Absolute proteomic quantification reveals design principles of sperm flagellar chemosensation

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Abstract

Cilia serve as cellular antennae that translate sensory information into physiological responses. In the sperm flagellum, a single chemoeffectant molecule can trigger a Ca2+ rise that controls motility. The mechanisms underlying such ultra-sensitivity are ill-defined. Here, we determine by mass spectrometry the copy number of nineteen chemosensory signaling proteins in sperm flagella from the sea urchin Arbacia punctulata. Proteins are up to 1,000-fold more abundant than the free cellular messengers cAMP, cGMP, H+, and Ca2+. Opto-chemical techniques show that high protein concentrations kinetically compartmentalize the flagellum: Within milliseconds, cGMP is relayed from the receptor guanylate cyclase to a cGMP-gated channel that serves as a perfect chemoelectrical transducer. cGMP is rapidly hydrolyzed, possibly via “substance channeling” from the channel to the phosphodiesterase PDE5. The channel/PDE5 tandem encodes cGMP turnover rates rather than concentrations. The rate-detection mechanism allows continuous stimulus sampling over a wide dynamic range. The textbook notion of signal amplification—few enzyme molecules process many messenger molecules—does not hold for sperm flagella. Instead, high protein concentrations ascertain messenger detection. Similar mechanisms may occur in other small compartments like primary cilia or dendritic spines.

Keywords cilia; electron tomography; fertilization; quantitative mass spectrometry; signaling

Subject Categories Neuroscience; Signal Transduction

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Introduction

Sensory systems command over exquisite sensitivity and precision. Photoreceptors in the eye can detect single photons (Baylor et al., 1979; Yau & Hardie, 2009; Gross et al., 2015); chemosensory neurons in the antennae of insects and vomero-nasal organs of mammals can detect a few or even single pheromone molecules (Kaislning, 1986; Leinders-Zufall et al., 2000); algae and sperm from marine invertebrates can register single chemoeffectant molecules (Boland et al., 1982; Starr et al., 1995; Kaupp et al., 2003). These sensory feats happen in specialized cellular compartments, called cilia or flagella: long, slender filaments that emanate from the cell body (Marshall & Basto, 2017). Cilia and flagella serve as antennae that translate chemical and physical stimuli into electrical signals that eventually evoke a cellular or behavioral response. In recent years, a concept of physiological significance has emerged that cilia and flagella are specialized cell organelles, whose ionic milieu and inventory of signaling proteins are unique (Corbit et al., 2005; DeCaen et al., 2013; Delling et al., 2013; Kaupp & Strünker, 2017; Balbach et al., 2018). Factors that may contribute to the exquisite sensory sensitivity of cilia or flagella are the inventory, concentrations, and topographical arrangement of signaling proteins, and the compartmentalization of cellular reactions. How each factor contributes to ultra-sensitivity and plays out in the minuscule reaction volume is largely unknown.

Here, we provide the first absolute quantification of the inventory of a flagellar chemosensory signaling pathway from sperm of the sea urchin Arbacia punctulata; this pathway controls chemotactic steering (Kaupp & Strünker, 2017). It is an optimal model system that meets all requirements for reliable absolute quantification. Sperm constitute a homogenous cell population, and flagella can be prepared purely. Moreover, most signaling proteins are
sperm-specific; therefore, potential contamination by other cell types does not compromise quantification. Importantly, most signaling proteins have been identified—from the chemoattractant receptor to the Ca^{2+} channel that controls chemotactic steering. Absolute mass spectrometric quantification using the QconCAT (quantification concatemers) technology (Scott et al., 2016) reveals astoundingly high copy numbers of all 11 signaling proteins in the flagellum. Compared with a typical cell soma, the enzyme-to-substrate ratio is reversed by several orders of magnitude. Using kinetic opto-chemical techniques, we probed the physiological consequences for the dynamics of cellular messengers. Quantification of signaling proteins and cellular responses provides new insights into the molecular underpinnings of chemosensory ultra-sensitivity.

Results

The cGMP-signaling pathway

Chemosensation in the flagellum of sea urchin sperm involves changes in the membrane potential (\(V_m\)) and the intracellular concentration of cGMP, cAMP, Na^+, Ca^{2+}, and H^+ (Fig 1A). A signaling pathway encodes these cellular events (Fig 1B) (for reviews, see; Kaupp & Alvarez, 2016; Wachten et al., 2017). The pathway is triggered by resact, a chemoattractant peptide that binds to a chemoreceptor guanylate cyclase (GC) and stimulates cGMP synthesis (Garbers, 1976; Singh et al., 1988; Kaupp et al., 2003; Pichlo et al., 2014). The rise of cGMP opens K^+-selective cyclic nucleotide-gated (CNGK) ion channels (Strünker et al., 2006; Galindo et al., 2007; Böning et al., 2009). The ensuing hyperpolarization activates a voltage-gated Na^+/H^+ exchanger (sNHE) (Lee, 1985; Windler et al., 2018) and a hyperpolarization-activated, cyclic nucleotide-gated (HCN) pacemaker channel (Gauss et al., 1998; Galindo et al., 2005). Na^+/H^+ exchange increases pHi, which primes CatSper Ca^{2+} channels to open during the recovery from hyperpolarization probably driven by HCN channels that carry an inward current (Seifert et al., 2015; Espíñal-Enríquez et al., 2017). Changes in free Ca^{2+} concentration ([Ca^{2+}]_i) control the swimming path.

The chemoattractant also stimulates cAMP synthesis (Hansbrough & Garbers, 1981; Harumi et al., 1992; Kaupp et al., 2003), presumably by a soluble adenylate cyclase (sAC) (Vacquier et al., 2014). cAMP modulates the activity of sNHE and HCN channels, which both carry a cyclic nucleotide-binding domain (CNBD) (Gauss et al., 1998; Wang et al., 2003; Windler et al., 2018). For signal termination,
a cGMP-specific phosphodiesterase (PDE5) (Su & Vacquier, 2006) breaks down cGMP and, finally, a Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchanger (NCKX) (Su & Vacquier, 2002) and/or a plasma membrane Ca\(^{2+}\)-ATPase (PMCA) (Gunaratne et al, 2006) restore resting Ca\(^{2+}\) levels.

Mass spectrometric (MS) analysis of purified flagella from *A. punctulata* sperm identified all previously known signaling components, but also additional proteins that might be involved in the signaling pathway (Appendix Table S1): (i) the Ca\(^{2+}\)-activated Cl\(^-\) channel TMEM16 of oocytic cilia, which has been predicted to exist in sperm from pharmacological and modeling studies (Guererro et al, 2013; Espinal-Enriquez et al, 2017); (ii) a dual-specific PDE10 that, unlike PDE5, can also hydrolyze cAMP, and, together with sAC, may control cAMP metabolism; (iii) K\(^+\)/Cl\(^-\) PDE10 that, unlike PDE5, can also hydrolyze cAMP, and, together with sAC, may control cAMP metabolism; (iii) K\(^+\)/Cl\(^-\) co-transporters KCC1 (SLC12A4) and/or KCC3 (SLC12A5); (iv) a Na\(^+\)/HCO\(_3\)\(^-\) co-transporter (SLC4A11); (v) a sperm-specific Na\(^+\)/K\(^+\)-ATPase \(x\) subunit; and (vi) two members (TMC 5 and 7) of a mechano-sensitive channel family (Zhao & Müller, 2015; Pan et al, 2018). Sperm respond to mechanical stimulation with a Ca\(^{2+}\) response (Kambara et al, 2011). The TMC channels may endow sperm with mechano-sensitivity. Using antibodies directed against their mammalian homologues, several transmembrane adenylyl cyclases (tmAC 1, 2, 5, and 9) were detected in sea urchin sperm (Beltrán et al, 2007; Vacquier et al, 2014). Although we identified 45 different peptides for sAC, we could not detect peptides for tmACs.

For absolute quantification, we selected 11 signaling proteins shown in Fig 1B along with their subunits, a total of 19 polypeptides (Appendix Table S2). The newly discovered proteins molecules, except for PDE10 and Na\(^+\)/K\(^+\)-ATPase, were not considered for absolute quantification because their function in chemotactic signaling remains to be elucidated.

