Controlling engineered P2X receptors with light

Benjamin N. Atkinson[1,2], Vijay Chudasama[1] and Liam E. Browne[2]

[1]Department of Chemistry, University College London, London WC1H 0AJ, UK

[2]Wolfson Institute for Biomedical Research and Department of Neuroscience, Physiology and Pharmacology, University College London, London WC1E 6BT, UK.

Correspondence to: liam.browne@ucl.ac.uk

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Abstract

This chapter details methods to express and modify ATP-gated P2X receptor channels so that they can be controlled using light. Following expression in cells, a photoswitchable tool compound can be used to covalently modify mutant P2X receptors, as previously demonstrated for homomeric P2X2 and P2X3 receptors, and heteromeric P2X2/3 receptors. Engineered P2X receptors can be rapidly and reversibly opened and closed by different wavelengths of light. Light-activated P2X receptors can be mutated further to impart ATP-insensitivity if required. This method offers control of specific P2X receptor channels with high spatiotemporal precision to study their roles in physiology and pathophysiology.

Keywords

P2X, ATP, ion channel, synthetic optogenetics, optochemicals, photoswitchable, azobenzene

1. Introduction

P2X receptors are trimeric transmembrane proteins that function as ATP-gated ion channels[1]. They have been implicated in afferent sensation, neuroeffector transmission, central control of respiration, and inflammation[2]. However, many of their roles in physiology and pathophysiology are not precisely understood, partly due to a lack of available tools to modulate their activity specifically. Optogenetic approaches employ light for the manipulation of genetically encoded light-sensitive proteins to study the function of molecules, synapses, cells and systems[3,4]. Using light for the remote control of biological processes provides high spatiotemporal precision.

P2X receptors can be engineered to impart optical control (Fig. 1) as previously demonstrated for homomeric P2X2 receptors and P2X3 receptors, and heteromeric P2X2/3 receptors[5]. A single cysteine mutation is introduced in each P2X receptor subunit to provide a new

functional handle within the protein channel. A photoswitchable tool compound, 4,4'-bis(maleimido)azobenzene (BMA), is then used to bridge cysteines between two of the mutated P2X receptor subunits *via* maleimide moieties at either end of the compound (Fig. 1a). This covalent attachment does not affect the P2X receptor function or its sensitivity to ATP, but crucially allows the channel to be opened and closed with different wavelengths of light[5]. Light at a wavelength of 440 nm isomerises the azobenzene at the centre of BMA molecule from *cis* to *trans*. The increased distance between the maleimide groups attached to the pore push the channel open (Fig. 1b). The location of the cysteine (*e.g.* rP2X2[P329C]) is crucial for effective channel opening. As thermal relaxation back to the *cis* state is relatively slow the channel is essential bistable when channel desensitisation is limited[5]. Illumination with 360 nm light reverses isomerisation of BMA, shortening the length of the BMA modification and thus facilitating the channel to close. Here we describe the methods (Fig. 2) for transient expression of mutant P2X receptors in HEK293T cells and protocols for chemical modification and light-switching of engineered P2X receptors during patch clamp recordings.

2. Materials

2.1 Cell culture and transient transfection

1. Cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine and 10% heat-inactivated Foetal Bovine Serum (*see* **Note 1**). Aliquot to 50 mL and store at 4 ºC.
2. Reduced serum medium: Opti-MEM, for example. Aliquot to 5 mL and store at 4 ºC.
3. Dulbecco's phosphate-buffer solution (DPBS).
4. Trypsin-EDTA: 0.05% solution in 5 mL aliquots and store at -20 ºC.

5. Lipofectamine 2000 transfection reagent. Store at 4 °C.
6. Mammalian DNA expression plasmids encoding wildtype and mutant P2X receptors (for example, rP2X2[WT] at 0.1 μg/μL and rP2X2[P329C] at 0.1 μg/μL), and a fluorescent reporter (for example, GFP at 0.05 μg/μL). Plasmids for expression of engineered homomeric P2X2 and P2X3 receptors, and heteromeric P2X2/3 receptors are available[5], including some that are further modified for ATP-insensitivity. Store at -20 °C.
7. Round borosilicate glass coverslips, 12 mm diameter (*see* **Note 2**).

