Optical control of trimeric P2X receptors and acid-sensing ion channels

Liam E. Brownea, João P. M. Nunesb, Joan A. Simc, Vijay Chudasamab, Stephen Caddickb, and R. Alan Northac

*Faculty of Life Sciences and Faculty of Medical and Human Sciences, University of Manchester, Manchester M13 9PL, United Kingdom; and bDepartment of Chemistry, University College London, London WC1H 0AJ, United Kingdom

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P2X receptors are trimeric membrane proteins that function as ion channels gated by extracellular ATP. We have engineered a P2X2 receptor that opens within milliseconds by irradiation at 440 nm, and rapidly closes at 360 nm. This requires bridging receptor subunits via covalent attachment of 4,4′-bis(maleimido)azobenzene to a cysteine residue (P329C) introduced into each second transmembrane domain. The cis–trans isomerization of the azobenzene pushes apart the outer ends of the transmembrane helices and opens the channel in a light-dependent manner. Light-activated channels exhibited similar unitary currents, rectification, calcium permeability, and dye uptake as P2X2 receptors activated by ATP. P2X3 receptors with an equivalent mutation (P320C) were also light sensitive after chemical modification. They showed typical rapid desensitization, and they could coassemble with native P2X2 subunits in pheochromocytoma cells to form light-activated heteromeric P2X2/3 receptors. A similar approach was used to open and close human acid-sensing ion channels (ASICs), which are also trimers but are unrelated in sequence to P2X receptors. The experiments indicate that the opening of the permeation pathway requires similar and substantial movements of the transmembrane helices in both P2X receptors and ASICs, and the method will allow precise optical control of P2X receptors or ASICs in intact tissues.

High-resolution structures are available for P2X receptors (closed: ref. 12; open: ref. 13) and ASICs (closed: refs. 14 and 15; open: ref. 16). In both these trimeric channels the second of the two transmembrane domains (TM2) of each subunit lines the permeation pathway (12–14, 16, 17), and the outermost ends of the TM2s undergo substantial lateral displacement when the channel opens (Fig. L4) (13, 16). We therefore reasoned that the energy provided by an azobenzene molecule undergoing cis to trans isomerization should be sufficient to force apart the TM2 domains and open the permeation pathway.

We synthesized a bis(maleimido)azobenzene (Fig. 1B) of molecular dimensions appropriate to react with two cysteines in different subunits: this produced a P2X2 receptor that was opened and closed by different wavelengths of light. We extended the studies to a second member of the P2X family (P2X3) and thus produced an optically controlled heteromeric P2X2/3 receptor. Despite the lack of primary similarity, including the TM2 domain (Fig. 1C), there are similarities between the overall conformational changes that underlie channel opening in P2X receptors and in ASICs (18). We anticipate that the introduction of these engineered channels into cells and animals, when combined with the covalent modification with bis(maleimido)azobenzene, will facilitate the further investigation of the physiological roles of P2X receptors and ASICs.

**Results**

**Synthesis of 4,4′-Bis(maleimido)azobenzene.** 4,4′-Bis(maleimido)azobenzene (BMA) (Fig. 1B) was synthesized from 4,4′-azodicaniol via a two-step protocol. Initially, 4,4′-azodicaniol was reacted with maleic anhydride to form 4,4′-bis(maleic acid)azobenzene. This species was then converted to 4,4′-bis(maleimido)azobenzene in 43% overall yield by the application of acetic anhydride and sodium acetate. The protocol afforded 4,4′-bis(maleimido)azobenzene (BMA) (Fig. 1B) in a yield of 43%.

**Significance**

P2X receptors are trimeric proteins in which an extracellular transmitter (ATP) binds to open a transmembrane ion permeation pathway. We have designed a thiol-reactive, light-sensitive azobenzene molecule that can attach between cysteines introduced by mutagenesis into different channel subunits. Such chemically modified channels can be opened within milliseconds by irradiation at 440 nm, and closed by light at 360 nm, in the absence of any extracellular ligand. A similar result was obtained for heterotrimeric P2X2/3 receptors and for structurally unrelated (but trimeric) acid-sensing ion channels. The work extends our understanding of the molecular mechanism of gating and provides a tool to investigate the role of P2X receptors and acid-sensing ion channels in intact cells and tissues.


