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Thermodynamically stable, photoreversible pharmacology in neurons with one- and two-photon excitation

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Abstract

Photoswitchable bioprobes enable bidirectional control of cell physiology with different wavelengths of light. Many current photoswitches use cytotoxic UV light and are limited by the need for constant illumination due to thermal relaxation in the dark. We have developed a photoswitchable tetrafluoroazobenzene(4FAB)-based ion channel antagonist that can be efficiently isomerized in two separate optical channels with visible light. Importantly, the metastable *cis* configuration showed very high stability in the dark over the course of days at room temperature. In neurons, the 4FAB antagonist reversibly blocks voltage-gated ion channels with violet and green light. Furthermore, photoswitching could also be achieved with two-photon excitation yielding high spatial resolution. 4FAB probes have the potential to enable long-term biological studies where both “on” and “off” states can be maintained in the absence of irradiation.

Keywords

thermodynamically stable; visible light; two-photon; photoswitch; intracellular

Starting in the 1970s, photochemically switchable agonists have been used to study integral membrane proteins involved in the control of ion gradients in living cells^[1–3]. In 1971 Erlanger and co-workers introduced azobenzene-based acetylcholine receptor potentiators that were inactivated by irradiation with UV light (ca. 330 nm), while drug activity was recovered by 420 nm illumination^[4]. In 1978 a second type of biophotoswitch was invented (called caged ATP^[5]), using photoremovable protecting groups, which were activated by UV light. Beginning in 2000, photochemical switches have attracted a good deal of renewed attention with the photocontrol of peptide conformation^[6] and ion channel activity^[7]. Both types of optical probes continue to be areas of intense investigation^[8–13].

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The core properties of such bioprobes are, ideally, the same^[8]. They should be: biologically inert; stable and soluble in physiological solution; photoactivated rapidly and efficiently with wavelengths that are biocompatible and transmitted through modern microscopes^[14]. Photoreversible switches, however, differ from caged compounds in that the former are bidirectional. Thus, these probes have an additional set of design considerations that are defined by this core property. Such probes should use two wavelengths of visible light to “flip the switch” that are chromatically independent^[11, 15], with switching occurring without fatigue between two states that are thermodynamically stable. Here, we describe the first biological deployment of a photoswitch that exemplifies this set of properties in living cells.

In 2012, Hecht and co-workers reported that tetrafluoroazobenzene (which we call “4FAB”) chromophores had a very interesting set of chemical properties that seemed near ideal for photoreversible bioprobes^[16]. In particular, they showed that the presence of the *ortho*-fluoro substituents introduced two new features to the traditional azobenzene photoswitch^[6, 7, 16]. First, a clean spectral separation in the visible region of the *trans* and *cis* configurations of the chromophore was reported such that *trans*-4FAB absorbed minimally in a region where the *cis*-4FAB absorbed maximally (ca. 410–420 nm). Secondly, and perhaps more importantly, with a half-life at RT of about 2 years^[16], the *cis*-4FAB configuration exhibited an astonishing thermal stability. The *cis* configuration of many other azobenzenes is, by contrast, highly unstable thermodynamically, with half-lives ranging from milliseconds^[17] to several seconds^[18] to tens or hundreds of minutes^[10, 18–20]. In contrast, we envisaged that the thermodynamic stability of 4FAB would allow us to pre-set a neurobiological probe into “on” or “off” states for the entire period of a long-term physiological experiment. Quaternary ammonium (QA) molecules have been important for the study of ion channels for more than 50 years^[21, 22], with photoswitchable and tethered forms originating in 1979^[23] and 1980^[24], respectively. Recently, several other photoswitchable QA molecules were made that advanced this technology^[7, 19, 20, 25]. Thus, we synthesized a bis-QA derivative of a 4FAB chromophore (4FAB-QA, **1**) to test in neurons. We found that both one- and two-photon (1P/2P) excitation can be used for efficient reversible photoswitching of **1** such that the voltage-gated ion channels can be modulated bidirectionally by violet and green irradiation. Moreover, the superb thermodynamic stability of the core 4FAB was maintained with **1** inside living neurons suggesting molecules like **1** could be significant additions to the photopharmacological arsenal. The synthesis of **1** is shown in Fig. 1a. Starting with the known^[16, 26] tetrafluoroazobenzene dicarboxylate **2**, two tertiary amine groups were installed through amidation with *N,N*-diethylethylenediamine using pyBOP, affording **3** in 68% yield. The amines were quaternized with iodoethane to yield the di-QA compound *trans*-**1**, which was purified by preparative HPLC to give the di-TFA salt in 20% yield.