2.2 Light-switching

1. Extracellular Solution: 147 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 13 mM D-glucose, pH 7.3. To 800 mL ultrapure water (*see* **Note 3**), add 8.59 g NaCl, 0.149 g KCl, 2.38 g HEPES and 2.34 g Glucose, and dissolve at room temperature with stirring (*see* **Note 4**). To this, add 2 mL of 1 M $CaCl_2$ solution and 1 mL of 1 M $MgCl_2$ solution. Adjust to pH 7.28-7.33 with dropwise addition of 5 M NaOH solution with stirring. Make this solution up to 1000 mL in a volumetric flask using ultrapure water. Store at 4 °C and dispose of after seven days.
2. Adenosine 5'-triphosphate (ATP) solution: 100 mM ATP, pH 7.3 in extracellular solution (*see* **Step 1**). Weigh 1 g adenosine 5'-triphosphate disodium salt hydrate and add extracellular solution filtered through a 200 μm membrane filter to yield 200 mM ATP, considering the hydrate number of the batch. Mix gently until fully dissolved (*see* **Note 4**), adjusting to pH 7.28-7.33 with dropwise addition of 5 M NaOH solution. Make to 100 mM using extracellular solution. Aliquot to 5 mL and store at -20 °C.
3. 10 mM BMA stock solution: 10 mM solution of (*E*)-4,4′-bis(maleimido)azobenzene (BMA) in dimethyl sulfoxide (DMSO). BMA can be synthesised according to the published procedure[5] (*see* **Note 5**) or commercial sources are also available (*see* **Note 6**). To make the stock solution, add 300 μL DMSO to solid BMA to yield a 10 mM orange solution with vortexing. Store at room temperature in the dark. Dispose of after 12 h.

4. Illumination System. A monochromator or multiple LEDs can be used to provide rapid switching (<3 ms) of narrow bandpass (14 nm full width at half maximum) blue light (440 nm, 7.5 $mW.mm^{-2}$) and near-ultraviolet light (360 nm, 2 $mW.mm^{-2}$). This can be applied through an optical fibre or a microscope fluorescence objective above the patched cell. The light source(s) should be computer-controlled to allow illumination to be timed accurately.

2.3 Electrophysiological recordings

1. Intracellular solution: 147 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.3. To 80 mL ultrapure water (*see* **Note 3**), add 0.859 g NaCl, 0.380 g EGTA, and 0.238 g HEPES, and dissolve at room temperature with stirring (*see* **Note 4**). Adjust to pH 7.28-7.33 with dropwise addition of 5 M NaOH solution with stirring. Make this solution up to 100 mL in a volumetric flask using ultrapure water. Filter through a 200 µm membrane filter, aliquot to 5 mL and store at -20 ºC.
2. Patch-pipettes. Use a pipette puller (*e.g.* Sutter P-97 or P-2000), to pull thin-wall borosilicate capillary glass (outer diameter = 1.50 mm, inner diameter = 1.17 mm, length = 10 cm) to give a 3-5 mm taper and 2-4 MΩ tip resistances when filled with intracellular solution (*see* **Step 1**).

3. Methods

All cell culture solutions are warmed to 37 ºC in a water bath at least 20 mins before use unless otherwise stated and manipulations are carried out under sterile conditions in a laminar flow hood. Light-switching of P2X receptor does not require electrophysiological recordings, these can be considered optional, however this method includes the details to carry out such experiments.