The authors declare no conflict of interest.

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1Present address: F. M. Kirby Neurobiology Center, Boston Children’s Hospital and Department of Neurobiology, Harvard Medical School, Boston MA 02115.

2To whom correspondence should be addressed. E-mail: r.a.north@manchester.ac.uk.

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azobenzene with only minor water contamination (2.7% by weight), but the product is highly hygroscopic.

Modification of P2X2[P329C] by BMA. Whole-cell recordings from cells expressing wild-type P2X2 showed typical inward currents (3.0 ± 0.4 nA, n = 14) in response to 2-s applications of ATP (100 μM). In P2X2 receptors with P329C mutation, after treatment with BMA for at least 10 min, blue light at 440 nm evoked an inward current (Fig. 1D); this was never observed in wild-type cells or with cysteine substitutions at nearby positions (L327C, I328C, I331C, or N333C). The sulfur atom was essential, because light did not elicit currents in P2X2[P329S] receptors. Changing the irradiation from 440 nm to near UV light at 360 nm rapidly terminated the inward current (Fig. S1).

Light (440 nm) evoked no inward currents in cells not treated with BMA. As the duration of exposure to BMA (10 μM) increased, the current elicited by the same 2-s light application also increased, and measurements showed that BMA associated with the channel according to a first-order reaction with k_on of approximately 250 M$^{-1}$s$^{-1}$ (Fig. S1A). BMA alone, without irradiation, had no detectable effect on the holding current during 10- to 12-min incubation (Fig. S1B). In subsequent experiments, we used 12-min pretreatment with BMA (10 μM).

A threefold increase in intensity (2.7–7.5 mW mm$^{-2}$) of blue light increased the rate at which the current developed [time constant changed from 121 ± 13 ms (n = 9) to 78 ± 8 ms (n = 9)] (Fig. S2A). Deactivation (at 360 nm) was also faster at 2.0 mW mm$^{-2}$ (τ = 559 ± 31 ms, n = 9) than at 0.7 mW mm$^{-2}$ (τ = 761 ± 40 s, n = 9) (Fig. S2A). These relatively small changes suggest that the intensity is near saturation under our experimental conditions with cells on the microscope stage.

Irradiation at 440 nm evoked significant current within a few milliseconds, and this became maximal with light application lasting approximately 300 ms (time constant 69 ± 11 ms, n = 4) (Fig. S2B). The rate of activation of P2X[P329C] receptors by light (time constant 78 ± 8 ms, n = 9) was similar to that using ATP (at 100 μM; time constant 98 ± 18 ms, n = 9) in the same cells, although under the present recording conditions we have not optimized the kinetics of ATP application. With maximal power (7.5 mW mm$^{-2}$ under the present conditions), maximal duration of light of 1 s, and maximal BMA exposure of 12 min, the currents evoked by light were typically approximately 35% (range 13–58%, n = 11) of those evoked by a maximal concentration of ATP (100 μM). ATP could elicit additional current after maximal light, but light had no effect when applied after maximal ATP. Currents at light-activated P2X2 receptors showed modest desensitization during prolonged light applications (Fig. S2C, Left) that was comparable to that reported for P2X2 receptors activated by ATP (19, 20). Deactivation was measured from the rate of current decay after a brief pulse of 440 nm light: the BMA trans state isomerized back to the cis state at 3.0% ± 0.3% s$^{-1}$ (n = 5) (Fig. S2C). The optimal wavelengths for activation and inactivation of the modified P2X2 receptor by light were 440 nm and 360 nm, respectively (Fig. S3), and highly reproducible responses were observed with repetitive illumination at these wavelengths.

ATP binding was not required for activation by light. Removal of the lysine residue at position 69 is well known to reduce dramatically the effect of ATP (20, 21), consistent with its direct interaction with the γ-phosphate oxygen atoms in the ATP-bound open channel structure (13). We introduced this mutation into the P293C channel and confirmed that ATP had no effect even at concentrations of 100 μM (Fig. 1D). However, the ability of light to activate this channel was unaltered.

