Light-Induced Depolarization of Neurons Using a Modified Shaker K⁺ Channel and a Molecular Photoswitch

James J. Chambers, Matthew R. Banghart, Dirk Trauner, and Richard H. Kramer. Light-induced depolarization of neurons using a modified Shaker K⁺ channel and a molecular photoswitch. *J Neurophysiol* 96: 2792–2796, 2006. First published July 26, 2006; doi:10.1152/jn.00318.2006. To trigger action potentials in neurons, most investigators use electrical or chemical stimulation. Here we describe an optical stimulation method based on semi-synthetic light-activated ion channels. These SPARK (synthetic photoisomerizable azobenzene-regulated K⁺) channels consist of a synthetic azobenzene-containing photoswitch and a genetically modified Shaker K⁺ channel protein. SPARK channels with a wild-type selectivity filter elicit hyperpolarization and suppress action potential firing when activated by 390 nm light. A mutation in the pore converts the K⁺-selective Shaker channel into a nonselective cation channel. Activation of this modified channel with the same wavelength of light elicits depolarization of the membrane potential. Expression of this modified channel in neurons allows light to reversibly hyperpolarize, and therefore silence, action potential firing. Here we have modified the K⁺ channel protein to make the pore nonselective in its permeability to cations, such that opening of the channel causes depolarization rather than hyperpolarization. Expression of this depolarizing SPARK (D-SPARK) channel causes reversible photo-stimulation, with the same wavelengths that cause photo-silencing of neurons expressing the original hyperpolarizing SPARK (H-SPARK) channel.

SPARK ion channels have two parts: a synthetic photoswitch that is covalently attached to a genetically engineered Shaker K⁺ channel protein. The photoswitch (MAL-AZO-QA) consists of a cysteine-reactive maleimide (MAL) group, an azobenzene (AZO) group, which is photoisomerizable, and a quaternary ammonium (QA) group, which is a blocker of the pore of K⁺ channels (Fig. 1A). The channel protein is engineered to allow attachment of the photoswitch to an extracellular cysteine positioned near the pore, and has mutations to render the channel constitutively active in the absence of the photoswitch. When the AZO is in its trans configuration, the QA can reach the pore, blocking ion flow. Photoisomerization to the cis form shortens the AZO removing the QA, unblocking the pore. Hence MAL-AZO-QA acts as an artificial light-sensitive gate for the channel. By extending and retracting the QA from the pore, different wavelengths of light turn the K⁺ current on and off (Fig. 1B).

Wild-type Shaker channels are highly selective for K⁺ over other cations (Hille 1992). To convert Shaker into a nonselective cation channel, we introduced a single point mutation into the pore-lining domain, converting valine-443 into glutamine (V443Q). Mutation of this residue, which is part of the conserved signature sequence of voltage-gated K⁺ channels, reduces the K⁺: Na⁺ permeability ratio of Shaker from <0.02 to about 0.70 (Heginbotham et al. 1994). Other than this mutation, the structure of the D-SPARK channel protein is identical to the H-SPARK protein (Fig. 1C).

METHODS

Chinese hamster ovary (CHO) cells were cultured on glass in 10% FBS in F12 media to ~70% confluency. Primary dissociated hippocampal cultures were prepared from embryonic day E18-19 Sprague-Dawley rat embryos and were cultured on polylysine-coated

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The resulting in depolarization of the membrane potential in cells expressing the trans form with visible light or through thermal relaxation. A schematic representation of the K⁺-selective H-SPARK channel. The QA group blocks the QA group (red oval) alters ion selectivity, allowing Na⁺ influx after UV photosimerization, resulting in depolarization of the membrane potential in cells expressing the D-SPARK channel.

FIG. 1. Light regulation of current flow in hyper- and depolarizing synthetic photoisomerizable azobenzene-regulated K⁺ (H-SPARK and D-SPARK channels. A: maleimide-azobenzene-quaternary ammonium (MAL-AZO-QA) photoswitch isomerizes to the cis form on exposure to UV light and returns to the trans form with visible light or through thermal relaxation. B: schematic representation of the K⁺-selective H-SPARK channel. The QA group blocks ion conduction when AZO is in its trans configuration (left). UV light induces photoisomerization, retracting the QA group, thus allowing K⁺ to flow out and hyperpolarize the cell. C: point mutation in the pore of the channel (red oval) alters ion selectivity, allowing Na⁺ influx after UV photosimerization, resulting in depolarization of the membrane potential in cells expressing the D-SPARK channel.

Glass coverslips in serum containing medium. Both mammalian cell types were grown in 7% CO₂ in air at 37°C. All animal care and experimental protocols were approved by the Animal Care and Use Committee at UC Berkeley.