3.1 Cell subculture

1. HEK293T cells are cultured in 25 cm^2 flasks containing 5 mL cell culture medium (*see* **Material 1.1**) in an incubator at 37 ºC with 5% CO_2. HEK293T cells that reach 90% confluence should be subcultured and plated for DNA plasmid transfection when required for the experiment.
2. Remove and discard the old culture medium from the 25 cm^2 flask.
3. Briefly rinse cells by carefully applying 1 mL warm DPBS along the side of the flask, minimising cell disturbance, and gently move the solution across the cell monolayer before removing and discarding the DPBS (*see* **Note 7**).
4. Add 1 mL warm trypsin-EDTA solution to the cell monolayer, gently move the solution across the cell monolayer and incubate for 2 mins at 37 ºC.
5. Confirm that the majority of cells have detached from the flask and add 5 mL of warm cell culture medium using a 10 mL stripette. Gently pipette up and down to release any remaining cells while being careful not to introduce air bubbles (*see* **Note 8**).
6. Transfer the HEK293T cell suspension to a 15 mL Falcon tube and centrifuge at room temperature at 90 x *g* for 5 mins.
7. Meanwhile, add 5 mL cell culture medium into two new 25 cm^2 flasks and add 1.8 mL cell culture medium into two 35 mm Petri dishes placed inside of a 100 mm Petri dish.
8. Take the centrifuged cells (*see* **Step 6**) and remove the medium so as not to dislodge the pellet at the bottom.
9. Add 5 mL of warm cell culture medium to the cell pellet using a 10 mL stripette and gently pipette up and down (~20-25 times) to a homogenous cell suspension, minimising air bubble formation.
10. Transfer 0.3 mL of the cell suspension into each of the 25 cm^2 flasks containing the previously added 5 mL (*see* **Step 7**) and ensure coverage by slowly moving the flask in a figure of eight motion (*see* **Note 9**).
11. To prepare cells for next-day transfection, transfer 0.4 mL of the cell suspension (*see* **Step 8**) into each of the two 35 mm Petri dishes (*see* **Step 7** and **Note 10**).

3.2 Transient transfection and plating of cells

1. Visually assess the pre-plated cells in Petri dishes (see **Method 3.1**, **Step 11**). The cells should be 60% confluent and healthy (*see* **Note 11**). Return the Petri dishes to the incubator to await transfection.
2. Allow the wildtype and mutant P2X receptor plasmids, fluorescent reporter plasmid, and lipofectamine 2000 to reach room temperature. Briefly vortex and minicentrifuge to draw liquid to the bottom of each tube.
3. Add 0.2 mL warm reduced serum medium into a sterile 1.5 mL tube and then 3 μL of lipofectamine 2000. Mix gently and incubate at room temperature for 5 mins.
4. Meanwhile, add 0.1 mL of reduced serum medium into two additional sterile 1.5 mL tubes. Each tube will contain fluorescent reporter plasmid and one of the two P2X receptor plasmids. Label accordingly.
5. Add 1 μL of the fluorescent reporter plasmid along with either 1 μL wildtype P2X receptor plasmid, or 1 μL mutant P2X receptor plasmid, to one of the two tubes from **Step 4**.
6. Remove the media from pre-plated cells. Briefly rinse in warm DPBS before immediately replacing with 800 μL warm reduced serum medium.
7. After the 5 min incubation is complete (*see* **Step 3**), transfer 0.1 mL of the lipofectamine 2000 mix to both of the tubes containing reduced serum medium and plasmids (*see* **Step 5**), and incubate for 20 mins at room temperature.
8. Into each 35 mm Petri dish containing pre-plated cells carefully add the contents of one of the lipofectamine-DNA tubes from **Step 7**. Slowly move the dishes in a figure of eight motion before returning to the incubator for 6 h.
9. Before the 6 h incubation is complete, transfer five pre-washed glass coverslips (*see* **Note 2**) into new 35 mm Petri dishes (*see* **Note 12**).

10. Add 1.8 mL of warm cell culture medium into each of the 35 mm Petri dishes containing coverslips. Ensure the coverslips are fully covered by pushing them to the bottom of the dish with a sterile pipette tip.
11. When the 6 h incubation (*see* **Step 8**) is complete, remove the all the reduced serum medium from the transfected cells in Petri dishes.
12. Immediately rinse with 175 µL of warm DPBS being careful not to detach cells.
13. Remove the DPBS and carefully add 175 µL of trypsin-EDTA solution, ensuring complete coverage of the cells. Incubate at 37 ^{o}C for 2 mins.
14. After 2 mins, check that the majority of cells have detached and add 1.8 mL of cell culture medium and gently pipette up and down >20 times to ensure an even suspension in the medium (*see* **Note 8**), avoiding the creation of air bubbles.
15. Carefully add the cell suspension dropwise over each cover slip in the Petri dishes. For whole-cell patch clamp, we use cells at approximately 10-30% confluence on the day of the recordings so that cells are not attached to one another (*see* **Note 13**).

