 recorded from sperm lacking functional CatSper channels *(CATSPER2*$^{-/-}$) in divalent-free Na^+-based bath solution (NaDVF) and in NaDVF containing 5 mM 8-CPT-cAMP (A) or 10 mM cAMP (C). Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV. **(B, D)** Steady-state current-voltage relationship from (A) and (C), respectively, with currents in extracellular solution containing Ca^{2+} and Mg^{2+} (HS) indicated as a control.



Supplementary Figure 3 cGMP analogs activate CatSper in human sperm. (A) Representative Ca^{2+} signals evoked by 8-CPT-cGMP and 8-Br-cGMP in sperm from healthy donors and averaged Ca^{2+} signal (lower panels, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(B)** Mean amplitudes of Ca^{2+} signals evoked by 8-CPT-cGMP and 8-Br-cGMP (n = 3) in sperm from healthy donors.



Supplementary Figure 4 The actions of PKA inhibitors in human sperm (A) Representative Ca^{2+} signals evoked by KT 5720, H89, and PKI 14-22 in sperm from healthy donors and averaged Ca^{2+} signal (lower panels, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(B and C)** Representative whole-cell currents at pH_i 7.3 in extracellular solution containing Ca^{2+} and Mg^{2+} (HS), in divalent-free Na^+-based bath solution (NaDVF), and in NaDVF containing 30 μM H89 (B) or 5 μM PKI 14-22 (C). Dotted black line: zero current level. Red traces: currents at +80 mV and -80 mV.



Supplementary Figure 5 The action of EPAC inhibitors in human sperm. (A, B) Representative Ca^{2+} signals evoked ESI 09, CE3F4, ESI 05, and HJC 0350 in sperm from healthy donors and averaged Ca^{2+} signal (lower panels, n = 3) in sperm lacking functional CatSper channels (*CATSPER2*$^{-/-}$). **(C)** Mean amplitudes of Ca^{2+} signals evoked by EPAC inhibitors ($n \geq 4$). **(D)** Ca^{2+} signals evoked by 8-CPT-cAMP (5 mM), progesterone (10 μM), PGE_1 (10 μM), and NH_4Cl (10 mM), in the absence and presence of ESI 09 (30 μM). **(E)** Mean amplitude of Ca^{2+} signals in the presence of ESI 09 (30 μM) relative the amplitude evoked in the absence of any drug ($n \geq 3$).