**Absolute quantification by QconCAT technology**

The abundance of proteins was quantified by selected reaction monitoring (SRM) using a synthetic, isotope-labeled protein as a standard (Rivers et al, 2007; Brownridge et al, 2011; Holman et al, 2012). For the synthesis of the standard protein, we selected three individual tryptic peptides from most of the 19 signaling proteins; a few proteins were covered by two or four peptides (Appendix Table S2). The presence of the peptides after trypsin digestion of the standard protein was verified by both MALDI-TOF and LC-ESI-MS/MS (SRM). These control experiments failed to retrieve three peptides (Appendix Table S2), which, therefore, were not used for quantification. Consequently, quantification of NCKX and CatSper3 rested on a single peptide, which might underestimate the protein abundance. For absolute quantification, we used the receptor GC, the most abundant protein of the flagellar membrane, as a reference protein. Previous work determined 200,000–400,000 GC molecules/flagellum by quantitative SRM (using a PSAQ-GC as a standard) (Pichlo et al, 2014), Coomassie Blue densitometry (Pichlo et al, 2014), and high-resolution electron tomography (Farci, 2017). We consider the GC density determined by electron tomography as the most precise estimate. Therefore, protein copy numbers were calculated assuming 400,000 GC molecules (Table 1); all protein ratios from SRM analyses are given in Dataset Table EV1. Moreover, as another internal benchmark of quantification, we included creatine kinase (CK) that, according to gel densitometry, is almost as abundant as the receptor GC (Pichlo et al, 2014). By SRM, we observed a GC:CK ratio close to unity, underscoring the high accuracy and suitability of using a synthetic labeled protein for MS determination of protein stoichiometries.

**Determination of flagellar volume by cryo-electron tomography**

For comparison, we calculated protein concentrations from copy numbers in the flagellum. The total volume inside motile cilia and flagella (\(V_t\)) contains three distinct compartments: the matrix volume \(V_{ma}\) that is accessible to diffusing or transported ciliary proteins, the volume occupied by the \([9 + 2]\) axonemal scaffold \(V_s\), and the lumen \(V_l\) of the nine doublet microtubules and the two singlet microtubules of the central pair complex. The microtubules are only accessible to small molecules like ions, nucleotides, and ligands (e.g., Taxol) (Fig 2). Thus, \(V_t = V_{ma} + V_s + V_l\), and the volume accessible to small molecules is \(V_{asm} = V_{ma} + V_l\) (Fig 2F and F\(^\circ\)). We measured these volumes using cryo-electron tomography reconstructions of rapidly frozen flagella for two evolutionary distant species, i.e., from the unicellular green algae *Chlamydomonas reinhardtii* and from sperm of the sea urchin *Strongylocentrotus purpuratus* (Carabajal-Gonzalez et al, 2013; Lin & Nicastro, 2018; preprint: Fu et al, 2019; Lin et al, 2019) (Fig 2A–D).

For calculating the \(V_t\), we measured the mean diameter (\(d\)) of intact flagella for *Chlamydomonas* as 247 ± 13 nm and for sea urchin sperm as 266 ± 13 nm (Fig 2E). Thus, the mean \(V_t\) per 1-µm flagellar length is for *Chlamydomonas* 0.0479 µm\(^3\) and for sea urchin sperm 0.0557 µm\(^3\); i.e., for an average length of sea urchin flagella of 40 µm, the total volume \(V_t\) is 2.2 fl (see Materials and Methods section for details). Overall, the sub-volumes per 1-µm flagellar length were very similar between the two species (Table 2). We used the matrix volume (for 40 µm long *S. purpuratus* flagella) \(V_{ma} = 1.7\) fl to calculate protein concentrations in Table 1. Surprisingly, volumes accessible to proteins \(V_{asm}\) and small molecules \(V_{asm}\) are 77 and 86% of the total volume.

**Signaling proteins are orders of magnitude more abundant than free messengers**

To compare protein abundance, Table 1 lists copy numbers, protein concentrations, and the densities of integral membrane proteins. We find that signaling proteins in the flagellum (Fig 1B) are up to 1,000-fold more abundant than the respective cellular messengers like H\(^+\), Ca\(^{2+}\), cAMP, and cGMP (Table 1). For example, the flagellum harbors 21,700 CNGK channels that are key targets of cGMP (Bönigk et al, 2009), and, at low and moderate chemoattractant stimulation of sperm, only ten to a few hundred cGMP molecules are synthesized by the receptor GC (Bönigk et al, 2009; Pichlo et al, 2014). The large number of CNGK channels suggests that this stage of signal transduction, i.e., capturing of cGMP, is highly efficient. The downstream targets of the CNGK channel, sNHE exchangers and HCN channels (Fig 1B) that are activated by hyperpolarization, are

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\(^a\)Correction added on 27 January 2020, after first online publication: “stoichiometry was” has been changed to “copy numbers were”.

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present at 54,000 and 28,000 molecules per flagellum, respectively. Remarkably, the sNHE is not involved in pH homeostasis at rest; only upon chemoattractant stimulation, it tightly couples membrane voltage and cAMP to generate rapid alkalization (latency only upon chemoattractant stimulation, it tightly couples membrane

| Proteins     | Molecules/Flagellum | Concentration (µM) | Density (molecules/µm²) |
|--------------|---------------------|--------------------|-------------------------|
| GC           | 400,000             | 400                | 11,976                  |
| CNGK         | 21,653 ± 4,038      | 21.7 ± 4           | 648                     |
| sNHE         | 53,979 ± 20,659     | 54.0 ± 20.7        | 1,616                   |
| sAC          | 6,718 ± 2,630       | 6.7 ± 2.6          | 201                     |
| HCN1         | 10,258 ± 901        | 10.3 ± 0.9         | 307                     |
| HCN2         | 18,337 ± 887        | 18.3 ± 0.9         | 549                     |
| CatSper1     | 1,853 ± 147         | 1.9 ± 0.1          | 56                      |
| CatSper2     | 2,125 ± 138         | 2.1 ± 0.1          |                         |
| CatSper3    | 587 ± 296           | 0.6 ± 0.3          |                         |
| CatSper4    | 1,208 ± 113         | 1.2 ± 0.1          |                         |
| CatSper β   | 1,690 ± 547         | 1.7 ± 0.5          |                         |
| CatSper γ   | 1,169 ± 1,395       | 1.2 ± 1.4          |                         |
| CatSper δ   | 2,071 ± 392         | 2.1 ± 0.4          |                         |
| PDE5         | 48,567 ± 6,525      | 48.6 ± 6.5         |                         |
| PDE10        | 4,211 ± 752         | 4.2 ± 0.8          |                         |
| NCKX         | 120,795 ± 41,520    | 120.8 ± 41.5       | 3,617                   |
| Ca²⁺-ATPase  | 1,991 ± 749         | 2 ± 0.7            | 60                      |
| Na⁺/K⁺-ATPase| 48,280 ± 16,788     | 48.3 ± 16.8        | 1,446                   |
| Creatine Kinase| 364,465 ± 29,428   | 364.5 ± 29.4       | 10,912                  |

*aMean ± SD from six animals.
*bCalculated from column 2, assuming a flagellar surface area of 33.4 µm² (r = 0.133 µm; l = 40 µm); only membrane-bound proteins are considered.
*cCalculated from column 2, assuming a flagellar surface area of 33.4 µm² (r = 0.133 µm; l = 40 µm); only membrane-bound proteins are considered.
*dCalculated from all CatSper subunits, except CatSperδ, assuming a 1:1 stoichiometry of subunits.
*Data from a single peptide only.

Tracking of cGMP by the CNGK channel and PDE5

The CNGK-mediated hyperpolarization is the first key signaling event; when hyperpolarization is abolished, downstream events are forestalled (Harumi et al., 1992; Pichlo et al., 2014; Seifert et al., 2015). For maximum efficacy, the CNGK channel must capture any cGMP molecule before it is wasted by hydrolysis or diffusion to the sperm head. Using rapid-mixing techniques and flash photolysis of caged cGMP (Hamzeh et al., 2019), we addressed two questions: How fast and efficiently does the CNGK channel entrap cGMP molecules, and how fast is the channel cleared from cGMP by PDE5 hydrolysis?

As read-out for cGMP dynamics, we recorded the CNGK-mediated $V_m$ changes using fluorescent potentiometric indicators FluoVolt and Di-8-ANEPPS, which detect changes in $V_m$ by photo-induced electron

Correction added on 27 January 2020, after first online publication: 48,500 has been corrected to 48,600.
transfer (PeT) and electrochromism, respectively. For the physiological cGMP-induced hyperpolarization, this read-out is likely contaminated by opening of HCN channels that carry a depolarizing inward Na\(^+\) current (Gauss et al., 1998). Therefore, we recorded \(V_m\) in the presence of 100 mM external [K\(^+\)]\(_o\) (100KASW), which clamps the reversal potential \(V_{\text{rev}}\) for CNGK to approximately \(-37\) mV, i.e., more positive than the resting voltage \(V_{\text{rest}}\) of approximately \(-50\) mV (Strübker et al., 2006; Seifert et al., 2015). Consequently, at high [K\(^+\)]\(_o\), CNGK opening should depolarize rather than hyperpolarize sperm. At \(V_m > -37\) mV, the open probability of HCN channels is low, and HCN channels are unlikely to contribute much to \(V_m\) (Gauss et al., 1998). Furthermore, high [K\(^+\)]\(_o\) abolishes changes in pH\(_i\), [Ca\(^{2+}\)]\(_i\), and cAMP evoked by the chemoattractant or cGMP (Pichlo et al., 2014; Seifert et al., 2015); therefore, putative pH-, Ca\(^{2+}\)-, or cAMP-regulated conductances, like HCN channels, are unlikely to interfere with changes in \(V_m\) evoked by CNGK activity.