3.3 Light-switching and electrophysiology

1. Allow light sources and electrophysiology equipment to warm up.
2. Allow the extracellular solution, intracellular solution and ATP solution to reach room temperature.
3. Meanwhile, pull glass electrodes required for the day (*see* **Material 2.3**).
4. Transfer 1 mL intracellular solution to a 2 mL syringe containing a 200 µm membrane filter and MicroFil.
5. Make a 30 µM ATP working solution by adding 3 µL of the ATP solution to 10 mL extracellular solution and vortex. Store on ice until required.
6. Prepare the perfusion system, setting up and testing separate flows of extracellular solution and 30 µM ATP working solution from **Step 5**.

7. Make a 1 mM BMA stock solution by diluting 20 µL of the 10 mM BMA stock solution in 180 µL DMSO and immediately vortex. Keep at room temperature and protect from light.
8. Immediately before the experiment, make a 10 µM BMA working solution by transferring 120 µL of the 1 mM BMA stock solution from **Step 7** into 11.88 mL extracellular solution in a Falcon tube and immediately vortex. No precipitate should be visible by eye. Protect from light.
9. Add the 10 µM BMA working solution to a third channel on the perfusion system (*see* **Note 14**), protect from light and test flows from all three channels (*see* **Note 15**).
10. From a Petri dish containing transfected cells (*see* **Method 3.2**), transfer a coverslip to the perfusion bath (*see* **Note 16**).
11. Under the microscope, identify an isolated, healthy, normal-sized fluorescent reporter-positive cell using epifluorescence with blue light excitation and green light emission.
12. Perfuse 10 µM BMA working solution over the cell for 12 mins.
13. Half-fill a pulled glass pipette with intracellular solution using the prepared syringe (*see* **Step 4**) and carry out whole-cell patch clamp recordings, filtering at 5 kHz and sampling at 10 kHz. Recordings may be made before, during and after the application of ATP or light is be applied (*see* **Note 17**).

4. Notes

1. Store Foetal Bovine Serum as 50 mL aliquots at -18 ^{o}C and avoid freeze-thaw cycles.
2. Submerge glass coverslips in ethanol overnight and air dry under a class II hood. Store in a sterile 35 mm Petri dish.
3. Ultrapure water is obtained by purifying deionised water to resistivity of 18.2 MΩ.cm at 25 ^{o}C.
4. Salts to do not dissolve immediately on adding water. The solution becomes homogenous nearing the target pH of 7.3.

5. A slight modification in the synthesis can be made, rather than column chromatography purification of the BMA may be out by recrystallisation from 1:1 Ethanol:1,4-Dioxane.
6. *Toronto Research* Chemicals (B497250), *Sigma Aldrich Partner Product* (ENAH049F1C70), *Enamine* (EN300-190205).
7. This step removes dead cells and dilutes any divalent cations contained in the media that can inhibit the action of trypsin.
8. The media will inactive the trypsin.
9. In our hands, HEK293T cells approximately double in number every 24 h and should be split twice a week at around 1/17. The volume of cell suspension in this step might need adjusting depending on actual cell growth rate and viable cell numbers.
10. The volume of cell suspension should give 60% confluence by the time of transfection. Again, this volume depends on actual cell growth rate and viable cell numbers.
11. For transfection, healthy cells will be well-adhered to the Petri dish and not completely rounded.
12. Typically, transfection of cells in one 35 mm Petri dish yields enough cells for the following day at 10-30% confluence in four 35 mm Petri dishes.
13. In our hands, transient transfection using this protocol provides cells for the following three days. However, there are fewer isolated cells on day three and cell shape can sometimes result in poor space clamping.
14. Alternatively, rather than perfusing BMA over the cells a coverslip can be placed in a 12-well plate along with 1 mL of the 10 μM BMA working solution for 12 minutes. If this option is employed it is important to minimise subsequent blue light illumination of cells during the identification of a transfected cells (*see* **Method 3.3**, **Step 11**). For example, very brief UV illumination is compatible with the excitation spectrum of GFP. In this case, **Steps 11** and **12** are no longer required.
15. To minimise photoisomerisation, reduce ambient lighting and cover the solution with blackout tape.

