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Engineering Light-Gated Ion Channels[†]

Matthew R. Banghart, Matthew Volgraf, and Dirk Trauner*

Department of Chemistry, University of California, Berkeley, California 94720

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ABSTRACT: Ion channels are gated by a variety of stimuli, including ligands, voltage, membrane tension, temperature, and even light. Natural gates can be altered and augmented using synthetic chemistry and molecular biology to develop channels with completely new functional properties. Light-sensitive channels are particularly attractive because optical manipulation offers a high degree of spatial and temporal control. Over the last few decades, several channels have been successfully rendered responsive to light, including the nicotinic acetylcholine receptor, gramicidin A, a voltage-gated potassium channel, an ionotropic glutamate receptor, α-hemolysin, and a mechanosensitive channel. Very recently, naturally occurring light-gated cation channels have been discovered. This review covers the molecular principles that guide the engineering of light-gated ion channels for applications in biology and medicine.

Ion channels control the electrical properties of cells by gating in response to a wide array of stimuli. To date, ion channels have been identified that are sensitive to changes in the concentration of ligands such as small molecules and ions, changes in membrane potential, temperature fluctuations, alterations in membrane tension, and most recently, exposure to visible light.

The ability of naturally occurring channels to command biological functions would be even greater if one could equip them with additional, artificial gating mechanisms. For instance, it is conceivable to develop an ion channel that responds to both ligands and light or to design voltage-gated channels that are also sensitive to artificial ligands. This would allow entirely new control mechanisms for the exploration and manipulation of biological systems. When applied to nervous systems, such an approach could even result in the development of "artificial senses".

Toward this end, molecular biology alone has a limited offering. However, clever integration of protein engineering with synthetic chemistry and nanotechnology could overcome these limitations and produce ion channels with novel gating mechanisms. Indeed, over the last decades, several light-gated ion channels have been reported following pioneering work in the 1980s.

We define a light-gated ion channel as a pore whose conductance reversibly changes in response to irradiation with UV or visible light. This definition excludes systems that respond to irreversibly photocaged ligands or those that are caged channel proteins themselves, although significant utility has been found for these approaches. The end result of such systems is similar, but they do not operate as a single functional unit, are usually not reversible, and have been extensively reviewed elsewhere (*1*). Similarly, fully synthetic photosensitive ion channels and lipids (*2*, *3*), although noteworthy, are excluded because of their limited application in the context of biological systems.

This review will focus on chemical modifications to physiologically relevant ion channels that render them light-sensitive. We will cover the existing examples based on the type of photoswitch employed, which largely coincides with their historical development, with an emphasis on principles of rational design. Major attention will be given to detailed structural models of the channel protein or pharmacology indicative of the potential for optical regulation. We will also highlight different methods of incorporating chromophores into channel proteins and modes of coupling photon absorption to channel gating.

Photochemistry. Although a range of ion channels have been rendered light-sensitive, a limited number of organic molecules that function as photoswitches have been employed (Figure 1). As shown in detail below, these switches control the gating through changing the local electric field, tethering components, moving blockers or agonists, or inducing conformational changes deep inside the protein to open a conductive pathway. The modes of photomodulation are largely determined by the nature of the photoswitch. Azobenzenes are often used for their large change in length and geometry upon trans−cis photoisomerization ($\Delta L \sim 7$ Å) (Figure 1A) and to a lesser extent for their change in dipole moment ($\Delta\mu \sim 3$ D) (*4*). However, spiropyrans undergo a dramatic increase in polarity upon photoinduced electrocyclic ring opening to the merocyanine form ($\Delta\mu \sim 15$ D) (Figure 1B), although the geometry change is less pronounced in comparison to that of azobenzenes (*5*). Retinal (Figure 1C) can also undergo trans−cis photoisomerization about one of several carbon−carbon double bonds, which is sensed by the protein to induce gating (*6*).

To afford rapid and complete on/off control of ion channel function in cells, any photoswitch should meet several general criteria. Photoswitching must occur on the time scale of protein and cellular function, generally somewhere between micro- and milliseconds using light intensities that

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* To whom correspondence should be addressed. Phone: (510) 643-5507. Fax: (510)-643-9480. E-mail: trauner@berkeley.edu.

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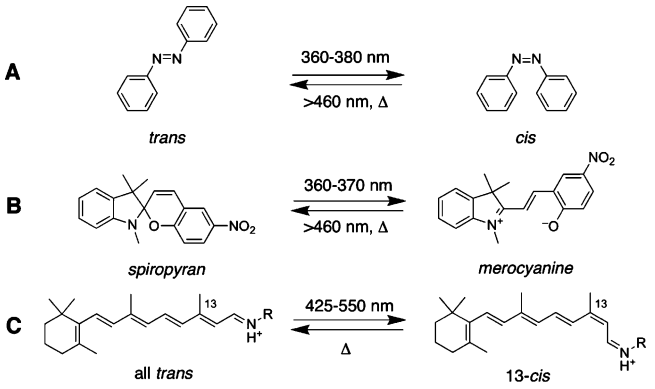

FIGURE 1: Photoswitches used in light-gated ion channels. (A) Azobenzenes undergo cis−trans isomerization in response to irradiation. Darkness and irradiation with visible light (>460 nm) favor the extended trans configuration. Irradiation with near-UV light (360−380 nm) favors the less stable, shorter cis configuration. (B) Spiropyrans/merocyanines undergo ring opening/closure upon irradiation. Darkness and irradiation with visible light (>460 nm) favor the less polar spiropyran configuration (closed form). Irradiation with near-UV light (360−370 nm) favors the more polar merocyanine configuration (open form). (C) Retinal, a naturally occurring photoswitch, forms an iminium ion with a lysine in channelrhodopsins. In the relaxed, nonconducting state, channelrhodopsins contain all-*trans*-retinal-iminium. Irradiation with visible light (425−550 nm) favors the 13-cis configuration to induce an unknown conformational change in the proteins that allow ion flux. Relaxation back to the all-trans configuration occurs spontaneously.

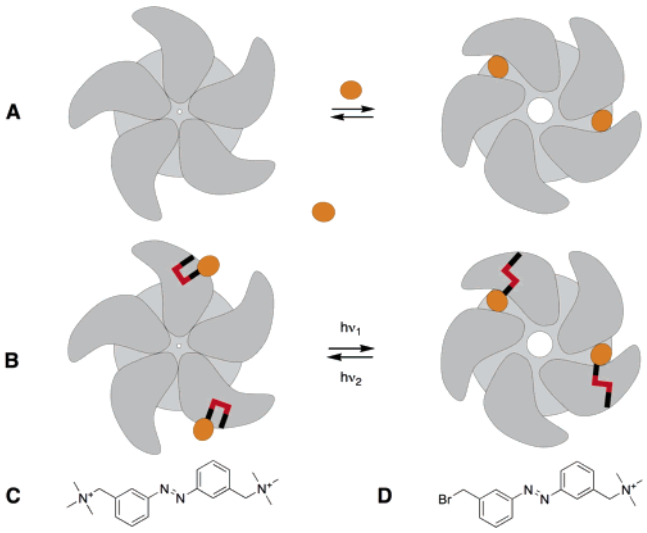

FIGURE 2: Light-gated nAChR. (A) Cartoon representation of the native function of nAChRs as viewed from the extracellular side of the cell membrane. Agonist binding at two sites between subunits induces a conformational change that leads to channel gating. (B) Design of a light-gated nAChR using a photoswitchable tethered agonist. An agonist is conjugated to the channel surface via an azobenzene linker such that the trans isomer activates the channel. (C) Structure of Bis-Q in the trans form. (D) Structure of Q-Br in the trans form.

