Photopharmacology

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Photochromic Blockers of Voltage-Gated Potassium Channels**

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Photochromic ligands (PCLs) can be optically switched between isomers that show different biological activities. As such, they offer an opportunity to convert ligand-actuated pathways into light-actuated pathways, thus making it possible to control a wide range of biological processes with light.

PCLs have been explored for various classes of target proteins, including enzymes,[1–3] ligand-gated ion channels,[4–6] and G-protein-coupled receptors.[7] For instance, photochromic agonists[5] and antagonists[8] for the nicotinic acetylcholine receptor, a ligand-gated ion channel, were described more than thirty years ago. More recently, we have introduced a photochromic version of glutamate that acts as a PCL on kainate receptors and can be used to trigger neuronal firing.[6] The PCL approach can be particularly effective in neural systems, where the nonlinear nature of cellular excitability can accentuate relatively small changes in efficacy or incomplete photoconversion between isomers.

We report herein a family of amphiphilic azobenzene molecules that target tetrameric voltage-gated ion channels (Figure 1a). Channels of this type are not gated by extracellular ligands but can be blocked by small molecules such as lipophilic cations.[9,10] Our molecules function as photochromic blockers of voltage-gated K^+ channels and act on the intracellular tetraethylammonium (TEA) binding site (Figure 1b). They can be applied from the extracellular side and have long-lasting effects in cells after a single, transient application. In excitable cells, they function as photochromic neuromodulators and can be used to optically control action potential firing.

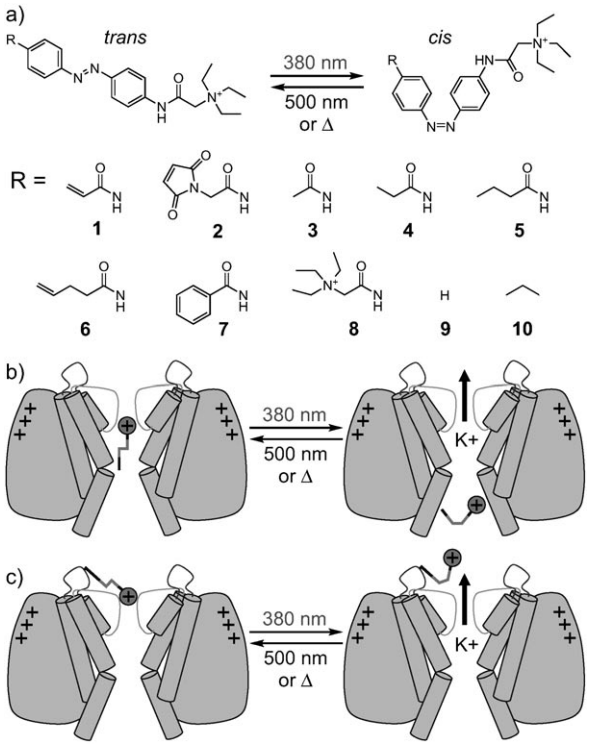

Figure 1. Photocontrol of K^+ channels. a) Structures and photoisomerization of photoswitchable K^+ channel blockers. b) A PCL for the internal TEA binding site. The linear *trans* isomer is a better blocker than the bent *cis* isomer. c) A PTL for the external TEA binding site. The extended *trans* isomer presents the blocking particle to the pore while the shorter *cis* isomer is unable to reach.

We have previously reported the molecule AAQ (Acrylamide-Azo-Quaternary ammonium, **1**), which was shown to photosensitize wild-type K^+ channels.[11] We had hypothesized that AAQ functions as a photoswitchable tethered ligand (PTL) at the external tetraethylammonium (TEA) binding site and that it would attach to native residues through affinity labeling (Figure 1c). However, our attempts to verify an interaction at this site were inconclusive (Figures S1 and S2 in the Supporting Information). Instead, our mechanistic studies indicate that AAQ is not covalently bound and acts as a PCL at the internal TEA binding site (Figure 1b).

