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Nanoengineering Ion Channels for Optical Control

Recombinant DNA technology and structural biology have made it possible to deduce the mechanisms of protein function. Such studies, and the recent advances in computer simulation, have made it possible to reengineer protein function and even design new proteins from scratch, with functions that did not exist previously (40). By covalently attaching synthetic chemical compounds to proteins in a site-directed manner, it is possible to engineer them to respond to new input signals. A promising avenue for development in this direction is to endow proteins with sensitivity to light—a new input signal that provides some powerful technological advantages. This review focuses on the covalent modification of ion channel proteins with synthetic photosensitive ligands that confer on them a direct sensitivity to light. The examples reported to date include ionotropic acetylcholine receptors (44), whose designs will be described in the first section. These synthetic photoswitchable ion channels share several features and properties that will be detailed in subsequent sections. 1) Receptor switching is based on the differential ability of trans and cis isomers of a photosensitive compound to permit ligand binding to a functional site on the protein. An examination will be made of the structural properties of the protein and the geometric relation between the ligand binding and tether attachment sites. 2) The modular structure of the compounds (reactant-switch-ligand) makes it possible to sculpt the optical control element by designing each of its modules. 3) The mechanism of conjugation of the photoswitch to the receptor follows an affinity label process, which accelerates the attachment reaction, can help with targeting in a complex tissue environment, and can be used to pattern attachment spatially. The design possibilities, the similarities and differences between the examined cases, and their applications provide a lesson on the opportunities in nanoengineering ion channels and other proteins bearing active and allosteric sites.

Azobenzene-based optical switches as protein actuators

One of the aims of nanotechnology is to obtain artificial nanoscale machines that obey external orders directed to a technological goal (e.g., to transduce the presence of an analyte into electrical signals measured by a sensor, to deliver a molecular cargo at a specific location or under specific physiological conditions, or to exert minute forces and very precise displacements on nanoscale objects). Although synthetic approaches to molecular machine design with a mechanical inspiration are leading to considerable advances (3, 9, 11, 37), the emerging field of biotechnology is taking a shortcut: developing nanotechnological devices based on biomolecules (2). Indeed, proteins can be viewed as highly efficient machines capable of performing many important classes of function, ranging from that of motors to structural elements, catalysts, and electronic and molecular carriers to information repositories. Proteins can be readily produced in relatively large amounts of identical entities and can be exploited technologically, even before understanding of their detailed molecular mechanism is complete. Within the cellular context, protein inputs and outputs operate in signaling chains, which detect events in the external and internal worlds, mediate intercellular communication, and regulate metabolism, development, division, movement, and survival.

To use proteins for technological applications, it is essential to gain external control of their function, preferably by means that differ from (and thus will not cross-react with) normal cellular signaling. One powerful approach has been to endow proteins with light sensitivity by means of synthetic photoisomerizable compounds that are coupled to the protein as a prosthetic “switch” group. These chemical photoswitches function as remote actuators that make it possible to turn protein function on and off. These can be used as counterparts to synthetic and protein-based optical reporters to obtain an all-optical control and detection of cell signaling from single cells to tissues to intact organisms.

Protein regulation with light has the advantage of being orthogonal in most tissues to natural inputs that the protein can receive (e.g., binding of ligands, substrates, or partner proteins), and it carries the additional benefit of allowing simple spatial-temporal control. Although synthetic photoregulation has targeted many protein classes (32), the rapid and spatially selective control provided by light is especially interesting in channel proteins, which drive cellular signaling on the millisecond time scale and have a complex spatial distribution in neurons that is essential for their physiological function. Photo-regulated proteins can also be especially useful in basic biochemical and biological studies, where it is advantageous to selectively engineer a protein in cells or whole organisms.

Several designs have been devised to link light-sensitive compounds with photosensitive proteins and tethers (4, 5, 44). These compounds or “on” or “off” tethers contain a photoisomerizable ligand and a covalent attachment moiety near its binding site (Fig. 1).
to selectively and noninvasively control a protein in the context of large complexes or whole cells.