are not harmful to cells. At a fixed light intensity, rates are dependent on the quantum yield of photoswitching and the extinction coefficient of the chromophore at the wavelength of irradiation (*7*). For bistable photoswitches such as azobenzenes and spiropyrans, it is also important to achieve high photoconversion between isomers, which relies on the ability to selectively excite one form or the other using different wavelengths (*4*). Generally, it is more important to obtain a complete "off" response than a complete "on" response because persistent partial activation of ion channels can be toxic to cells. Photoswitches tend to thermally relax back to their lowest energy isomer in the absence of irradiation (*4*, *5*). Therefore, the lowest energy isomer is often chosen as the "off" state. Artificial photoswitches such as azobenzenes and spiropyrans can be rapidly converted to the "off" state with light, whereas the naturally occurring retinal chromophore can only be photoswitched in one direction.

Nicotinic Acetylcholine Receptor. The nicotinic acetylcholine receptor (nAChR[1]) plays a crucial role in the peripheral nervous system as the only ionotropic receptor at the neuromuscular junction. Acetylcholine released from motor neuron terminals opens this ligand-gated channel, which results in a depolarization of muscle cells. Long before the advent of cloning and site-directed mutagenesis, a pioneering biophysical investigation of the muscle nAChR by Erlanger, Wasserman, and Lester resulted in the first light-gated ion channel. At the time, the muscle nAChR was the best characterized ion channel and the ability to render it light sensitive provided an opportunity to study the kinetics of channel gating with unprecedented temporal resolution (*8*).

nAChRs are pentameric cation channels composed of a transmembrane pore-forming domain coupled to an extracellular ligand-binding domain, both characteristic of the Cys-loop family. Several X-ray structures of a homologous acetylcholine-binding protein in complex with various agonists and antagonists offer a view of ligand binding that involves a cation−pi interaction between ammonium ions and electron-rich aromatic side chains (*9*−*11*). This event is mechanically coupled to a poorly understood conformational change in the transmembrane domain to allow ion flow through the pore (*12*) (Figure 2A).

Efforts to render nAChRs light-sensitive were derived from studies with a noncovalent photoswitchable agonist. Relying on prior pharmacology, a symmetric meta-substituted azobenzene bearing two benzylic trimethylammonium ions (Bis-Q) (Figure 2C) was prepared and found to activate the receptor in *Electrophorus* electroplaques (*13*). The *cis* and *trans*-azobenzene isomers demonstrated different efficacies for activating the receptor, with *trans*-Bis-Q acting as a full agonist. Photoisomerization between the two states using millisecond light flashes produced rapid changes in the concentration of trans isomer, resulting in reversible induction of inward currents (*14*).

In an insightful extension of this technology, a covalent photoswitchable agonist bearing one benzylic trimethylam-

[1] Abbreviations: 7-TM, seven-transmembrane helix domain; αHL, α-hemolysin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Bis-Q, azobenzene bis-quaternary ammonium (Figure 2C); ChR, channelrhodopsin; ClC, chloride channel; DTT, dithiothreitol; GABA, γ-amino butyric acid; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HEK293, human embryonic kidney cells; iGluR, ionotropic glutamate receptor; LBD, ligand binding domain; LiGluR, light-activated ionotropic glutamate receptor; MAG, maleimide azobenzene glutamate (Figure 5C); MAQ, maleimide azobenzene quaternary ammonium (Figure 4C); MscL, mechanosensitive channel of large conductance; nAChR, nicotinic acetylcholine receptor; Na_V, voltage-gated sodium channel; NMDA, *N*-methyl-D-aspartate; NTD, *N*-terminal domain; Pap, phenylazo-phenyl alanine (Figure 3G); P2X, ionotropic purinoceptor type 2X; Q-Br, azobenzene quaternary ammonium ion benzylic bromide (Figure 2D); SPARK, synthetic photoisomerizable azobenzene-regulated K^+ channel; TMD, transmembrane domain.

monium ion and one electrophilic benzylic bromide (Q-Br) (Figure 2D) was prepared (*13*). Previous studies with non-photoswitchable covalent agonists and antagonists had demonstrated the presence of a cysteine residue in the vicinity of the ligand binding site that could be affinity labeled with electrophilic acetylcholine analogues to produce constitutively active or inactive receptors, respectively (*15*, *16*). Prior to alkylation, the reduction of a disulfide with DTT was required to liberate the free thiol(s). *trans*-Q-Br was found to label this cysteine as evidenced by a persistent depolarizing current after washout. Comparison to the current elicited by acetylcholine prior to reduction identified *trans*-Q-Br as a full agonist. Photoswitching the azobenzene between isomers reversibly activated the channel, presumably because of changes in the local concentration of quaternary ammonium ion within reach of the aromatic binding pocket (Figure 2B). Notably, covalently bound *cis*-Q-Br did not activate the receptor to a detectable degree (*8, 13, 17*).

Rapid photoactivation with covalently tethered Q-Br eliminates the contribution of agonist diffusion when investigating the kinetics of channel activation, which was previously an insurmountable limitation. Thus, Q-Br firmly established the existence of a rate-limiting conformational change in the receptor after agonist binding. Furthermore, comparison of the wavelength dependence of photostationary states with the degree of channel activation was used to investigate the agonist stoichiometry required to activate the modified channels (*8, 14*).

Although the role of nAChRs at the neuromuscular junction is well understood, the function of various nAChR subtypes in the brain and their involvement in disease states such as nicotine addiction and Alzheimer's disease is currently the subject of intensive investigation. We anticipate that a refinement of this ground-breaking system, using genetically engineered channels with strategically placed cysteines, would alleviate the need for subtype-selective ligands because only these channels would become photosensitive. Such an approach would offer a high degree of spatial and temporal control for the investigation of different nAChR subtypes in the nervous system.

Gramicidin A. The next effort toward developing a light-gated ion channel did not surface until nearly 10 years later, when chemists turned their attention to a pentadecapeptide derived from *Bacillus brevis* known as gramicidin A. Commonly used against certain gram-positive bacteria, gramicidin was one of the first antibiotics to be produced commercially. Mechanistic studies showed that gramicidin's antibiotic properties are derived from its pore-forming properties. As a result, the channel has been studied extensively, providing a deep understanding of its structure and function. Because of their small size, gramicidin analogues are readily synthesized using standard peptide-coupling techniques. As such, various modes of regulating gramicidin channels with light have been explored (*18*).

The ion channel properties of gramicidin derive from its unique sequence of alternating D- and L-amino acids bearing hydrophobic side chains, an *N*-terminal aldehyde, and a *C*-terminal ethanolamide. Upon insertion into lipid bilayers, gramicidin adopts a right handed β-helical structure spanning one-half of the membrane, with the side chains contacting lipids to form a monovalent cation-selective conduction pathway in the helix lumen. To pass ions across the membrane, two gramicidin monomers must dimerize through lateral diffusion in an *N*- to *N*-terminal fashion. This form of "gating" produces characteristic flickering conductances as monomers dimerize to form membrane-spanning pores and then rapidly dissociate (*18*) (Figure 3A).