Potassium channels are not only blocked by alkyl ammonium ions at the external tetraethylammonium (TEA)

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binding site, but also at the internal entrance to the selectivity filter.[12–15] Charged blockers of the internal but not external TEA binding site exhibit "open-channel block", wherein pore occupancy does not occur until after the voltage gate has opened.[9,10,16] This effect is most easily observed in Shaker IR (Sh-IR) and other channels that lack the fast-inactivating N-terminal peptide.[17,18] In response to step depolarization, some ionic current initially flows through the unbound channel, but this flow quickly decays over tens of milliseconds as the blocking molecules bind to the pore.

Figure 2a shows the current responses to depolarizing voltage steps of a HEK293 cell expressing Sh-IR that has been treated with 400 μM AAQ. Under irradiation at 380 nm, the

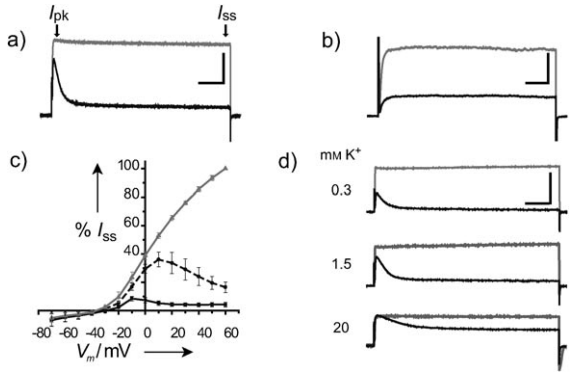

Figure 2. AAQ is an open-channel blocker of the Sh-IR internal TEA-binding site. a, b) Whole-cell current responses to 200 ms depolarizing voltage steps from −70 to +40 mV under irradiation at 380 nm (gray line) or 500 nm (black line). a) Open-channel block is apparent in AAQ-treated Sh-IR channels (scale bar: 6 nA, 25 ms). b) Open-channel block is absent in MAQ-treated SPARK channels (scale bar: 0.5 nA, 25 ms). c) Steady-state *I*/*V* curves under 380 nm (gray line), 420 nm (black dashes) and 500 nm (black line) irradiation. *I*/*V* curves from each of four cells were normalized to the current measured at 380 nm and +60 mV. d) Dependence of AAQ block on $[K+]_o$. Whole-cell current responses of a cell to depolarizing voltage steps from −70 to +40 mV under irradiation at 380 nm (gray line) or 500 nm (black line) at the indicated $[K^+]_o$ value. Similar results were obtained in two other cells (Scale bar: 5 nA, 50 ms.)

channels are not blocked by AAQ (gray trace). However, when the channels are blocked by AAQ under irradiation at 500 nm, an initial transient current remains (I_{pk}), which rapidly decays so that nearly all of the steady-state current (I_{ss}) is blocked (black trace). This effect is not observed during blockade of SPARK channels,[19] which contain an extracellular cysteine residue for covalent attachment of the maleimide analogue MAQ (**2**; Figure 2b).

The data obtained suggest that AAQ acts predominantly at the internal TEA binding site of Sh-IR, while MAQ acts on the external TEA binding site of the SPARK channels.[20,21] A more extensive characterization of the open-channel block observed with AAQ is provided in Figure S3 in the Supporting Information. These experiments directly demonstrate the requirement for channel opening to occur before AAQ is able to block the pore, and reveal that pore occupancy is correlated with the frequency of channel opening.

The current–voltage (*I*/*V*) curve further indicates that AAQ acts at the internal TEA binding site and shows that block by AAQ is distinctly voltage-dependent, so that I_{ss} is blocked more effectively at more depolarized membrane potentials (Figure 2c). Under irradiation at 380 nm, the current increases linearly with voltage once the channels are fully activated (gray line). Under irradiation at 500 nm, the current is predominantly blocked at all membrane potentials (black line). However, irradiation at 420 nm, which produces only partial conversion to the *cis* isomer, reveals voltage-dependent block (dashed black line), as indicated by the decline in I_{ss} at potentials more positive than +10 mV (Figure S4a in the Supporting Information shows the raw current responses). This result is typical of positively charged intracellular K^+ channel blockers.[10,22] Just as depolarization provides a driving force for positively charged K^+ ions to flow in the outward direction, internal alkyl ammonium ions are driven into their binding site within the membrane electric field and block more effectively as membrane depolarization is increased.