Light and ATP Activate P2X2 Channels with the Same Properties. Single-channel currents activated by ATP were somewhat smaller in amplitude in P293C receptors than in wild-type channels (1.7 pA vs. 3.0 pA at −120 mV) (Fig. 2A). However, in the P293C channel treated with BMA, there was no difference between the unitary currents activated by light and those activated by ATP (Fig. 2B). P2X2 receptors show marked inward rectification, and this is dependent on expression level (22, 23). We found that for comparable levels of expression, the light-activated current and the ATP-activated current showed similar rectification (Fig. 2C).

Calcium permeability is an important feature of P2X receptors (19, 20, 23, 24). Fig. 2D shows that the relative permeability to calcium (P$_{Ca}$/P$_{Na}$) was similar for channels whether gated by light or by ATP. For P2X2[P329C] receptors gated by light, P$_{Ca}$/P$_{Na}$ was 2.6 ± 0.1 (n = 6), and for ATP-activated currents this was 2.5 ± 0.1 (n = 6). This calcium permeability is the same as observed for wild-type P2X2 receptors activated by ATP (P$_{Ca}$/P$_{Na}$ = 2.7 ± 0.1, n = 10) (24). Several P2X subtypes become permeable to large organic cations such as N-methyl-D-glucamine when activated by ATP, and this pore dilation can also be studied by measuring the uptake of fluorescent dyes (20, 25). We found that exposure of P2X2[P329C] receptors to blue (440 nm) light for 2 s elicited significant entry of YO-PRO-1, a dye that becomes fluorescent on binding to nucleic acid (Fig. S4). By this criterion, the permeation pathway of the light-activated P2X2 receptor also resembles that of the native channel opened by ATP.

The homotrimeric P2X receptor presents three identical ATP binding sites, but occupancy of only two of these is sufficient to drive channel opening (26). We constructed concatenated cDNA that encoded three joined subunits and introduced the P293C mutation into the first and/or second and/or third subunit. The concatenator that contained three P293C substitutions (denoted C-C-C) was activated by blue (440 nm) light (mean current was 456 ± 122 pA, n = 9, with light, and 2,147 ± 315 pA, n = 10, with ATP), whereas the wild-type concatenator that contained three prolines

**Fig. 1.** Light activation of P2X2 receptors. (A) Models of rat P2X2 receptor showing closed (Left) and open (Right) conformations. (Upper) Space-fill of trimeric holoprotein. (Lower) Ribbon representation of TM domains, from extracellular side. The positions of P329 are indicated in green. (B) Approximately 250 M cells expressing wild-type P2X2 showed typical inward currents. (C and P329C/K69A 1360 pA. (D) Changing the irradiation from 440 nm to near UV light at 360 nm rapidly terminated the inward current (Fig. S1).
Light-Gated Homomeric P2X3 and Heteromeric P2X2/3 Channels. Two features distinguish ATP-evoked currents at homomeric P2X2 and P2X3 receptors (20, 27). First, P2X3 receptors show rapid desensitization: currents evoked by 30 μM ATP decline almost to zero within less than 2 s during a continued application. Second, αβmeATP activates P2X3 receptors at concentrations that have no effect at P2X2 receptors (27). Coexpression of P2X2 and P2X3 subunits in HEK293 cells results in the formation of receptors that are activated by αβmeATP and that show little desensitization during a 2-s application, and this phenotype allows the P2X2/3 heteromeric current to be distinguished from the homomeric forms (27).

