Kashikar et al., http://www.jcb.org/cgi/content/full/jcb.201204024/DC1

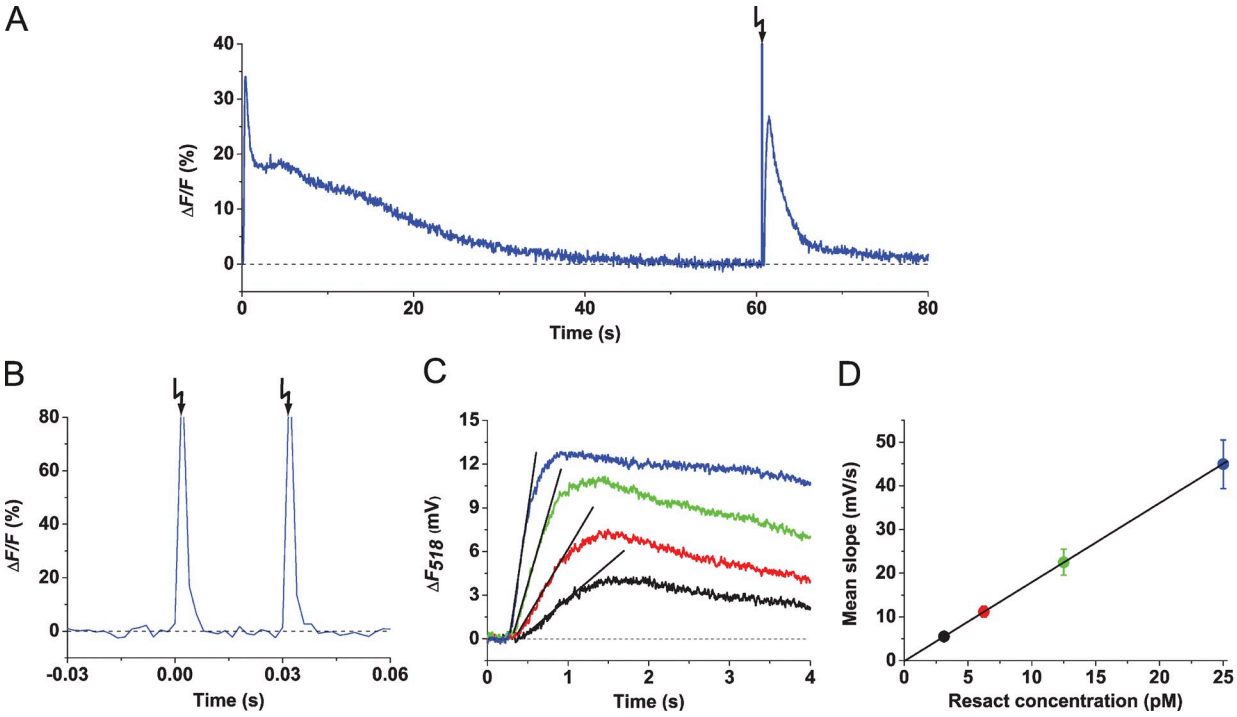


Figure S1. **Scheme of the sampling and resetting experiments using caged resact.** (A) Caged resact on its own is ∼1,000-fold less potent in initiating a signal compared with normal resact. For example, the signal evoked by 100 nM caged resact alone is equivalent to a signal evoked by ∼100 pM resact. We refer to this property as "residual activity" of caged resact. To perform sampling and resetting experiments, the Ca^{2+} signal evoked by the residual activity was allowed to return to the basal level (at 60 s) before a light flash was delivered. A flash of light released resact from the caged form and evoked a Ca^{2+} signal. The vertical line at 60 s represents the flash artifact. (B) Illustration of the temporal accuracy of paired stimuli: two flashes were set apart by 30 ms. Because the photomultiplier does not recover rapidly from the surge, a flash artifact of 5–20 ms was observed. Such flash artifacts illustrating the delivery of UV flashes are shown. Arrows on top depict the onset of first and second flash, respectively. (C and D) Calibration of the residual and the flash-induced signals from caged resact. (C) For calibration, we relied on the dose–response relation of the Ca^{2+} signals evoked by different concentrations of resact (in picomolars): 3.125 (black), 6.25 (red), 12.5 (green), and 25 (blue). The initial slope of the Ca^{2+} signals (depicted as black lines) was calculated as millivolts per second. (D) Linear regression of the plot of different resact concentrations against the respective slopes of the Ca^{2+} signal ($R^2 = 0.99$; $n = 3$; color coding as in C). To determine the residual activity of caged resact or the resact released by photolysis of caged resact, the slope of the Ca^{2+} signals was fit by a linear regression. Such a calibration assay was performed for each experiment. Arrows indicate time of flashes.