Three distinct approaches have been investigated to render gramicidin sensitive to light. In each case, single channel recordings from artificial lipid bilayers were utilized to reveal changes in conductance associated with photoisomerization. The first studies reported by Schreiber and Heinemann modulated dimerization and subsequent pore formation by tethering two monomers at their *N*-termini with a photoresponsive linker (*19*). Previous work by Schreiber showed that monomers could be covalently linked to form channels with extremely long conductance lifetimes (*20, 21*). By using a symmetric *meta*-azobis(benzeneacetic acid) linker (Figure 3C) attached directly to the *N*-termini of two monomers, a scenario was envisaged in which the *cis*-azobenzene would bring two monomers in close proximity to facilitate conduction, but the trans state would keep them apart, inhibiting functional channel formation (Figure 3B). Single channel recordings indicated that, indeed, irradiation with UV light to enrich the cis state did increase the frequency of occurrence of a high conductance channel that displayed an extended mean open time. Surprisingly, however, two additional conductance states were observed prior to irradiation when the sample was entirely in the trans configuration, which were attributed to unanticipated oligomers (*19*). Subsequently, Arseniev and co-workers refined this approach by incorporating a longer and more flexible linker between the azobenzene derivative *para*-azodianiline and the gramicidin *N*-terminus. In this case, the single-channel conductance was dramatically reduced, but oligomerization was not observed, which might be accounted for by the less restricting linker (*22*).

The next modification of gramicidin exploited the susceptibility of cation channels to blockade by ammonium ions. Using single channel recordings, Woolley and colleagues found that gramicidin channels bearing amines linked to the *C*-terminal ethanolamide displayed several conductance levels after dimerization. Careful analysis showed that each conductance level correlated with a different degree of transient block depending on the cis or trans geometry about a carbamate bond in the tether. Notably, the degree of block generated by a single ammonium ion depends on the direction of ion flow; blocking the entrance to the pore is more effective than blocking the exit so that four conductance levels were observed (i.e., trans/trans, trans/cis, cis/trans, and cis/cis with respect to the carbamates) (*23*).

This approach was quickly extended to the incorporation of either *meta*- or *para*-bis(aminomethyl) azobenzene tethers (Figure 3E). Molecular modeling suggested that the azobenzene cis isomers should present the blocking ammonium ion to the mouth of the channel pore while the azobenzene trans isomers should be too long and rigid to allow for effective block (Figure 3D). With both *meta*- and *para*-azobenzene derivatives, photoconversion to the shorter azobenzene cis isomers produced a channel displaying various degrees of block, again corresponding to the carbamate bond geometry (cis or trans). Conversely, the extended azobenzene trans isomers showed very little block at all (*24*).

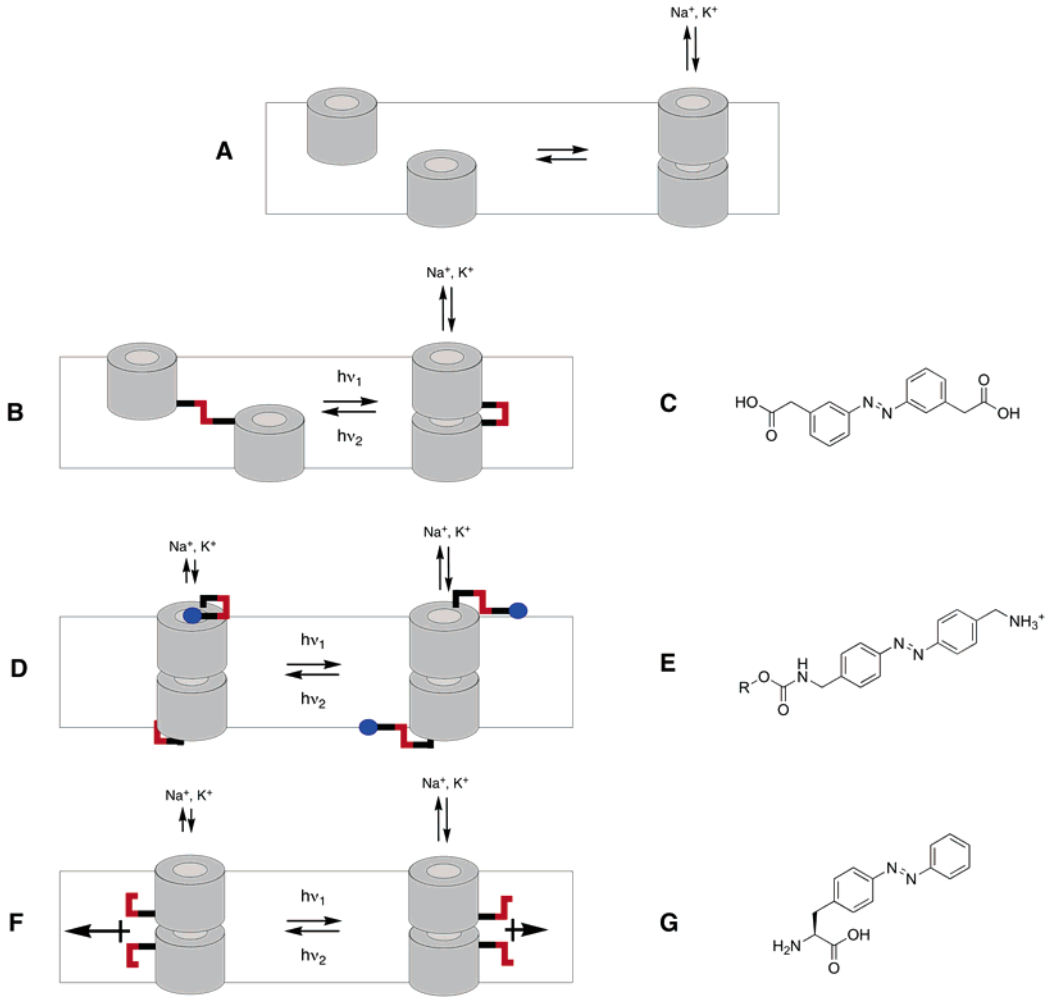

FIGURE 3: Light-gated gramicidin A. (A) Cartoon representation of the native function of gramicidin A. Two monomers dimerize in the cell membrane to form a functional cation channel. (B) Design of the first light-gated gramicidin A using a photoswitchable cross-linker. Two monomers are linked by an azobenzene that induces dimerization in the cis form. (C) Structure of the *meta*-azobis(benzeneacetic acid) cross-linker in the trans form. (D) Design of a light-gated gramicidin A using a photoswitchable tethered blocker. An ammonium ion pore blocker is conjugated to the *C*-terminal ethanolamide via an azobenzene linker such that the cis isomer blocks the channel. (E) Structure of the *para*-bis(aminomethyl)azobenzene tethered blocker in the trans form. (F) Design of a light-gated gramicidin A using photoswitchable ion−dipole interactions. An azobenzene-bearing amino acid is substituted for a valine such that the cis isomer impedes conductance. (G) Structure of phenylazo-phenylalanine (Pap) in the trans form.