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**Figure 2. Determination of flagellar volumes.**

A–D Longitudinal (A and C, proximal view on left) and cross-sectional (B and D, proximal-to-distal view) tomographic slices of representative intact flagella from wild-type *Chlamydomonas reinhardtii* (*C.r.* flagella (A and B) and sea urchin *Strongylocentrotus purpuratus* (*S.p.*) sperm (C and D). Note that in *Chlamydomonas*, the flagellar membrane (m) is surrounded by a glycocalyx (g). Other labels: central pair complex (c) and doublet microtubule (d). Scale bar, 100 nm.

E Diameter of intact flagella from *Chlamydomonas* and sea urchin sperm measured on cross-sectional views of cryo-tomographic reconstructions. Numbers indicate average diameters ± SD (\(n = 84\) for sea urchin (*S.p.*) and \(n = 18\) for *Chlamydomonas* (*C.r.*)).

F, F\('\) Schematic diagrams of *Chlamydomonas* flagella in cross-sectional view. The yellow area (F\('\)) indicates the matrix volume \(V_{\text{ma}}\) that is accessible to proteins, and the cyan area (F\('\)) represents the volume that is accessible to small molecules \(V_{\text{asm}}\) (i.e., the combination of the matrix volume and the microtubule lumen).
Sperm were loaded with DEACM-caged cGMP. This neutral hydrophobic compound readily equilibrates across the cell membrane; therefore, photorelease of cGMP inside cells can be calibrated precisely. Changes in $V_m$ were followed with the voltage-sensitive PeT dye Fluovolt (Miller et al., 2012; Hamzeh et al., 2019). Sperm were rapidly mixed in a stopped-flow apparatus with 100KASW and then stimulated with a brief flash of light that photo-released cGMP. A 2-ms flash (releasing 52 nM of cGMP) gave rise to an almost instantaneous depolarization that relaxed to baseline levels in < 1 s (Fig 3A). A fit of the rise and relaxation of the $V_m$ signal using equation (1) (in Materials and Methods—data analysis and simulations) yielded time constants $\tau_{\text{rise}} = 16.6 \pm 7.3$ ms and $\tau_{\text{relax}} = 384 \pm 107$ ms ($n = 3$ experiments) (Fig 3A). The mean $\tau_{\text{rise}}$ is compatible with estimates of the rate of cGMP binding to CNGK ($\tau_{\text{binding}} = 1–2$ ms) or the RC time constant of the membrane (5–20 ms), which represent lower bounds of $\tau_{\text{rise}}$ (Materials and Methods section).

Because CNGK channels are not voltage dependent and do not desensitize in the presence of cGMP (Böning et al., 2009), the relaxation time $\tau_{\text{relax}}$ might reflect the clearance of CNGK due to the removal of cGMP. We tested this presumption using a DEACM-caged form of 8-Br-cGMP (Hagen et al., 2003), a hydrolysis-resistant analogue that also activates CNG channels (Zimmerman et al., 1985; Kaupp & Seifert, 2002). Release of 8-Br-cGMP by a light flash evoked a rapid depolarization that, by contrast to cGMP, was persistent and not relax. Therefore, the rate of cGMP dissociation provides an upper bound for $\tau_{\text{relax}}$. The calculated dissociation rate is $k_{\text{off}} = 0.68/s$ or $440$ nM/s of cGMP.

What limits the rate of cGMP hydrolysis: the rate of cGMP dissociation from CNGK or the catalytic power of PDE5? We determined the $V_m$/light energy relation using 2-s light pulses over a 50-fold range of light energies. Invariably, during the light pulse, $V_m$ rose to a stable plateau that was graded with increasing light energy (Fig 3C and D), suggesting that PDE5 is exactly counterbalancing steady CNGK release. The steady-state amplitude of $\Delta V_m$ increased linearly for low light energies, consistent with the idea that PDE5 is constitutively active (Rybalkin et al., 2003) (Fig 3C and D). At the end of a light pulse, $V_m$ returned to $V_{\text{rest}}$ with a characteristic time $\tau_{\text{relax}}$ that was constant for a 50-fold range of cGMP-photolysis rates and that was similar to $\tau_{\text{relax}}$ of signals evoked by short light flashes (220 ms vs. 384 ms) (Fig 3C). The independence of $\tau_{\text{relax}}$ from cGMP levels suggests that dissociation of cGMP from CNGK determines the rate of $V_m$ recovery rather than PDE5’s catalytic power. We scrutinized this notion by mathematical calculation and numerical simulations.

### Estimates of CNGK turnover kinetics

We calculated the $V_m$ recovery kinetics for three different reaction schemes (Fig 4A) (see Materials and Methods section and Appendix). Scheme 1 involves the CNGK channel alone; scheme 2 involves the PDE alone; and scheme 3 involves CNGK and PDE together, recapitulating the in vivo situation. Schemes assume that CNGK must first dissociate from CNGK before PDE5 can degrade it. Therefore, the rate of CNGK dissociation provides an upper bound for $\tau_{\text{relax}}$. The calculated dissociation rate is $k_{\text{off}} = 0.68/s$ or $440$ nM/s of cGMP.
\[ \tau_{\text{off}} = 1.48 \text{ s}, \text{assuming } k_{\text{on}} = 2.6 \times 10^7/\text{M/s} \text{ and a constant of half-maximal activation } K_{1/2} = 2.6 \times 10^{-8} \text{ M} \text{ (Bönigk et al., 2009; Peuker et al., 2012). Accounting for the effect of ionic strength on second-order rate constants (Peuker et al., 2012) and assuming that the ionic strength of seawater and the cytosol is similar, } k_{\text{on}} \text{ rates might be twofold larger (} k_{\text{on}} = 5 \times 10^7/\text{M/s} \text{; } \tau_{\text{off}} = 0.75 \text{ s}) \text{ (Fig 4B). Intriguingly, the shortest estimate of } \tau_{\text{off}} \text{ is about twofold to fourfold longer than experimental } \tau_{\text{relax}} \text{ for } V_m \text{ recovery.}

Second, we estimated a lower bound for \( \tau_{\text{relax}} \) if the turnover number \( k_{\text{cat}} \) of PDE5 were rate-limiting (Fig 4A, PDE alone). For constitutively active PDE5 (\( k_{\text{cat}} = 24/\text{s} \) and \( N_{\text{PDE5}} = 48,600 \)), the time constant of cGMP hydrolysis was estimated to be 131 ms (Materials and Methods section) (Fig 4B). Thus, the hydrolytic power of PDE5 is not rate-limiting.

Finally, we simulated cGMP hydrolysis under \textit{in vivo} conditions, i.e., in the presence of both PDE5 and CNGK. In this scenario, cGMP hydrolysis was considerably slowed down (\( \tau = 9.6 \text{ s} \) (Fig 4B), primarily due to rebinding of cGMP to the high-affinity site of CNGK. We searched the parameter space for values of \( k_{\text{cat}} \) (PDE) and \( k_{\text{off}}/k_{\text{cat}} \) (CNGK) that are compatible with experimental \( \tau_{\text{relax}} \) (Fig 4C). To achieve \( \tau_{\text{relax}} \) high values for both \( k_{\text{on}} \) (CNGK) and \( k_{\text{cat}} \) (PDE5) are required (Fig 4D, gray area). Such high \( k_{\text{on}} \) and \( k_{\text{cat}} \) values have not been observed for CNBD domains and for sea urchin or mammalian PDE5, respectively (Kaupp et al., 2003; Rybalkin et al., 2003). In conclusion, given the high CNGK concentration and the high \( k_{\text{cat}} \) value, hydrolysis of cGMP free in solution, for a wide range of parameters, fails to explain the extraordinary fast cGMP breakdown. To solve this conundrum, we propose substrate “channeling” between CNGK and PDE as a mechanism that might accelerate cGMP dissociation from the channel and, ultimately, hydrolysis (see Discussion).

