Biophysical Characterization of Genetically Encoded Voltage Sensor ASAP1: Dynamic Range Improvement

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ABSTRACT Recent work has introduced a new fluorescent voltage sensor, ASAP1, which can monitor rapid trains of action potentials in cultured neurons. This indicator is based on the Gallus gallus voltage-sensitive phosphatase with the phosphatase domain removed and a circularly permuted GFP placed in the S3-S4 linker. However, many of the biophysical details of this indicator remain unknown. In this work, we study the biophysical properties of ASAP1. Using the cut-open voltage clamp technique, we have simultaneously recorded fluorescence signals and gating currents from Xenopus laevis oocytes expressing ASAP1. Gating charge movement and fluorescence kinetics track closely with each other, although ASAP1 gating currents are significantly faster than those of Ciona intestinalis voltage-sensitive phosphatase. Altering the residue before the first gating charge removes a split in the ASAP1 QV curve, but preserves the accelerated kinetics that allow for the faithful tracking of action potentials in neurons.

The hunt for a robust, genetically encoded voltage indicator (GEVI) has been ongoing for the last several decades (1). Although some rely on rhodopsin constructs (2), alternative GEVI designs capitalize on modifications of stereotypical S4-type voltage sensors like ArcLight and VSFP2.3 (3,4). Accelerated sensor of action potentials 1 (ASAP1) (5), is based on a mutated voltage-sensing domain of Gallus gallus (GgVSD) voltage-sensitive phosphatase, where a circularly permuted GFP (cpGFP) (6) is added in the S3-S4 linker, making the fluorophore extracellular. The first arginine of GgVSD is mutated to a glutamine (R153Q), shifting the voltage dependence. Previous studies have demonstrated the ability of ASAP1 and its subsequent versions to follow action potentials in neurons in vitro (5) and in vivo (7). Yet, a deeper understanding of the mechanism of ASAP1 could generate a more versatile GEVI. By incorporating rationally chosen mutations, we can evaluate the degree to which this voltage-sensitive phosphatase resembles, functionally and structurally, the extensively investigated Ciona intestinalis voltage sensitive phosphatase (CiVSP). This way we can determine the nature of its accelerated kinetics, and engineer faster and more robust voltage indicators. We expressed ASAP1 in Xenopus laevis oocytes and simultaneously recorded gating currents (Fig. 1A) and fluorescence signals (Fig. 1B) using the cut-open voltage clamp method (8). ASAP1 displays a striking split in both electrophysiological and optical signals, indicating the presence of an intermediate state (Fig. 1C). The fluorescence signal shows high fidelity to the movement of the gating charge. Both the kinetics of fluorescence and gating charge movement have a slow and a fast component, where the majority of the kinetics is determined by the fast component (Table S1). The fast component of the gating charge time constants ($\tau_{fast}$) and the fluorescence signal are in the 1–4 ms range (Fig. 1D). Another important metric for all GEVIs is the time it takes for the fluorescence to change in response to a change in voltage, called the fluorescence lag. For ASAP1, this lag is in the submillisecond range and represents a large improvement compared with other GEVIs (Fig. 2).

The split in the QV creates a plateau between $-100$ and 0 mV, which occurs in both the QV and FV and points to a stable intermediate state. This clearly limits the dynamic potential of ASAP1 and subsequent versions like ASAP2f by limiting the fluorescence read-out in the physiological voltage range (Fig. S1). A large split in the QV is not a property shared by other voltage-sensitive...
phosphatases (9), thus we hypothesized that this phenomenon might be related to the addition of the GFP in the S3-S4 linker. Indeed, removal of the cpGFP from ASAP1, also known as GgVSD R153Q, leads to a sensor with a QV curve fitted by a two-state model (a single Boltzmann). Fig. 3 shows that the kinetics and steady-state features of GgVSD and ASAP1 are drastically different from one another as a result of the addition of a cpGFP to the S3-S4 linker. We reasoned that addition of the cpGFP led to an acceleration in gating current kinetics not seen in other voltage-sensitive phosphatases (9,10).