Both cell line and primary cell culture were transfected with 0.8 μg DNA per 12 mm coverslip encoding either eGFP or eGFP-tagged Shaker H4 channels, with the following mutations: Δ6–46, L366A, E422C, V443Q (for D-SPARK only), and T449V. The eGFP was tagged to the N terminal of the Shaker gene. Transfections with CaPO₄ were carried out at 10–13 days in vitro for the primary culture and electrophysiological recording was performed ~48 h later (Dudek et al. 2003). Coverslips containing cells were treated for 15 min with 300 μM MAL-AZO-QA at 37°C in an extracellular recording solution containing (in mM) 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 5 HEPES, 2.5 CaCl₂, and 10 glucose at pH 7.4. The concentration of DMSO in the bath did not exceed 0.1%. Patch pipettes (4–8 MΩ) were filled with (in mM) 10 NaCl, 135 K-glucionate, 10 HEPES, 2 MgCl₂, 2 Mg-ATP, and 1 EGTA at pH 7.4. After washout of MAL-AZO-QA with extracellular solution, whole patch was established. Voltage-clamp configuration was used to generate I-V data and then the configuration was changed to current clamp and the membrane potential was recorded. Initial recordings were made at the resting potential to evaluate the effects of light on spontaneous activity in neurons. Pulse protocols and measurements were carried out with pCLAMP 8.0 software, a DigiData 1200 series interface, and an AxoPatch 200A amplifier (Axon Instruments). Samples were taken at 10 kHz, and the data were filtered at 1 kHz. Seals with a leak current of >200 pA were not included in the analysis. Cells were irradiated using a Lambda-LS illuminator containing a 125-W xenon arc lamp (Sutter Instruments) equipped with narrow-band-pass (±10 nm) filters through a Fluor ×20, 0.5 n.a. objective lens (Nikon).

Cell viability assays were performed according to the protocol provided by the vendor of the Live/Dead Kit (Invitrogen). Variability among data are expressed as means ± SD.

RESULTS

To characterize the electrophysiological properties of D-SPARK channels, CHO cells transfected with the GFP-tagged D-SPARK protein were treated with 300 μM MAL-AZO-QA for 15 min before establishing whole cell patch recordings. Steady-state current-voltage (I-V) curves show that exposure to 390- and 505-nm light, increased and decreased the conductance, respectively, and that the reversal potential of the light-regulated conductance was near 0 mV (Fig. 2A). No light-induced effect was observed when control cells were tested (Fig. 2B). Light had similar effects on cells expressing H-SPARK, but the reversal potential was near −70 mV as expected for a K⁺-selective channel (Fig. 2C). A point mutation (L366A) was introduced into the S4 domain of both H- and D-SPARK to shift the activation potential to −70 mV compared with −35 mV for wild type (Lopez et al. 1991). This mutation along with disruption of fast (N-type) inactivation renders H-SPARK constitutively active at physiological membrane potentials (> −65 mV), but the channel can be closed at potentials less than −65 mV. We observed a surprising difference between the voltage-dependent gating of H- and D-SPARK channels. D-SPARK remained open at membrane potentials down to −100 mV. Apparently the combination of a pore mutation and a voltage-sensor mutation disrupts the normal voltage control over the permeation pathway (Molina et al. 1998).

In current-clamp mode, CHO cells that are expressing D-SPARK and treated with MAL-AZO-QA depolarize when illuminated with 390-nm light, and hyperpolarize when illuminated with 505-nm light, reaching steady state within several seconds (Fig. 2D). Channels opened with 390-nm light closed spontaneously in the dark, but the rate of closure is slow (tens of seconds) (M. R. Banghart, D. L. Fortin, R. H. Kramer, and D. Trauner, unpublished data). When cells were current clamped to a membrane potential similar to the resting potential of many cells (~40 mV), opening and closing the channels had opposite effects on cells expressing D- and H-SPARK channels (Fig. 2E). Cells expressing D-SPARK depolarized by ~9 mV on exposure to 390-nm light, whereas cells expressing H-SPARK hyperpolarized by ~8 mV. Switching to 505-nm light reversed the effect in both cells. Cells expressing GFP alone showed no significant change in membrane potential on exposure to light.

D-SPARK channels could also be expressed and photo-switched in mammalian neurons. The gene encoding the D-
SPARK channel protein was transfected into hippocampal neurons in culture using the calcium phosphate transfection method (Dudek et al. 2003). After ~48 h, neurons expressing GFP-tagged D-SPARK could be identified by their fluorescence. To covalently attach the photoswitch to the channels, the cultures were treated with 300 μM MAL-AZO-QA for 15 min at 37°C. After washing away unreacted MAL-AZO-QA, whole cell patch-clamp recordings were established. Under voltage clamp, we found that transfected cells showed an increase in membrane conductance on exposure to 390-nm light and a decrease on exposure to 505-nm light. Under current clamp, 390-nm light depolarized transfected neurons and triggered bursts of action potentials, and 505-nm light reversed the depolarization, halting firing (Fig. 3). Neurons expressing GFP alone were insensitive to light.