Simulations provided additional insight regarding the potential role of the allosteric GAF-A domain that is conserved in \textit{A. punctulata} PDE5. Mammalian PDE5 displays basal activity that is about threefold enhanced by binding of cGMP to the GAF-A domain (Rybalkin et al., 2003). The cGMP-stimulated activity scheme yielded the same kinetics as the basal activity-only scenario: For physiological stimulation levels, the enhanced activity of a few PDE5 molecules is negligibly small compared with the basic activity of ten thousand of active PDE5 molecules. Although GAF-A domains display a relatively high cGMP affinity (\( K_D \approx 200 \text{ nM} \); Francis et al., 2011), they do not compete with CNGK or the catalytic PDE domain for cGMP, because its \( k_{\text{on}} \) is almost 1,000- and 100-fold lower than \( k_{\text{on}} \) for cGMP binding to the CNGK channel and to the catalytic PDE5 domain, respectively (Appendix Table S3). Thus, putting the kinetic argument aside, even at equilibrium, the CNGK channel captures \( \geq 90 \% \) of cGMP molecules.

**Control of \( V_m \) recovery by HCN channels**

In many cells, HCN channels counteract hyperpolarization and, thereby, serve several different functions. In the retina, HCN channels extend the frequency response and operational light range of rod and cone photoreceptors (Fain et al., 1978; Barrow & Wu, 2009). In neurons and heart cells, HCN channels sustain oscillatory activity and modulate resting \( V_m \) (Pape, 1996). In sperm, the precise function of HCN channels has been elusive. Two different HCN channel subunits (HCN1 and HCN2) exist in sea urchin sperm (Gauss et al., 1998; Galindo et al., 2005). Do these subunits form homomeric or heteromeric channels? 

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Figure 4. Discrete-event simulations of cGMP dynamics.
A Schemes illustrate three different situations defined by chemical reactions (2) to (5) in the Materials and Methods section. Colors of schemes refer to the simulations shown in panel (B).
B Characteristic decay of \( V_m \) in experiments shown in Fig 3A and C (orange, \( \tau_{\text{relax}} = 0.384 \text{ s} \) ) is compared with cGMP hydrolysis by PDE5 in the absence of CNGK channels (blue; characteristic time \( \tau = 0.131 \text{ s} \) ), dissociation of cGMP from CNGK at the rate \( k_{\text{off}} \) (yellow; \( \tau = 0.748 \text{ s} \) ), and cGMP hydrolysis in the presence of CNGK channels and PDE (violet; \( \tau = 9.73 \text{ s} \) ). Simulations were done using those parameters listed in Appendix Table S3. COMP is given in molecules.
C Simulations of the rate of cGMP hydrolysis upon varying the catalytic rate \( k_{\text{cat}} \) of PDE and the \( k_{\text{on}} \) rate of cGMP binding to the CNBD of the CNGK channel. The gray plane indicates the experimental relaxation time (\( \tau_{\text{relax}} \)). The black dot represents the kinetic argument: \( 90\% \) of cGMP molecules. In neurons and heart cells, HCN channels sustain oscillatory activity (Fain et al., 1978) and modulate resting \( V_m \) (Pape, 1996). In sperm, the precise function of HCN channels has been elusive. Two different HCN channel subunits (HCN1 and HCN2) exist in sea urchin sperm (Gauss et al., 1998; Galindo et al., 2005). Do these subunits form homomeric or heteromeric channels?
heteromeric channels! What is their physiological function? We detected in the flagellum 10,000 and 18,000 copies of HCN1 and HCN2 channel subunits, respectively. Do HCN channels counteract the chemoattractant-induced hyperpolarization? We compared the rate of \( V_m \) recovery after weak and strong cGMP stimulation, i.e., when HCN channel activity is low and high, and for both depolarizing (100KASW) and hyperpolarizing (ASW) responses. For strong hyperpolarization, \( V_m \) completely recovered within 110 ms, while a large fraction of CNGK channels was still open (Fig 5A, compare blue and red traces). Thus, HCN channels clearly outperform CNGK channels. For weak stimuli, i.e., for small depolarization or hyperpolarization, the \( V_m \) response relaxed with similar time constants of about 300 ms (Fig 5A, compare pink and navy traces). The 1.5-fold larger amplitude of the hyperpolarizing compared with the depolarizing response (100KASW) and hyperpolarizing (ASW) responses. For strong stimuli, i.e., for small depolarization or hyperpolarization, the \( V_m \) response relaxed with similar time constants of about 300 ms (Fig 5A, compare pink and navy traces). The 1.5-fold larger amplitude of the hyperpolarizing compared with the depolarizing signal is due to the larger electrochemical driving force. We systematically determined the recovery time over a wide range of stimulus strength (Appendix Fig S1A). The recovery time was similar (\( \tau_{\text{relax}} = 280 \) ms) (Fig 5B inset) when \( p \) 30 cGMP molecules (equivalent to \( \leq 3 \) chemoattractant molecules) were produced, and hastened gradually with increasing hyperpolarization, i.e., HCN channel activation (Fig 5B). In conclusion, HCN channels accelerate recovery—except for weak stimuli.

We scrutinized this conclusion by inhibiting either cGMP hydrolysis or HCN activity. The relaxation times of hyperpolarizing responses evoked by photolysis of caged 8-Br-cGMP or caged cGMP were similar (Fig 5C), except for a depolarizing overshoot. Similarly, inhibition of PDE activity by high concentrations of IBMX (1 mM) did not retard recovery (Fig 5D). The depolarizing voltage overshoot was abolished either in the absence of \( \text{Ca}^{2+} \) (Fig 5D) or when 8-Br-cGMP was used as a stimulus (Fig 5C), suggesting that recovery is first driven by a \( \text{Na}^+ \) inward current carried by HCN channels, followed by \( \text{Ca}^{2+} \) currents carried by CatSper channels. Finally, inhibition of the HCN channels by the drug ZD7288 (Postea & Biel, 2011) strongly slowed down recovery (Fig 5E and F). In the presence of the drug, \( V_m \) signals also became smaller (Fig 5E), because the drug also shifted \( V_{\text{rest}} \) to more negative values, which compressed the

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**Figure 5. Recovery from hyperpolarization**.

A. Time course of cGMP-evoked changes in \( V_m \) in 100KASW (red, 500 nM cGMP; pink, 9 nM cGMP) and normal ASW (blue, 500 nM cGMP; navy, 9 nM cGMP). The time constants of recovery after cGMP release (9 nM were 289 ms (depolarization) and 303 ms (hyperpolarization). Signals in panels (A-C) were detected using the potentiometric probe FluoVolt; dashed magenta lines indicate the timing of the photolysis flash.

B. Representative measurement of the dependence of recovery-time constant on the amount of released cGMP. Inset: Decay time was constant for low stimulation levels equivalent to \( \leq 30 \) cGMP molecules or \( \leq 3 \) resact molecules.

C. Voltage response evoked by photolysis of DEACM-caged cGMP (blue) or 8-Br-cGMP (red). The light energy was adjusted such that \( V_m \) response amplitudes became similar; for 8-Br-cGMP, about five times more light energy was required. Light pulse 10 ms.

D. cGMP-evoked \( V_m \) response in ASW (black), in the presence of IBMX (1 mM) (red), and in \( \text{Ca}^{2+} \)-free ASW ([\( \text{Ca}^{2+} \]) \leq 500 \) nM) in the presence of IBMX (1 mM) (blue). Signals in panels (D-F) were detected using the potentiometric probe Di-8-ANEPPS in the ratio mode.

E. \( V_m \) responses evoked by cGMP in the presence (blue) and absence (red) of the HCN channel blocker ZD7288 (50 \( \mu \)M).

F. Normalized responses from panel (E).

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*Correction added on 27 January 2020, after first online publication: panels D, E and F have been corrected and the order of panels D and E in the legend has been reversed.*
operational range of hyperpolarization (Appendix Fig S1B). Of note, in the presence of ZD7288, the recovery time is similar to that at 100KASW, i.e., in the absence of HCN channel activity. Collectively, these results show that except for low stimulation levels, HCN channel activation controls the time of recovery from hyperpolarization.

Components of Ca\(^{2+}\) signaling

Three different signaling proteins control Ca\(^{2+}\) homeostasis: the Ca\(^{2+}\) channel CatSper, a NCKX exchanger, and a Ca\(^{2+}\)-ATPase (Fig 1B). Their quantitative stoichiometry provides several insights. First, CatSper in mammalian sperm comprises four homologous pore-forming subunits (CatSper 1-4) (Navarro et al., 2008) and at least five auxiliary subunits (CatSper β, γ, δ, ε, and ζ) (Liu et al., 2007; Wang et al., 2009; Chung et al., 2011, 2017). CatSper1-4, β, γ, and δ were also detected in Arbacia sperm (Seifert et al., 2015). Except for CatSper3, each of the subunits was equally abundant, suggesting that each subunit contributes one copy to a large CatSper complex. The CatSper 3 quantification relied on one peptide only; thus, the estimate is less reliable than for the other CatSper subunits.

