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Engineering Light-Regulated Ion Channels

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INTRODUCTION

Optical regulation provides unrivaled spatial and temporal precision for the control of protein function. We describe a strategy to engineer photosensitivity onto ion channels based on small photoisomerizable molecules. Photosensitivity is targeted to the ion channel of interest by combining the photoisomerizable moiety with a specific agonist or antagonist. Inclusion of a cysteine-reactive maleimide group enables covalent attachment of the photoswitch to a genetically engineered cysteine on the surface of the ion channel. Exposure to different wavelengths of light triggers changes in the properties of the photoswitch that enable control of protein function.

BACKGROUND INFORMATION

Optical control of ion channel function offers several advantages over traditional pharmacological regulation. Light can be turned on and off rapidly, allowing control of channel activity with exceptional temporal precision. Because light can be projected precisely in space, ion channel function can also be controlled at specific locations within a cell or tissue. In addition, the ability of light to penetrate tissue enables control of ion channel function from afar, minimizing invasiveness and potential damage to the structure under study.

Several strategies have been developed to control the function of ion channels and receptors with light, including the use of caged molecules (Ellis-Davies 2007) and the development of chimeric proteins that combine the protein of interest with a naturally light-sensitive protein (Airan et al. 2009). This article focuses on a third strategy: the use of small photoswitchable molecules to photosensitize genetically engineered or native ion channels (Fig. 1). These molecules contain distinct chemical moieties responsible for regulation of protein function (the ligand), photoisomerization (the photoswitch), and, if applicable, covalent attachment (the reactive moiety). Light absorption induces changes in the properties of the photoisomerizable moiety that are harnessed to trigger biophysical, and ultimately cellular, events. The modular nature of photoswitch molecules allows flexibility in the design of each functional group, yielding a combinatorial tool kit for optical regulation of ion channel function.

PHOTOSWITCHABLE TETHERED LIGANDS (PTLs)

The Photoswitch

Azobenzene is ideal for use as a photoswitch molecule because it can be photoisomerized repetitively with a high quantum yield and no detectable photobleaching (Renner and

Moroder 2006). The *cis* and *trans* azobenzene isomers differ in length by as much as 3 Å (Fig. 1A); this can be exploited to use azobenzene as a mechanical lever that maneuvers a functional group between two different positions depending on the wavelength of illumination. The *trans* (i.e., extended) azobenzene isomer is predominant after prolonged incubation in darkness because it is more thermodynamically stable than the *cis* isomer. Exposure to near-ultraviolet (UV) light (~360–400 nm) triggers photoisomerization to the *cis* state. The reverse *cis*-to-*trans* conversion occurs spontaneously in darkness but can be accelerated by exposure to visible light (~460–600 nm). Photoconversion between isomers occurs within picoseconds (Lednev et al. 1996; Nägele et al. 1997; Ihalainen et al. 2007).

Complete photoconversion to either *cis* or *trans* azobenzene cannot be achieved because the two isomers have overlapping absorption spectra. The relative amounts of *cis* and *trans* isomers at a given wavelength depend on the quantum yield for the isomerization processes and the lifetime of the *cis* isomer, both of which vary with chemical substitution to the azobenzene core, light intensity, temperature, and the environment surrounding the molecule (Yager and Barrett 2006). Typically, exposure to near-UV or visible light yields populations of azobenzene molecules containing up to ~85% *cis* or ~90% *trans* isomer, respectively (James et al. 2001). Near full conversion (>99%) to the *trans* state can be reached only after prolonged incubation in darkness (Renner and Moroder 2006).

The Reactive Moiety

Current strategies for photosensitizing ion channels with photoswitch-containing molecules rely on cysteine–maleimide chemistry. Surface-exposed endogenous cysteine(s) are first replaced with nonreactive amino acids using site-directed mutagenesis. Individual cysteine residues are then introduced on the surface of the protein and assayed for reactivity with the photoswitch-containing molecule. Maleimide reacts rapidly and irreversibly with cysteines, although the local environment of cysteines can influence their reactivity. The recent introduction of chemical approaches that target distinct genetically encoded protein tags (Marks and Nolan 2006) provides potential alternatives for orthogonal labeling if removing endogenous cysteines has a negative impact on ion channel function.

