The CatSper channel: a polymodal chemosensor in human sperm

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The sperm-specific CatSper channel controls the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and, thereby, the swimming behaviour of sperm. In humans, CatSper is directly activated by progesterone and prostaglandins—female factors that stimulate Ca^{2+} influx. Other factors including neurotransmitters, chemokines, and odorants also affect sperm function by changing $[\text{Ca}^{2+}]_i$. Several ligands, notably odorants, have been proposed to control Ca^{2+} entry and motility via G protein-coupled receptors (GPCRs) and cAMP-signalling pathways. Here, we show that odorants directly activate CatSper without involving GPCRs and cAMP. Moreover, membrane-permeable analogues of cyclic nucleotides that have been frequently used to study cAMP-mediated Ca^{2+} signalling also activate CatSper directly via an extracellular site. Thus, CatSper or associated protein(s) harbour promiscuous binding sites that can host various ligands. These results contest current concepts of Ca^{2+} signalling by GPCR and cAMP in mammalian sperm: ligands thought to activate metabotropic pathways, in fact, act via a common ionotropic mechanism. We propose that the CatSper channel complex serves as a polymodal sensor for multiple chemical cues that assist sperm during their voyage across the female genital tract.

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Introduction

In mammalian sperm, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is controlled by sperm-specific Ca^{2+} channels (CatSper, cation channel of sperm) (Quill *et al*, 2001; Ren *et al*, 2001; Kirichok *et al*, 2006; Lishko *et al*, 2010). The CatSper channel complex comprises four homologous α subunits (CatSper 1–4) (Navarro *et al*, 2008; Kirichok and Lishko, 2011) and at least three auxiliary subunits: CatSper β , CatSper γ , and CatSper δ (Liu *et al*, 2007; Wang *et al*, 2009; Chung *et al*, 2011). In human sperm, progesterone and prostaglandins—two important ingredients of the oviduct (Schuetz and Dubin, 1981)—directly activate CatSper channels without involving classical nuclear receptors or G protein-coupled receptors (GPCRs; Lishko *et al*, 2011; Strünker *et al*, 2011). The progesterone-induced Ca^{2+} influx has been implicated in sperm capacitation, chemotaxis, hyperactivation, and acrosomal exocytosis (Harper *et al*, 2003; Oren-Benaroya *et al*, 2008; Publicover *et al*, 2008). Ca^{2+} influx stimulated by prostaglandins evokes acrosomal exocytosis and affects motility (Aitken and Kelly, 1985; Schaefer *et al*, 1998) but their function in chemotaxis and sperm hyperactivation is not known.

Chemosensation in sperm appears to be complex. A plethora of chemicals including vitamin D, chemokines, small peptides, the gas NO, neurotransmitters, analogues of cyclic nucleotides, and odorants (De Jonge, 2005; Eisenbach and Gijalas, 2006; Florman *et al*, 2008; Kaupp *et al*, 2008; Suarez, 2008) affect sperm motility and/or acrosomal exocytosis *in vitro*. However, the underlying signalling mechanisms are ill defined. These chemicals have been proposed to activate various ionotropic and metabotropic receptors that are reportedly expressed in mammalian sperm (Meizel, 2004; Naz and Sellamuthu, 2006).

Several ligands for GPCRs have been proposed to control sperm function via cAMP-signalling pathways that regulate $[\text{Ca}^{2+}]_i$. A case in point concerns odorants that might serve as chemoattractants for sperm. Odorants have been proposed to activate olfactory receptors, stimulate the synthesis of cAMP by transmembrane adenylyl cyclases (tmACs), and, thereby, open unknown Ca^{2+} channels (Spehr *et al*, 2003, 2004; Neuhaus *et al*, 2006; Veitinger *et al*, 2011). This notion has received strong support by the observations that (i) membrane-permeable analogues of cyclic nucleotides evoke an increase of $[\text{Ca}^{2+}]_i$ (Kobori *et al*, 2000; Ren *et al*, 2001; Machado-Oliveira *et al*, 2008) and (ii) odorant-induced Ca^{2+} influx is impaired by inhibitors of tmACs and antagonists of odorant receptors (Spehr *et al*, 2003, 2004; Veitinger *et al*, 2011).

Here, we show by patch-clamp recording, Ca^{2+} fluorimetry, and cAMP radioimmunoassay (RIA) that structurally diverse odorants directly activate the CatSper channel in human sperm by a mechanism that does not involve metabotropic receptors and cAMP signalling. Moreover, several other GPCR ligands that reportedly stimulate cAMP-signalling

pathways in mouse sperm fail to elevate cAMP levels in human sperm. Finally, membrane-permeable analogues of cAMP and cGMP directly activate CatSper from the outside. These results resolve several long-standing issues concerning odorants, cyclic nucleotides, and Ca^{2+} signalling in human sperm and provide new insight into the function of CatSper channels as target for female factors.

Results

Bourgeonal stimulates Ca^{2+} entry via CatSper channels

In human sperm loaded with the fluorescent Ca^{2+} indicator Fluo-4, the odorant bourgeonal stimulated a dose-dependent increase of $[\text{Ca}^{2+}]_i$ (Figure 1A and B; Spehr *et al*, 2003, 2004; Veitinger *et al*, 2011). At bourgeonal concentrations $\leq 100 \mu\text{M}$, the Ca^{2+} signal consisted of two components: a rapid Ca^{2+} transient followed by a slower sustained Ca^{2+} increase (Figure 1A). At higher concentrations, bourgeonal evoked a rapid, sustained elevation of $[\text{Ca}^{2+}]_i$ (Figure 1A). The Ca^{2+} signal was abolished at low (30 nM) extracellular $[\text{Ca}^{2+}]$ (Supplementary Figure S1A); thus, the Ca^{2+} signal rests upon Ca^{2+} entry (Spehr *et al*, 2003). The sustained component might reflect Ca^{2+} release from intracellular stores that is triggered by Ca^{2+} influx (Publicover *et al*, 2007; Bedu-Addo *et al*, 2008), or bourgeonal might also inhibit Ca^{2+} export by Ca^{2+} -ATPase or $\text{Na}^+/\text{Ca}^{2+}$ exchange.