16. Minimise the time the Petri dish is spent outside of the incubator and the time the coverslip is transferred from the Petri dish to the perfusion bath.
17. A typical light-switching protocol might involve illumination of the cells for 2 s with 440 nm light followed immediately with 2 s illumination with 360 nm light before returning to dark. However, at the suggested optical powers (*see* **Material 2.2**), a 440 nm light pulse as short as 100 ms gives 80% of the maximum light activated current[5].

5. References

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Figures

Figure 1

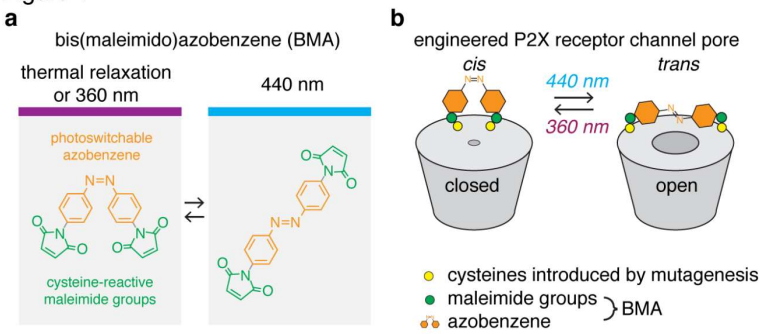

Figure 2

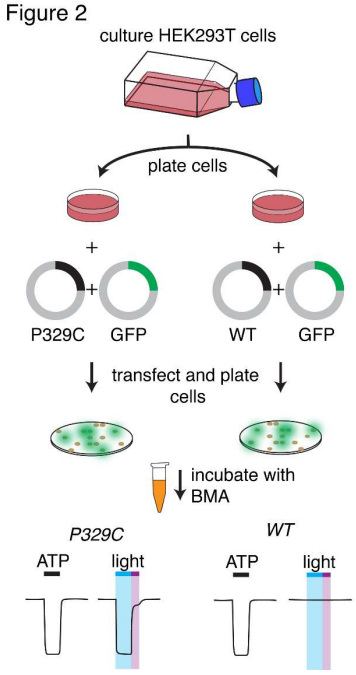

Figure captions

Fig 1. (**a**) Light activation of engineered P2X receptors, for high spatiotemporal control of their activity in cells. Photoswitching is carried out using bis(maleimido)azobenezene (BMA), a compound that contains two cysteine-reactive maleimide groups bridged by a photoisomerisable azobenzene. BMA is in a *cis* state in the dark or under near-UV light (360 nm) and rapidly and reversibly converts to an extended *trans* state under blue light (440 nm). (**b**) BMA covalently attaches to introduced cysteines at the pore of trimeric P2X receptor

channels. The bridged P2X receptor subunits are pushed apart as BMA enters a *trans* state (440 nm light), leading to channel opening. The channel can be closed by returning BMA to a *cis* state (360 nm light).

Fig. 2. Simplified workflow for the generation of engineered P2X receptors that can be rapidly and reversibly opened and closed with light. HEK293T cells are cultured, plated for transient transfection with a P2X receptor plasmid DNA (P329C and WT as an example) and fluorescent reporter plasmid DNA (GFP as an example), and plated for experiments. fluorescent reporter-positive cells are identified, incubated with BMA, and recorded using whole-cell patch clamp electrophysiology. Engineered P2X receptor channels respond to ATP and can be rapidly opened and closed with two different wavelengths of light.