Residues in the hydrophobic plug of ASAP1 are similar to those in CiVSP, such as I126 (11). Moreover, gating and fluorescence kinetics in ASAP1 are very similar to each other. Therefore, we hypothesized that alterations of these similar hydrophobic plug residues in ASAP1 would result in accelerated gating. However, although some of the mutations effectively altered the biophysical properties of ASAP1 (Fig. S2), the analogous mutations in CiVSP did not improve the fluorescence signal or accelerate sensing kinetics. This may be due to the cpGFP in the linker.
Recent work (12) in the voltage-gated potassium channel Shaker has demonstrated the importance of nonsensing residues in position 361 for tuning the voltage dependence of the voltage-sensing domain, and we have used this knowledge to further improve on ASAP1. Changing leucine 158 to a tyrosine removed the intermediate state in the QV curve, but preserved the accelerated kinetics and large signal (Fig. 4, A–C). The $\tau_{\text{fast}}$ of the gating charge and the fluorescence were $1.95 \pm 0.29$ and $1.74 \pm 0.23$ ms (mean $\pm$ SE) at 60 mV, respectively. Change of this magnitude in the QV and FV curves within the physiological range generates a larger dynamic range than ASAP1 (Fig. 4 D). We named this new variant of ASAP1, ASAP-Y.

![FIGURE 4 Biophysical characterization of ASAP-Y and comparison to ASAP1. (A) Normalized QV curves for ASAP1 (circle) and ASAP-Y (square). (B) A comparison of the activation $\tau_{\text{fast}}$ of the gating charge for ASAP1 and ASAP-Y. (C) Normalized QV (circle) and 1-FV$_{\text{normalized}}$ (square) curves for ASAP-Y. (D) A comparison of the $\Delta F/F$ % in a physiological range of ASAP1 (circle) and ASAP-Y (square). (ASAP1: $n = 8$; ASAP-Y: $n = 6$). To see this figure in color, go online.](image)

![FIGURE 5 ASAP-Y can follow action potentials in rat dorsal root ganglion neurons. (A) Representative traces of action potentials from dorsal root ganglion neurons transfected with ASAP1 (left) and ASAP-Y (right). Corresponding fluorescence traces are shown below as $\Delta F/F$ (%). (B) A table comparing the $\Delta F/F$ per 100 mV and deactivation $\tau$ of ASAP1 and ASAP-Y fluorescence. ASAP1: $n = 11$; ASAP-Y: $n = 13$.](image)
To determine the effectiveness of our new tool, we transfected rat dorsal root ganglion neurons with ASAP1 and ASAP-Y and recorded action potentials using whole-cell patch clamp. ASAP-Y has comparable transfection efficiency and photobleaching properties to ASAP1 (Fig. S3). We observed fluorescence read-outs of action potentials with both ASAP1 and ASAP-Y and demonstrated increased signal amplitude with ASAP-Y, even though the voltage range spanned by the action potentials in both cases was similar (Fig. 5 A). This result is expected when comparing the FV curves of ASAP1 and ASAP-Y (Figs. 1 D and 4 C). Furthermore, ASAP-Y displayed faster kinetics on the return to baseline than ASAP1 (Fig. 5 B).

We found that the fast kinetics of ASAP1 and ASAP-Y are due to the addition of the cpGFP in the S3-S4 loop. This observation is in line with the reported crystal structures of CiVSP in the resting and activated states (13), where it was found that the position of this loop changes drastically during activation.

In conclusion, a rational design method has allowed us to improve upon an existing GEVI to create ASAP-Y. This is, to our knowledge, a novel optical voltage probe with a steeper fluorescence response in the physiological range of voltage changes.

SUPPORTING MATERIAL

Supplemental Materials and Methods, three figures, and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)31133-5.

AUTHOR CONTRIBUTIONS

F.B. and E.E.L.L. designed research, analyzed data, and wrote the manuscript. E.E.L.L. performed research. F.B. contributed analytic tools.