We found that blue light (440 nm) activated homomeric P2X3 [P320C] receptors pretreated with BMA, and these currents desensitized rapidly during sustained (2 s) light application in a manner similar to that observed for application of ATP (or αβmeATP) (Fig. 3A). The light-activated currents peaked at 129 ± 17 pA (n = 5) and declined to 9 ± 2 pA at 2 s (n = 5) (Fig. 3B). Currents activated by αβmeATP (30 μM) peaked at 1,160 ± 234 pA (n = 5) and declined at 2 s to 270 ± 79 pA (n = 4). Light had no effect on wild-type P2X3 receptors (Fig. 3A). Coexpression of P2X2 and P2X3 receptors resulted in a slowly desensitizing heteromeric current in response to αβmeATP (27, 28) (Fig. 3B). This phenotype was also mimicked by light application after BMA treatment. The peak amplitude of the light-activated currents (90 ± 27 pA, n = 8) was less than that observed by αβmeATP (571 ± 79 pA, n = 8), but the close similarity in the time course of the evoked currents strongly indicates that light is activating a heteromeric P2X2/3 receptor (Fig. 2B).

Expression in PC12 Cells. PC12 cells express P2X2 receptors (19, 29). Whole-cell recordings made 48 h after transfection with GFP showed robust depolarizations (30–50 mV) in response to ATP (30 μM) but no effect of αβmeATP (30 μM) or light (Fig. 4). Cells transfected with P2X3[P320C] receptor cDNA and pretreated with BMA responded to ATP, to αβmeATP (30 μM: 95% ± 17% of depolarization evoked by ATP, n = 7), and to light (40% ± 10% of that caused by ATP, n = 8) (Fig. 4A), whereas cells transfected with wild-type P2X3 receptors responded to both αβmeATP and ATP but not to light (Fig. 4B). The sustained depolarization elicited by αβmeATP is typical of that observed in heteromeric P2X2/3 receptors, either by heterologous expression or in native afferent neurons (20, 27). This indicates that transfection with P2X3 receptor cDNA leads to the production of P2X3 subunits that coassemble with endogenous P2X2 subunits and that light sensitivity can be conferred by this coassembly when these P2X3 subunits contain the P320C substitution.

(continued P-P-P) was not activated by light but responded normally to ATP (2,383 ± 185 pA, n = 10) (Fig. 5S). We found that the introduction of two cysteine residues also produced robust responses to light (384 ± 33 pA, n = 30) and ATP (2,689 ± 118 pA, n = 30). The response to light was not dependent on the positions of the cysteines in the concatemer (P-C-C, C-P-C, or C-C-P) (P = 0.13, one-way ANOVA). Two of the three forms with a single P329C substitution also gave small responses to light. This was most notable for the form with cysteine in the first of the three concatenated subunits and may reflect aberrant (“heads-up”) channel formation from three N-terminal subunits or from minimal breakdown of the concatemers as observed and discussed previously (26).
Light-Activated ASIC1a Channels. There is no amino acid sequence relatedness between ASIC and P2X receptors. Their ectodomains exhibit quite different folds, and the sequences of their TM2 domains also show very limited conservation (18). However, comparison of open and closed structures suggests that the outer part of TM2 undergoes substantial lateral movements during channel opening in each case (18) (Fig. 5). Part of TM2 undergoes substantial lateral movements during the iris-like rearrangement that underlies channel opening (13, 16, 18), with distances that are similar in magnitude to those predicted for the cis and trans photosomers of BMA. We predicted that BMA could bridge two cysteines so that in the cis state the β-carbons of the cysteines would be approximately 13 Å apart, and in the trans state they would be approximately 22 Å apart. In our homology models of the P2X2 receptor, the β-carbons of TM2 residue P329 are separated by 12 Å in the closed channel and 23 Å in the open channel. P329 is oriented toward other P329 residues in adjacent subunits and is solvent-exposed. We found that bridging two of the three cysteines was sufficient to open and close trimeric P2X2 receptors, consistent with the finding that only two intact agonist-binding sites (and by inference only two ATP molecules) are required for P2X receptor activation (26). It is noteworthy that at rest (the closed channel state) the BMA was in its higher-energy cis isomer, suggesting it is constrained in this form by the protein to which it is conjugated.