CatSper is the principle Ca^{2+} channel in mouse and human sperm (Kirichok *et al*, 2006; Lishko *et al*, 2010). We reasoned that Ca^{2+} entry stimulated by bourgeonal involves CatSper. In fact, the CatSper inhibitors NNC 55-0396 (Lishko *et al*, 2011; Strünker *et al*, 2011) and mibefradil (Strünker *et al*, 2011) suppressed and slowed down the bourgeonal-evoked Ca^{2+} signals (Figure 1C): the signal amplitude was reduced by $70 \pm 20\%$ (10 μM NNC 55-0396, four experiments) and $65 \pm 11\%$ (30 μM mibefradil, three experiments). We did not test higher concentrations, because at ≥ 10 and $\geq 30 \mu\text{M}$,

NNC 55-0396 and mibefradil, respectively, evoke Ca^{2+} signals themselves (Strünker *et al*, 2011) probably by intracellular alkalization (Supplementary Figure S2).

The activation of CatSper by progesterone is sensitized in capacitated sperm (Strünker *et al*, 2011). We therefore examined whether bourgeonal-induced Ca^{2+} signals differ in capacitated and non-capacitated sperm. Capacitation was assessed by an FITC-CD46 assay probing the acrosome reaction (Supplementary Figure S3). The amplitude and waveform of bourgeonal-induced Ca^{2+} signals were similar in sperm incubated under capacitating or non-capacitating conditions (Figure 1A and D). However, the constant of half-maximal activation, $K_{1/2}$, was smaller in capacitated sperm ($K_{1/2} = 68 \pm 32 \mu\text{M}$, five experiments; Figure 1B) than in non-capacitated sperm ($K_{1/2} = 102 \pm 44 \mu\text{M}$, four experiments); thus, capacitated sperm are more sensitive to bourgeonal.

The CatSper channel is promiscuously activated by a variety of odorants

The odorant undecanal reportedly acts as a competitive antagonist that inhibits the bourgeonal-evoked Ca^{2+} influx (Spehr *et al*, 2003, 2004; Veitinger *et al*, 2011). We failed to reproduce this result: in the presence of undecanal, the Ca^{2+} response evoked by bourgeonal was enhanced rather than abolished (Figure 1E). Moreover, undecanal itself evoked Ca^{2+} signals that were similar to those evoked by bourgeonal (compare Figure 1A and F) and that were also due to Ca^{2+} influx (Supplementary Figure S1B). The dose-response relationship for undecanal yielded a $K_{1/2}$ of $28 \pm 6 \mu\text{M}$ (four experiments) (Figure 1G). Finally, NNC 55-0396 (10 μM) and mibefradil (30 μM) suppressed the amplitude of the undecanal-evoked Ca^{2+} signals by $85 \pm 23\%$ (four experiments) and $74 \pm 34\%$ (three experiments), respectively (Figure 1H). Other odorants like cyclamal ($K_{1/2} = 318 \pm 138 \mu\text{M}$, four experiments) and helional ($K_{1/2} = 98 \pm 15 \mu\text{M}$, four experiments) also evoked Ca^{2+} signals that were inhibited by

Figure 1 Odorant-induced Ca^{2+} signals in human sperm. (A) Ca^{2+} signals induced by bourgeonal. $\Delta F/F_0$ (%) indicates the percent change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before application of bourgeonal. (B) Dose-response relationship of the signal amplitudes from (A) ($K_{1/2} = 65 \mu\text{M}$). (C) Bourgeonal-induced Ca^{2+} signals in the presence of the CatSper channel inhibitors NNC 55-0396 and mibefradil. (D) Ca^{2+} signals induced by bourgeonal in non-capacitated sperm. (E) Ca^{2+} signals induced by bourgeonal and simultaneous application of bourgeonal and undecanal. (F) Ca^{2+} signals induced by undecanal. (G) Dose-response relationship of the signal amplitudes from (F) ($K_{1/2} = 30 \mu\text{M}$). (H) Undecanal-induced Ca^{2+} signals in the presence of the CatSper channel inhibitors NNC 55-0396 and mibefradil.

NNC 55-0396 and mibefradil (Supplementary Figure S4). These results suggest that diverse odorants activate CatSper channels and, thereby, stimulate Ca^{2+} entry.

Odorants enhance CatSper currents

To scrutinize these findings by an independent technique, we recorded membrane currents from human sperm by whole-cell patch clamping. The pipette solution did neither include ATP nor GTP to prevent metabotropic signalling via G proteins. In standard extracellular solution containing Ca^{2+} and Mg^{2+} (HS), little or no currents were evoked by stepping the membrane voltage (V_m) from 0 to ± 80 mV (Figure 2; Lishko *et al*, 2010, 2011; Strünker *et al*, 2011). However, monovalent CatSper currents could be recorded in divalent-free solution (NaDVF) (Figure 2; Lishko *et al*, 2010, 2011; Strünker *et al*, 2011). Mean inward currents at -60 mV were -96 ± 58 pA, range -30 to -236 pA (20 experiments). Bourgeonal ($80 \mu\text{M}$) potentiated monovalent currents by 2.3 ± 0.4 -fold (-60 mV) and by 1.8 ± 0.3 -fold ($+60$ mV) (six experiments) (Figure 2A and B). The reversal potential (V_{rev}) of monovalent CatSper currents and bourgeonal-evoked currents was indistinguishable (26.5 ± 5 mV, eight experiments each; Figure 2B). Moreover, mibefradil ($30 \mu\text{M}$) completely inhibited both CatSper and bourgeonal-evoked currents (Figure 2A and B). Undecanal ($50 \mu\text{M}$) also potentiated monovalent currents by 2.1 ± 0.2 -fold (-60 mV) and by 1.7 ± 0.2 -fold ($+60$ mV) (six experiments) (Figure 2C and D). Currents evoked by undecanal displayed a V_{rev} of 27.5 ± 5 mV (seven experiments),

similar to the V_{rev} of CatSper, and were completely inhibited by mibefradil (Figure 2C and D). We conclude that CatSper mediates the odorant-induced Ca^{2+} influx in human sperm.