The Ligand

The ligand chosen in the design of the PTL defines which protein will become photosensitized. To be effective, there must be a “handle” on the ligand, such that it can be extended and conjugated to the azobenzene moiety without destroying the ability of the ligand to bind and activate the target protein. The success of affinity labeling as a technique to characterize enzyme function suggests that ligands can be modified with reactive groups and still maintain at least some biological activity (Silman and Karlin 1969; Wold 1977).

When designing PTLs, it can help to introduce flexible linkers between the different functional moieties to generate molecules slightly longer than the optimal distance between the ligand binding and attachment sites (Krishnamurthy et al. 2007). Flexible linkers impart conformational flexibility that might be necessary for tethering but can also compromise the effectiveness of the ligand by increasing the entropic cost of binding (Chung et al. 2009). The presence of flexible linkers can also introduce uncertainty by making the average length

of the molecule different from its extended length (Kramer and Karpen 1998; Green et al. 2001). However, the increasing availability of computational tools, including molecular simulation and docking software, combined with the growing number of solved three-dimensional (3D) protein structures should aid in the rational design of PTL/target protein interactions.

TARGETED PROTEINS

Nicotinic Acetylcholine Receptor

Erlanger and colleagues were the first to use azobenzene-containing molecules to regulate the activity of the nicotinic acetylcholine receptor (nAChR) (Bartels et al. 1971). They synthesized 3-(α-bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene bromide (QBr), a photoisomerizable agonist that attaches covalently to a previously reduced natively encoded cysteine on the surface of the nAChR. Once tethered, QBr activates the nAChR only in its *trans* configuration (Fig. 1B). The ability to toggle rapidly between activating and nonactivating QBr using light minimized nAChR desensitization allowing the kinetics of receptor activation to be studied with great temporal accuracy (Lester et al. 1980).

Ionotropic Glutamate Receptor

If available, protein structure information can guide the development of PTLs and engineering of target ion channels. For example, a light-activated ionotropic glutamate receptor (LiGluR) was designed based on the X-ray structure of the ligand-binding domain of the ionotropic glutamate receptor-6 (iGluR6) bound to the agonist (2S,4*R*)-4-methyl glutamate (Mayer 2005). Binding of an agonist causes the ligand-binding domain to engulf the ligand, except for a small cavity through which a molecular tether that includes an azobenzene moiety and a maleimide reactive group can be threaded (Volgraf et al. 2006). A cysteine was engineered into iGluR6 such that maleimide–azobenzene–glutamate-2 (MAG-2), a PTL for this receptor, reaches the ligand-binding domain and activates the receptor only in its *cis* configuration (Fig. 1C; Volgraf et al. 2006). Activation of the resulting LiGluR with near-UV light depolarizes neurons and induces action potential firing (Szobota et al. 2007). A shorter PTL, MAG0, activates iGluR in its *cis* and *trans* configurations, depending on the position of the cysteine attachment site. Molecular dynamics simulations revealed that the site of attachment for MAG0 influences the occupancy of the ligand-binding domain and its closure after binding of the PTL, thus determining which photo-isomer is “active” (Numano et al. 2009).

Voltage-Gated Potassium Channel

The engineering of the synthetic photoisomerizable azobenzene-regulated K^+ (SPARK) channel (Banghart et al. 2004) was also guided by the abundant biophysical and structural information available on voltage-gated K^+ channels. In particular, a 4 Å difference in the length of tethered tetraethyl-ammonium pore blockers (11 Å vs. 15 Å) attached to an engineered cysteine (E422C) made the distinction between ineffective or complete blockage of the channel (Blaustein et al. 2000). Based on this, the PTL maleimide–azobenzene–quaternary ammonium (MAQ) was synthesized to photoregulate the activity of a Shaker K^+ channel. Once MAQ is tethered to its target cysteine (E422C), the blocking quaternary

ammonium can reach the pore of the channel only in its extended (~17 Å) *trans* configuration but not in its short (~10 Å) *cis* form (Fig. 1D,E). Additional mutations were engineered into the channel to abolish fast inactivation (Hoshi et al. 1990) and shift the voltage sensitivity of activation (Lopez et al. 1991), generating a channel with a high resting K^+ conductance that hyperpolarizes and silences neurons illuminated with near-UV light. Illumination with visible light induces photoisomerization to the *trans* configuration and closes the chemical gate, restoring normal membrane potential and action potential firing (Banghart et al. 2004). Mutation of another amino acid in SPARK (V443Q) generates a nonspecific depolarizing cation channel (D-SPARK) by altering the relative permeability of the channel for Na^+ ions (Heginbotham and MacKinnon 1992). Exposure to near-UV light triggers action potential firing in MAQ-treated neurons expressing D-SPARK that can then be halted by illumination with visible light (Chambers et al. 2006).