As K^+ ions move through the permeation pathway of K^+ channels in a single file, high concentrations of external K^+ ($[K^+]_o$) electrostatically repel intracellular charged blockers to accelerate their exit rate from the channel and thereby reduce their blocking potency.[23,24] Accordingly, the extent of AAQ block correlates inversely with the extracellular potassium concentration $[K^+]_o$, as revealed by the current values shown in Figure 2d. After establishing a voltage clamp in standard external buffer ($[K^+]_o = 1.5$ mM) and measuring I_{ss} under irradiation at 380 and 500 nm, cells were locally perfused with solutions containing 0.3 mM and 20 mM $[K^+]_o$. We controlled for the change in maximal current, which results from the altered K^+ driving force, by measuring currents at 380 nm, which completely unblocks the channels. This trend of lower efficiency was consistent across the range of voltages that activate Sh-IR (Figure S4b in the Supporting Information).

Consistent with this mode of action, direct application of AAQ to the internal TEA binding site in both the inside-out patch (Figure 3) and whole-cell recordings (Figure S5a in the Supporting Information) also produced photoswitchable open-channel block. Because inclusion in the patch pipette does not permit solution exchange at the cytosolic interface, inside-out patches were pulled from HEK293 cells expressing Sh-IR to allow AAQ application, followed by washout. In this

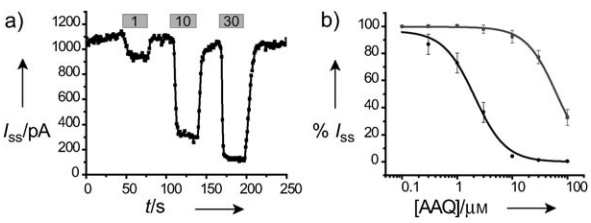

Figure 3. AAQ is a PCL for the internal-TEA binding site of Sh-IR. a) I_{ss} recorded from inside-out patches as AAQ was applied at the indicated concentrations (μM). b) Dose-response relationships for AAQ applied to inside-out patches under 500 nm (black line) and 380 nm (gray line) irradiation.

case, current block by AAQ was relieved within several seconds of washout (Figure 3a), which indicates that covalent binding did not occur under these conditions. Dose–response curves could therefore be generated by irradiating patches under 380 and 500 nm light in the presence of different concentrations of AAQ. Summary data for photostationary states enriched in *trans*- and *cis*-AAQ are shown in Figure 3b, which reveals a 30-fold difference in block potency (IC_{50}(500 nm) = (2.0 ± 0.2) μM, IC_{50}(380 nm) = (64 ± 2.1) μM, n = 3–5 patches). Because the photoswitchable block was observed without any indication of covalent modification, these experiments demonstrate that AAQ can act as a PCL to block the internal TEA binding site.

AAQ is an acrylamide and therefore contains an electrophilic functional group, yet our data indicated that analogues that lack an electrophile would function in a similar manner. To test this model and identify photochromic blockers with improved biophysical properties, we prepared a series of analogues (Figure 1a, **3**–**10**). To facilitate partitioning into the membrane, several analogues contain aliphatic "tails" of increasing hydrophobicity (**3**–**7**). In contrast, the doubly charged, symmetric analogue **8** was expected to exhibit poor membrane penetration. In other members of the series, the amide tail was either removed completely (**9**) or replaced with a propyl group (**10**) to mimic the length of **3**, which was the shortest amide examined. Prior to screening, UV/Vis spectroscopy confirmed that irradiation under 380 nm and 500 nm light produced nearly identical photostationary states, which consisted of at least 80% *cis* and *trans* isomers for 380 and 500 nm irradiation, respectively (data not shown, details for chemical synthesis are given in the Supporting Information)

Strikingly, after a brief extracellular application followed by washout, all of the new compounds that contain a hydrophobic tail (**3**–**7**, **9**, **10**) were found to function as open-channel blockers and persistently blocked Sh-IR currents for the duration of a typical recording (5 min). I_{ss} measurements from cells treated with either AAQ, **7**, or **10** under repeated 380 nm and 500 nm irradiation are shown in Figure 4a–c. Step responses to depolarization for each new compound are shown in Figure S5 in the Supporting Information.