Several strategies have been devised to control the activity of ion channels with light (20), including free photolabile "caged" ligands (23), photoisomerizable ligands (5, 29, 45), and tethered photoisomerizable ligands (4, 5, 44). This review is focused on tethered photoisomerizable ligands or "optical nano-toggles," which contain at one end a ligand (agonist, antagonist, or blocker), at the other end a reactive group that mediates covalent attachment to the protein near its binding site, and in the middle a photoisomerizable linker. The entire compound is attached site specifically to the target protein at either a native amino acid or at an amino acid introduced by mutagenesis (often a cysteine, which provides the conjugation selectivity of thiol chemistry). The photoisomerizable moiety and the ligand located at its end exert differential effects on the protein on absorption of light at two wavelengths. Photoisomerization back and forth between two states of the photoisomerizable moiety, in response to the two wavelengths of light, changes the location in space of the ligand such that it can only bind in one of the isomeric states. The ligand can be a blocker or an activator, and the binding site can be an active site in a functional domain or an allosteric site in a regulatory domain.

Azobenzene (38, 39) is the photoisomerizable moiety that is most extensively used as synthetic photoswitch, especially in ion channel applications (20). The dark, thermally relaxed trans isomer of azobenzene adopts an extended configuration that is ~0.7 nm longer than the higher energy cis or "bent" isomer. Illumination with near-ultraviolet (UV) irradiation at wavelengths preferred for absorption by the trans isomer (380 nm) leads to...
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accumulation of the cis isomer, and visible light irradiation at at wave-lengths preferred for absorption by the cis isomer (500-600 nm) switches it back to the trans form. Photoisomerization cycles can be repeated many times without detectable photodestruction or loss of responsiveness. A population of azobenzene molecules typically dis-played a trans/cis equilibrium at the optimal UV wavelength, and the fraction is reduced to a minimum of ~15% cis at the optimal visible wavelength, with full conversion to the trans form only after thermal relaxation. Wavelengths intermediate between the optimal and those of the two states, resulting in a wavelength-dependent photostationary state. The photoisomerization lifetime of azobenzene is in the picosecond range (41) with a quantum yield of 0.2-0.4 (8), which allows for rapid kinetic studies. In the dark, cis-trans thermal relaxation depends on substitutions in the azobenzene, and rates between 50 ns and 18 h have been reported (14, 45).

Nicotinic acetylcholine receptor

The nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction was the first channel to be nano-engineered with photochromic compounds (Figure 1A) (5). The design was based on the previous discovery that tethered agonist compounds can be conjugated to native cysteines in the vicinity of the ACh binding site of the receptor, yielding permanently active receptors, with the degree of activation proportional to tether length (26, 42). Using a tethered ligand compound of similar length, but bearing an azobenzene in the tether (QBr; Figure 1B), allowed the agonist to bind the receptor and activate it in the trans configuration, whereas in the cis configuration the tether was too short for the agonist to bind (5). Photoisomerization and subsequent induction of nAChR currents could be achieved reversibly with millisecond light pulses (12, 28, 30) but it was found that QBr acts as a partial agonist and that photocurrents decreased over time. In these studies, the photoisomerizable tether length was not varied and conjugation sites other than the native cysteines were not accessible. The arrival of cloning techniques (34) helped identify the putative conjugating residues as C192 or C193 (24, 25), and further progress in the field included the finding of Homozygous receptors and slowly desensitizing mutants (36). However, the photoswitchable nAChR has scarcely been revisited in the last 30 years. Nevertheless, at a time where structural biology was in its infancy, the studies of the photoswitchable nAChR provided evidence that the rate-limiting step of activation is not the initial encounter between agonist and receptor but rather some subsequent conformational rearrangements in the receptor (28).