The kinetics of P2X receptor activation were similar whether activated by a maximal concentration of ATP or by blue light. This indicates that the cis-to-trans photoisomerization was not rate limiting and that the activation rate of P2X receptors is determined by the gating rearrangement rather than agonist-binding events. P2X2 receptors typically show a maximum probability of opening (p_o) of approximately 0.6 when activated by high concentrations of ATP (30). In the present experiments the P329C mutation had a unitary conductance that was approximately 60% that of the wild-type channel, but we do not know the effects of this mutation on maximal p_o. Our observation that light-induced currents were up to 50% of ATP-activated currents implies that BMA conjugation followed by light may drive P2X2 channels to open probabilities similar to those achieved by ATP.

The observation that light could activate the P2X2[K69A/P329] receptor indicates that endogenous ATP is not involved. However, it would be of interest to know whether the conformational change associated with light-induced channel opening nonetheless involves protein rearrangement at the ATP binding pocket. This might be studied by using a competitive antagonist, but the molecular mechanism of such compounds at P2X2 receptors is still poorly understood.

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**Fig. 4.** Light activates heteromeric P2X2/3 receptors in PC12 cells. (A) PC12 cells transfected with P2X3[P320C] depolarized by 440 nm irradiation and switched off by 360 nm light. These cells respond to αβmeATP with a sustained depolarization, indicative of heteromeric P2X2/3 receptors. (B) Cells transfected with wild-type P2X3 receptors do not respond to light but are depolarized by αβmeATP. (C) Native PC12 cells (mock transfected with GFP) show responses to ATP (P2X2 receptors) but no effect of light or αβmeATP. ATP and αβmeATP concentrations 30 μM.

**Fig. 5.** Optical control of trimeric ASIC1 channels. (A) Pore of trimeric chick ASIC1 channels, depicted in desensitized (Left, Protein Data Bank ID 3IJ4) and open (Right, Protein Data Bank ID 4FZ1) states, showing transmembrane domains viewed from the outside. The residue corresponding to G430 is shown in green. (B) CHO cells expressing wild-type human ASIC1a subunit cDNAs show large inward currents in pH 5.3 but no effect of light (pre-incubated with BMA). (C) G430C and I428C ASIC1b receptors showed light-activated currents. For G430C, light induced currents were 15% ± 9% (n = 5) the amplitude of currents evoked by pH 5.3, and for I428C this was 10% ± 1% (n = 4). Traces to peak current amplitude induced by pH 5.3 (amplitudes were as follows: wild type, 3,866 pA; G430C, 1,986 pA; I428C, 3,148 pA) or by light (amplitudes were as follows: G430C, 969 pA; I428C, 448 pA).

**Discussion.** To confer light sensitivity on P2X and ASIC proteins, we bridged two transmembrane helices with a photoisomerizable conjugate (BMA) that switches rapidly and reversibly to open or close the channel, depending on the wavelength of light that is applied. A similar strategy has been carried out for certain other proteins, such as restriction enzymes and DNA-binding proteins (11), but in those cases the azobenzene bridged residues are used to render the molecule nonfunctional by inducing disorder. In contrast, here we apply appropriate mechanical forces to the protein to gain optical control of two discrete functional states.
The approach was extended to P2X3 receptors with the equivalent mutation, P2X2C. The distinctive rapid desensitization was maintained in this construct irrespective of whether agonist or blue light was used for activation. Previous work has shown key roles for transmembrane and juxtamembrane cysteolic domains (31). Heteromeric P2X2/3 receptors were formed where both P2X3 and P2X2 subunits are both expressed together in primary adherent cells. This heteromeric channel, when heterologously expressed, is formed by two P2X3 subunits and one P2X2 subunit (28). In accordance with this stoichiometry, the P2X3[P2X2C] subunit assembled to form light-gated heteromeric P2X2/3 receptors when coexpressed with wild-type P2X2 subunits. This displayed the slower desensitization that is characteristic for the heteromeric P2X2/3 receptor. The presence of two P2X3 subunits in the trimer would be consistent with our interpretation that the cis-trans conformational change between only two TM2s is sufficient for channel opening.