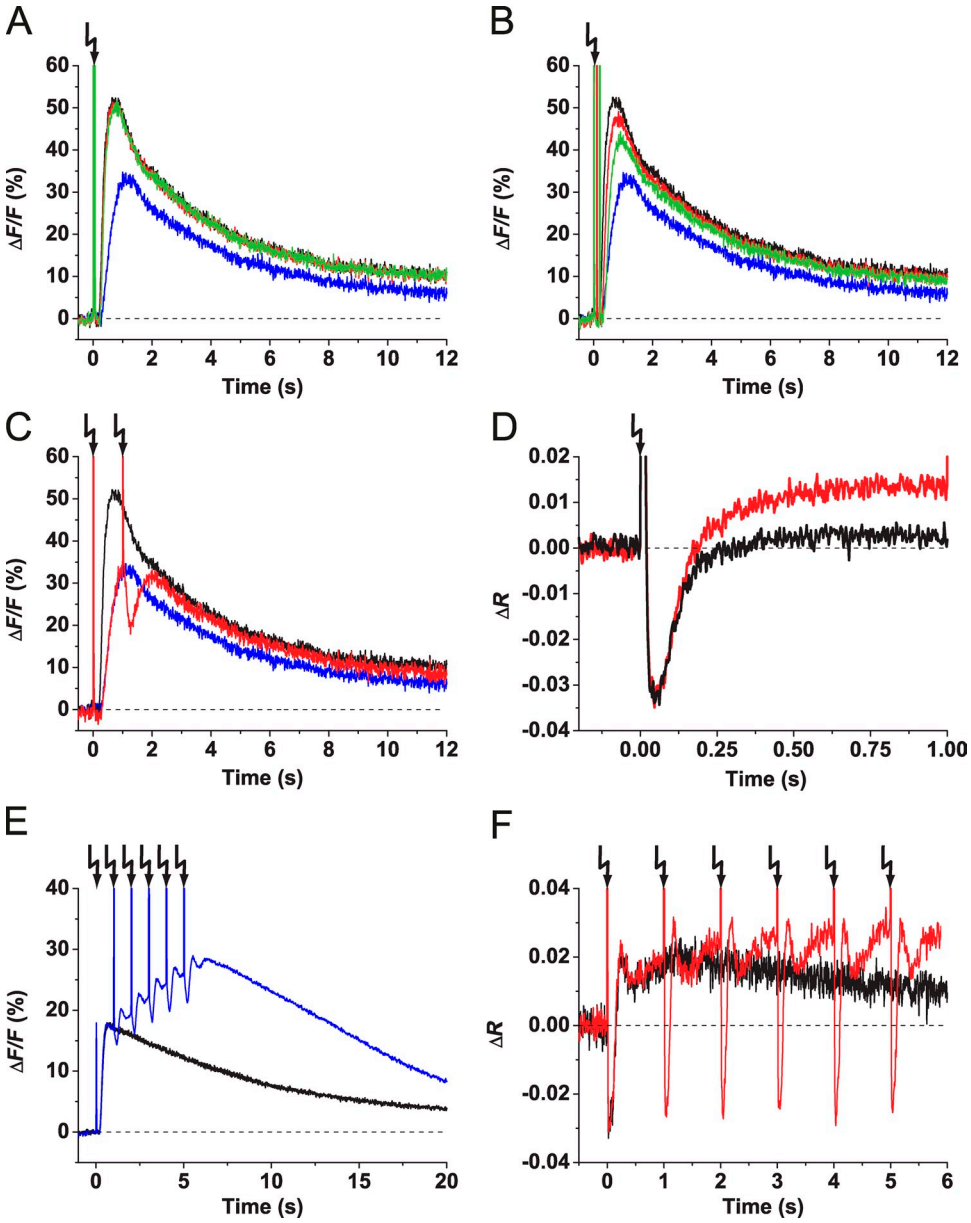


Figure S2. **Illustration of sampling and resetting experiments.** (A and B) Time course on a longer time scale of Ca^{2+} signals produced by paired stimuli (as in Fig. 2, A and B). Paired stimuli of 12.5 pM resact were delivered by flash photolysis of 100 nM caged resact. The time origin of the first flash was shifted to coincide to $t = 0$. (A) Single 50% flash (blue), single 100% flash (black), and paired 50% flashes with a Δt of 50 ms (red) are shown. (B) Single 50% flash (blue), single 100% flash (black), and paired 50% flashes with a Δt of 100 ms (red) and 200 ms (green) are shown. (C) The second stimulus on top of the first Ca^{2+} signal produces a decrease in $[Ca^{2+}]_i$. Ca^{2+} signals evoked by paired stimuli using 100 nM caged resact. Single 50% flash (blue) and two 50% flashes separated by 1 s (red) are given; for comparison, a single 100% flash (black) is shown. The time origin of the first flash was shifted to coincide with $t = 0$. (D) V_m signals were evoked by paired identical stimuli of cGMP with a Δt of 1 s (as in Fig. 3 B). For comparison of the two V_m signals, the t axis was shifted by the flash interval $-\Delta t$ and V_m before the respective flashes were set equal: first signal (red) and second signal (black). (E) Ca^{2+} signals produced by repetitive stimuli of cGMP (5% flash) with a Δt of 1 s each (blue trace) in *S. purpuratus* sperm. A Ca^{2+} signal evoked by a single 5% flash (black trace) is also shown. (F) V_m signals produced by repetitive stimuli of cGMP (5% flash) with a Δt of 1 s each (red trace) in *S. purpuratus* sperm. A V_m signal evoked by a single 5% flash (black trace) is also shown. Arrows indicate time of flashes.