Voltage-gated potassium channel (SPARK)

The photoswitching strategy employed with nAChR was revisited three decades later and applied to a voltage-dependent potassium channel (Figure 1C) (4). As in nAChR, the design was based on previous knowledge of a blocking quaternary ammonium compound tetraethylammonium (TEA) on a passive linker that permanently blocked the outer end of the channel pore when conjugated to a cysteine introduced at external residue 422 (7). In this case, the tether length threshold for channel blockade had been precisely characterized using compounds with spacer lengths between 0.7 and 4.5 nm and revealed that an increase in compound length between 1.1 and 1.2 nm produced an abrupt increase in block efficacy (7). Despite the lack of an atomic structure of K+ channels at the time, these results suggested that, including an azobenzene group in the tether, the 0.7-nm length change induced by photoisomerization would be enough to reversibly promote channel block with light. Indeed, a compound comprising a maleimide (cysteine reactive) group at one end, an azobenzene in the linker, and a quaternary ammonium blocking group at the other end (MAQ) (Figure 1D) was found to conjugate E422C and block the channel in the trans state, and blocking was relieved in the cis state under UV illumination (4). In this modified K+ channel, termed SPARK, the extended configuration of azobenzene allows the ligand to reach the binding site, whereas the short form does not (Figure 1C), as was the case in nAChR, described above. Since opening of a K+ channel results in membrane hyperpolarization, SPARK allowed the photo-inhibition of neuronal firing, providing the first demonstration of optical control of neuronal activity not involving caged compounds (4). SPARK was further converted into a photoswitchable, excitatory channel by mutational disruption of the ion selectivity filter, which rendered it permeable to Na+, leading to membrane depolarization when the channel was unblocked and triggering action potentials in neurons in response to light (13). Since the molecular design of SPARK was based on previously established functional data, variations on MAQ length or on the position of the cysteine (E422C) were not explored.

Ionotropic glutamate receptor (LiGluR)

In the development of the photoswitchable glutamate receptor, the lack of functional data on tethered ligands was compensated for by knowledge of the detailed anatomical structure of ligand binding domain (LBD) of AMPA (1) and kainate receptors (33). A systematic approach was thus used for the first time in which a tethered agonist model compound was initially designed to test whether the bound ligand remains accessible to the solvent throughout the conformational changes in the binding pocket (44). This model free compound was based on the known pharmacological agonist of the kainate receptor ionotropic glutamate receptor 6 (iGluR6), (25,4R)-4-allyl glutamate (35), whose structure and position in the LBD suggested a site for extending a tether off of lowering affinity. After verifying that the new compound acts not interfere with changes in compound length, but canno
a tether off of the glutamate without lowering affinity or agonist potency. After verifying that the model compound acts as a full agonist (i.e., it does not interfere with the conformational changes in the LBD), a complete compound featuring gabememic, azobenzene, and a glutamate end group (MAG) was synthesized (FIGURE 1F). To find the optimum site in the protein for photoswitch attachment, 11 exposed residues around the glutamate binding site were mutated individually to cysteine and photoresponses were evaluated after MAG conjugation. Several positions gave rise to light-activation (Numano et al., unpublished observations), with L439C displaying the largest responses (FIGURE 1E) (44). Other geometrical variations that were tested besides

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**FIGURE 2.** LiGluR photoswitching mechanism

A: when MAG is conjugated to L439C in LiGluR, the local effective concentration of the glutamate group near its binding site is low with azobenzene in trans and high in cis (21). B: two MAG derivatives with different tether length and tether model compound used to validate the nanotoggle design strategy in this case (21, 44). C: titration of photoresponse inhibition using a competitive antagonist allows determination of local effective concentration of MAG (D) (21). In agreement with a photoswitching mechanism driven by changes in local effective concentration, comparison of MAG compounds with different tether lengths reveals a lower basal activation in trans (21) and lower residual photoresponses at high antagonist concentration (44). D: the tether length in cis (l_cis) and trans (l_trans) relative to the distance d_0 between tether attachment and ligand binding sites, gives rise to different regimes of the current photoswitching mechanism: when either d_0 > l_trans or d_0 < l_cis, the agonist cannot bind, and no photoswitching is obtained. When l_trans > d_0 > l_cis, the ligand can bind in trans but cannot reach the site in cis, as in SPARK and the photoswitchable nAChR. When l_cis < d_0, photoswitching is driven by local effective concentration as in LiGluR. B–F are reproduced with permission from Ref. 21.
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the attachment site included spacers added to MAG to lengthen the linker between the ligand and the azobenzene (21). In these studies, steady state photosponses were obtained by blocking desensitization with concanavalin A (conA), and it was verified that neither the presence of the cysteine attachment site nor conjugation of the MAG photoswitch to the receptor altered its affinity for glutamate (21), indicating that the photoswitch provides a true orthogonal means for receptor activation. The device was termed LiGluR for ligand- and light-activated iGluR. Regarding the photoswitching mechanism, it was found that, for the first (intermediate length) MAG compound reported (44) attached at site L439C, the ligand was bound in the glutamate binding pocket in cis (leading to receptor activation and channel opening) and unbound in trans (leading to deactivation and closure). The cation-selective pore of LiGluR and its depolarizing capabilities were eventually applied to optically control firing in neurons. Using millisecond light pulses, it was possible to elicit single action potentials at high frequency and reliability in cultured hippocampal neurons, and light could be used to control specific behaviors by regulating activity in specific subsets of neurons in live zebrafish (43).