MODIFICATIONS TO EXISTING LIGHT-GATED CHANNELS

Tuning the Photoswitch

Cis azobenzene reverts spontaneously to the *trans* configuration in the dark, a process known as thermal relaxation. Thus, continuous illumination is required to maintain the *cis* configuration on modified proteins. In the case of LiGluR, the protein environment surrounding the PTL stabilizes the *cis* state (Gorostiza et al. 2007). Because this might not be the case for other proteins, it can be desirable to design azobenzene-based molecules with enhanced thermal stability. Modification of the nature and position of substituents on the azobenzene core can decrease the rate of thermal relaxation from milliseconds to days (Forber et al. 1985; Pozhidaeva et al. 2004; Chi et al. 2006; Sadovski et al. 2009).

Existing PTLs undergo maximal photoisomerization to the *cis* configuration with exposure to near-UV light. Because short-wavelength light is scattered by living tissue, it can be advantageous to shift the action spectra of PTLs from near-UV toward longer wavelengths by modifying the azobenzene core to shift its spectral sensitivity (Chi et al. 2006; Sadovski et al. 2009). Similarly, it can be useful to modify current PTLs for use in deep tissue by improving their two-photon absorption cross-section. Although the two-photon absorption cross-sections of several azobenzene-containing molecules have been investigated (De Boni et al. 2005), these photoswitches have not been incorporated into the design of PTLs. Also, nonlinear optics have not yet been used to regulate ion channels modified with azobenzene-containing PTLs, but this is an important future goal.

Engineering Ion Channel Targets

The introduction of previously characterized trafficking motifs in light-regulated ion channels can enable better control of biological function. For example, SPARK channels localize to the cell body and axon when expressed heterologously in neurons. This localization is adequate for the photocontrol of action potential firing (Banghart et al. 2004). However, depending on the study, it might be more efficacious to target SPARK to dendrites, using a dendritic localization motif from Kv4.2 (Rivera et al. 2003) or a myosin-binding domain (Lewis et al. 2009), to silence inputs to the SPARK-expressing neuron. The cytoplasmic loop linking domains II and III of voltage-gated Na^+ channels (Garrido et al.

2003; Lemaillet et al. 2003) can be used to target proteins to the axon initial segment. Targeting light-regulated depolarizing channels such as D-SPARK and LiGluR to the axon initial segment will mimic the way spikes are initiated naturally in vivo and could be more likely to induce normal downstream events, such as synaptic plasticity.

In addition to providing a means to photoregulate biological functions (e.g., neuronal firing), the use of PTLs also enables functional studies of target proteins in cells and tissues. This can be particularly useful in cases in which few or no specific pharmacological regulators of the protein of interest exist (e.g., between different members of a K^+ channel subfamily). Traditional gene knockouts have provided a wealth of information about ion channel function but sometimes result in unintended lethality or induce compensation from related channels. Replacement of a native ion channel with a cysteine-containing variant in combination with a PTL provides a "chemical knockout" strategy. As a proof-of-concept for this method, we are using the previously characterized MAQ to photosensitize various K^+ channels containing a cysteine at a position analogous to E422C in Shaker. Because of the divergence of loop sequences between different K^+ channels, cysteine scanning might be required to identify the optimal tethering position. The chemical knockout strategy is not limited to K^+ channels; the modular nature of PTLs allows flexibility in the design of each functional group. The choice of ligand and the introduction of the cysteine attachment site will determine which ion channel/receptor is targeted.