Second, the NCKX exchanger is one of the most abundant signaling molecule, only second to the receptor GC (Table 1). By contrast, the PMCA pump is 50-fold less abundant, showing that Na\(^+\)/Ca\(^{2+}\)/K\(^-\) exchange is the major if not only mechanism of Ca\(^{2+}\) clearance from the flagellum. In fact, PMCA is more abundant in the head than the flagellum (Gunaratne et al., 2006). The NCKX exchanger is also about 50-fold more abundant than the CatSper channel. This high ratio is required because of the vastly different transport numbers of ion channels and ion exchangers: Ca\(^{2+}\) channels can carry >1,000 ions/s (Hille, 2004), whereas NCKX exchangers can transport about 100–300 Ca\(^{2+}\) ions/s (Jalilou et al., 2016).

NCKX transporters use inward Na\(^+\) and outward K\(^-\) gradients for Ca\(^{2+}\) extrusion (Cervetto et al., 1989; Schnetkamp, 2013). The exchange is electrogenic (4Na\(^+\):1Ca\(^{2+}\)::1K\(^-\)) and therefore enhanced at negative membrane potentials (Cervetto et al., 1989). The involvement of Na\(^+\) and K\(^-\) gradients and the electrogenic mechanism suggests rapid ion-exchange kinetics and very low Ca\(^{2+}\) levels at rest. NCKX is primarily expressed in sensory neurons, in particular in rod and cone photoreceptors (Schnetkamp et al., 2014). The resting [Ca\(^{2+}\)]\(_i\), in rods is 100 nM and, during hyperpolarization, can reach levels as low as 5–10 nM (Woodruff et al., 2002). We assume that [Ca\(^{2+}\)]\(_i\), in sperm can also adopt such low levels.

Third, its high density argues that NCKX can restore basal Ca\(^{2+}\) levels in the flagellum before Ca\(^{2+}\) diffuses to the head compartment. We tested this presumption by recording cGMP-evoked Ca\(^{2+}\) changes, using the fluorescent Ca\(^{2+}\) indicator GFP-certified FluoForte, in the flagellum and the head separately. A flash of light, producing 28 cGMP molecules (equivalent to approximately 3 resact molecules), evoked a short Ca\(^{2+}\) pulse in the flagellum, but no significant change in the head (Fig 6A). For stronger stimulation, a small delayed Ca\(^{2+}\) signal in the head became apparent (Fig 6B). In conclusion, for low stimulation levels, the Ca\(^{2+}\) response is restricted to the flagellum; for higher stimulus strengths, the signal partially propagates from the flagellum to the head.

Changes in pH\(_r\) are compartmentalized

A voltage-activated Na\(^+\)/H\(^+\) exchanger (SLC9C1 or sNHE) is the only molecule known in sperm to regulate pH\(_r\). Its excessively high density (Table 1) explains why changes in pH\(_r\) occur on a millisecond time scale (Seifert et al., 2015; Windler et al., 2018). We examined whether cGMP-evoked changes in pH\(_r\), similar to Ca\(^{2+}\) signals, are restricted to the flagellum. Changes in pH\(_r\) were measured, using the fluorescent pH indicator pHrodo Red, in the flagellum and the head separately (Fig 6C and D). The pH\(_r\) rose rapidly in the flagellum and declined again within 3 s (Fig 6C). By contrast, the alkalization in the head was much smaller and slower. After about 4 s, the pH\(_r\) in flagellum and head was equal and slightly elevated. This result is consistent with the idea that Na\(^+\)/H\(^+\) exchange in the flagellum elevates pH\(_r\) (Windler et al., 2018) and that this change slowly propagates to the head.

Discussion

In a typical cell soma, signaling proteins are less or about equally abundant than their respective substrates; processing of many substrate molecules by a single active enzyme lies at the heart of cellular signal amplification. For example, in the ERK and AKT pathways concentrations of signaling proteins range between 10 and 1,000 nM (Adlung et al., 2017). By contrast, the enzyme-to-substrate ratio in the sperm flagellum is reversed: Signaling proteins are up to 1,000-fold more abundant (micromolar range) than their free substrates (nanomolar range). Simply put, a large number of enzymes, transporters, and channels compete for few substrate molecules, and a single enzymatic turnover by a minute fraction of signaling proteins suffices to initiate or upend a cell response. Clearly, this minuscule reaction vessel is designed for a specific purpose: low-noise, perfect molecule detection rather than signal amplification.

The high-affinity CNGK channel acts as a close-to-perfect cGMP detector that avidly absorbs cGMP molecules and translates binding events into an incremental voltage response. Binding happens faster than spreading of cGMP along the flagellum by unrestricted diffusion (about 1 s) (Pichlo et al., 2014). Furthermore, PDES is unlikely to kinetically compete for cGMP binding. Thus, the single-molecule sensitivity of sperm ultimately rests not only on the exquisitely high density and affinity of the receptor GC (Pichlo et al., 2014), but also on the CNGK channel as well.

The target proteins of cAMP, Ca\(^{2+}\), and H\(^+\) are also orders of magnitude more abundant than the free messengers themselves. The high density serves two different functions. Rapid sequestering of cellular messengers attenuates their diffusional exchange between flagellum and head. Kinetic compartmentalization enhances antennal sensitivity and shields acrosomal exocytosis in the head, which is also controlled by cAMP, Ca\(^{2+}\), and pH\(_r\) (Buffone, 2016), from signaling events in the flagellum. In addition, the time derivative of the Ca\(^{2+}\) response d [Ca\(^{2+}\)]/dt rather than absolute [Ca\(^{2+}\)], determines the curvature of the swimming path (Alvarez et al., 2012). Therefore, kinetic control of the Ca\(^{2+}\) influx/efflux balance is key to chemotactic navigation. Transport rates of solute carriers are orders of magnitude slower than those of ion channels. The 60-fold higher NCKX density compared with CatSper ascertains that the Ca\(^{2+}\) rise can be rapidly counterbalanced. The EMBO Journal 39: e102723 | 2020 9 of 18
kinase A (PKA) system. The regulatory subunit R binds cAMP with extremely high affinity ($K_D = 2–10$ nM); consequently, cAMP recycling is expected to be slow. The decade-long riddle (Ramírez-Sarmiento, 2017) was recently unraveled by proposing a “substrate-channeling” mechanism: cAMP, PDE8, and RI form a ternary complex; cAMP is transferred between active sites of RI and PDE8 by restricted diffusion without release into the cytosol (Krishnamurthy et al, 2014, 2015; Ramírez-Sarmiento, 2017; Tulsian et al, 2017). The allosteric active-site coupling tweaks cAMP dissociation from RI and, thereby, accelerates hydrolysis. Notably, PDE8 is only active in the RI-cAMP-PDE8 complex (Tulsian et al, 2017), a mechanism that prevents breakdown of free cAMP. Two lines of evidence suggest that CNGK and PDE5 form a similar “channeling” complex (Fig 7A): cGMP turnover is much faster than estimated from reaction kinetics in free solution, and the CNGK/PDE5 ratio is 1:1 (assuming PDE5 forms dimers via the GAF-B domain; Francis and Corbin (2010)). Future work needs to establish this mechanism for PDE5. The “channeling” mechanism renders cGMP binding to CNGK essentially irreversible; before cGMP has a chance to dissociate, it is hydrolyzed. Within the framework of chemosensation physics (Berg & Purcell, 1977; Endres & Wingreen, 2008), the inner surface of the flagellar membrane constitutes a perfect absorber rather than a monitoring surface that allows dissociation and rebinding of a ligand. The sensing precision of a perfect absorber is 2.5-fold enhanced compared with a monitoring surface (Endres & Wingreen, 2008).