Mechanism of light gating

The photoswitching mechanism of these nanoengineered ion channels is based on the different length of the azobenzene tether in the trans or extended configuration (under visible illumination or in the dark), and the cis or bent configuration (under near UV illumination), the latter being shorter by ~0.7 nm. When azobenzene groups are used to tether a ligand (agonist or blocker), the accessibility of the ligand to the binding site can be regulated with light, but the detailed mechanism is apparently different in nAChR, SPARK, and LiGluR. In nAChR and SPARK, the extended configuration of azobenzene allows the ligand to reach the binding site, whereas the short form does not. In LiGluR, the ligand is not bound in the trans configuration because the attachment site lies on the outer surface of the clamshell LBD, and in trans the glutamate extends out into the water, whereas in the cis state the azobenzene linker bends and enables the glutamate to turn the corner into the glutamate binding pocket and dock there and activate.

In the photoswitchable K+ channel SPARK, inhibition of the photoswitchable agonist bis-Q (5). This difference was attributed to the higher local effective concentration of the conjugated QBr, although accurate quantification was complicated by voltage-dependent, partial noncompetitive antagonism of dTC and by other blockade processes that were not identified (10). Using an nAChR pore blocker, QX-222, photosresponsive generated in tethered and free agonists could be blocked with the same sensitivity, as expected for the pore being located far from the ligand binding site and for different agonists to open and close the channel for different fractions of time, without altering the structure of the mouth of the channel where QX-222 binds (28).

In the photoswitchable K+ channel SPARK, no pharmacological interference of photoswitching has been reported. The tethered TEA blocker has a relatively low affinity in the T449V version of Shaker that (22), and should, in principle, be displaced by other pore blockers, e.g., peptide toxins.

In the case of LiGluR, inhibition of MAG photocurrents by the competitive antagonist DNOX was used to estimate the effective ligand concentration for cis- and trans-MAG (21).

DNOX binds in the same site as glutamate but does not allow for closure of the clamshell LBD, thus not activating the channel by itself and preventing glutamate from doing so. LiGluR photocurrent inhibition was titrated against DNOX and compared with known concentrations of a freely diffusible model compound based on MAG but lacking the maleimide and half of the azobenzene linker. This data indicated that the photoswitch attaches covalently nor photo-switches but contains the glutamate and enough of the linker to project into bulk water out of the binding pocket and thus model the bound state of MAG. Based on DNOX competition with the model compound and with covalently attached MAG in the two states of the photoswitch, the estimate of the effective concentration of the glutamate end of the MAG near the binding pocket was 12 and 0.3 mM for cis- and trans-MAG, respectively, conjugated to the receptor (FIGURE 2, C AND D) (21). A version of MAG with a longer linker (addition of a second glycine spacer) between the azobenzene and the glutamate (i.e., able to sweep through a larger spatial volume) had a threefold lower effective local concentration in the cis state and a lower basal activation in the trans state and allowed for fuller block of the photosponses at high antagonist concentrations (FIGURE 2, E AND F) (21).