Complementary to this, Woolley and co-workers set out to explore another method of optical manipulation. In general, ion−dipole interactions have been implicated in facilitating ion conduction through cation channels (*25*, *26*). Although the side chains in gramicidin do not contact the permeation pathway, incorporation of unnatural amino acids with large dipole moments significantly affects channel conductance properties (*27*). To render the pore dipole photosensitive, the valine at position 1, which resides in the center of the lipid membrane at the dimer interface, was replaced by phenylazo-phenyl alanine (Pap) (*28*) (Figure 3G). Concurrent with the change in double bond geometry, the dipole moment of the azobenzene chromophore increases by 3 Debye upon photoisomerization to the cis configuration (*4*). As predicted, Pap−gramicidin dimers underwent reversible changes in conductance upon photoisomerization such that the trans/trans and cis/cis dimers showed the highest and lowest single channel conductances, respectively (Figure 3F). In this case, only three of the four possible conductance states were observed because the trans/cis and cis/trans states were indistinguishable (*28*). More recently, a hemithioindigo chromophore was evaluated at the same position, yielding similar results (*29*).

One initial motivation for photoregulating gramicidin channels was to construct light-sensitive membranes that could be used in artificial memory storage devices. Although gramicidin derivatives that have been engineered to gate in response to other stimuli are beginning to find use as molecular sensors (*30*), photosensitive gramicidin has not yet found such utility and has not been applied in the context of living cells. Regardless, the gramicidin scaffold has proved a valuable model system for exploring several ways to optically control ion flow.

Voltage-Gated Potassium Channel. Expanding the tethered ligand approach, Trauner, Isacoff, and Kramer were able to render the Shaker voltage-gated potassium channel light-sensitive (*31*). Potassium channels represent particularly attractive targets for optical regulation. Voltage-gated potassium channels play a crucial role in the nervous system of higher organisms as regulators of membrane potential and as “molecular brakes” during action potential propagation. Because potassium channels are ubiquitously expressed, the

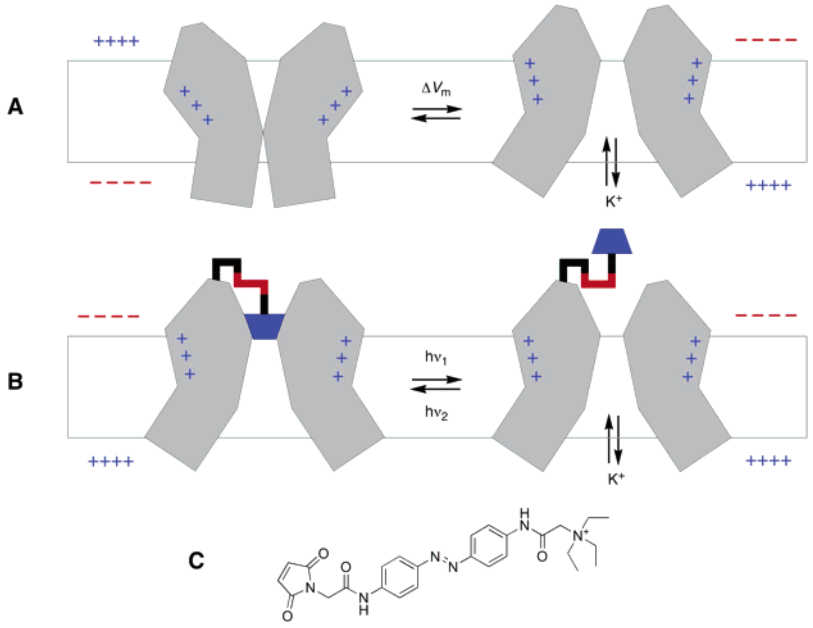

FIGURE 4: Light-gated potassium channel. (A) Cartoon representation of the native function of voltage-gated potassium channels. A positively charged voltage sensor moves within the membrane upon membrane depolarization to induce channel gating. (B) Design of a light-gated potassium channel using a photoswitchable tethered blocker. A tetraethylammonium ion pore blocker is conjugated to the channel surface via an azobenzene linker such that the trans isomer blocks the channel. (C) Structure of MAQ in the trans form.

ability to render them light-sensitive presents an opportunity for optical control of neural activity independent of the neurotransmitter types utilized synaptically.

Potassium channels are tetrameric ion channels composed of at least two transmembrane α-helices per subunit and a selectivity filter near the extracellular surface. Most potassium channel subunits contain four additional transmembrane alpha helices that confer voltage sensitivity to channel gating, characteristic of the voltage-gated ion channel superfamily (*32*). Several X-ray structures and functional studies offer a view of channel gating, which involves bending of the innermost S6 helix at a conserved hinge to allow intracellular ions access to the selectivity filter (*33*−*35*) (Figure 4A). Additional structural rearrangements and gating movements may occur at the selectivity filter, but the details are poorly understood.

The possibility for optical regulation of potassium channels was foreshadowed by an elegant experiment with non-photoswitchable tethered blockers. Validating the X-ray structures with biochemical experiments, Miller and Blaustein prepared a series of "molecular tape-measures" that were designed to probe the dimensions of the Shaker potassium channel. Each molecule contained a general pore-blocking tetraethyl ammonium derivative connected to an electrophilic maleimide through a peptidic tether whose length was varied. Because the tetraethylammonium ion was known to bind and block potassium channels at the extracellular entrance to the selectivity filter, the "tape-measures" were evaluated for their ability to block current flow when attached to a genetically introduced cysteine residue (E422C) on the channel surface. Strikingly, an increase in tether length of only 5 Å made the difference between zero and saturated block (*36*).

These sharp geometric constraints were crucial to rendering the channel light-sensitive. Building on this scaffold, the peptidic tether was simply replaced with *para*-azodianiline, which is known to change in length by approximately 7 Å upon photochemical isomerization. A series of photoswitchable analogues with varying linker lengths was prepared and then screened against the E422C Shaker mutant in inside-out patch clamp recordings from *Xenopus* oocytes. This screen identified the molecule showing the largest dynamic range of current when reversibly photoswitched between isomeric states. This molecule, termed MAQ (Figure 4C) for the maleimide (M), azobenzene (A), and quaternary ammonium (Q) components, was found to block Shaker channels when applied in the trans configuration following washout. Photoisomerization to the cis isomer resulted in a significant current increase, corresponding to a reduction in the local concentration of quaternary ammonium ion at the pore entrance (Figure 4B). This process was found to be completely reversible and dependent on the wavelength of illumination. Accordingly, these channels were named SPARK channels for **S**ynthetic **P**hotoisomerizable **A**zobenzene **R**egulated $\mathbf{K}^+$ channels (*31*).

In addition to introducing a cysteine for the attachment of MAQ, several mutations were applied to Shaker to minimize the effects of endogenous gating mechanisms, thereby isolating MAQ photoisomerization as the dominant gating event. First, fast or N-type inactivation was eliminated by removing the *N*-terminal ball and chain (*37*). Second, slow or P-type inactivation was reduced by mutating Thr 449 to a Val (T449V) (*38*). Finally, a mutation in the voltage-sensing region (L366A) was used to shift the voltage dependence of gating to negative values by ∼30 mV (*39*). This last mutation was particularly relevant for applying SPARK channels in neurons, where the resting potential of the cell (∼ −60 mV) limits the impact of optical gating because the voltage gates are mostly closed.