Odorants do not activate a cAMP-signalling pathway

We examined whether bourgeonal stimulates the synthesis of cAMP as predicted by an olfactory signalling pathway. Bourgeonal failed to elevate cAMP levels in human sperm (Figure 3A; Veitinger *et al*, 2011). We scrutinized our experimental conditions and entertained several eventualities that might explain this unexpected failure. Different measures that elevate cAMP levels in mammalian sperm also enhance cAMP levels under our experimental conditions. In high (25 mM) HCO_3^- medium, the inhibition of cAMP hydrolysis by isobutylmethylxanthine (IBMX) and the stimulation by adenosine elevated cAMP levels by 7-fold and 4.5-fold, respectively (Figure 3A). A further increase of HCO_3^- , which potentially ($K_{1/2} \sim 10$ mM) stimulates a soluble AC (sAC; Litvin *et al*, 2003; Kamenetsky *et al*, 2006), did not elevate cAMP (Figure 3A), indicating that sAC is either fully activated or desensitized in the high HCO_3^- medium. In fact, sperm bathed in low (4 mM) HCO_3^- and stimulated with 50 mM HCO_3^- displayed a sizeable 4-fold cAMP increase (Figure 3B). Low extracellular $[\text{Ca}^{2+}]$ ($< 1 \mu\text{M}$) did not affect resting cAMP levels (Figure 3B), but abolished activation of the sAC by HCO_3^- (Figure 3B; Carlson *et al*, 2007).

In kinetic experiments, we studied the time course of the HCO_3^- -stimulated cAMP synthesis. cAMP levels commenced

Figure 2 Electrophysiological characterization of whole-cell CatSper currents from human sperm. Currents were recorded at pH_i 7.3 in the absence of intracellular divalent ions. The membrane voltage was stepped from -80 to $+80$ mV in steps of 10 mV from a holding potential of 0 mV. (A) Currents in extracellular solution containing Mg^{2+} and Ca^{2+} (HS) and monovalent CatSper currents in divalent-free Na^+ -based bath solution (NaDVF); perfusion with $80 \mu\text{M}$ bourgeonal potentiated monovalent currents (NaDVF + Bg). CatSper currents and currents evoked by bourgeonal were completely blocked by $30 \mu\text{M}$ mibefradil (NaDVF + Bg + Mib). (B) Current-voltage relationship from (A). (C) Currents in solution containing Ca^{2+} and Mg^{2+} (HS) and CatSper currents in divalent-free solution (NaDVF). Perfusion with $50 \mu\text{M}$ undecanal potentiated monovalent currents (NaDVF + Un). Currents were completely blocked by $30 \mu\text{M}$ mibefradil (NaDVF + Un + Mib). (D) Current-voltage relationship from (C).

Figure 3 Bourgeonal does not activate a cAMP-signalling pathway. **(A)** Changes in total cAMP concentration in human sperm bathed in high (25 mM) bicarbonate (HCO₃⁻) evoked by (in mM): 50 HCO₃⁻, 0.5 IBMX; (in μ M) 50 bourgeonal, and 50 adenosine (5 measurements from 2 donors). The red lines indicate the 95% confidence intervals (CIs). Overlapping CIs indicate that there is no significant difference between conditions. **(B)** cAMP changes in sperm bathed in low (4 mM) bicarbonate evoked by (in mM): 50 HCO₃⁻, 0.5 IBMX; (in μ M) 50 bourgeonal, 50 adenosine, 30 chloro-adenosine, 500 SQ22536, 100 MDL12330A; (in nM) 100 FPP, 10 angiotensin II, 1.5 calcitonin (5–133 measurements from 4 to 16 donors). Data comprise data from Strünker *et al* (2011) combined with data from additional experiments.

to rise with a latency of >2 s that probably reflects the time required for HCO₃⁻ to enter the cell. Within 60 s cAMP rose to a peak and then slowly declined to lower levels (Supplementary Figure S5). After 25 min, cAMP levels were still 2–3-fold elevated. In sperm that were bathed for $\geq$ 2 h in 4 mM or 25 mM HCO₃⁻, resting cAMP levels were similar (0.082 ± 0.042 pmol cAMP/10⁶ sperm, 310 measurements from 16 donors, versus 0.031 ± 0.024 pmol cAMP/10⁶ sperm, 7 measurements from 2 donors), supporting the notion that SAC activity is downregulated via PKA by negative feedback (Nolan *et al*, 2004; Burton and McKnight, 2007). We were concerned that a similar negative feedback might hamper the activation of tmACs. However, bourgeonal also failed to stimulate cAMP synthesis in a low (4 mM) HCO₃⁻ medium (Figure 3B).

cAMP levels were determined >15 s after mixing sperm with bourgeonal. We wondered if a bourgeonal-evoked cAMP response is transient and cAMP levels decline to basal levels within 15 s. We tested for this caveat by inhibition of cAMP hydrolysis. In fact, simultaneous application of HCO₃⁻ and IBMX elevated cAMP levels beyond values reached by IBMX or HCO₃⁻ alone (Figure 3B; Supplementary Figure S5; Strünker *et al*, 2011). Importantly, the HCO₃⁻/IBMX-induced cAMP increase persisted on a higher level for at least 25 min (Supplementary Figure S5). In contrast, the cAMP increase was similar in sperm stimulated either by IBMX alone or by bourgeonal/IBMX (Figure 3B), arguing against a transient cAMP response stimulated by bourgeonal.

Support for an olfactory cAMP-signalling pathway rested primarily on pharmacological studies using two inhibitors of tmACs—SQ22536 and MDL12330A (Spehr *et al*, 2003, 2004; Veitinger *et al*, 2011). We examined whether these drugs affect cAMP levels. Incubation of sperm with SQ22536 or MDL12330A did not lower resting cAMP levels (Figure 3B). Surprisingly, SQ22536 rather elevated cAMP (Figure 3B). There is also evidence in mouse sperm that SQ22536 elevates cAMP. The drug accelerates the flagellar beat (Schuh *et al*, 2006), which is another, yet indirect, measure of a rise of cAMP.