Expression of the D-SPARK protein without MAL-AZO-QA present did not affect the viability of hippocampal neurons in culture. The percentage of neurons successfully transfected with GFP-tagged D-SPARK or GFP alone were the same (0.5% in both cases), and there was no significant difference in the fraction of each that tested positive for DEAD Red nuclear staining (Table 1). In fact, culturing transfected neurons in the presence of Shaker channel blockers, including 4-aminopyridine, dendrotoxin-K, or charybdotoxin, did not alter viability, and therefore was not necessary. We also found that D-SPARK expression had no significant effect on the resting membrane potential or on input resistance. This was surprising because D-SPARK channels are expected to be constitutively open before adding the photoswitch. Furthermore, after the addition of the photoswitch, the membrane potential should hyperpolarize as the open cation channels are blocked. Our recordings from D-SPARK-expressing neurons suggested that the resting potential of MAL-AZO-QA-treated cells was slightly more hyperpolarized (by ~5 mV) as compared with untreated cells (Table 1). However, the difference was not statistically significant (P = 0.26 by t-test). There are several possible explanations for the lack of a clear depolarization in D-SPARK-expressing neurons and the lack of a clear hyperpolarization in the subset of those neurons that were treated with MAL-AZO-QA. First, some of the D-SPARK channels may be present on distal parts of the neuron, limiting their contribution to their resting potential measured at the cell body. Second, hippocampal neurons may compensate for D-SPARK expression by homeostatically altering expression levels of other channels (Turrigiano and Nelson 2004). Finally, it is also possible that D-SPARK subunits co-assemble with native K⁺ channel subunits in neurons to produce heteromeric channels whose properties differ from those of homomeric

FIG. 2. Properties of H- and D-SPARK channels expressed in Chinese hamster ovary (CHO) cells. Steady-state I-V curves showing the effect of photoswitching. A: cells transfected with GFP-tagged D-SPARK channels and treated with MAL-AZO-QA elicit more current when stepped to any given voltage when 390 nm light is used for illumination (open circles) when compared with 505 nm light (closed triangles). B: control cells transfected with GFP plasmid and similarly treated with MAL-AZO-QA do not respond to light. C: cells expressing H-SPARK channels and treated with MAL-AZO-QA also conduct more current on exposure to UV light. D: current-clamp recording from a CHO cell showing repeatable depolarization by exposure to 390 nm light (white bar) and hyperpolarization in 505 nm light (black bar). E: D-SPARK channels closed slowly in the dark (hashed bar) through thermal relaxation of the photoswitch. E: average membrane potential changes elicited by light (n = 10 CHO cells for each treatment).
physiological parameters measured in neurons prior to MAL-AZO-QA photoswitch addition

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<thead>
<tr>
<th></th>
<th>Input resistance, MΩ</th>
<th>Resting membrane potential, mV</th>
<th>Viability (exclusion of DEAD Red), %</th>
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<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td>n=139</td>
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<tr>
<td>D-SPARK</td>
<td>171.1 ± 104.0</td>
<td>-51.2 ± 10.4</td>
<td>89.9 alive (n = 139)</td>
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<tr>
<td>D-SPARK with AZO-QA</td>
<td>186.7 ± 94.1</td>
<td>-56.1 ± 8.7</td>
<td>ND</td>
</tr>
<tr>
<td>GFP Only</td>
<td>196.2 ± 100.5</td>
<td>-53.3 ± 7.2</td>
<td>91.4 alive (n = 128)</td>
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Values are means ± SD. Values in parentheses are numbers alive. MAL, maleimide; AZO, azobenzene; QA, quaternary ammonium; ND, not determined.
vector to drive H-SPARK expression in the intact rat retina. Addition of MAL-AZO-QA, which penetrates effectively into the retina, imparts light sensitivity onto retinal ganglion cells. Recently researchers using virally expressed Channelrhodopsin have demonstrated the use of this tool for the activation of populations of retinal ganglion cells directly by light. Ganglion cells were caused to fire trains of action potentials on visual stimulus of the correct wavelength, 460 nm for the expressed channel (Bi et al. 2006). We have recently built a light-activated glutamate receptor (LiGluR), by substituting QA with glutamate and expressing a mutant form of the GluR6 receptor (Volgraf et al. 2006). Illumination with 390-nm light opens this channel and depolarizes neurons in which it is expressed; hence it could also be used to build a virtual ON cell. Because the photoswitch is the same in H-SPARK, D-SPARK, and LiGluR, the same wavelengths of light that silence virtual OFF cells expressing H-SPARK should activate virtual ON cells expressing D-SPARK or LiGluR. In wholly different experiments to those discussed here, we have obtained data that demonstrate that the photoswitch can penetrate intact tissue and thus should not limit the applicability of this approach to preparations other than dissociated culture. This would greatly simplify the task of regulating the activity of retinal ganglion cells, assuming the appropriate channel type could be expressed selectively in the appropriate cell type. The identification of ON and OFF cell specific proteins may lead to discovery of cell-type specific gene regulatory elements, providing a potential means for selectively expressing the light-activated channels in their appropriate neuronal populations.

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