Comparing the results on MAG-activated effective concentration in LiGluR to a simple model of a single agonist molecule enclosed in a hemisphere with radius equal to the molecular length (C(mol/l) = 3/2πrL²Nₐ), and using r = ~1.5 nm, Ltrans = 2.5 nm, Nₐ = 6 x 10²³ molecule/mol, the concentrations obtained are 4.1 x 10⁻⁶ M for cis and 7.9 x 10⁻⁷ M for trans, which is higher than the estimated competition constant for MAG and their ratio is consistent with the theoretical ratio predicted for the photoswitching mechanism.

A generalized photoswitching scheme of the azobenzene linkage isomerizes in bulk water with temperature, and the presence of different lengths of the azobenzene linker bend and enable the ligand to be more conformationally constrained or not. For MAG, the distance of the trans state is longer than the cis state and trans-MAG is taken into consideration (like a “biacetyl” required for the cis-MAG inside the clamshell). The highest right direction isomerized isomerized.

1. If ltrans < d0, binding affinity for the tethered ligand will be higher than the cis form, and higher than the cis form, and the trans isomer can arise. The azobenzene linker isomerizes with the ligand bound.
2. If ltrans > d0, binding affinity for the tethered ligand will be lower than the cis form, and the cis isomer can arise. The azobenzene linker isomerizes with the ligand bound.
3. If ltrans > d0, and the cis isomer is the major species, then the photoswitching mechanism can be used to alter the affinity of the agonist for the receptor.
4. If ltrans > d0, and the trans isomer is the major species, then the photoswitching mechanism can be used to alter the affinity of the agonist for the receptor.

The photoswitching mechanism does not take advantage of the flexibility of the azobenzene linker, but it provides a method to change the effective concentration of the agonist in the receptor, which can be used to alter the affinity of the agonist for the receptor.
compared with blockers on a 40-fold site as glutamate (i.e., governed by reach as in nAChR-blocking states (SPARK) or of LBDs in non-photoswitchable tethered ligand complex moving the attachment site (the clamshell) to point the MAG in the right direction.

A generalized view of photoswitching mechanisms is depicted in the scheme of FIGURE 2G as a function of the tether length (distance between ligand and reactive end in the cis and trans isomers, $d_c$ and $d_{trans}$, respectively) and the relative distance between the attachment (conjugation) site and binding pocket ($d_b$). Four situations can arise. 1) If $d_c > d_{trans}$, the ligand cannot bind regardless of the isomerization state and the receptor remains inactive. 2) If $d_c > d_{trans}$, only the trans isomer can bind and the receptor is thus photoswitchable, with the ligand bound under visible light. 3) If $d_c > d_{trans}$, binding of the cis isomer is favored over the trans, and the receptor is again photoswitchable, now with the ligand bound under UV light. 4) If $d_c > d_{trans}$, ligand binding should again be difficult regardless of the isomerization state and give rise to inactive receptors. Due to steric limitations in the proximity between the attachment and the ligand binding sites, regime 4 may arise only with relatively long photoswitchable compounds.

The simple model presented above does not take into account the local flexibility of the photoswitchable compounds, nor does it consider the specific geometry of the protein surface where the binding and conjugation sites are located, something that is especially likely to be an issue in clamshell domains, where the ligand binding pocket is deep inside a cavity that closes on the ligand during activation. It also does not consider changes in ligand affinity due to isomerization itself, which have been reported in free compounds (5, 45). Correspondingly, introducing cysteines farther from the glutamate binding site in LiGluR should give rise to an “inverted” behavior of ligand binding in trans and unbinding in cis, i.e., governed by reach as in nAChR-QBr and SPARK. It should also be possible to obtain similar effects by elongating QBr and MAQ tethers and by shortening the MAG tether.