A general trend was observed in which potency correlates with tail length and hydrophobicity. Table 1 shows the lowest concentrations required to produce more than 95% block of I_{ss} at +40 mV. The doubly charged analogue **8** did not block channels after extracellular treatment (tested at concentrations up to 2 mM), although inclusion in the patch pipette did afford photoswitchable block (results not shown). Benzoyl-Azo-QA (BzAQ, **7**), was found to block more than 95% of I_{ss} at a concentration of only 25 μM. A dose–response curve obtained from inside-out patches (Figure 4d) indicates that *trans*-BzAQ has a similar affinity to *trans*-AAQ when applied directly to the internal TEA binding site (IC_{50}(500 nm) = (1.3 ± 0.2) μM, IC_{50}(380 nm) = (83 ± 20) μM, n = 4). Therefore, its enhanced potency is most likely due to better membrane partitioning rather than increased affinity for the blocking site.

In contrast to the amides **3**–**7**, compound **9** exhibited comparable block in both the *cis* and *trans* forms (Figure S5 in the Supporting Information). This result suggests that interactions between the tail and channel protein might account for the differences in affinity between the isomers. Indeed, Propyl-Azo-QA (PrAQ, **10**), preferentially blocks when it is in the *cis* form (Figure 4c). External treatment with 40 μM of compound **10** resulted in approximately 50% block of I_{ss} with the *cis* form, but no obvious block by the *trans* isomer was observed, as judged by the lack of rapid current decay upon

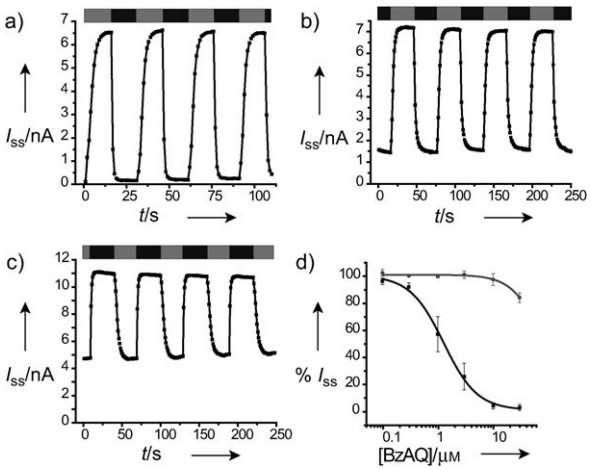

Figure 4. PCLs persistently photosensitize Sh-IR. I_{ss} recorded over time from a cell treated with a) 400 μM AAQ, b) 20 μM BzAQ (**7**), and c) 40 μM PrAQ (**10**) under 380 nm (gray bars) and 500 nm (black bars) irradiation. d) Dose–response relationships for BzAQ (**7**) applied to inside-out patches under 500 nm (black line) and 380 nm (gray line) irradiation.

Table 1: Structure–activity relationships.

Compound	μM[a]	% Block[b]	Active isomer
1 (AAQ)	400	>95	*trans*
2 (MAQ)	400[c]	0	–
3	1000	>95	*trans*
4	800	>95	*trans*
5	300	>95	*trans*
6	200	>95	*trans*
7 (BzAQ)	25	>95	*trans*
8	2000	0	*trans*
9	1000	ND[d]	*trans* = *cis*
10 (PrAQ)	40	≈50	*cis*

[a] Concentrations reported for **1**–**6** and **8** were determined by first identifying a dose that produced greater than 95% block. The concentration was reduced in 100 μM increments until less than 95% block was observed. For compounds **7** and **10**, 5 μM increments were used to determine the reported values. At each reported concentration, determinations were repeated in three to five cells from two separately treated coverslips. [b] For compounds **1**–**9**, the block percentage was calculated as the ratio between the I_{ss} values measured under irradiation at 500 nm (block) and 380 nm (reference). For compound **10**, the wavelengths were reversed. In each case, unblock was estimated as complete by the lack of open-channel block, as shown in Figure S5 in the Supporting Information. [c] Treatment of HEK293 cells with MAQ at concentrations greater than 400 μM did not result in stable recordings. [d] Although substantial open-channel block was observed, the lack of unblock at either wavelength prevented calculation of the block percentage. ND = not determined.

irradiation at 500 nm. However, at higher concentrations, blocking by the *trans* isomer was observed (data not shown). As the thermally stable *trans* isomer is inactive, optimized *cis*-blocking analogues of PrAQ will reduce cellular exposure to UV light and avoid unintentional channel blocking during PCL application in the dark.