SPARK channels offer a unique opportunity to manipulate the electrical activity of neurons with light. Overexpression of potassium channels has previously been shown to reduce neuronal activity by preventing the membrane potential from reaching action potential threshold due to excess outward potassium conductance (*40*, *41*). Accordingly, expression of

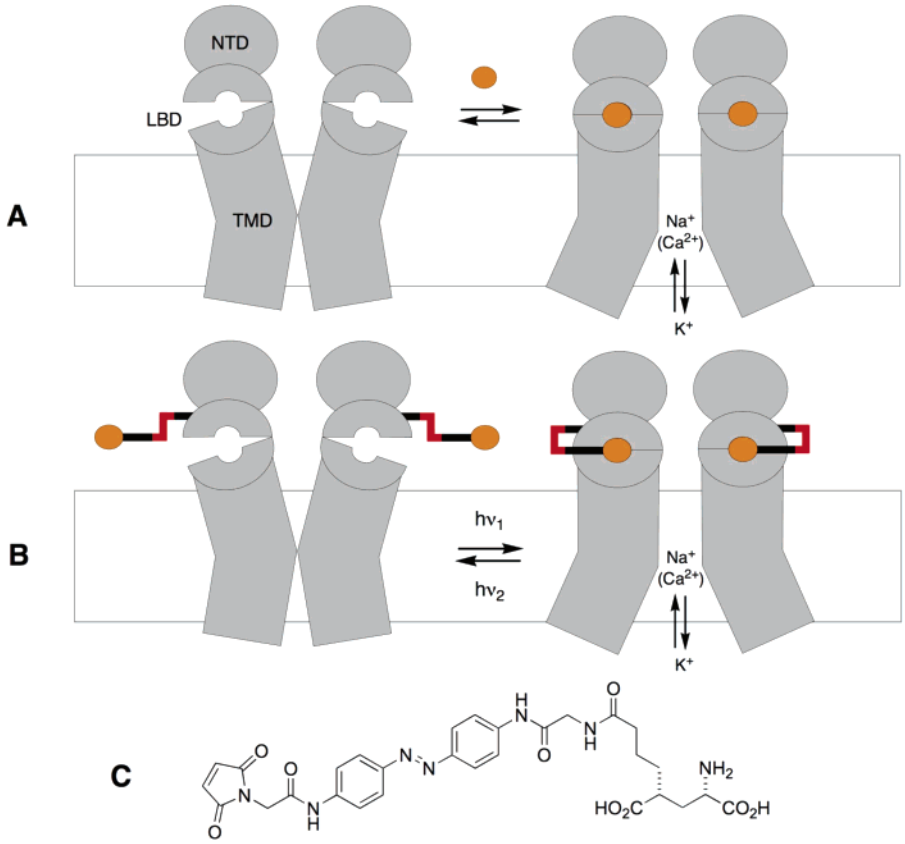

FIGURE 5: Light-gated iGluR. (A) Cartoon representation of the native function of iGluRs. Agonist binding in the ligand-binding domain (LBD) induces a conformational change in the transmembrane domain (TMD) that leads to channel gating. (B) Design of a light-gated iGluR (LiGluR) using a photoswitchable tethered agonist. A glutamate analogue is conjugated to the channel surface via an azobenzene linker such that the cis isomer activates the channel. (C) Structure of MAG in the trans form.

SPARK channels in dissociated hippocampal neurons, followed by treatment with *trans*-MAQ allowed neurons to fire action potentials as normal using their native ion channels while SPARK channels remain blocked. Opening the SPARK channels with UV light resulted in an excess outward potassium conductance, effectively silencing electrical activity as assayed by whole-cell current clamp recordings (*31*).

One drawback to the SPARK system is a requirement for exogenous gene expression to provide a site for attaching the photoregulatory element, MAQ. As a result, one future effort is geared toward photoregulating potassium channels that are native to the cells under study. In pursuit of this goal, a photoswitchable affinity labeling approach is being explored in which various electrophiles are used to target endogenous reactive nucleophiles on the channel surface (*42*). Furthermore, changing the ion selectivity of Shaker channels using previously characterized mutations (*43*) converts SPARK channels to depolarizing or excitatory channels, further demonstrating the malleability of this system (*44*).

Ionotropic Glutamate Receptor. The success of azobenzene-based tethered ligands and their utility in complex cellular systems encouraged Trauner, Kramer, and Isacoff to extend this concept to other important ion channels (*45*). Specifically, ionotropic glutamate receptors (iGluRs), the major mediators of excitatory synaptic transmission in the central nervous system, proved ideal candidates because of their well characterized pharmacology and the availability of 3D structural data.

iGluRs comprise a family of ligand-gated cation channels categorized as either AMPA (iGluR1−4), kainate (iGluR5−7, KA1−2), or NMDA receptors, according to their pharmacological profile. iGluRs exist as either homo- or heterotetramers *in vivo*. Each subunit possesses three domains: a transmembrane pore-forming domain (TMD), an extracellular ligand-binding domain (LBD), and an *N*-terminal domain (NTD) that controls subunit oligomerization. Functionally, ligand binding and subsequent closure of the clamshell-like LBD is directly coupled to channel gating (*46*) (Figure 5A). Extensive structural data are available for the LBD, including crystal structures in complex with a number of agonists and antagonists (*47*, *48*). The structures not only lay the groundwork for understanding the pharmacology of these receptors, in particular the phenomenon of partial agonism (*49*), but also enable their rational re-engineering.

A crystal structure of iGluR6 bound with the agonist (2*S*,4*R*)-4-methyl glutamate revealed that kainate receptors (iGluR5−6) possess an exit tunnel connecting the binding pocket and extracellular surface (*48*). The exit tunnel provided the basis for a light-activated iGluR by indicating that a glutamate analogue could be threaded through the tunnel and attached to an appropriately positioned mutant cysteine residue on the exterior of the LBD via a photoswitchable tether. Once attached, the glutamate analogue would be held at arm's length in the trans form of the photoswitch but could reach the LBD in the cis form, thus activating the channel (Figure 5B).

The full-length tethered agonist termed MAG (Figure 5C) was designed to include a maleimide (M), an azobenzene linker (A), and a glutamate headgroup (G). An iGluR6 mutant possessing a cysteine at amino acid position 439