The structure of the ASIC1 indicates that G430 is also oriented toward the other G430 residues in adjacent subunits (14, 16). The observation of light-evoked currents indicates that the opening mechanism for ASICs and P2X receptors at the level of the transmembrane domains is functionally similar (18). We also observed light-evoked currents from mutation I428C, which is predicted to face away from the other I428C residues in adjacent subunits. This may be explained by differences between chick and human ASIC1 structures, but we cannot exclude the possibility that BMA may be bridging to an endogenous cysteine (e.g., C49, C59, C61, and C440) that are sufficiently close enough to I428C.

The expression of a nonnative light-gated protein (such as the channelrhodopsins) can address important questions about the cells and systems into which it is introduced. However, there are also advantages to control directly the activity of the native protein under investigation. First, the conjugated BMA is relatively stable in the trans isomer, so that the biological sample is illuminated only transiently. Second, the photosomerizable conjugate can be tuned depending on the application; for example, by switching at longer wavelengths of light (32). Third, it is possible to exploit properties of the native channel, such as calcium permeability. Ion channels used currently as optogenetic tools show relatively lower calcium permeability [such as the calcium-permeable channelrhodopsin CatCh (33); the 8-thiocyano-ATP (35, 36) or the photoaffinity agonist BzATP (37, 38)] to afford (E)-4,4′-bis(maleimido)azobenzene as an orange solid (86 mg, 22 mmol, 43%) (42) with some water (12 mg or 13% by weight). The water content was reduced to 2.3 mg (2.7% by weight) by drying the isolated solid under high vacuum at 150 °C for 72 h.

**Materials and Methods**

**Synthesis of (E)-4,4′-Bis(maleimido)azobenzene.** To a solution of 4,4′-azodianiline (106 mg, 0.50 mmol) in N,N-dimethylformamide (1 mL) was added maleic anhydride (103 mg, 1.05 mmol). The mixture was stirred for 1 h at room temperature, then added warm dichloromethane (20 mL) and dried under vacuum. The orange solid was suspended in dichloromethane (20 mL) and filtered through a Büchner funnel. The orange filter cake was washed with dichloromethane (20 mL) and dried under vacuum. The orange solid was suspended in acetic anhydride (2 mL) and the mixture was diluted with dichloromethane (2 × 30 mL). The combined organic layers were washed with brine (10 mL), dried (MgSO4), filtered, and concentrated under reduced pressure. The resultant crude residue was purified by flash column chromatography on silica with dichloromethane/EtOAc (20:1) to afford (E)-4,4′-bis(maleimido)azobenzene (86 mg, 22 mmol, 43%) (42) with some water (12 mg or 13% by weight).

**BMA Conjugation, Electrophysiology, and Light-Switching.** BMA was dissolved in DMSO to give 1 mM stock solution: this was stored in the dark at room temperature for use within 48 h. Stock was diluted in the standard extracellular solution at 10 μM. Recordings were at room temperature. For whole-cell voltage clamp experiments, patch pipettes were pulled from thin-wall borosilicate glass capillaries (Harvard Apparatus); they had resistances of 2–4 MΩ when filled with (in mM): 147 NaCl, 10 EGTA, and 10 Heps. Current-voltage relationships were obtained from voltage ramps (−60 mV to +60 mV in 500 ms). Permeability ratios were determined by measuring the reversal potentials first in an extracellular solution containing (in mM): 145 NaCl, 13 glucose, and 10 Heps (adjusted to pH 7.3 using NaOH), and then in a solution containing (in mM) 110 CaCl2, 13 glucose, and 10 Heps (adjusted to pH 7.3 using Ca(OH)2). Reversal potentials were measured during voltage ramps (−20 to +20 mV in 500 ms). Currents elicited by protons were measured using a calibrated pH meter (Sensel). Permeability ratios were determined by measuring the reversal potentials first in an extracellular solution containing (in mM): 145 NaCl, 2 KCl, 2 MgCl2, 1 MgCl2, 10 Mes, and 13 glucose (pH 5.3 with NaOH). The holding potential was −60 mV for whole-cell recordings. For outside-out recordings, pipettes were pulled from thick-walled borosilicate glass (World Precision Instruments) and had resistance of 10–20 MΩ when filled with solution containing (in mM): 145 NaCl, 10 Heps, and 10 EGTA. The holding potential was −120 mV for single-channel recordings. Recordings of membrane potential PC-12 cells used pipettes (resistances 5–8 MΩ) filled with solution containing (in mM): 125 K gluconate, 15 KCl, 5 NaCl, 2 MgCl2, 5 EGTA, and 10 Heps. The resting voltage was held at −60 mV. The standard extracellular solution contained (in mM): 147 NaCl, 2 KCl, 2 CaCl2, 1 MgCl2, 10 Heps, and 13 glucose (pH 7.3, 285–315 mOsM). Chemicals were purchased from Sigma-Aldrich.
Illumination for light switching used a rapid wavelength-switching (~3 ms) xenon lamp monochromator (Polychrome IV, TILL Photonics) connected through the epifluorescence port of the microscope (Nikon TE200). Light was directed to a Fluor 40x/0.75 N.A. objective (Nikon) using a short-pass 532-nm edge filter in the exciter, and a 520-nm edge dichroic (Semrock), which also enabled the light to be switched off rapidly by switching to longer wavelengths. The monochromator was controlled using the EPC10 amplifier. Light illumination intensity was determined using an optical power meter (Newport).