Photochromic Ligands

The use of PTLs involves engineering target proteins to contain a cysteine attachment site followed by heterologous expression of the protein in cells. However, if genetic manipulation is not available or not desirable, photosensitive chemicals that target native proteins can provide an alternative one-component system for photosensitization. We have described photoswitchable molecules that confer photosensitivity to endogenously expressed K^+ channels in genetically unadulterated cells or tissues. These molecules contain a pore-blocking quaternary ammonium, a photoisomerizable azobenzene and an electrophilic group for attachment to the channel. In particular, acryl–azobenzene–quaternary ammonium (AAQ) efficiently photosensitizes K^+ channels expressed natively in neurons and enables control of neuronal excitability in dissociated cultures, slices, and semi-intact neuronal preparations (Fortin et al. 2008). Further characterization suggested that AAQ acts as an open channel blocker and thus at the internal tetraethylammonium-binding site of K^+ channels (Banghart et al. 2009). Replacement of the acryl moiety with a series of nonreactive aliphatic tails results in nontethered photoswitch-containing molecules whose potency for K^+ channel blocking generally correlates with tail length and hydrophobicity (Banghart et al. 2009). Similar nontethered agonists have been generated for the control of the nAChR and iGluRs (Deal et al. 1969; Bartels et al. 1971; Volgraf et al. 2007). Unlike photolysis of caged molecules, which consumes the starting material and cannot be reversed, nontethered azobenzene-containing ligands can be toggled repeatedly between *cis* and *trans* configurations to turn ion channel function on and off without genetic modification.

PRACTICAL CONSIDERATIONS FOR USING PTL-MODIFIED ION CHANNELS

Light

We have successfully regulated the activity of PTL-modified ion channels using lasers and other light sources such as mercury and xenon arc lamps equipped with band-pass filters. Our typical light output is in the low milliwatts per square centimeter (mW/cm^2) range or below (measured with a Newport light power meter and normalized to the illuminated area at the sample plane). For azobenzene-based PTLs, the intensity of light influences the relative amount of *cis* and *trans* photo-isomers generated at a given wavelength and the rate at which photoequilibrium is reached.

Cysteine–Maleimide Reactivity

Maleimides react specifically and rapidly with cysteine residues; for example, *N*-ethyl maleimide reacts within seconds at neutral pH (Gregory 1955). Cysteine residues are deprotonated and thus most reactive at slightly alkaline pH, which can, however, accelerate the nonspecific hydrolysis of maleimide. For efficient labeling of cysteine-containing ion channels with PTLs, we typically apply the PTL in pH 7.4–8 saline solution. The redox state of cysteine residues will also affect the efficiency and extent of maleimide–cysteine labeling. When photoregulating K^+ channels expressed in HEK293 cells, pretreatment with a reducing agent (1 mMothreitol or 1 mM *tris* (2-carboxyethyl)phosphine) is essential for efficient tethering, presumably because the high levels of cystine in Dulbecco's minimum essential-based growth media oxidize surface-exposed cysteine residues. If ion channel function requires disulfide bonds, follow the reducing treatment with a "resting" incubation in normal saline solution to allow native disulfide pairs to reform before PTL treatment.

CONCLUSION

The method described here for engineering light-regulated ion channels and receptors is based on exogenously applied synthetic photoisomerizable molecules. The versatility of the photoswitch approach has already been demonstrated with the development of PTLs and photochromic ligands for different classes of proteins, including an ionotropic glutamate receptor (Volgraf et al. 2006) and voltage-gated K^+ channels (Banghart et al. 2004; Fortin et al. 2008). Based on the increasing availability of structural data and extensive pharmacological knowledge of various ion channels and receptors, extensions of this approach to other proteins are likely forthcoming.