A far-reaching consequence of rapid cGMP binding to CNGK and “channeling” to PDE5 is that the mean resting-free cGMP concentration is almost zero and that the cGMP action happens on the membrane surface rather than the cytosol. Several back-of-the-envelope estimates illustrate this conclusion. The free cGMP concentration is determined by the rates of synthesis and hydrolysis. To maintain a free cGMP concentration of 1 nM (equivalent to one cGMP molecule/flagellum) in the face of a basal hydrolysis rate of 2.6/s requires the synthesis of 2,103 cGMP molecules/s by 210 active GC molecules/s (calculated from a turnover rate of 72 cGMP/s/GC and a lifetime of 150 ms of active GC; Pichlo et al, 2014). Such basal activity would be equivalent to 30 pM of the chemoattractant; at this concentration, 210 chemoattractant molecules/s hit the flagellum; Berg, 1993; Pichlo et al, 2014). When PDE activity is blocked, the resting cGMP level should rise to high micromolar concentrations. In fact, elevated cGMP levels were not observed in such experiments (Kaupp et al, 2003), arguing that non-stimulated GC activity at rest is negligible. Moreover, such high background activity would seriously compromise if not abolish single-molecule detection of sperm. For comparison, single-photon sensitivity in rod photoreceptors is limited by spontaneous thermal activation of roughly one rhodopsin molecule/min (Luo et al, 2011). Finally, at chemical equilibrium with

![Figure 6. Compartmentalization of Ca$^{2+}$ and pH signals. Changes in relative fluorescence $\Delta F/F_0$ recorded from the head and the flagellum are shown in blue and black, respectively.](image-url)
1 nM free cGMP, approximately 800 cGMP molecules are bound to CNGK channels [calculated from $K_D = 26$ nM (Bönigk et al., 2009) and $c_{\text{CNGK}} = 22$ μM; Table 1]. Because binding of a single cGMP molecule can open CNGK (Bönigk et al., 2009), 800 CNGK channels would be permanently open at rest. By comparison, the opening of maximally 300 CNGK channels by the chemoattractant saturates the $V_m$ response (Strünker et al., 2006). In conclusion, free cGMP concentration is virtually zero at rest.

We propose that the GC/CNGK/PDE5 triad is designed to encode rates of cGMP production rather than free cGMP concentrations. Over a 40,000-fold range of cGMP synthesis rates, the free cGMP concentration increases almost linearly (Fig 7B). Of note, even for strong stimulation, a rate-encoding mechanism never saturates the CNGK channel with cGMP and allows cells to operate over a wide dynamic range. Another important benefit of rate-detection is the speed with which a cell response can be initiated: No binding equilibrium has to be reached, and no diffusional times have to be awaited. A cell can rapidly and continuously sample a stimulus landscape and respond in real time. The mechanism is optimal to probe a chemical gradient on periodic paths with 1–2 Hz frequency.

Finally, our study also illustrates fundamental limits to track cellular messenger concentrations in small compartments using genetically encoded or chemical sensors. The sensitivity of available cAMP/cGMP sensors (ranging between 70 nM; Mukherjee et al. (2016) and 3 μM; Halls and Canals (2018)) would be not sufficient to detect changes in free cyclic nucleotide concentrations in sperm. Enhancing a sensor’s ligand affinity creates another dilemma because sensors become inherently slow and are expected to smear 20 times by centrifugation for 30 s at 75 × g and 4°C through a 40-μm mesh of a cell strainer (BD Biosciences, USA). Flagella were

Materials and Methods

Preparation of flagella

The collection of *A. punctulata* sperm and the preparation of flagella were as described in Seifert et al. (2015) with one modification: Instead of shearing with a 24-G needle, the sperm suspension was sheared 60 times by centrifugation for 30 s at 75 × g and 4°C through a 40-μm mesh of a cell strainer (BD Biosciences, USA). Flagella were...
washed in artificial seawater (ASW), containing (in mM): 423 NaCl, 9.27 CaCl₂, 9 KCl, 22.94 MgCl₂, 25.5 MgSO₄, 0.1 EDTA, and 10 HEPES, adjusted to pH 7.8 with NaOH, and stored as pellet.

**Analysis of solubilization efficacy by Coomassie staining and Western blotting**

The precise determination of copy numbers and protein stoichiometries relies on the efficacy of protein solubilization by detergents. Therefore, we tested for different solubilization conditions the fraction of proteins in the supernatant and the pellet by SDS–PAGE and Western blotting of selected membrane-associated proteins (Appendix Fig S3). Flagella were resuspended in solubilization buffer [10 mM Tris–HCl, pH 7.6; 140 mM NaCl, 1 mM EDTA, 1% dodecyl-maltopyranoside (DDM), protease inhibitor cocktail 1:500 (P8340, Sigma-Aldrich, USA)]. The protein concentration determined by BCA assay was 1.5 μg/μl. The solubilize was split into three samples for parallel incubation. Tubes 1 and 2 were incubated on ice for 30 min, and tube 3 was incubated on ice for 120 min. Flagella in tube 2 were sonicated three times for 30 s (Sonifier 450, Branson, Danbury, CT). After incubation, supernatant and pellet were separated by centrifugation (10,000 × g at 4°C).

To compare the different solubilization conditions, proteins in the supernatant and pellet were separated on a 7.5% SDS gel for Coomassie staining or on an 8% SDS precast gel (SurePAGE, Censcript, NJ, USA) for Western blotting. Each lane was loaded with the equivalent of 8 μg of flagellar protein. Pre-stained Protein Marker VI (Applied Chem, Darmstadt, Germany) was used as molecular weight marker.

Proteins were transferred onto an Immobilon FL PVDF membrane (Merck Millipore, Darmstadt, Germany) and consecutively probed with various antibodies. After each probing, the Western blot was analyzed using the Odyssey Imaging System (LI-COR, Bad Homburg, Germany). All figure panels were taken from the same Western blot. Figures were prepared using CorelDraw X6 and Photo-Paint X6 software (both from Corel Corporation). Primary antibodies were monoclonal: rat GCN 3D10 (anti-GC, 1:200) (Pichlo ApCNGK (repeat 4), 1:20) (Bönigk et al, 2009), and consecutively probed with the identity of the previously selected 53 peptides by manual pairwise comparison of retention times during LC and by comparison of fragment ions of the respective sample and standard peptides. For three peptides, no correct light/heavy pairs were observed; these peptides were excluded from quantitative analysis (Table 2). Ultimately, 50 peptides were used for protein quantification.

**Quantification of chemotaxis signaling proteins by selected reaction monitoring (SRM) mass spectrometry**

For quantification, an aliquot of 20 μg sperm flagella protein was spiked with either 0.15, 1.5, or 15 pmol isotope-labeled standard protein to account for different concentrations of sperm target proteins. The standard protein/sample mixtures were subjected to 12.5% (v/v) SDS–PAGE. The SDS–PAGE was stopped after the sample had migrated about 1 cm into the separation gel, and the gel was stained with Coomassie Blue. The about 1-cm-long gel piece was cut into 1–2 mm-sized pieces. In-gel digestion was performed
as described (Pichlo et al., 2014) with 6.25 ng/µl trypsin in a NH₄HCO₃ buffer (40 mM at pH 8.6).

The LC-MS/MS system consisted of a TSQ Vantage Triple Quad MS (Thermo Fisher Scientific, USA) interfaced with a nanoflow Acquity UHPLC system (Waters, USA). LC used an Acquity UPLC M-Class Symmetry C18 trap column (5 µm particle size, 180 µm × 20 mm, 2D, V/M) and an Acquity UPLC M-Class HSS T3 analytical column (1.8 µm particle size, 75 µm × 150 mm) (Waters, USA). LC conditions (66-min multistep gradient from 1% to 99% acetonitrile/0.1% formic acid at a flow rate of 0.4 µl/min) were as described (Pichlo et al., 2014). SRM parameters for the TSQ Vantage and data analysis with Skyline 3.7 were as described (Pichlo et al., 2014) with the following changes: The two most intense and specific fragment ions (transitions) were used for quantification, and a measuring time window of ± 2.5 min from each peak apex and a 3-s cycle time was set. For optimal ion transmission, the following S-lens RF values were fixed: 53 at 182.082 m/z, 109 at 508.208 m/z, and 220 at 997.398 m/z. The collision energy was first predicted by Skyline 3.7 and then individually optimized for each precursor ion. Heavy-isotope incorporation was determined by in-solution digestion of the standard protein, followed by SRM analysis as described above. Heavy-isotope incorporation was 97% (arginine) and 98% (lysine).