We found that the important spectral separation of the $n\text{-}\pi^*$ absorptions^[16] is preserved for *trans*- and *cis*-**1** (Fig. 1b) in physiological buffer (Fig. 1c). Using 533 and 405 nm lasers we found that the photostationary state (PSS) composition was 88% *cis* and 94% *trans*, respectively (Fig. S1). These values compare favorably with other azobenzene-QA PSS values of >80% using UV and green light^[19]. A solution of **1** kept at 25 °C for 2 days showed no thermal relaxation of the *cis* isomer (Fig. 1c, inset). Thus, 4FAB-QA

photochemistry essentially preserves all the important photophysical properties reported for the core chromophore^[16, 27]. However, “favorable” chemical properties of optical probes are often not maintained when tested on living cells^[28, 29]. Therefore, we evaluated the “photopharmacological” efficacy of **1** in neurons natively expressing voltage-gated ion channels targeted by QA molecules. Single pyramidal neurons in the CA1 hippocampal region in acutely isolated mouse brain slices were filled via a patch pipette with a low concentration of **1** (0.1 mM). Current flow through voltage-gated ion channels was induced by step depolarizations and the current amplitude monitored over time. Irradiation with violet and green light from 405 and 530 nm LEDs, respectively, caused reversible modulation of the steady state potassium current amplitude (Fig. 2, for details see Fig. S2 and Supplemental Discussion) consistent with the *trans* form of **1** blocking open ion channels much more effectively than the *cis* configuration (Fig. 2a). Notably, photoswitching could be performed over 100 transitions (Fig. 2d), demonstrating that the reported lack of photocycle fatigue in cuvette^[16] was preserved inside cells.

Next, we evaluated the thermal stability of both *cis*- and *trans*-**1** in a biological context. A solution of **1** was irradiated with green light to generate the *cis* PSS (79% *cis* by HPLC, Fig. S1) and was stored in the dark. After 3.5 hours, a neuron was filled via whole-cell patch clamp with this solution and the potassium current amplitude was monitored for 10 min, followed by irradiation with green light which *caused no further unblocking* of the current (Fig. 3a) demonstrating that the extreme thermodynamic stability of *cis*-4FAB could be used in a biological context and inside cells. Further cycles of bidirectional switching with violet and green light showed chromophore integrity and cell viability. After 2 additional hours, a second cell was loaded with the same stock solution of *cis*-**1** and exhibited the same pattern of response as the first cell (Fig. 3a). These results are in stark contrast to the only other 4FAB probe tested with neurons in which no *cis* thermal stability was observed^[30]. The solution used for cell filling was then frozen for 2 days, thawed and used for a similar physiological experiment, *with identical results* (Fig. 3a). Additionally, we confirmed the expected stability of the *trans* isomer by performing analogous experiments starting with *trans*-**1** (Fig. 3b). Even though the thermal stability of photoswitches has been reported previously, such properties have not been exploited in any biological experiments^[31]. The experiments shown in Fig. 3 demonstrate for the first time that a photoswitch can be preset to its thermodynamically unstable isomer and used over several days for cellular physiology without any loss of the initial activity.

Finally, we explored if the 4FAB chromophore itself could be used for photoswitching using 2P excitation. First, we tested 2P switching in either direction in a cuvette. Irradiation of a solution of *cis*-**1** with a 780 nm laser could indeed induce *cis* to *trans* photoisomerization (Fig. 4a). Other wavelengths in the 750–810 nm range were found to be less effective. Irradiation of a solution of *trans*-**1** with 950–1000 nm light did not produce noticeable isomerization in a cuvette, likely due to low power available at these wavelengths, but 900 nm did induce *trans* to *cis* isomerization (Fig. 4a). Encouraged by this, we tested 2P photoswitching in living cells. A neuron was filled with **1** pre-irradiated with green light. Continuous 2P laser scanning of the cell body with 780 nm light for ~70 s (see supplement) caused block of the steady state potassium current, indicating highly effective, 2P-driven *cis*

to *trans* isomerization (Fig. 4c,d). Following this, the cell was illuminated with green light, which unblocked the current back to the original amplitude, and cycles of violet and green light could consistently modulate current. Next, we tested 2P *trans* to *cis* isomerization by filling a neuron with **1** pre-irradiated with violet light. Scanning the soma with 1000 nm light increased the current amplitude consistent with 2P *trans* to *cis* isomerization which was reverted by irradiation with violet light (Fig. 4e,f, similar effects were observed with 900 and 950 nm, data not shown).