Several other ligands for GPCRs do also not stimulate cAMP signalling

We also tested other ligands—angiotensin, calcitonin, fertilization promoting peptide (FPP), and adenosine—that reportedly activate cAMP signalling in mouse sperm via GPCRs (Fraser and Duncan, 1993; Adeoya-Osiguwa *et al*, 1998; Adeoya-Osiguwa and Fraser, 2003; Mededovic and Fraser, 2004; Burnett *et al*, 2010). In low (4 mM) HCO₃⁻ medium, angiotensin, calcitonin, and FPP did not elevate cAMP levels (Figure 3B). In contrast, adenosine and chloro-adenosine elevated cAMP levels by 6.7- and 3.2-fold, respectively (Figure 3B). Whether the cAMP increase by adenosine involves GPCRs is unclear (Burnett *et al*, 2010). The tmAC inhibitors SQ22536 and MDL12330A did not suppress the cAMP increase evoked by adenosine in mouse (Schuh *et al*, 2006) and in human sperm (Figure 3B). Moreover, the combination of IBMX and adenosine or chloro-adenosine did not elevate cAMP levels beyond the level reached by each substance alone (Figure 3B). We conclude that adenosine and chloro-adenosine inhibit PDEs rather than activate GPCRs. A similar conclusion has been reached for the action of adenosine in mouse sperm (Schuh *et al*, 2006, 2007). In summary, besides odorants, various other agonists thought to control cAMP signalling via GPCRs, in fact, do not elevate cAMP levels in human sperm. Angiotensin, calcitonin, and FPP also did not evoke Ca²⁺ signals in human sperm (Supplementary Figure S6). Most importantly, also adenosine did not elevate [Ca²⁺]_i (Supplementary Figure S6), supporting the notion that cAMP does not stimulate Ca²⁺ entry (Strünker *et al*, 2011).

Activation of CatSper by odorants does not involve a metabotropic pathway

Notwithstanding the failure of bourgeonal and tmAC inhibitors to elevate and lower cAMP levels, respectively, we examined whether SQ22536 and MDL12330A impair bourgeonal-induced Ca²⁺ signals. In a previous report, incubation of sperm with SQ22536 (200 μ M) completely inhibited bourgeonal-evoked Ca²⁺ signals (Veitinger *et al*, 2011).

Figure 4 Pharmacology of inhibitors for transmembrane adenylyl cyclases (tmACs) and phospholipase C (PLC). (A) Ca^{2+} signals induced by bourgeonal in the presence of the tmAC inhibitor SQ22536. (B) Mean amplitude of bourgeonal-induced Ca^{2+} signals in the absence and presence of 200 μ M SQ22536 (four experiments). (C–E) Ca^{2+} signals induced by bourgeonal (C), progesterone (D) and ammonium chloride (E) in the presence of the tmAC inhibitor MDL12330A. (F) Relative inhibition of the signal amplitudes by 100 μ M MDL12330A (≥ 4 experiments). (G) Whole-cell membrane currents in extracellular solution containing Ca^{2+} and Mg^{2+} (HS). Monovalent CatSper currents in divalent-free Na^+ -based bath solution before (NaDVF) and after intracellular alkalization by ammonium chloride (10 mM) (NaDVF + NH_4Cl) in the absence and presence of MDL12330A (100 μ M). MDL12330A completely inhibited basal and alkaline-activated monovalent CatSper currents. Currents were recorded at pH_i 7.3 in the absence of intracellular divalent ions. (H) Ca^{2+} signals induced by the PLC inhibitor U73122.

We failed to reproduce this result: Ca^{2+} signals were similar in the absence and presence of SQ22536 (Figure 4A and B); SQ22536 itself did not change $[\text{Ca}^{2+}]_i$ at concentrations $\leq 400 \mu\text{M}$ (Supplementary Figure S7A). In the presence of MDL12330A (100 μM), the amplitude of the bourgeonal-evoked Ca^{2+} signal was strongly attenuated ($82 \pm 26\%$, seven experiments) (Figure 4C and F). In contrast to SQ22536, MDL12330A affected $[\text{Ca}^{2+}]_i$ on its own (Supplementary Figure S7B). MDL12330A not only inhibits tmACs, but is also known to block Ca_v channels, TRP channels, and store-operated Ca^{2+} channels (Lee *et al*, 1985; Palfreyman *et al*, 1989; Rampe *et al*, 1989). The drug has been widely used as a tool to directly inhibit Ca^{2+} entry in somatic cells (Rampe *et al*, 1987; van Rossum *et al*, 2000; Snetkov *et al*, 2001; Wang, 2003). We wondered whether MDL12330A also blocks CatSper. The CatSper channel is directly activated by progesterone and intracellular alkalization (Lishko *et al*, 2010, 2011; Strünker *et al*, 2011). MDL12330A suppressed Ca^{2+} signals evoked both by progesterone (Figure 4D and F; $97 \pm 2\%$ inhibition, four experiments) and by intracellular alkalization (Figure 4E and F; $93 \pm 3\%$ inhibition, four experiments). The constant of half-maximal inhibition of alkaline-evoked Ca^{2+} signals was $19.2 \pm 8.2 \mu\text{M}$ (three experiments) (Supplementary Figure S7C and D). Moreover, in patch-clamp experiments, MDL12330A completely abolished CatSper currents (Figure 4G). Thus, MDL12330A and SQ22536 are not suitable to probe cAMP-mediated Ca^{2+} signalling in sperm: suppression of odorant-evoked Ca^{2+} signals by MDL12330A reflects inhibition of CatSper channels rather than inhibition of tmACs; and SQ22536 does not inhibit bourgeonal-evoked Ca^{2+} signals and elevates rather than lowers cAMP levels. In summary, CatSper activation by bourgeonal does not involve tmACs and cAMP.

It has been reported that olfactory receptors can activate phospholipase C (PLC; Klasen *et al*, 2010). Therefore, we wondered whether odorants might act via a PLC rather than a tmAC pathway. The PLC inhibitor U73122 has been used at micromolar concentrations to interfere with thermotaxis and the acrosome reaction in human sperm (Bahat and Eisenbach, 2010; Chavez *et al*, 2011). However, at nanomolar concentrations ($K_{1/2} = 0.98 \pm 1.1 \mu\text{M}$, three experiments), the drug itself evoked Ca^{2+} signals in human (Figure 4H; Supplementary Figure S7E) and in mouse sperm (Chavez *et al*, 2011). U73122 is a steroid derivative that might directly activate CatSper channels. Moreover, U73122 is not very specific; in somatic cells, the drug inhibits Ca^{2+} pumps, releases Ca^{2+} from intracellular stores, activates and inhibits ion channels, and binds to GPCRs (Mogami *et al*, 1997; Hughes *et al*, 2000; Macmillan and McCarron, 2010). Thus, U73122—like MDL12330A and SQ22536—is ill suited to investigate Ca^{2+} signalling in sperm. Consequently, we did not test whether the drug inhibits bourgeonal-induced Ca^{2+} signals.