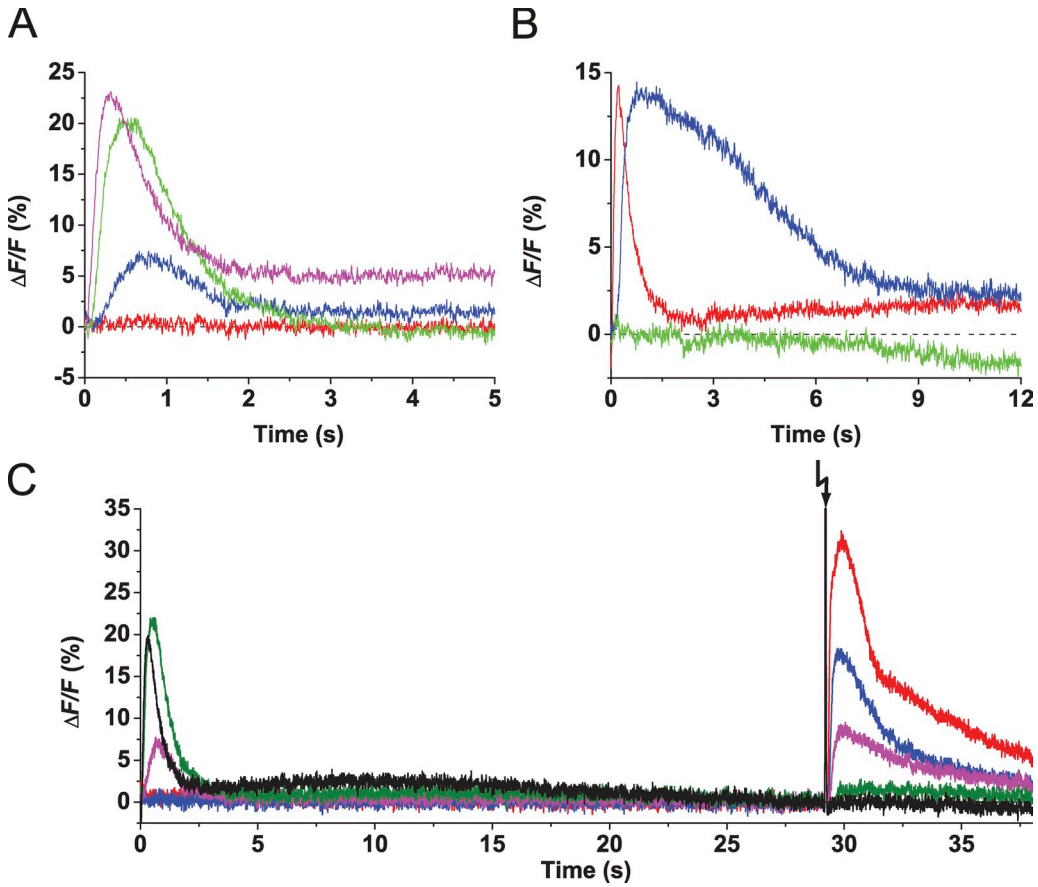
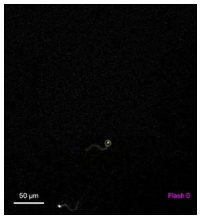


Figure S3. **Unspecific effects of Na^+/Ca^{2+} exchanger blocker KB-R7943 mesylate in sea urchin sperm.** (A) Ca^{2+} responses generated by mixing different concentrations of KB-R7943 mesylate (EMD) with sperm: 5 µM (red), 10 µM (blue), 25 µM (green), and 50 µM (magenta). The blocker on its own produced a rapid and transient Ca^{2+} response. (B) Ca^{2+} signal evoked by 25 pM resact (blue), 50 µM KB-R7943 (red), and 25 pM resact + 50 µM KB-R7943 (wherein, sperm were incubated first with the drug for 1 min and then mixed with resact; green). The blocker abolishes the resact-induced response. (C) Ca^{2+} signals evoked by cGMP 29.2 s after incubation with different concentrations of KB-R7943: 0 µM (control; red), 5 µM (blue), 10 µM (magenta), 25 µM (green), and 50 µM (black). The blocker also abolishes cGMP-induced responses in a dose-dependent manner. Moreover, within 1 min, the drug completely abolishes sperm motility (Su and Vacquier, 2002). All these effects most likely are unrelated to its inhibition of the NCKX exchanger; except for the brief transient Ca^{2+} increase, the drug displayed no long-term effect on $[Ca^{2+}]_i$. We conclude that this drug is not suitable for the study of Ca^{2+} homeostasis in sperm and possibly other cells. Arrows indicate time of flashes.



Video 1. **Resetting of Ca^{2+} signals in single moving sperm.** A sperm cell is loaded with the Ca^{2+} indicator Fluo-4 AM and caged cGMP. The cell is repeatedly stimulated by releasing cGMP from its caged derivative with six consecutive UV flashes. After each flash, the $[Ca^{2+}]_i$ rapidly decreases and rises anew. The recording was performed using an epifluorescent microscope (IX71). Frames were acquired at 30 frames/s using a back-illuminated EM CCD camera (DU-897D). Photolysis of caged compounds was achieved using a mercury lamp (U-RFL-T). The irradiation time was controlled by a mechanical shutter (VS25). Laser stroboscopic illumination (488-nm wavelength and 2-ms pulse) was achieved using an acousto-optical tunable filter (AA Opto-Electronic Company). The fluorescence was filtered by a 500-nm long pass filter (500 ALP; Omega Optical, Inc.). Superposition of the scale bar and flash number to the original video was performed using MATLAB, and editing was finalized using VideoStudio Pro X4 (Corel Corporation).