Currents were recorded with an EPC10 amplifier using Patchmaster software (HEKA). The data were low-pass filtered at 3 kHz and sampled at 1 or 5 kHz unless stated otherwise. Agonists and other compounds were applied using an RSC-160 Rapid perfusion system (Bio-Logic) and Perfusion Pencil (Di- timer). The RSC-160 was triggered by the EPC10 amplifier. Agonist concentrations were 100 μM ATP and 30 μM α-meATP, unless stated otherwise.

Confocal Laser-Scanning Fluorescence Microscopy. Fluorescent images were acquired using a Nikon laser-scanning C1 confocal microscope, using an Ar-ion 488-nm laser line with 515/30 nm emission filter. Photobleaching was <5% over the duration of experiments. YO-PRO-1 was used at 1 μM. Excitation for image acquisition was not carried out until after light switching had been carried out, so as not to isomerize the BMA. Light switching was carried out as described above. The C1 confocal, RSC-160, and monochromator were triggered using outputs of the EPC10 amplifier.

Molecular Models. Closed and open molecular models of the rat P2X2 receptor were generated with MODELER 9.10 (44) using the zebrafish P2X4.1 crystal structures (Protein Data Bank accession nos. 4DW0 and 4DW1) as templates. MolProbity (45) was used to assess lowest-energy models, which were subsequently energy minimized using AMBER. The models showed 98.9% (closed model) and 97.4% (open model) of residues in the allowed regions of the Ramachandran plot. The ASIC structures shown are the chick ASIC1 closed (314) and open (4F2Z) crystal structures. Protein images were produced using UCSF Chimera 1.62 (46).

Data Analysis. Electrophysiological data were analyzed using FitMaster (HEKA), asxGraph X (Molecular Devices), Kaleidagraph 4 (Synergis), and Prism 4 (GraphPad) software. Dose–responses were fit with the Hill equation (I/Imax = [A]/(IC50 + [A]^n)), where [A] is the agonist concentration causing current I, IC50 is the agonist concentration causing half the maximal current Imax, and n is the Hill coefficient. Relative calcium permeabilities were calculated from the reversal potential of the ATP-evoked or light-evoked current (Erev, mV) from PNa/PNa = [Na]_i/[1 + exp(x)]/exp(x)/4Ca^{2+} where [Na]_i = 151.7 and [Ca]_i = 112 mM, and x = E_{rev}/RT (F is the Faraday constant, R is the gas constant, and T is the absolute temperature). Amplitudes of single-channel currents were measured by all-points amplitude histograms fit to two Gaussian distributions. Fluorescent images were analyzed using EZ-C1 Viewer (Nikon) and were low-pass filtered with a 3 × 3 pixel median filter using ImageJ. Pooled data are given as the mean ± SEM. Tests for statistical significance were performed using nonparametric ANOVA.

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