We further investigated the signalling pathway for bourgeonal by kinetic Ca^{2+} fluorimetry and patch-clamp recordings. Cellular responses involving metabotropic pathways often display a latency. For example, olfactory neurons respond to odorants with a latency of ~ 200 ms (Firestein and Werblin, 1989; Lowe and Gold, 1993; Kaupp, 2010). However, there are notable examples of ultrafast metabotropic signalling pathways: for example, phototransduction in insect photoreceptors involving a PLC occurs within < 10 ms (Gonzalez-Bellido *et al*, 2011). Such almost instantaneous responses usually involve ionotropic chemoreceptors or mechanoreceptors (Gillespie and Muller, 2009; Kaupp, 2010). We determined the latency of bourgeonal-induced Ca^{2+} signals by rapid mixing of odorants and sperm in a stopped-flow

Figure 5 Activation of CatSper by odorants does not involve a metabotropic pathway. (A) Bourgeonal-induced Ca^{2+} signals recorded in a stopped-flow apparatus. (B) Ca^{2+} signals from (A) shown on an extended time scale. Intracellular Ca^{2+} concentration rose within time resolution of the stopped-flow apparatus (36.6 ms). (C) Whole-cell membrane currents in extracellular solution containing Ca^{2+} and Mg^{2+} (HS). Monovalent CatSper currents in divalent-free Na^+ -based bath solution before (NaDVF) and after perfusion with 50 μ M bourgeonal (NaDVF + Bg). Currents were recorded at pH_i 7.3 in the absence of intracellular divalent ions. The pipette solution contained GDP β S (250 μ M) to preclude activation of G proteins.

apparatus (Figure 5A and B). After mixing with bourgeonal, $[\text{Ca}^{2+}]_i$ rose with no detectable latency (time resolution of the mixing device was 36.6 ms) (Figure 5B), arguing against an olfactory pathway and indicating that bourgeonal directly activates CatSper.

To rule out that CatSper is activated by a GPCR pathway, we recorded monovalent CatSper currents in the presence of intracellular GDP β S (250 μ M), which has been shown to almost completely abolish G protein action on ion channels and tmACs (Eckstein *et al*, 1979; Holz *et al*, 1986; Cuevas and Adams, 1997; Nörenberg *et al*, 1997; Wu and Barish, 1999; So and Kim, 2003; Gerevich *et al*, 2005; Aman *et al*, 2007; Drdla *et al*, 2009). However, GDP β S did neither change basal monovalent CatSper currents (-65 ± 27 pA at -60 mV; range -27 to -141 pA; 30 experiments) nor prevent stimulation of CatSper currents by odorants (Figure 5C; Supplementary Figure S8). In the presence of the drug, bourgeonal and undecanal potentiated CatSper currents at -60 mV by 1.9 ± 0.4 -fold (four experiments) and 1.7 ± 0.2 -fold (eight experiments), respectively. Similarly, GDP β S did not prevent activation of CatSper by progesterone and PGE1 (Supplementary Figure S8). In summary, odorants directly activate CatSper without involving a metabotropic pathway and synthesis of second messengers.

Analogues of cyclic nucleotides activate human CatSper

cAMP enhances the frequency of the flagellar beat (Esposito *et al*, 2004; Schuh *et al*, 2006; Xie *et al*, 2006). It is contentious, however, whether the control of the flagellar beat by cAMP involves a rise of $[\text{Ca}^{2+}]_i$. Elevation of cAMP levels in mouse and human sperm by either HCO_3^- or adenosine, or by photorelease of cAMP from caged cAMP fails to increase $[\text{Ca}^{2+}]_i$ (Wennemuth *et al*, 2003; Carlson *et al*, 2007; Strücker *et al*, 2011). In contrast, extracellular application of 8-Br-cAMP and 8-Br-cGMP stimulates an increase of $[\text{Ca}^{2+}]_i$ (Kobori *et al*, 2000; Ren *et al*, 2001; Liu *et al*, 2007; Xia *et al*, 2007; Machado-Oliveira *et al*, 2008; Xia and Ren, 2009) that is abolished in CatSper $^{-/-}$ mice (Ren *et al*, 2001; Xia *et al*, 2007; Xia and Ren, 2009), suggesting that Ca^{2+} enters via CatSper and that CatSper is controlled by cyclic nucleotides. How can we reconcile the two sets of conflicting results?

We examined whether 8-Br-cNMPs stimulate Ca^{2+} signals in human sperm (Figure 6A and B). 8-Br-cGMP and 8-Br-cAMP evoked a rapid and transient Ca^{2+} signal at concentrations ≤ 3 and 10 mM, respectively; at higher concentrations,

the transient was followed by a sustained elevation of $[\text{Ca}^{2+}]_i$. At 30 nM extracellular $[\text{Ca}^{2+}]$, the Ca^{2+} signal evoked by 8-Br-cGMP was abolished (Supplementary Figure S9). The $K_{1/2}$ for 8-Br-cGMP was 7.9 ± 1.8 mM (three experiments) (Figure 6C). 8-Br-cAMP was less potent, which prevented us from determining a complete dose-response curve. The Ca^{2+} signals were suppressed by NNC 55-0396 and mibefradil (Figure 6D–F), indicating that Ca^{2+} enters the cell via CatSper. As a control, neither cAMP nor cGMP (≤ 1 mM) stimulated Ca^{2+} influx (Figure 7A and B), indicating that CatSper is not activated by physiologically relevant concentrations of extracellular cAMP and cGMP. ATP (≤ 1 mM) also failed to stimulate Ca^{2+} signals (Figure 7C and D), and in patch-clamp experiments, ATP failed to evoke membrane currents (Figure 7E). This indicates that CatSper is insensitive to ATP and that human sperm do not express functional P2X2 receptors that exist in mouse sperm (Navarro *et al*, 2011).