Table S1. **Mass spectrometric analysis of flagellar membrane protein preparation from *A. punctulata* sperm**

m/z (measured)	Molecular weight (determined)	Molecular weight (theoretical)	Peptide
523.7663	1,045.5180	1,045.4941	K.HGELSSNFR.R
401.5239	1,201.5499	1,201.5952	K.HGELSSNFRR.Q
601.8182	1,201.6218	1,201.5952	K.HGELSSNFRR.Q

List of tryptic peptides identified for NCKX. Dots indicate tryptic cleavage sites. m/z, mass per charge.

Table S2. **Mass spectrometric analysis of flagellar membrane protein preparation from *S. purpuratus* sperm**

m/z (measured)	Molecular weight (determined)	Molecular weight (theoretical)	Peptide
523.7558	1,045.4970	1,045.4941	K.HGELSSNFR.R
601.8078	1,201.6011	1,201.5952	K.HGELSSNFRR.Q
734.8661	1,467.7176	1,467.7147	R.FDDGAFGPIPPHAK.F
587.6455	1,759.9147	1,759.9216	R.KLPPLQAHELSEENR.K
587.6470	1,759.9193	1,759.9216	K.LPPLQAHELSEENRK.I
945.0108	1,888.0070	1,888.0166	R.KLPPLQAHELSEENRK.I
982.5498	1,963.0851	1,963.0850	R.IAINGGTNIALLNPADQLR.F
804.4379	2,410.2918	2,410.2870	K.ISVLSNQSLAVPGNNHIFHHVK.H
847.1353	2,538.3840	2,538.3819	R.KISVLSNQSLAVPGNNHIFHHVK.H

List of tryptic peptides identified for NCKX. Dots indicate tryptic cleavage sites. m/z, mass per charge.

Table S3. **Mass spectrometric analysis of flagellar membrane protein preparation from *S. purpuratus* sperm**