To determine if these novel PCLs function in neurons in the same way as AAQ, we examined the most potent analogue BzAQ (**7**) in dissociated hippocampal cultures, a preparation in which AAQ was previously found to photosensitize endogenous K^+ after preincubation at 300 μM.[11] Current–voltage curves from neurons recorded in the whole-cell voltage clamp configuration showed that the K^+ channels that contribute to I_{ss} in neurons are modulated after extracellular treatment with 20 μM BzAQ (Figure 5a). From this data, the I_{ss} value at +40 mV was compared after irradiation at 380 and 500 nm, and revealed that on average, $(35.5 \pm 4.7)\%$ ($n = 10$) of I_{ss} is blocked by BzAQ under these conditions. In the current clamp mode, which allows the recording of action potentials, BzAQ was found to depolarize the cellular membrane potential when switched from *cis* to *trans*, which was sufficient to induce action potential firing (Figure 5b). Furthermore, similarly to AAQ,[11] BzAQ did not affect depolarizing Na^+ currents in neurons (data not shown) and did not photosensitize voltage-gated Na^+ or Ca^{2+} channels expressed in GH3 cells (Figure S6 in the Supporting Information). Taken together, these data indicate that BzAQ is an effective photomodulator of neural activity, which has features similar to AAQ,[11] but has the advantage of increased potency and avoids the potential toxicity and immunogenicity associated with reactive reagents.

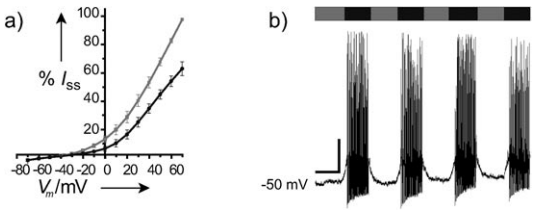

Figure 5. BzAQ photoregulates endogenous K^+ channels in dissociated hippocampal neurons to modulate neural activity. a) Steady-state *I*/*V* curves under 380 nm (gray line) and 500 nm (black line) irradiation recorded from neurons treated with 20 μM BzAQ. Recordings from four individual cells were normalized to the current measured at 380 nm and +70 mV. b) Current clamp recording from a neuron showing the induction of action potential firing in response to 500 nm light (black line). Similar results were obtained in at least three additional cells. Scale bar: 10 mV, 10 s.

The results presented here do not explain how the blocker molecules are retained by cells for long periods of time. While AAQ can effectively photosensitize Sh-IR in HEK293T cells for just over one hour, dissociated hippocampal neurons respond to light at least 24 hours after exposure to AAQ (Figure S7 in the Supporting Information). It is likely that retention occurs by tight association with the plasma membrane, as is the case with a structurally related styryl class of fluorophores, which includes the endosomal marker FM1-43[25] and many voltage-sensitive dyes.[26] It has been proposed that classic local anesthetics access the internal binding site of voltage-gated sodium channels through hydrophobic pathways.[27] Regardless of the exact mechanism of retention, the phenomenon of long-lasting block is not unique to our photochromic blockers. An array of hydrophobic ammonium ions such as the lidocaine derivative Tonocaine, and tetrapentylammonium (TPeA), have been shown to become trapped after crossing the cell membrane and block voltage-gated sodium and potassium channels for many minutes after washout.[28,29]

AAQ was originally designed to covalently bind at the external TEA binding site. Although the results presented here do not rule out covalent modification of Sh-IR altogether, they do suggest that AAQ predominantly acts as a PCL at the internal TEA binding site instead. This result has allowed us to define an entire class of photochromic ligands that have a new mode of function and explore their structure-activity relationships in detail. Given the many structural, functional, and pharmacological similarities of voltage-gated ion channels, the principles established herein should also enable the development of additional PCLs for voltage-gated Na^+ and Ca^{2+} channels. Our photochromic neuromodulators have already proven themselves as useful tools in neurobiology and could have therapeutic value, for instance in attempts to restore vision.

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