Another important issue concerning the light gating mechanism of these channels is their stoichiometry, i.e., how many switches govern the activity of a single channel. In nAChR-QBr, only one sulfhydryl is alkylated per receptor, as was already seen with non-photoswitchable tethered ligands (15, 16, 42). Each receptor is thus controlled by a single tethered QBr, in contrast with the two free agonist molecules that are required to fully activate the receptor (12, 28). Here, the role of the reducing agent applied before QBr conjugation was ruled out, and the altered stoichiometry was attributed to QBr attachment (28). In LiGluR, comparison of the agonist- and light-induced currents after conjugating MAG to completion at high concentrations and long exposures was in agreement with one MAG molecule attached to each subunit of the tetrameric receptor, based on almost a degree of agonism that roughly agreed with the photostationary state at the optimal wavelength for isomerizing to the cis conformation (21). In SPARK, four MAQ molecules (at 100 nm) lead an ensemble of molecules to distribute themselves in various fractions between the two states. This results in a wavelength-dependent photostationary state and can be used to obtain an analog control of current amplitude through LiGluR (FIGURE 3A) in a manner reminiscent of current clamping. In channels that contain more than one photoswitch, like SPARK and LiGluR, the intermediate wavelengths will lead to mixtures of tethered blockers in the blocking and non-blocking states (SPARK) or of LBDs in the liganded/activated and unliganded/resting states.

Adjusting photoswitch properties by design

An intrinsic advantage of synthetic photoswitchable tethered ligand compounds is their flexibility and modularity. These molecules comprise three modules or moieties: 1) a ligand group that can bind reversibly to an active site or an allosteric site in a protein, 2) a photosomerizable group or switch whose properties (length,
angle, dipolar moment) are modified with light, and 3) a reactive group that conjugates to selected chemical groups present in proteins, anchoring the tethered ligand compound. All three of the modules can be adjusted by chemical substitution. The effect of the ligand and its affinity can be changed taking as a reference the binding and effect of free pharmacologically active compounds like agonists, competitive antagonists, and blockers. The conjugation chemistry can be selected to meet specific requirements, as discussed in the next section. In addition, modification of the protein itself provides further flexibility for the design by adjusting the attachment site (introducing cysteine residues at different locations relative to the binding site) and the affinity or efficacy of a given ligand (mutating residues in the binding pocket). But perhaps the most unique feature of the modular design of photoswitches is that it makes it possible to adjust the optical properties of the switch independently, since it is not embedded and tightly coupled to a protein binding pocket like natural chromophores (e.g., retinal in opsins).

Photoswitches based on azobenzene are the most widely employed due to their large length change on isomerization and chemical robustness (low photobleaching rate), although others have been reported like spiropyran (27), stilbene (18), and hemithioindigo (31). In azobenzene, UV illumination produces a maximal population of cis isomers, and visible light results in a mainly trans population. Intermediate wavelengths between the maximum and minimum fractional occupancy of the cis state provide analog control of the balance between cis and trans, and thus the percent activation of the channel (21). Pulses of light lasting only a millisecond, and having intensities that are typically used in confocal microscopy (i.e., which are well tolerated by cells and live animals), are long enough to open and close the channels and trigger significant changes in membrane potential, and thus to elicit action potentials (43). The photoisomerization rate of azobenzene is faster still, in the picosecond range (41), with a quantum yield of 0.2–0.4 (8), which has allowed kinetic studies of gating mechanisms (28, 30). In the dark, cis-trans relaxations depend on substituents in and around the azobenzene, and rates have been reported between milliseconds to days for spontaneous relaxation into the more stable trans state. Thus slow-relaxing azobenzene switches display persistent activation in dark, which can be viewed as a sort of “molecular memory” of the state (cis or trans) that has been imprinted with light, and which can elicit persistent action potentials.

**FIGURE 3.** Current injection into cells by means of LiGluR
A: current injection into cells by means of LiGluR can be adjusted in an analog way with the illumination wavelength. Under voltage clamp at –60 mV (left), light between 500 and 380 nm results in currents between 0 to several hundreds of pA, respectively. Under current clamp (right), these currents allow depolarizing the same cell between ~70 and ~30 mV. B: since the MAG relaxation time constant is 18 min (21), short (5 s) UV pulses are enough to maintain LiGluR open for several minutes in the dark. When applied to hippocampal neurons, UV light-driven depolarization triggers action potential firing that is sustained until cells are illuminated again with visible light. A and C are reproduced with permission from Ref. 43.
can elicit persistent inward current (FIGURE 3B) and action potential firing (FIGURE 3C) from LiGluR after a short activating light pulse. Despite the high energy barrier to thermal isomerization from the higher energy cis state back to the trans state in the dark, the transition can still be driven rapidly by light (FIGURE 3, B AND C). In addition, it is also possible to adjust the absorption spectra of azobenzene to a certain extent (14), although these changes may affect relaxation rates.