**Calculation of copy numbers and statistical analysis**

All SRM data were manually inspected to ensure correct peak identification. Furthermore, from three different amounts of 0.15, 1.5, or 15 pmol standard protein per 20 µg flagella protein, individual L/H ratios were selected to exclude extreme values. The peak area of each light (sperm sample) and heavy standard peptide was calculated using Skyline 3.7 and exported to an Excel sheet for further data processing. The total area of each heavy peptide was corrected for the percentage of heavy-isotope incorporation. For example:

| Total area heavy | Heavy-isotope incorporation | Total area heavy corrected |
|------------------|----------------------------|---------------------------|
| 5,558,109        | 0.071                      | 5,724,108                 |

The total area of each light peptide was corrected for the percentage of light-isotope incorporation in the isotope-labeled standard protein:

| Total area light | Total area heavy corrected | Light-isotope incorporation | Total area light corrected |
|------------------|----------------------------|-----------------------------|----------------------------|
| 556,239          | (5,724,108)                | 0.029                       | 390,240                    |

The ratios between the corrected peak areas of each light and heavy peptide were calculated using Excel. Based on these ratios, the numbers of molecules of the light peptides were calculated using Avogadro’s number. For the different peptides assigned to the same protein, the median and the standard deviation were calculated. For each replicate, the ratios between the different proteins, e.g., guanylate cyclase-to-creatine kinase ratio, and the mean ± SD of all replicates belonging to the same animal were calculated. Representative results are presented in Dataset Table EV2, and the underlying RAW files and Skyline analyses have been made publicly available at ProteomeXchange with identifier PXD015502. Furthermore, the ratios between signaling proteins and the mean ± SD of the replicates belonging to the same animal are provided in Dataset Table EV1.

### Processing of electron microscopy images

For the measurement of flagellar diameters and axonemal protein volumes, the following previously published cryo-electron tomography and subtomogram averaging data were used: the three-dimensional (3D) reconstructions of intact wild-type *Chlamydomonas reinhardtii* and sperm flagella from the sea urchin *Strongylocentrotus purpuratus* (Lin & Nicastro, 2018) for flagellar diameter, the 3D structures of the averaged DMT-associated (Lin et al., 2019) and central pair complex repeats (preprint: Fu et al., 2019) of wild-type *Chlamydomonas*, as well as the averaged DMT-associated repeats (Lin & Nicastro, 2018), and central pair complex repeats of sea urchin sperm flagella (Carabajal-Gonzalez et al., 2013).

The IMOD software (Kremer et al., 1996) and ImageJ (Schneider et al., 2012) were used for visualization of the tomographic slices and measurement of the diameters (measured at the inside of the flagellar membrane), respectively. Only tomograms with straight and non- or mildly compressed flagella were used for diameter measurements. In total, 6 tomograms of *Chlamydomonas* flagella and 19 tomograms of sea urchin sperm flagella were selected, and their diameters were measured at three different positions: proximal end, midpoint, and distal end.

The UCSF chimera package (Pettersen et al., 2004) was used for estimating the volumes occupied by axonemal proteins using the subtomogram averages of the 96-nm DMT-associated repeat (*V*₂₃₅₇) and the [3 × 32 nm] central pair complex repeat (*V*₇₁₂). The isosurface-rendering threshold for volume measurements was determined using the average density of 1.43 g/cm³ (Quillin & Matthews, 2000) for proteins and normalizing the threshold so that the measured molecular mass of microtubule protiformaments and dynein heads matched the calculated molecular masses for a 96-nm long protiformament (~1,200 kDa) and dynein head domain (~350 kDa). For instance, the surface threshold for the averaged 96-nm DMT-associated axonemal repeat of *Chlamydomonas* was set to 125.1, resulting in estimated molecular weights for the IDA a dynein head and protofilament B7 of 343 and 1,210 kDa, respectively.

### Volume calculations

The measured average diameters (d = 2r) of the *Chlamydomonas* and sea urchin sperm flagella were used to calculate the total volumes *V*l for a flagellar length (l) as *V*l = π × r² × l. The volumes of the axonemal proteins were calculated for 96-nm length as *V*s = (9 × *V*₂₃₅₇) + *V*₇₁₂ and then scaled to 1-µm flagellar length. For measuring the luminal volumes *V*l inside the DMTs and central pair microtubules, we “filled” the microtubule lumen in the subtomogram averages of the 96-nm DMT-associated repeat and the [3 × 32 nm] central pair complex repeat by setting the pixel values inside the microtubules to zero (i.e., black like protein) using a mask or the IMOD command imodmop. Using the same chimera thresholds as for estimating the volumes *V*₂₃₅₇ and *V*₇₁₂ before, we estimated the volumes of the “filled” averages *V*₁₂₇₃₅₇ and *V*₂₇₁₂ and calculated *V*l = (9 × *V*₂₃₅₇) + *V*₇₁₂ - *V*l for 96-nm length, which was then scaled to 1-µm flagellar length.
Stopped-flow techniques

Resact- and cGMP-induced changes in membrane voltage were measured with a voltage-sensitive dye (FluoVolt, Thermo Fisher) in a stopped-flow device (SFM-400; Bio-Logic) as described previously (Hamzeh et al., 2019). Dry sperm were suspended 1:6 (v/v) in loading buffer containing ASW and 5 μM fluorescent dye. After incubation for at least 20 min at 18 °C, the sample was diluted 1:20 with ASW in the presence of 15 μM caged DEACM-cGMP and 0.05% Pluronic F127 (Sigma-Aldrich). In the stopped-flow device, sperm were rapidly mixed 1:1 with either ASW or ASW containing 191 mM K+. Mixing with 191 mM K+ ASW yielded a final K+ concentration of 100 mM (referred to as 100KASW). Fluorescence was excited with a Spectra X Light Engine (Lumencor). Emission was recorded by photomultiplier modules (H9656-20; Hamamatsu Photonics). Data acquisition was performed with a data acquisition board (PCI-6221; National Instruments) and Bio-Kine software v. 4.49 (Bio-Logic). The excitation light was passed through a BrightLine 513/17 nm filter (Semrock). The excitation light was operated at 1–10 kHz. Emission was recorded through a 542/20 nm filter by a photomultiplier module and filtered through a lock-in amplifier. Signals represent the average of at least two recordings and are depicted as the percent change in fluorescence (ΔF) relative to the mean of the first 5–10 data points before the onset of the signal (Fo). The control (ASW) ΔF/Fo signal was subtracted from the resact- or cGMP-induced signals. The data obtained were analyzed and plotted using OriginPro 9.0 (OriginLab Corporation).

Caged compounds and flash photolysis

Caged cGMP was photolyzed using a 365-nm LED (M365LP1, Thorlabs). The LED was coupled to a liquid light guide and delivered to the cuvette (FC-15 Bio-Logic). The fluorescence background resulting from the 365-nm LED was removed using lock-in detection. The waveform, timing, and triggering of the photolysis light were controlled through the interface of a self-made LabVIEW program using a DAQ card (NIUSB-625; National Instruments). The photolytic release of cGMP was calibrated via the increase in fluorescence up upon photolysis (Hamzeh et al., 2019).

Data analysis and simulations

Simulations of cGMP dynamics in the flagellum were based on the following reaction schemes:

| Reaction | Description |
|----------|-------------|
| cGMP →<sub>k<sub>rel</sub></sub> cGMP | (1) |
| CNGK + cGMP →<sub>k<sub>on</sub>,CNGK</sub> CNGK · cGMP | (2) |
| PDE<sub>i</sub> + cGMP →<sub>k<sub>cat</sub>,PDE<sub>i</sub></sub> PDE<sub>i</sub> | (3) |

These reactions correspond to the release of cGMP from its caged derivative (reaction 1), binding (unbinding) of cGMP to/from the CNGK channel (reaction 2), binding (unbinding) of cGMP to/from the allosteric GAF-A domain of the inactive PDE (PDE<sub>i</sub>) to produce active PDE (PDE<sub>a</sub>), reaction 3), cGMP binding to the catalytic domain of PDE of either PDE<sub>i</sub> or PDE<sub>a</sub> (reaction 4), and cGMP hydrolysis by PDE (reaction 5). Reaction rates were taken from previous studies (see Appendix Table S3). The catalytic rate for hydrolysis (<i>k<sub>cat</sub></i>) of cGMP by PDE<sub>i</sub> is assumed to be about one-third of that of PDE<sub>a</sub>, as reported for mammalian PDES (Rybalkin et al., 2003). The apparent <i>k<sub>on</sub></i> for cGMP binding to the catalytic domain of PDE (reaction 4) was estimated assuming that the reaction is irreversible (<i>k<sub>off</sub></i> ~0/s), and using the Michaelis–Menten relation: <i>K<sub>M</sub></i> = (<i>k<sub>on</sub></i> + <i>k<sub>cat</sub></i>)/<i>k<sub>off</sub></i>, wherein <i>K<sub>M</sub></i> is the Michaelis–Menten constant. The maximal catalytic rate (<i>k<sub>cat</sub></i>) was estimated from cGMP measurements of sperm stimulated with saturating resact concentrations (Appendix) (Kaupp et al., 2003).

Discrete-event simulations were performed using the direct Gillespie method (Gillespie, 1977). Stochastic reaction rates were derived from the deterministic rates listed in Appendix Table S3. The on rates for the second-order reactions 2, 3, and 4 were normalized by the flagellar volume as described (Gillespie, 1977). The flagellum was approximated by a cylinder 40 μm in length and a radius of 125 nm. Turnover rates were obtained by fitting of a mono-exponential decay function to the average of 100 simulations.