While 2P excitation of azobenzenes has not been extensively studied^[32], it has been reported that the entire 820–900 nm range can have similar effects on both *cis* and *trans* configurations^[17]. In contrast, amino-substituted azobenzenes have been reported to undergo unidirectional *trans* to *cis* photoswitching using non-linear excitation, with very rapid thermal isomerization accounting for chromophore reversion on the millisecond time scale^[17, 33]. There is significant disparity between these reports of the ratio of the absolute value of the 2P/1P effects for the same azobenzene system. One report finds a 2P/1P ratio of 40%^[17], while the other reports 12%^[33]. We have found a 2P/1P ratio of $31 \pm 5\%$ for the *cis* to *trans* isomerization of **1** (Fig. 4d) and of $78 \pm 7\%$ for the *trans* to *cis* reaction (Fig. 4f), demonstrating that 4FAB undergoes efficient chromatically independent two-color, 2P switching in both directions. It should be noted that the area of the cell illuminated with 2P irradiation is smaller than the area irradiated by 1P light (Fig. 4b), meaning that a smaller fraction of **1** in the cell is excited by the 2P laser and thus the biological 2P/1P ratio is limited by this constraint. Recently, Hecht and Kovalenko used 2P excitation of the π - π^* state of benzyl-4FAB with visible light at 640 nm^[34]. We suggest that 2P excitation in the NIR region probably causes direct excitation to the S₁ state of n- π^* absorption bands of the *cis*- and *trans*-forms of the 4FAB chromophore.

Given its excellent photochemical properties, there have been surprisingly few applications of the 4FAB chromophore^[30, 35–38], with the only neurobiological application^[30] unexpectedly showing no thermodynamic stability of the *cis* configuration with cells. In contrast, our work firmly establishes that the superb thermal stability of *cis*-4FAB^[16, 27] is preserved inside cells, and can be used for photoswitching for multiple cycles of two-color, wavelength independent excitation in living cells using visible light. Importantly, we could also use 2P excitation to enable subcellular bidirectional photopharmacology with wavelengths in the therapeutic window^[10, 12]. These are distinct improvements over other azobenzenes^[19] and other spectrally modified QA probes^[20]. Thus, 4FAB probes have the potential to enable long-term biological studies where both “on” and “off” states can be maintained in the absence of irradiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Synthesis of **1**, photoisomerization and PSS spectra. a) Reagents and conditions: (a) *N,N*-diethylethylenediamine, pyBOP, DIPEA, DMF, 68%. (b) EtI, 20%. b) Photoreversible *cis-trans* isomerization of **1** using violet and green light. c) Absorption spectra of all *trans*-**1** in HEPES (pH 7.3) (black dots), and the PSS for 533 nm (violet) and 405 nm (green) lasers. HPLC analysis indicated 88% *cis* and 94% *trans*, respectively. A solution of *cis*-**1** in HEPES (pH 7.3) was stable over 2 days at RT (inset).

Figure 2.

Bidirectional photocontrol of voltage-gated ion channels in neurons with visible light. a) The linear *trans* configuration is a better ion channel blocker than the bent *cis*. b) Current responses of a hippocampal CA1 neuron to voltage steps with **1** (0.1 mM) loaded via the patch pipette. Voltage steps during either violet or green light blocked and unblocked voltage-gated ion channels. c) Reversible block of steady state voltage-gated potassium currents. d) Multiple rounds of photoswitching could be effected in a hippocampal CA1 neuron without any detectable fatigue (50 cycles shown, the change in baseline is due to small changes in access resistance over the course of the experiment).

Figure 3.

Long-term thermodynamic stability of **1** in physiological solution. a) A solution of **1** (0.1 mM) was pre-irradiated with a green LED to generate the *cis* PSS and stored in the dark. At set time points the solution was used to load hippocampal CA1 neurons via whole-cell patch clamp. Irradiation with green light did not produce a change in current amplitude indicating stability of the *cis* isomer (top). Application of violet and green light produced subsequent block and unblock of voltage-gated ion channels. The solution was stored at -4°C and showed the same stability when used 2 days later (bottom left). b) A solution of all *trans*-**1** (0.1 mM), which was used to fill a neuron as in a), showed that initial irradiation with violet light did not induce any further block of the current response.

Figure 4.

Bidirectional photoswitching of **1** using 2P excitation. a) A solution of *cis*-**1** in HEPES (pH 7.3) was irradiated with 780 or 900 nm light from a Ti:sapphire laser focused into a quartz cuvette with a lens (10x, 0.25 numerical aperture). The extent of isomerization was followed by UV-vis spectroscopy. b) Cartoon illustrating the different areas of 1P and 2P neuronal irradiation used in c-f. c) Starting from *cis* PSS, 2P irradiation at 780 nm (red) induced current block which green light unblocked and violet light blocked. Black bar represents no illumination. d) Comparison of violet and 780 nm photocurrents from the same cells ($n = 11$). e) Same as (c) but starting from *trans* PSS. Scanning the soma with 1000 nm (dark red) evoked unblock of current. f) Comparison of green and 1000 nm photocurrents from the same cells ($n = 7$).