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REFERENCES

Supplemental Information

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Supplemental Figure 1 Biophysical characterization of ASAP2f.
A) Comparison of the ΔF/F of ASAP1 (bright red, circles) to ASAP2f (dark red, squares). B) Comparison of the QV curves of ASAP1 (bright red, circles) to ASAP2f (dark red, squares). C) Comparison of QV normalized and 1-FV normalized (inverted fluorescence) of ASAP2f gating (blue, circles) and fluorescence (red, squares). D) The weighted τ’s of ASAP2f activation gating (blue, circles) and fluorescence (red, squares). E) A comparison of fluorescence lag of ASAP1 (bright red, circles) vs ASAP2f (dark red, squares). F) Table of activation τ at 60mV of ASAP2f. (ASAP1 n=8, ASAP2f n = 6)
Supplemental Figure 2 CiVSP-R217X-motivated mutation in ASAP1.
A) A comparison of the QV\textsubscript{off} with several residues at position 153 ASAP1 (blue, circle), Q153D (white, circle), Q153E (light blue, square), Q153R (light blue, triangle). B) \(V_{1/2}\) of ASAP1 153X constructs. (ASAP1 n=11, Q153D n=5 , Q153E n=4, Q153R n=5)
Supplemental Figure 3 Photobleaching properties of ASAP1 and ASAP-Y.
Cultured DRG neurons expressing ASAP1 (red, circles) or ASAP-Y (light blue, squares) were illuminated continuously with 488nm light at 1.5W/cm². Each neuron was imaged every 10 seconds for 10 minutes. Fluorescence was normalized to 1.0 at t=0. Photobleaching time constants (τ) were estimated from single exponential fits with ASAP1 1.852 min and ASAP-Y 2.282 min. ASAP1, n=7, ASAP-Y, n=8
Supplemental Table 1 Activation Kinetics of ASAP1 and ASAP-Y.
A comparison of activation kinetics of gating charge and fluorescence of ASAP1 and ASAP-Y at 60mV. Data are presented as mean±S.E.M. ASAP1, n=8, ASAP-Y, n=6.

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<th>ASAP1</th>
<th>ASAP-Y</th>
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<td>τ_{fast} (ms)</td>
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<td>τ_{slow} (ms)</td>
<td>52.1±3.22</td>
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<td>Percentage fast</td>
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Supplemental References

**Methods**

**Cell culture**
Glass-bottomed culture dishes (Cellvis, Mountain View, CA) were thoroughly cleaned, rinsed with water, dried, and sterilized with UV light. They were then incubated with poly-L-lysine (PLL, Thermo Fischer Scientific) for 15 min at room temperature, after which they were rinsed with sterile water several times and stored until use.

Dorsal root ganglia were isolated from P1-P3 Sprague-Dawley rats following decapitation and were immediately placed in DMEM medium (Thermo Fisher Scientific) on ice. Ganglia were rinsed multiple times with Earle’s Balanced Salt Solution (EBSS, Thermo Fisher Scientific) then digested with EBSS + 0.25% trypsin (Thermo Fisher Scientific) for 20 min at 37°C with shaking. Following digestion, the cells were centrifuged and the supernatant removed and replaced with EBSS + 10% Fetal bovine serum (FBS, Thermo Fisher Scientific) to inhibit remaining trypsin. The digested ganglia were then extruded through three glass pipettes of decreasing size to mechanically disperse the cells. The cells were centrifuged a final time and the supernatant replaced with DMEM + 5% FBS. Cells were seeded into the previously prepared PLL-treated culture dishes and allowed to sit undisturbed for 30 min to facilitate DRG cell adhesion to the PLL-treated glass. Finally, the dishes were flooded with DMEM + 5% FBS + 100 U/ml penicillin (Thermo Fisher Scientific) + 100 ug/ml streptomycin (DRG media) and incubated at 37°C with 5% CO₂ until use.

**Molecular biology**
ASAP1 for Xenopus laevis expression was created from the pcDNA3.1/Puro-CAG-ASAP1 which was a gift from Michael Lin (Addgene plasmid # 52519) (St-Pierre et al., 2014). ASAP-Y was identical to ASAP1 with an additional point mutation at L158Y. Using site-directed mutagenesis by polymerase chain reaction, we added L158Y to this construct and verified it via sequencing. ASAP2f for Xenopus laevis expression was created from ASAP1 with the removal of A146 and the change of residue 147 from an alanine to a serine as described previously (Yang et al., 2016).

For oocyte expression, DNA was prepared using the NucleoSpin Plasmid kit (Macherey-Nagel, Bethlehem, PA) and linearized with XbaI (New England Biolabs, Ipswich, MA). Linearized cDNA was transcribed to RNA with the mMESSAGE mMACHINE Sp6 kit (Life Technologies, Carlsbad, CA). Oocytes were injected with 50 ng of RNA and incubated at 18°C in solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, at pH 7.4 with 10 mg/L of gentamicin. Recordings were made 1-2 days following injection.