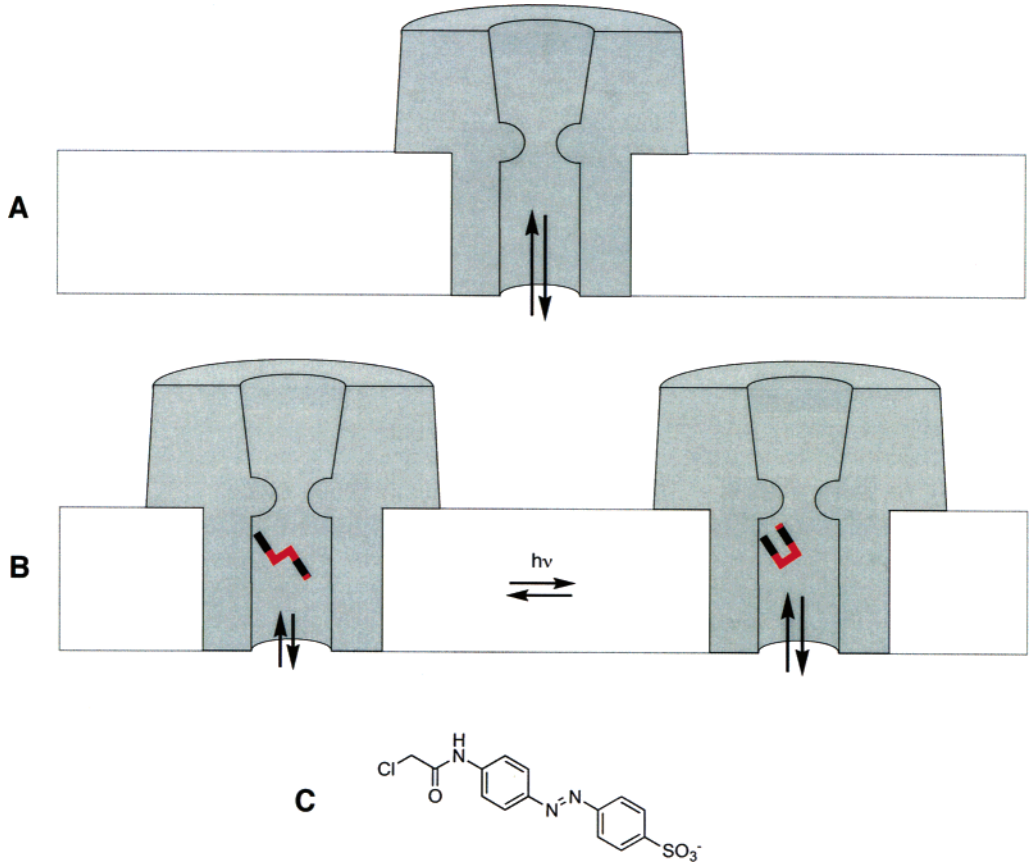

FIGURE 6: Light-gated α-Hemolysin. (A) Cartoon representation of the native function of αHL. The fully assembled heptamer is constitutively open. (B) Design of a light-gated αHL (P_{AZO}) for the study of single azobenzene photoswitching. A sulfonated azobenzene was conjugated to the channel lumen such that the trans isomer impedes conductance more than the cis isomer. (C) Structure of the cysteine-reactive sulfonated azobenzene in the trans form.

(L439C) was shown to covalently bind *cis*-MAG as indicated by persistent channel activation after washout. Subsequent isomerization to the trans form of the azobenzene led to a significant reduction in current (45).

The iGluR6-L439C-MAG construct, termed LiGluR, was investigated using whole-cell recordings from HEK293 cells. As in the case of SPARK, the wavelength of light can finely tune the degree of activation and deactivation of the receptor and, therefore, the amount of current injected into the cell (*45*). At a peak wavelength of 380 nm, the tethered agonist yielded approximately half-maximal current with respect to a saturating glutamate response. Subsequent studies have shown that, despite this submaximal response, dissociated hippocampal neurons equipped with LiGluR are rendered light sensitive with good temporal control of action potential firing (*50*).

The ability to re-engineer the clamshell domain of iGluRs using tethered agonists and photoswitches opens the possibility for further modification using tethered antagonists. In this configuration, individual iGluR subtypes could be reversibly knocked out with good spatial and temporal control, a feat not currently possible with soluble antagonists (*1*). Extension of the technology to other iGluR subtypes, including other kainate and NMDA receptors that also possess an exit tunnel, may be envisioned. Meanwhile, re-engineering other LBDs to possess an artificial exit tunnel would expand this approach to the control of other pharmacologically relevant targets, for instance AMPA receptors (iGluRs1−4).

α-*Hemolysin.* The most recent addition to the growing list of light-sensitive ion channels is based on the well studied α-hemolysin (αHL) (*51*). This heptameric pore-forming protein from the human pathogen *Staphylococcus aureus* possesses a large (∼15 Å) water-filled channel permeable to anions, cations, small molecules (*52*), and even biopolymers (*53*) (Figure 6A).

αHL has long been re-engineered for non-native applications. Early work by Bayley and co-workers using caged amino acids resulted in a phototriggered αHL whose assembly was irreversibly initiated with light (*54*). Subsequently, αHL was applied as a "nanoreactor" or stochastic sensor for the study of a number of single-molecule processes (*55*). These studies employed single-channel recordings from artificial bilayers to monitor changes in conductance as modulated by a number of processes within the lumen of αHL. Examples include the binding of divalent metal ions (*56*) and small organic molecules (*57*, *58*) as well as the kinetics of a three-step reaction on the single-molecule level (*59*).

Most recently, Bayley developed a light-sensitive αHL by appending a single azobenzene to the lumen of the transmembrane channel, which reversibly modulated ionic conductance upon photoisomerization (*51*). This configuration provided the opportunity to study single-molecule dynamics of azobenzene photoswitching with unprecedented detail using electrophysiology as a means of detecting isomerization.

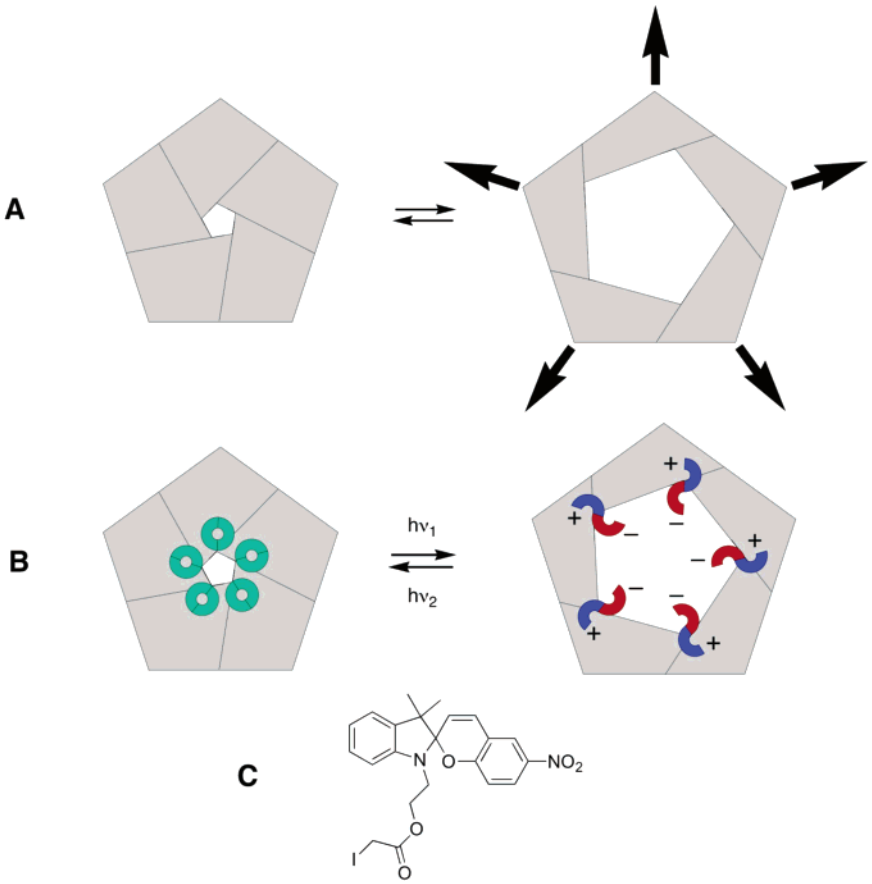

FIGURE 7: Light-gated MscL. (A) Cartoon representation of the native function of MscL viewed from the extracellular side of the cell membrane. Membrane tension disrupts a hydrophobic gate to open the 30−40 Å pore. (B) Design of a light-gated MscL using photoswitchable electrostatic interactions. A relatively nonpolar spiropyran is conjugated to the pore lumen such that photoconversion to the more polar merocyanine form weakens the hydrophobic gate to induce pore opening. (C) Structure of the cysteine-reactive spiropyran.