We studied the action of 8-Br-cGMP on CatSper currents (Figure 8). 8-Br-cGMP enhanced monovalent currents. The V_{rev} of CatSper currents (30 ± 3 mV, nine experiments) and currents evoked by 8-Br-cGMP (28.6 ± 3 mV, nine experiments) was similar. Mibefradil blocked the currents completely (Figure 8A and B), indicating that 8-Br-cNMPs activate CatSper. We tested whether 8-Br-cGMP acts from the inside or from the outside. Currents in standard HS and divalent-free solutions (NaDVF) recorded in the presence of 5 mM cGMP (Figure 8C and D) or 8-Br-cGMP (Figure 8E and F) in the pipette solution were similar to control currents recorded in the absence of intracellular cyclic nucleotides (Figure 8G). However, superfusion of sperm with 8-Br-cGMP enhanced monovalent CatSper currents, no matter whether cGMP or 8-Br-cGMP was included in the pipette solution (Figure 8C–F). Under all conditions tested, currents evoked by extracellular 8-Br-cGMP were completely blocked by mibefradil (Figure 8A–G). In sperm stimulated with 8-Br-cGMP, $[\text{Ca}^{2+}]_i$ rose without detectable latency (< 36.6 ms) and 8-Br-cGMP potentiated CatSper currents also in the presence of intracellular GDP β S (Supplementary Figure S9C). Thus, 8-substituted analogues of cyclic nucleotides directly activate CatSper channels through a site that is accessible from the extracellular space only. This explains why extracellular cNMP analogues but not intracellular cAMP elevate $[\text{Ca}^{2+}]_i$. Moreover, this result supports the notion that CatSper is promiscuously activated by diverse small molecules.

Figure 6 Ca^{2+} signals evoked by 8-Bromo analogues of cyclic nucleotides. Signals induced by 8-Br-cGMP (A) or 8-Br-cAMP (B). (C) Dose-response relationship for 8-Br-cGMP based on the signal amplitudes from (A) ($K_{1/2} = 6.5$ mM). (D) 8-Br-cGMP-induced Ca^{2+} signals in the presence of the CatSper inhibitors NNC 55-0396 or mibefradil. (E) 8-Br-cAMP-induced Ca^{2+} signals in the presence of NNC 55-0396 or mibefradil. (F) Relative inhibition of the signal amplitudes by NNC 55-0396 and mibefradil. Inhibition of signal amplitudes > 100% indicates that 8-Br-cGMP evoked a decrease in $[\text{Ca}^{2+}]_i$ in the presence of the inhibitors.

Figure 7 Cyclic nucleotides and ATP do not stimulate Ca^{2+} influx. (A) Time course of $[\text{Ca}^{2+}]_i$ after stimulation with cAMP and cGMP. (B) Signal amplitudes evoked by cAMP, cGMP, and ammonium chloride (NH_4Cl) (four experiments). (C) Time course of $[\text{Ca}^{2+}]_i$ after stimulation with ATP. (D) Signal amplitudes induced by ATP and NH_4Cl (four experiments). (E) Whole-cell membrane currents in extracellular solution containing Ca^{2+} and Mg^{2+} (HS) before and after perfusion with ATP (100 μM). ATP did not stimulate membrane currents. Monovalent CatSper currents in divalent-free Na^+ -based bath solution (NaDVF) served as a positive control. Currents were recorded at pH 7.3 in the absence of intracellular divalent ions.

Even ligands for TRPM8 channels that evoke Ca^{2+} entry in human sperm (De Blas *et al*, 2009) might target CatSper. The TRPM8 agonist menthol stimulates a rapid Ca^{2+} signal (De Blas *et al*, 2009; Supplementary Figure S10A) that is inhibited by NNC 55-0396 and mibefradil (Supplementary Figure S10B and C). In support of the notion that CatSper mediates menthol-induced Ca^{2+} entry, icilin, a potent TRPM8 agonist ($K_{1/2} = 0.36$ μM ; McKemy *et al*, 2002), did not elevate

$[\text{Ca}^{2+}]_i$ in human sperm at concentrations up to 100 μM (Supplementary Figure S10D).

Discussion

Chemicals as diverse as steroids, prostaglandins, odorants, menthol, and analogues of cyclic nucleotides activate the human CatSper channel without involving metabotropic

Figure 8 Whole-cell CatSper currents are potentiated by extracellular 8-Br-cGMP. Currents were recorded at pH_i 7.3 in the absence of intracellular divalent ions. Voltage was stepped from -80 to +80 mV in steps of 10 mV from a holding potential of 0 mV. (A) Currents in extracellular solution containing Ca²⁺ and Mg²⁺ (HS) and monovalent CatSper currents in divalent-free Na⁺-based bath solution (NaDVF). Extracellular 8-Br-cGMP (5 mM) potentiated monovalent currents (NaDVF + 8-Br-cGMP). Currents were completely blocked by 30 μM mibefradil (NaDVF + 8-Br-cGMP + Mib). (B) Current-voltage relationship from (A). (C) Currents recorded as described in (A), with 5 mM cGMP included in the pipette solution. (D) Current-voltage relationship from (C). (E) Currents recorded as described in (A), with 5 mM 8-Br-cGMP included in the pipette solution. (F) Current-voltage relationship from (E). (G) Mean current amplitudes at -60 mV (4–10 experiments) recorded in Ca²⁺ and Mg²⁺ containing solution (HS), in divalent-free solution (NaDVF), after perfusion with 5 mM 8-Br-cGMP (NaDVF + 8-Br-cGMP), and after perfusion with 5 mM 8-Br-cGMP and 30 μM mibefradil (NaDVF + 8-Br-cGMP + Mib). The currents were recorded either in the absence or in the presence of 5 mM cGMP or 8-Br-cGMP in the pipette solution. Extracellular but not intracellular application of cyclic nucleotides potentiated CatSper currents.

signalling pathways. Activation happens through promiscuous, extracellularly accessible site(s) either on the channel itself or on associated proteins. Our results resolve several controversial issues and have important bearings on future studies of Ca²⁺ signalling in sperm.

Are there tmACs in sperm?