Fitting of depolarizing V<sub>m</sub> signals

Depolarization signals resulting from 2-ms flashes in the presence of 100KASW were fitted with the equation:

\[
V_m(t) = A(1 - e^{-1/\tau_{on}})e^{-t/\tau_{off}}
\]

Estimate of time constant of CGMP binding

The time constant of cGMP binding to CNGK was calculated using:

\[
\tau_{on} = \frac{1}{k_{on}[CNGK]}
\]

Fitting of light-evoked V<sub>m</sub> responses

A macroscopic heuristic model was used for fitting voltage data. The change in free cGMP concentration ([cGMP]) was derived for the mass-balance equation:

\[
\frac{d[cGMP]}{dt} = k_{rel} - k_{on,PDE}[cGMP][PDE] - k_{on,CNGK}[cGMP][CNGK] + k_{off,CNGK}[CNGK · cGMP]
\]

where in <i>k<sub>rel</sub></i> represents the rate of cGMP release by light and [PDE], [PDE·cGMP], and [CNGK·cGMP] are the free concentrations of PDE, PDE bound to cGMP, and CNGK bound to cGMP,
respectively. For simplicity, we assume that [CNGK-cGMP] and $V_m$ are proportional to [cGMP] and that binding of cGMP to PDE is irreversible. Additionally, for low light levels, [PDE] and [CNGK] can be approximated by a constant.

Equation (3) was solved for a square pulse stimulation (Fig 3C). All fits were performed using nonlinear least-square methods. For a square release waveform, the release rate can be approximated by

$$k_{rel} = \begin{cases} k & t_{on} < t < t_{off} \\ 0 & \text{otherwise} \end{cases},$$

(4)

where in $k$ is a constant and $t_{on}$ and $t_{off}$ correspond to the time points where the UV light was switched on and off, respectively. Solving equation (3) yields the following:

$$V_{rmn} = \begin{cases} A(1-e^{-kt_{eff}}) & t_{on} < t < t_{off} \\ Ae^{-kt_{eff}} & \text{otherwise} \end{cases},$$

(5)

with $A$ and $k_{eff}$ being constants.

**Time constant of cGMP binding to CNGK**

The time constant of cGMP binding to the CNGK channel was estimated to be between 1 and 2 ms (equation 2) using a lower and upper bounds of $k_{on}$. A $k_{on} = 2.6 \times 10^7/M/s$ was determined for a CNBD of similar affinity (Peuker et al., 2012). The $k_{on}$ is moderately dependent on ionic strength; it is maximally twofold larger at the high ionic strength of ASW compared with a Ringer solution (Peuker et al., 2012). CNGK concentration is 21.6 μM. This exercise shows that cGMP binding is at least an order of magnitude faster than $\tau_{rise}$ of cGMP-induced $AV_m$.

**Estimate of membrane time constant**

The $\tau_{BC}$ of sperm was estimated to be 5–20 ms assuming an input resistance of mouse sperm of about 10–20 Ω (Navarro et al., 2007; Zeng et al., 2013). As membrane capacitance, we used $C_m = 1 \text{ pF}$. In mouse, $C_m$ was measured to be 2.5 pF (Navarro et al., 2007). Thus, the $C_m$ is estimated to be 1.25 pF in sea urchin sperm, i.e., half of the value measured in mouse sperm, whose flagellum is about twofold longer (100 μm) than that of $A. punctulata$ sperm. The time constant of CNGK activation is not known, but is of the order of a few milliseconds for ion channels (Hille, 2004). Thus, $\tau_{rise}$ of cGMP-induced $AV_m$ agrees well with these estimates.

**Estimate of the rate of cGMP by PDE5**

The rate $k_{cat}$ for PDE5 was estimated based on previous studies where the cGMP kinetics was evaluated by quench-flow techniques on intact sperm. In brief, sperm were mixed with saturating resact concentrations (250 mM) and the suspension was allowed to age. After different time intervals, the suspension was mixed with a denaturing agent (perchloric acid) stopping all biological reactions, and the cGMP concentration in the cell was determined using a radioimmunoassay (Kaupp et al., 2003). The total cGMP synthesized could be estimated from experiments performed in the presence of the PDE5 inhibitor IBMX to be about 110 pmol cGMP per 10⁸ cells, i.e., about 6.6 × 10⁸ cGMP molecules per sperm cell (or 660 μM), assuming a flagellar volume of 1.7 fl. At such high cGMP concentrations, all binding domains for cGMP are fully saturated. From the time difference between synthesis and total cGMP(t) in the cell, a hydrolysis rate of about 40 pmol cGMP per 10⁸ cells in 0.2 s can be derived. This rate is equivalent to a $k_{cat}$ of 24/s. This rate is larger than that observed for mammalian PDE5 (approximately 4/s) (Francis & Corbin, 2010). Of note, this estimate for $k_{cat}$ could underestimate the catalytic rate of the sea urchin PDE5, if enzyme inhibition by IBMX was incomplete.

**Single-cell Ca²⁺ and pH, measurements**

Sperm cells were suspended 1:100 (v/v) in ASW supplemented with 0.5% Pluronic F127 (Sigma-Aldrich) and either 30 μM GFP-certified FluoForte-AM (Enzo Life Sciences; Ca²⁺ imaging) or 20 μM pHrodo Red (Molecular Probes; pH imaging). Samples were incubated for 45 min at room temperature; 10 min prior to the measurements, DEACM-caged cGMP (15 μM) was included in the incubation. Fluorescence was recorded from single cells with an inverted microscope (Olympus IX71) equipped with a 20× objective lens (UPlanSApo 20×, 0.75 NA; Olympus) and two dichroic mirrors to image sperm that were loaded with GFP-certified FluoForte (520LP; FF520-Di02; Semrock) or pHrodo Red (560 DCXR; Chroma). For sharp imaging of the rapidly moving sperm flagellum, 2-ms pulses of excitation light were used (Spectra X Light Engine; Lumencor). For Ca²⁺ imaging, Teal light was used in combination with the excitation band-pass filter (513/17; FF01-513/17; Semrock) and a long-pass filter (BLP01-532R; Semrock). Due to the large difference in emission intensity between head and flagellum, imaging with pHrodo Red resulted either in a poor dynamical range for imaging of the flagellum or in a saturating head signal. To overcome this problem, we used two alternating excitation wavelengths with different pHrodo Red excitation efficacies: Green excitation (Spectra X Light Engine; Lumencor) in combination with a band-pass excitation filter (ET545/30; Chroma) was used to image the sperm flagellum, and Teal excitation (Spectra X Light Engine; Lumencor) in combination with an excitation filter (FF01-513/17 nm; Semrock) for imaging of the head. Emission light from pHrodo Red was filtered using a band-pass filter (BA575-625; Olympus). Emitted light was collected using a back-illuminated electron-multiplying charge-coupled device camera (DU-897D; Andor Technology). Photolysis of DEACM-caged cGMP was accomplished with either 365 nm LED (10 ms flashes; M365LP1, Thorlabs) or the 390-nm LED from the Spectra X Light Engine (20-ms flashes; Lumencor). Quantification of fluorescence signals was done using custom-made software written in MATLAB (MathWorks) (Alvarez et al., 2012). Signal smoothing was done using a moving average of three points, corresponding to a time window of 208 ms (pH imaging) or 107 ms (Ca²⁺ imaging).

**Data availability**

 Shotgun proteomics data have been uploaded to PRIDE with identifier PXD015332, http://www.ebi.ac.uk/pride/archive/projec ts/PXD015332. Targeted proteomics data can be accessed at PRIDE with identifier PXD015502 and PANORAMA with URL https://panoramaweb.org/arbacia_flagella.url.

Expanded View for this article is available online.
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Author contributions

UBK, CT, HH, LA, RS, and TS contributed to the design of the project; CT and AP performed and analyzed mass spectrometry experiments; HH, UBK, TS, and RS performed stopped-flow experiments; WB performed sequence analysis; HGK performed and analyzed mass spectrometry experiments; HH, UBK, TS, and RS wrote the manuscript. All authors edited the manuscript. The authors declare no competing financial interests.

Conflict of interest

The authors declare that they have no conflict of interest.

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programmed numerical simulation; LA, FL, and RP performed Ca

performed stopped-flow experiments; WB performed sequence analysis; HGK performed and analyzed mass spectrometry experiments; HH, UBK, TS, and RS wrote the manuscript. All authors edited the manuscript. The authors declare no competing financial interests.

Conflicts of interest

The authors declare that they have no conflict of interest.

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