To this end, an αHL monomer containing a single mutant cysteine was modified with a sulfonated azobenzene equipped with a chloroacetamide (Figure 6C). The sulfonate group added aqueous solubility and maximized the effect of photoswitching on channel conductance. The modified monomer was then reassembled with wild-type subunits and purified by SDS−PAGE to isolate αHL heptamers with a single modified subunit. This fully assembled construct was designated P_{AZO} (*51*).

Once P_{AZO} was inserted into a planar lipid bilayer, two conductance states were observed, corresponding to *cis*- and *trans*-azobenzene (Figure 6B). Irradiation with 330 nm light induced switching between conductance levels; however, all transitions ceased when the light was removed, indicating that the cis isomer failed to thermally relax. This was in contrast to the half-life of thermal relaxation measured in bulk solution, which was on the order of minutes. Thus, the conjugation of the azobenzene chromophore to the channel pore created a bi-stable switch that could be reversibly isomerized in the presence of UV light, yet could be effectively locked in a cis or trans state in the absence of irradiation. The origin of the cis isomer stabilization is not yet understood (*51*).

The development of a light-sensitive αHL represents a unique application for chemically modified light-gated ion channels. In this case, the goal was not to directly control protein or cellular function with light but instead to use the ion channel as a means to study azobenzene photoisomerization at the single-molecule level. In principle, this concept could be extended not only to the study of other photoswitches within αHL but also to other light-gated channels, in particular for investigating how chromophore properties are altered after bioconjugation.

Mechanosensitive Channel. Harnessing the power of Nature's molecular machines is an important goal of nanomedicine. As such, an intriguing application of light-gated ion channels includes light-mediated drug release for use in protein-based devices. Toward this end, Feringa and Meijberg recently reported a light-actuated nanovalve derived from the mechanosensitive channel of large-conductance (MscL) of *Escherichia coli* (*60*).

Mechanosensitive channels are found in both prokaryotic and eukaryotic systems, regulating osmotic imbalances imposed by high osmolarity environments. Upon the onset of hypo-osmotic shock, these channels gate in response to changes in membrane tension, with channel conductances up to 2.5 nS (*61*) (Figure 7A). The prokaryotic homopentameric MscL possesses a pore of 30−40 Å diameter that has previously been shown to permit the passage of ions, small organic molecules, and even small proteins (*62*, *63*). Therefore, MscL is a promising platform for the development of drug-release technologies.

MscL channels possess a hydrophobic gate within the pore that effectively locks the channel in a closed state in the absence of membrane tension. In designing a light gate for MscL, Feringa and Meijberg exploited gain-of-function

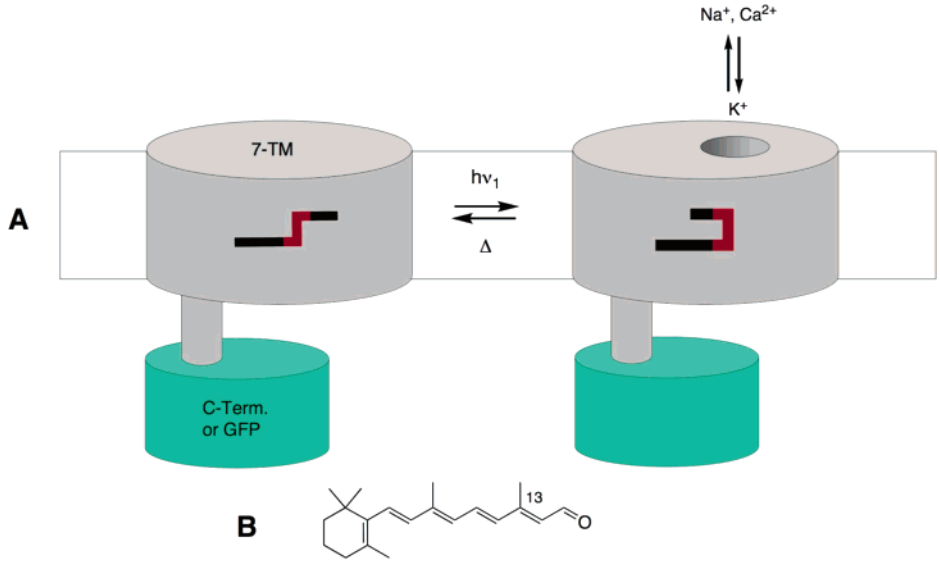

FIGURE 8: Channelrhodopsin-2. (A) Cartoon representation of the function of ChR2. Isomerization of the all-*trans*-retinal chromophore causes a conformational change in the transmembrane domain to induce channel gating. Replacement of the *C*-terminal domain with green-fluorescent protein (GFP) does not alter channel properties. (B) Structure of the all-*trans*-retinal chromophore.

mutations in the MscL pore known to induce spontaneous channel gating without the influence of membrane tension. Specifically, mutation to polar or charged residues at amino acid Gly22 significantly weakens this hydrophobic interaction, providing access to an expanded subconducting state (*64*−*66*).

Central to the design of an optical gate within the pore of MscL was the ability to introduce charges at position 22 upon irradiation with light. To this end, a cysteine introduced at position 22 (G22C) was alkylated with a caged carboxylic acid. Irradiation with 346 nm light revealed that a cysteine-bound carboxylic acid induced channel opening in an irreversible fashion (*60*).

Reversible optical control was conferred to MscL using the G22C mutant and a cysteine-reactive spiropyran photoswitch (Figure 7C) (*60*). The spiropyran moiety is well suited for the control of MscL gating because of the polarity difference between the spiropyran (closed form) and the more polar merocyanine (open form) (*5*). As designed, the spiropyran maintains the hydrophobic gate, leaving the modified MscL channel closed. However, upon irradiation with 366 nm light, an electrocyclic ring opening unmasks the merocyanine moiety (Figure 7B). The optically induced polarization of the channel pore, thus, promotes channel activation through the previously described subconductance state. Reversion to the spiropyran is achieved by irradiation with visible light (>460 nm), effectively closing the channels (*60*).

This strategy was successful in remotely controlling MscL activity, as demonstrated in single-channel recordings from reconstituted liposomes. Analysis of the switching cycles after photoswitch conjugation revealed incomplete conversion to the merocyanine state after the first cycle, perhaps because of a change in the protein structure. In addition, the rate of optical gating was relatively slow compared to those of other photoswitchable systems. Nonetheless, the ability of the light-actuated MscL to control the efflux of drug-like molecules across a membrane was demonstrated by the release of a fluorescent dye from liposomes within minutes of UV irradiation (*60*).