For more than two decades, ligands of GPCRs including odorants have been proposed to control sperm via cAMP-signalling pathways. However, a growing body of evidence now suggests that mouse and human sperm, in fact, are lacking functional tmACs. First, several tmAC^{-/-} mice (AC1^{-/-}, AC5^{-/-}, AC6^{-/-}, and AC8^{-/-}) display no obvious fertility phenotype. Although tmAC3^{-/-} mice are subfertile (Livera *et al*, 2005), subfertility might reflect faulty sperm development (Livera *et al*, 2005) and mating behaviour (Wang *et al*, 2006). Second, forskolin and its congeners fail to elevate cAMP levels (Aitken *et al*, 1986; Rojas and Bruzzone, 1992; Rojas *et al*, 1993; Jaiswal and Conti, 2003; Hess *et al*, 2005; Strünker *et al*, 2011); see however Baxendale and Fraser (2003) and Livera *et al* (2005). In countless studies of many cell types from worm to humans, forskolin has proven its worth as a powerful and reliable stimulator of most if not all tmAC isoforms (Kamenetsky *et al*, 2006). Therefore, the failure needs to be taken seriously. Third, in sperm lacking the sAC, no cAMP synthesis can be detected, cAMP levels are below detection threshold (Xie *et al*, 2006), and inhibition of cAMP hydrolysis by IBMX fails to increase flagellar beat frequency (Schuh *et al*, 2006). Moreover, in tmAC3^{-/-} and wild-type sperm, basal cAMP levels are similar (Livera *et al*, 2005). Finally, several ligands for GPCRs that reportedly activate tmACs in mouse sperm (Fraser and Duncan, 1993; Adeoya-Osiguwa *et al*, 1998; Adeoya-Osiguwa and Fraser, 2003; Mededovic and Fraser, 2004), in fact, fail to raise cAMP levels in human sperm. Altogether, these observations are hard to reconcile with the presence of functional tmACs in sperm. Alternatively, tmACs together with PDE, PKA, and its substrates might be organized in sparse signalling complexes. Such local cAMP signalling might escape detection by techniques that measure total cAMP content. For the time being, until local cAMP signalling in sperm has been unequivocally demonstrated with spatio-temporal resolution, we argue that cAMP levels in both mouse and human sperm are controlled by sAC (Schuh *et al*, 2006; Xie *et al*, 2006).

Does cAMP stimulate Ca²⁺ entry?

Another issue concerns the relationship between cAMP and Ca²⁺ entry. Membrane-permeable analogues of cyclic nucleotides stimulate Ca²⁺ entry in mouse and human sperm (Kobori *et al*, 2000; Ren *et al*, 2001; Liu *et al*, 2007; Xia *et al*, 2007; Machado-Oliveira *et al*, 2008; Xia and Ren, 2009). In contrast, several measures that increase intracellular cAMP levels—HCO₃⁻, IBMX, photorelease from caged cAMP, and adenosine—fail to stimulate a Ca²⁺ influx (Wennemuth *et al*, 2003; Carlson *et al*, 2007; Strünker *et al*, 2011; Supplementary Figure S6). Our finding that extracellular 8-Br-cGMP and 8-Br-cAMP, but not intracellular cAMP and cGMP, directly activate human CatSper provides an explanation for these seemingly contradictory findings. However, there might be differences among mammalian species. In mouse sperm, no direct action of 8-Br-cNMPs on CatSper was observed (Kirichok *et al*, 2006). Different experimental conditions and drug concentra-

tions might account for this difference. Alternatively, mouse CatSper might not be directly activated by cyclic nucleotide analogues. Such a species difference has been shown for progesterone, which activates human but not mouse CatSper (Lishko *et al*, 2011). Moreover, in bovine sperm, intracellular photorelease of cNMP derivatives in the flagellum evokes a rapid and robust Ca^{2+} influx (Wiesner *et al*, 1998), and in sperm of marine invertebrates, a well-established cGMP-signalling pathway controls Ca^{2+} entry (Kaupp *et al*, 2003; Matsumoto *et al*, 2003; Strünker *et al*, 2006; Darszon *et al*, 2008; Bönigk *et al*, 2009). In summary, there is no evidence that a rise of cyclic nucleotides elevates $[\text{Ca}^{2+}]_i$ at least in mouse and human sperm.

How many different Ca^{2+} -permeable channels exist in sperm?

Another issue is concerned with the type of Ca^{2+} channels that mediate Ca^{2+} entry. Four different channel types—CatSper, CNG channels, various voltage-activated Ca_v channels, and TRP channels—have been proposed to pass Ca^{2+} into sperm (Publicover *et al*, 2007; Darszon *et al*, 2011). However, only CatSper has been shown to be required for fertilization: Mice deficient in either one of the pore-forming subunits (CatSper 1–4) or the auxiliary CatSper δ subunit are infertile due to impaired Ca^{2+} signalling and motility (Qi *et al*, 2007; Chung *et al*, 2011). In humans, mutations in *CatSper* genes have been correlated with male infertility (Hildebrand *et al*, 2010). Finally, CatSper is the only voltage-activated Ca^{2+} channel that can be detected by patch-clamp recordings from ejaculated human and epididymal mouse sperm (Kirichok *et al*, 2006; Navarro *et al*, 2008; Xia and Ren, 2009; Lishko *et al*, 2010, 2011; Kirichok and Lishko, 2011; Strünker *et al*, 2011). Currents carried by other Ca_v channels have only been recorded from precursor cells or testicular sperm (Martinez-Lopez *et al*, 2009; Xia and Ren, 2009). The only other Ca^{2+} -permeable ion channel that has been functionally characterized is a purinergic P2X2 receptor present in mouse sperm (Navarro *et al*, 2011). However, this purinergic channel is functionally absent in human sperm.

Pharmacology of sperm

The study of signalling pathways in sperm has heavily rested upon pharmacological tools. Our results regarding three inhibitors and a GPCR agonist are alarming. Adenosine, which activates adenosine receptors in somatic cells, probably inhibits PDE activity and thereby elevates cAMP. SQ22536, a tmAC inhibitor, enhances rather than lowers cAMP levels probably also by inhibiting PDE activity. MDL12330A, another tmAC inhibitor, potentially blocks CatSper channels. U73122, a PLC inhibitor used at micromolar concentrations for motility assays, provokes on its own sizeable Ca^{2+} signals at nanomolar concentrations. In Ca^{2+} fluorimetry, but not patch clamping, also the pharmacology of CatSper inhibitors is complex, because at high concentrations, these drugs evoke Ca^{2+} signals on their own. These examples caution against rash interpretations of results obtained by pharmacological intervention, even more so, if drug concentrations are used that are orders of magnitude larger than those required to specifically inhibit the function of the respective target. The problem is particularly severe in sperm: because of the miniscule flagellar volume, even minute

changes in the activity of a few CatSper channels or Ca^{2+} transport systems can evoke sizeable Ca^{2+} signals.