m/z (measured)	Molecular weight (determined)	Molecular weight (theoretical)	Peptide
360.1870	718.3594	718.3585	K.AVMWGR.N
368.1839	734.3531	734.3534	K.AVMWGR.N
377.2177	752.4208	752.4181	K.QHLLDK.V
379.7166	757.4187	757.4156	K.MIIPER.D
387.7127	773.4107	773.4105	K.MIIPER.D
393.2368	784.4590	784.4555	R.LQQQIR.V
432.2337	862.4529	862.4509	K.TGTLTTNR.M
433.7408	865.4670	865.4657	R.RTELYGK.N
459.2582	916.5017	916.4953	R.AQILWMR.G
465.2443	928.4739	928.4767	R.DRWPVEK.Y
467.2532	932.4918	932.4902	R.AQILWMR.G
468.2458	934.4771	934.4793	K.GASEIMLSK.C
476.2451	950.4756	950.4743	K.GASEIMLSK.C
556.2875	1,110.5605	1,110.5604	K.MLHDNNLVR.H
564.2875	1,126.5603	1,126.5553	K.MLHDNNLVR.H
589.2855	1,176.5564	1,176.5557	R.MVTGDNVNTAR.S
595.7762	1,189.5378	1,189.5397	K.QGDDALVMEGR.E
597.2839	1,192.5533	1,192.5506	R.MVTGDNVNTAR.S
603.7752	1,205.5359	1,205.5347	K.QGDDALVMEGR.E
606.8356	1,211.6566	1,211.6510	R.SSPTDKHTLVK.G
608.8323	1,215.6501	1,215.6459	R.KQDAINEISAK.Y
634.3555	1,266.6965	1,266.6932	K.LESEHTIAVIR.A
684.8516	1,367.6886	1,367.6867	R.ILPAEQQGEMPR.Q
460.2501	1,377.7285	1,377.7266	R.VVHAFQSGLQHR.I
692.8490	1,383.6834	1,383.6816	R.ILPAEQQGEMPR.Q
701.3856	1,400.7566	1,400.7664	K.VIYIANNVPVDGK.A
732.8568	1,463.6991	1,463.6991	K.LDESSLTGESDAIK.K
745.8922	1,489.7697	1,489.7599	K.SMSTIVPLPEGGFR.M
753.8822	1,505.7498	1,505.7548	K.SMSTIVPLPEGGFR.M
761.8822	1,521.7499	1,521.7497	K.ADVGFAMGLAGTDVAK.E
765.4395	1,528.8645	1,528.8613	R.KVIYIANNVPVDGK.A
769.8769	1,537.7393	1,537.7447	K.ADVGFAMGLAGTDVAK.E
774.3512	1,546.6878	1,546.6933	K.LEMEEAAIGNNDNK.R
780.3922	1,558.7699	1,558.7661	K.MIIPERDEGISDGK.S
788.3872	1,574.7599	1,574.7610	K.MIIPERDEGISDGK.S
796.9055	1,591.7965	1,591.7941	K.LDESSLTGESDAIKK.G