Mechanism of photoswitch conjugation

Most nanotoggles (QBr, MAQ, and MAG) are designed with thiol-reactive moieties to target them to extracellular cysteine residues in the channel, either native (as in the case of the nAChR) or introduced by site-directed mutagenesis (SPARK and LiGluR). The extracellular location of the attachment sites that have been used in all of the photoswitched channels made it possible to perfuse membrane-impermeant compounds that likely attach to all exposed cysteine ions relative to the affinity or specificity of the affinity labeling (mutating the cysteine pocket). But a feature of azobenzene is that it can be employed as a switch that can be adjusted through a maximal change on submillisecond timescales (41), with a maximal change on timescales that are rapid enough to affect actions and triggers of membrane proteins in milliseconds and trigger persistent action potentials (8), which are known to be critical for gating Na⁺ in the dark, cis-trans photoisomerization of azobenzene, and which were reported to cause days for the membrane to return to the more low-relaxing configuration that they display persistently (e.g., the persistent memory of the nanotoggles that has been reported).

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An interesting issue for the development of light-activated channels is the ability to adjust the absorption spectra of azobenzene to a certain extent (14), although these changes may affect relaxation rates. The mechanism of photoswitch conjugation involves the following steps:

**A**: At low MAG concentrations (<100 nM), conjugation to the ligand binding domain (LBD) of iGluR6-L439C is preceded by binding of the glutamate end of MAG, i.e., it occurs by affinity labeling. Thus conjugation can be hindered by visible light, which favors the trans configuration and puts the reactive maleimide away from the cysteine. Under UV light, conjugation is favored.

**B**: MAG conjugation can also be hindered by occupying the LBD under excess glutamate.

**C**: Calcium imaging traces of an iGluR6-L439C expressing cell under the indicated conditions. Visible (green) light hinders MAG conjugation and no photoswitching is observed, and UV (purple) light favors conjugation giving rise to photoswitching.

**D**: MAG conjugation is also hindered under excess (300 µM) glutamate, whereas MAG alone can conjugate and produce photoswitching.

**E**: Use of affinity labeling to spatially constrain MAG conjugation to UV-irradiated regions.

**F**: Photoswitch responses measured by whole-cell patch clamp are fivefold higher in regions irradiated with UV during MAG incubation.

**G**: Tethered photoswitchable ligands bearing strong electrophilic groups (e.g., acrylamide in AAQ compound used in SPARK variants) can conjugate to a variety of nucleophilic residues. Specificity is achieved by affinity labeling, since the only nucleophiles that can be conjugated are those that reach from the position where the photoswitchable ligand is bound. A-F are reproduced with permission from Ref. 21. G-H are reproduced with permission from Ref. 19.
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how the photoswitchable tethered ligand compound conjugates to the protein. The question arises because these compounds have one end that reacts selectively with an extracellular cysteine and another end that has high affinity for the ligand binding site, leading to the possibility of affinity labeling, whereby docking of the ligand and at the binding site would place the tether end near the intracellular cysteine and favor conjugation. Indeed, in nAChR, it was found that 100 nM trans-QBr (the active isomer in nAChR) conjugates much faster than cis-QBr (the inactive isomer) (28). A similar effect was observed in LiGluR, where 100 nM cis-MAG (the active isomer) conjugated more efficiently than trans-MAG (FIGURE 4, A-D) (21). In addition, trans-QBr was found to compete with carbamylcholine for binding to nAChR (5), and conjugation of trans-QBr could be inhibited by the competitive antagonist di-tubucurarine (dTC) (28). Correspondingly, in LiGluR, free glutamate inhibited the conjugation of cis-MAG (active isomer) (21) by occupying the binding pocket and preventing docking of the glutamate end of MAG (FIGURE 4, A-D). Related effects had been already observed with the non-photoswitchable tethered compounds that preceded the development of photoswitchable nAChRs (15, 16) and K+ channels (6). Overall, they suggest that the effective concentration of the cysteine-reactive end in the vicinity of the target cysteine is enhanced when the ligand end of the molecule docks in the binding site. Such affinity labeling has been widely exploited to identify drug-target interactions and the binding site on the target, as well as to measure drug affinity and selectivity (17). Variants of MAQ used in SPARK (4) have been recently developed that further exploit affinity labeling by using electrophilic reactive groups to target native nucleophilic, non-cysteine residues, so that phototargeting can be avoided (FIGURE 4, G AND H) (19). Such compounds endow optical excitability to endogenous potassium channels, thus allowing optical control of neuronal firing without the need for genetic manipulation. One of the compounds, termed AAQ (Acrylamide Azobenzene Quaternary ammonium), comprises a TEA ligand, an acrylamide acryl-