For neuronal mammalian cell expression, DNA was prepared using the NucleoBond Xtra Midi Plus kit (Macherey-Nagel). DNA was then transfected into rat DRG neurons had been previously plated on glass coverslips at low density, using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA). To transfect, each dish had mixture of OptiMem (Thermo Fisher Scientific), Lipofectatmine 2000, and 6 ug of DNA added drop-wise. After five hours, the cells were gently rinsed with DRG media and incubated at 37°C with 5% CO₂, 24-48 hours prior to recording.

**Electrophysiology**
For oocytes, simultaneous recordings of ASAP1 gating currents and fluorescence responses were performed using the cut-open oocyte voltage-clamp technique (Stefani and Bezanilla, 1998) in combination with a photodiode to measure temporal changes in fluorescence emission (Cha and Bezanilla, 1998). Gpatch, an in-house program, controlled an SB6711 digital signal processor-based board (Innovative Integration, Simi Valley, CA) with an A4D4 board (Innovative Integration, Simi Valley, CA). Oocytes were held under voltage-clamp with a Dagan CA-1B amplifier (Minneapolis, MN) and current was filtered at 5 kHz. ASAP1 was sampled at 50 kHz and subsequent constructs’ fluorescence emissions were collected through an Olympus LUMPlan FL N 40X/0.8 NA water-immersion objective by a PIN-020A photodiode (UDT Technologies, Torrance, CA), amplified by a patch clamp amplifier L/M-EPC-7 by LIST Medical Electronic (Darmstadt, West Germany) with a filter of 10 kHz, and then integrated over each sampling period using a home-built integrator circuit. ASAP1 and subsequent constructs were excited via a ThorLabs LED controller triggering a 470 nm LED (ThorLabs, Newton, New Jersey) that was passed through a filter cube housing a 480/40 excitation filter, a 505 long-pass dichroic, and a 535/50 emission filter (Chroma, Bellows Falls, VT). All recordings were performed at around 18°C, with an external solution containing (in mM) 115 N-methyl-D-glucamine/methanesulfonic acid (NMG/MES), 10 HEPES, and 2 Ca(OH)\textsubscript{2} and an internal solution containing (in mM) 115 NMG-MES, 10 HEPES, and 2 EGTA. Both solutions were set to pH 7.5. Microelectrodes were pulled on a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA, model P-87) and were filled with 3 M CsCl\textsubscript{2} and had a resistance of ~0.2–0.8 M\textOmega.

For neuronal mammalian cell expression, ASAP1 and ASAP-Y fluorescence from dorsal root ganglion neurons was recorded by an Evolve 128 EMCCD camera (Photometrics, Tucson, Arizona) attached to an Olympus IX71 inverted microscope (Center Valley, Pennsylvania) with a 40X/0.6 NA microscope objective (Olympus). Excitation was provided by a 473 nm DPSS laser (Shanghai Dream Lasers Technology Co., Ltd., Shanghai, China). Typical excitation intensities were around 1.5W/cm\textsuperscript{2} through the 40X objective listed above. Fluorescence was observed through a T495lpxt dichroic and ET500lp emission filter (Chroma Technology Corp., Bellows Falls, VT).

Patch clamp was performed with an Axopatch 200A integrating patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Both the electrophysiological and optical equipment were controlled using in-house software. Recordings were performed at around 18°C, in a solution containing (in mM) 132 NaCl, 4 KCl, 1.2 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 10 HEPES, 5.5 Glucose, pH 7.4 with NaOH. Patch pipettes pulled on Sutter model P-2000 (Sutter Instrument Co., Novato, CA) were filled with a solution containing (in mM) 10 NaCl, 130 KF, 4.5 MgCl\textsubscript{2}, 10 HEPES, 9 EGTA, 2 ATP, pH 7.3.

**Data analysis**

Data analysis was performed in MATLAB (The MathWorks, Inc., Natick, MA), as well as in-house software. The time constants of gating charge were taken using double exponential fits to the rising phase of the integration of the gating current. Kinetics of fluorescence traces were first taken from double exponential fits to the trace; when fit with two exponentials, fluorescence traces obtained from off pulses in oocytes only had one meaningful time constant, and thus were refit with one exponential. For the DRGs, time constants of the fluorescence deactivation were
taken using an exponential fit to the decay of the fluorescence signal from the action potential. For clarity of comparison, all kinetics were reported as $\tau_{\text{fast}}$, which denotes the faster and larger amplitude component of the fluorescence traces. Q-V curves were calculated by normalizing the integral of the gating current following a sloped baseline subtraction to remove leak current. (Villalba-Galea et al., 2008)

**Method references**