This light-actuated nanovalve may provide a robust system for the spatially controlled release of liposomal cargo in living tissue. Subsequent studies have revealed the generality of this approach in controlling MscL channel gating using other elements responsive to environmental triggers such as pH-sensitive switches (*67*). Biologically derived nanocomponents show promise in the development of not only drug-delivery vehicles but also a variety of small molecule sensors (*68*).

Channelrhodopsin. The systems described so far in this review all contain artificial light gates added to channels that are normally not photosensitive. Very recently, however, two naturally occurring light-gated cation channels have been described. On the basis of the rapid kinetics of the phototactic and photophobic response of the green alga *Chlamydomonas reinhardtii*, it was suggested that a monolithic photoreceptor/ion channel rather than a G-protein coupled receptor (GPCR) was involved (*69*). This proposal was validated when two photosensitive ion channels were cloned and characterized: the light-gated proton channel channelrhodopsin-1 (ChR1) and the light-gated cation channel channelrhodopsin-2 (ChR2). Both of these channels directly couple the photoisomerization of a retinal chromophore (Figure 8B) to the passive conductance of ions (*70*, *71*).

The ChRs resemble type 1 GPCRs insofar as they contain predicted *N*-terminal seven-transmembrane helix domains (7-TM) as well as extended intracellular *C*-terminal domains (Figure 8A). It has been determined that truncated ChRs comprising the *N*-terminal halves encode the fully functional light-gated ion channel, which has enabled the construction of ChR2-GFP chimeras (*72*). The covalently linked all-*trans*-retinal chromophore of the ChRs absorbs in the visible range (ChR1 around 500 nm (*70*) and ChR2 around 460 nm (*71*)). Upon absorption of a photon, all-*trans*-retinal probably isomerizes to 13-*cis*-retinal as in the related sensory rhodopsins and bacteriorhodopsins. Apparently, this movement is directly mechanically coupled to the opening of a pore. The predicted 13-*cis*-retinal intermediate relaxes spontaneously without the need for dissociation and metabolic processing,

resulting in the closure of the pore (*73*). Although both ChRs have been expressed and characterized in exogenous cellular systems, ChR2 possesses a much larger conductance and, owing to its sodium permeability, is able to effectively depolarize cell membranes (*71*). Therefore, it is not surprising that ChR2 has rapidly found applications in neurobiology. To date, four research teams have used ChR2-GFP chimeras for the rapid and spatially precise control of action potentials in a number of neural systems, including dissociated and slice cultures of mammalian hippocampal neurons, *C. elegans* muscle cells, intact embryonic chick spinal cords, and mammalian retina (*74*−*78*).

Although the engineering of ChRs is still in its infancy, some interesting directions have already been explored. Apart from the relatively straightforward step of replacing the *C*-terminus of ChR2 with GFP, efforts have been made to increase the conductance. Borrowing heavily from studies conducted on bacteriorhodopsin, a single mutation critical for bacteriorhodopsin proton pumping resulted in larger currents when applied to ChR2 (*76*). Although spectral tuning has proven a difficult endeavor in other microbial rhodopsins (*79*), ChR2 could still be re-engineered to shift its absorption spectrum. X-ray crystal structures, many of which exist for the related microbial rhodopsins (*80*−*82*), could help to elucidate key protein/chromophore contacts responsible for the ChR action spectra and allow for rational design of channelrhodopsins with altered spectral properties. In addition, synthetic chemistry could change the nature of the chromophore. The recent success in generating red-shifted bacteriorhodopsins using azulenic, fluorinated, and merocyanine-based retinal analogues may provide a basis for the development of functionally altered ChRs (*83*).

Several avenues for improving ChR2 in neurobiological applications still remain completely unexplored. For example, it may be possible to alter the rates of activation and inactivation, which could improve the fidelity of action potential firing at high frequencies. Furthermore, modifying ion selectivity could lead to channels with lower Ca^{2+} permeability or even a complete inversion of polarity by producing K^+ selective, hyperpolarizing channels.

CONCLUSIONS

Light-gated ion channels hold great promise as tools for controlling the function of excitable cells or artificial membrane vesicles. Optical control of ion channel function is particularly intriguing because it offers the opportunity to govern the electrical excitability of cells and other membrane structures with a high degree of temporal and spatial control. In the context of the nervous system, an obvious application is to use light-gated ion channels to control action potential firing while investigating neuronal circuits, mechanisms of learning and memory, or the activity-dependence of development (*84*). Light-gated ion channels may prove useful for restoring function downstream of neural damage, particularly in the retina, with hopes of creating some form of artificial vision (*78*). Additionally, using a chemical genetic approach wherein only mutant cysteine-containing channels are rendered light-sensitive, the molecular roles of various channel subtypes might be explored without the need for selective pharmacological agents. Light-gated ion channels can be incorporated into artificial lipid bilayers and liposomes, which could be utilized as artificial memory storage devices or as a way to optically induce targeted drug delivery. Furthermore, biophysical investigations of the kinetics of ion channel opening may be facilitated by optical activation, which eliminates the limiting process of diffusion when applying ligands in solution. Along these lines, using single-protein pores as nanosensors of photoisomerization may provide unprecedented insight into the function of photoswitches on the single-molecule level.

From a more chemical perspective, exploring new modes of bioconjugation could extend the reach of light-gated ion channels in living cells. Attachment of the photoswitches has thus far been restricted to labeling endogenous or genetically encoded cysteine residues with common electrophiles, such as maleimide and haloacetamides. While this strategy is convenient when using reconstituted channels, such reagents are generally unselective for the target protein and can be toxic to living cells. However, affinity labeling with tethered ligands at low concentrations offers some selectivity and even promotes the reactivity of endogenous nucleophiles other than cysteine for targeting wild-type channels (*42*). As an alternative, genetically encoded affinity epitopes remain unexplored in the context of light-gated ion channels. Depending on technological advancements to be useful in living tissue, artificial amino acids or sugar analogues that display bioorthogonal reactive groups, such as ketones, azides, and alkynes, offer good chemoselectivity, and their creative use could lead to novel modes of photomodulation (*85*).

Several features of the chromophores could be refined as well. Because most photoswitches are triggered by UV light, red-shifted photoswitches that operate fully in the visible range are needed. Having a range of such photoswitches would not only reduce the phototoxicity associated with UV irradiation but also facilitate the use of light-gated channels in conjunction with a variety of common fluorophores (*84*). In general, changing the substituents on the chromophore not only leads to altered wavelength sensitivity but also affects the thermal stability and extent of photoconversion between isomers (*4*). For this reason, a significant challenge remains in designing a photoswitch that performs well by all criteria. Finally, two-photon switches operating at wavelengths that can cause little harm while deeply penetrating tissue remain undeveloped.

The light-gated ion channels developed to date are derived from very different families of channel proteins, such as Cys-loop receptors, voltage-gated channels, and glutamate receptors. Therefore, other members of these families (e.g., $GABA_A$ receptors, Na_V channels, and NMDA receptors) or entirely new types of ion channels (e.g., P2X or ClC) could be subject to a similar approach.

Ion channels are in many respects ideal targets for manipulation. They can be probed with exquisitely sensitive assays (e.g., electrophysiology and fluorescence imaging), can be addressed from the extracellular side, and have a fast and pronounced impact on cellular function. The lessons learned from working with ion channels, however, should be generally applicable to other proteins, such as enzymes, metabotropic receptors, transporters, or molecular motors.

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