CatSper might be a polymodal sensor for chemical cues

We show that the CatSper channel complex or associated protein(s) can accommodate diverse chemicals. Therefore, we suspect that ligands thought to act via GPCRs or ligand-gated ion channels, in fact, activate CatSper. The potency of structurally diverse CatSper agonists (Supplementary Figure S11) differs by orders of magnitude, ranging from nanomolar to millimolar concentrations (Figure 9). Notably, the physiological factors progesterone and prostaglandins represent by far the most potent CatSper agonists. However, we note that CatSper activation by menthol, cyclamal, and helional needs to be confirmed by patch-clamp recordings. Although the activation of CatSper by odorants, 8-Br-cNMPs, or menthol is physiologically irrelevant, it points to a potential source of artifacts that needs to be considered in future studies when testing sperm for native factors from the oviduct.

Promiscuous binding sites for small ligands exist in other ion channels. For example, the cardiac human ether-a-go-go-related-gene K^+ channel (HERG) is inhibited by structurally diverse molecules that bind to a few amino-acid residues in transmembrane segment S6 (Zou *et al*, 2010; Windisch *et al*, 2011). Crystal structures of acetylcholine receptors reveal a common binding site for general anaesthetics. The site hosts functionally and structurally diverse compounds at different depths along the binding cavity (Nury *et al*, 2011). Finally, the TRPV1 channel is modulated by different chemical and physical stimuli (Vriens *et al*, 2009; Baez-Nieto *et al*, 2011). The channel is directly activated by lipophilic compounds such as capsaicin and other vanilloids. Various lipids, such as conjugates of biogenic amines and oxygenated eicosatetraenoic acids, derived from the fatty-acid pool, also activate TRPV1. Moreover, TRPV1 is activated by acidic extracellular pH, heat, and membrane depolarization. In neurons of dorsal root ganglia, the polymodal TRPV1 channel integrates various noxious stimuli (Stucky *et al*, 2009).

We envisage a similar principle operating in CatSper channels: The complex regulation of CatSper by pH, voltage, and ligands might be functionally important. On their voyage across the oviduct, sperm are exposed to a series of chemical milieus that differ in viscosity, temperature, pH, HCO_3^- , progesterone, and possibly several other factors. These factors enable sperm to locate and fertilize the egg. We propose that CatSper is a polymodal ‘stimulus integrator’

Figure 9 Normalized dose–response relationships for various compounds that activate CatSper. Each data point represents the mean of $n \geq 3$.

that translates the chemical and physical code of each microenvironment into spatio-temporal Ca^{2+} patterns and, therefore, is key for navigation of sperm over long distances to reach the site of fertilization. It needs to be studied whether female factors act synergistically or even antagonistically on CatSper. Moreover, it needs to be studied whether agonists evoke stereotypic or unique motility responses.

Materials and methods

Materials and reagents

NNC 55-0396, mibefradil, U73122, SQ22536, and MDL12330A were purchased from Tocris (MN, USA). Bourgeonal was from Enzo Life Sciences (NY, USA). 8-Br-cAMP and 8-Br-cGMP were obtained from Biolog (Bremen, Germany). All other reagents were obtained from Sigma-Aldrich (MO, USA).

Sperm preparation

Human semen samples were obtained from healthy volunteers with their prior consent. Sperm were handled and purified as described (Strünker *et al*, 2011). In brief, sperm were purified by a 'swim-up' procedure in human tubular fluid (HTF⁺) 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, 2.78 glucose, 21 HEPES, and 25 NaHCO_3 adjusted to pH 7.3–7.4 with NaOH. After washing, 3 mg/ml human serum albumin (HSA) (Irvine Scientific, CA, USA) was added to the HTF⁺ (making it HTF⁺⁺). Before experiments, sperm were incubated for at least 2 h in HTF⁺⁺ at 37°C. Under these conditions, sperm become capacitated. All Ca^{2+} measurements and patch-clamp recordings were done with capacitated sperm unless stated otherwise. To obtain non-capacitated sperm, samples were purified and incubated in HTF lacking HCO_3^- and HSA.

Measurement of changes in $[\text{Ca}^{2+}]_i$

Changes in $[\text{Ca}^{2+}]_i$ were measured in sperm loaded with Fluo-4 (Invitrogen, OR, USA). Measurements were performed either in 384 multi-well plates (Greiner Bio-One, Frickenhausen, Germany) in a fluorescence plate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany) at 29°C or in a rapid-mixing device in the stopped-flow mode (SFM400; Bio-Logic, Claix, France) at 37°C (Kilic *et al*, 2009; Strünker *et al*, 2011). After loading, sperm were washed and resuspended in HTF⁺. Ligands or inhibitors were dissolved in HTF⁺. Sperm were incubated with NNC 55-0396, mibefradil, SQ22536, and MDL12330A for ~5 min prior to application of agonists.

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Patch-clamp recordings

We recorded from sperm in the whole-cell configuration as described (Strünker *et al*, 2011). Seals between pipette and sperm were formed either at the cytoplasmic droplet or in the neck region in standard extracellular solution (HS) containing (in mM): 135 NaCl, 5 KCl, 1 MgSO_4 , 1 CaCl_2 , 5 glucose, 1 Na-pyruvate, 10 lactic acid, 20 HEPES adjusted to pH 7.4 with NaOH. Monovalent 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 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 V_{rev} of CatSper currents was ~8 mV less positive as reported previously (Strünker *et al*, 2011), because we modified the composition of the intracellular solution (e.g., 130 mM Cs-aspartate instead of 121 mM Cs-methanesulphonate). Data were not corrected for liquid junction potentials.

Determination of cAMP content

Sperm in HTF⁺⁺ containing 25 or 4 mM NaHCO_3 were mixed 1:1 (v/v) with substances dissolved in the same buffer. The cAMP content was determined by RIAs (^{125}I -labelled cAMP; IBL, Germany) (Strünker *et al*, 2011).

Data analysis

Statistical analysis and fitting of the data were performed using Prism 5 (GraphPad Software, Inc.), OriginPro 8.1G SR3 (OriginLab Corporation), or Clampfit 10.2 (Molecular Devices). All data are given as mean ± standard deviation (number of experiments).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CB, NG, IW, NK, MN, MK, AM, and TS designed and performed experiments. TS and UBK conceived the project. TS and UBK wrote the manuscript. All authors revised and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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