540.2960	1,617.8662	1,617.8549	R.KSMSTIVPLPEGGFR.M
817.9377	1,633.8608	1,633.8498	R.KSMSTIVPLPEGGFR.M
550.9591	1,649.8553	1,649.8447	K.KADVGFAMGLAGTDVAK.E
828.9281	1,655.8416	1,655.8366	R.EVVAVTGDGTNDAPALK.K
833.9252	1,665.8358	1,665.8396	K.KADVGFAMGLAGTDVAK.E
849.9583	1,697.9021	1,697.9022	K.ELTCIALVGIEDPVR.N
576.2952	1,725.8638	1,725.8798	K.TSPTHGINGLQFDIAR.R
583.3038	1,746.8896	1,746.8900	R.HLLVEGISVNSSYSSR.I
595.6529	1,783.9368	1,783.9316	R.EVVAVTGDGTNDAPALKK.A
904.9085	1,807.8025	1,807.8047	K.MFVETPGNNEIDSEAR.H
912.9071	1,823.7996	1,823.7996	K.MFVETPGNNEIDSEAR.H
940.9767	1,879.9389	1,879.9415	K.EASDIILTDDNFTSIVK.A
956.4827	1,910.9509	1,910.9520	R.NEVPPAIADCQSAGITVR.M
959.0010	1,915.9875	1,915.9891	K.YGDLLPADGVVIQSNDLK.L
694.6927	2,081.0563	2,081.0575	K.GVERDPMLLSGTHVLEGSGK.M
700.0254	2,097.0545	2,097.0525	K.GVERDPMLLSGTHVLEGSGK.M
737.3914	2,209.1523	2,209.1525	K.KGVERDPMLLSGTHVLEGSGK.M
738.3997	2,212.1774	2,212.1774	R.AGDVAQTVVQDIVVGDVCLIK.Y
763.6761	2,288.0066	2,288.0015	R.SSEPEHLPSMTPIDEDHPDR.V
769.0066	2,303.9980	2,303.9964	R.SSEPEHLPSMTPIDEDHPDR.V
839.0481	2,514.1224	2,514.1220	K.SLSNSVHEFMSPDNTYDIETTK.Q
844.3796	2,530.1169	2,530.1170	K.SLSNSVHEFMSPDNTYDIETTK.Q
928.7553	2,783.2441	2,783.2457	R.SSEPEHLPSMTPIDEDHPDRVDHK.R

List of tryptic peptides identified for Ca^{2+}-ATPase. Dots indicate tryptic cleavage sites. The underlined M means that in this peptide, the Met was oxidized (+16). m/z, mass per charge.

Reference

Su, Y.H., and V.D. Vacquier. 2002. A flagellar K^+-dependent Na^+/Ca^{2+} exchanger keeps Ca^{2+} low in sea urchin spermatozoa. *Proc. Natl. Acad. Sci. USA*. 99:6743–6748. http://dx.doi.org/10.1073/pnas.102186699