amido moiety that can react with the numerous nucleophilic groups present in proteins (FIGURE 4D). Although acrylamide conjugation is often sensitive at physiological pH, extracellular binding of the TEA ligand to the pore of potassium channels produces an increased effective acrylamide concentration in the vicinity of the pore that favors conjugation at a precise distance from the pore. Once AAQ is conjugated, it can be operated as MAQ: shortening of the azobenzene tether under UV light retracts the TEA ligand from the pore and allows ion conduction (cell hyperpolarization), and extension of the tether under visible light blocks the channel and drives cell depolarization. AAQ was used to photocontrol action potential firing in intact neural circuits, notably in rat slices where introduction of foreign genes is not widely used (19). A novel application of the state dependence of affinity labeling (preferred conjugation for the activating isomer) was demonstrated with LiGluR (21) by patterning MAG conjugation over an otherwise uniform set of cells expressing GluRI6-L439C. This effect was achieved by masking light into regions of 380 nm (favoring the active cis-MAG isomer and thus conjugation) and 500 nm (favoring trans-MAG and hindering conjugation) (FIGURE 4, E AND F) (21). The same concept has been recently applied to “paint on” AAG with light onto untransfected cells (19).

Conclusion

The chemical modification of ion channels and receptors with synthetic photoswitchable compounds has been applied to nicotinic acetylcholine receptors, voltage-gated potassium channels, and non-competitive glutamate receptors. As more instances are reported and their properties are characterized in detail, basic nanooengineering principles are emerging: Functional switching is based on the “differential effect” of trans and cis isomers on docking of a tethered ligand to a binding pocket, with the detailed mechanism depending on the distance between the ligand and binding and tether attachment sites relative to the length of the entire photoswitch. Tethers that are short in relation to the anchoring-binding distance give rise to a “reaching” mechanism, i.e., trans-bound, cis-unbound ligand as in the photoswitchable nAChR and SPARK. Tethers that are long in relation to the anchoring-binding distance give rise to an “effective concentration” mechanism in which the shorted cis isomer samples a smaller volume than the trans isomer and thus the end ligand has a higher effective concentration in cis, as in the case of LiGluR. The modular structure of the compounds (reactant-switch-ligand) makes it possible to adjust by design each of the modules. Reactive ends can be cysteine selective (as in maleimide or methanethiosulfonate) or else they can be widely reactive with nucleophilic residues (as in acrylamide or epoxide); photoswitch absorption spectra and thermal relaxation rates can be adjusted with chemical substitutions, and ligands can range from agonists to antagonists, blockers and modulators. Finally, the conjugation mechanism of the photoswitch to the receptor follows an affinity labeling process, which can be exploited to achieve region-selective conjugation with light and receptor-specific conjugation without the need for selective reactive groups. Systematic testing of several properties of the switch (e.g., length) and protein (e.g., attachment position) makes it possible to adapt new protein targets to novel nanotoggles. This strategy should be applicable to the optical control of active sites and allosteric sites in both membrane and cytoplasmic proteins.

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