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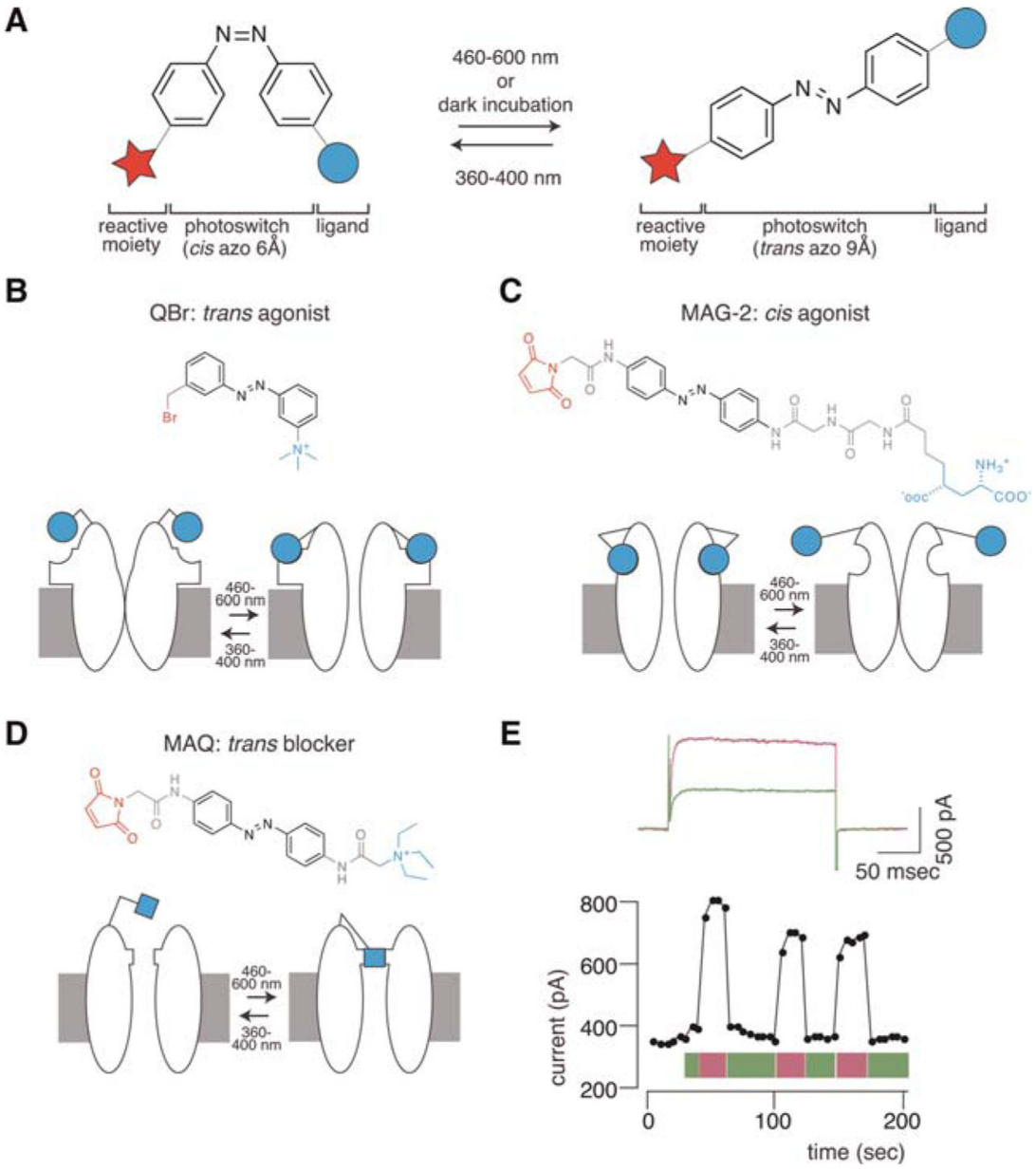

FIGURE 1.

Strategies for engineering light-regulated ion channels with azobenzene-containing molecules. (*A*) A photo-switchable tethered ligand (PTL) consists of a reactive moiety (red), a ligand (blue), and the photoswitch (here azobenzene [azo], black). Azobenzene undergoes *trans* to *cis* isomerization under 360- to 400-nm light. Visible light (>460 nm) or prolonged time in darkness returns the molecule to the *trans* state. The end-to-end distance between the para positions on the *trans* and *cis* azobenzenes is ~9 Å and 6 Å, respectively. (*B*) Tethered QBr is a *trans*-acting agonist of the nAChR. The receptor is activated when the agonist reaches its binding site under 460- to 600-nm light, whereas illumination with 360- to 400-nm light retracts the agonist, abrogating ion conduction through the channel. (*C*) MAG-2 is a *cis*-acting iGluR6 agonist that tethers to a genetically engineered cysteine. The agonist

reached its binding site only in the *cis* configuration (i.e., under 360- to 400-nm light). (*D*) MAQ reacts with an engineered cysteine on the surface of a Shaker K^+ channel and acts as a photoisomerizable tethered blocker, blocking ion conduction in the *trans* but not in the *cis* configuration. (*E*) (*Top*) Tethering MAQ to a genetically engineered cysteine (E422C) in a modified Shaker channel enables photoregulation of ion conduction. Under 500-nm light (green), MAQ is in the *trans* form; the QA group reaches the pore of the channel and blocks ion flow. Photoisomerization to the *cis* configuration with 380-nm light (violet) retracts the blocker, allowing ion flow. (*Bottom*) MAQ-treated Shaker channels can be regulated repetitively and persistently with light.