Lysosomal alkalinization, lipid oxidation, and reduced phagosome clearance triggered by activation of the P2X7 receptor

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ABSTRACT Lysosomal enzymes function optimally at low pH; as accumulation of waste material contributes to cell aging and disease, dysregulation of lysosomal pH may represent an early step in several pathologies. Here, we demonstrate that stimulation of the P2X7 receptor (P2X7R) for ATP alkalinizes lysosomes in cultured human retinal pigmented epithelial (RPE) cells and impairs lysosomal function. P2X7R stimulation did not kill RPE cells but alkalinized lysosomes by 0.3 U. Receptor stimulation also elevated cytoplasmic Ca2+; Ca2+ influx was necessary but not sufficient for lysosomal alkalinization. P2X7R stimulation decreased access to the active site of cathepsin D. Interestingly, lysosomal alkalinization was accompanied by a rise in lipid oxidation that was prevented by P2X7R antagonism. Likewise, the autofluorescence of phagocytosed photoreceptor outer segments increased by lysosomal alkalinization was restored 73% by a P2X7R antagonist. Together, this suggests that endogenous autostimulation of the P2X7R may oxidize lipids and impede clearance. The P2X7R was expressed on apical and basolateral membranes of mouse RPE; mRNA expression of P2X7R and extracellular ATP marker NTPDase1 was raised in RPE tissue from the ABCA4 mouse model of Stargardt’s retinal degeneration. In summary, P2X7R stimulation raises lysosomal pH and impedes lysosomal function, suggesting a possible role for overstimulation in diseases of accumulation.—Guha, S., Baltazar G. C., Coffey, E. E., Tu, L.-A., Lim, J. C., Beckel, J. M., Patel, S., Eysteinsson, T., Lu, W., O’Brien-Jenkins, A., Laties, A. M., Mitchell, C. H. Lysosomal alkalinization, lipid oxidation, and reduced phagosome clearance triggered by activation of the P2X7 receptor. FASEB J. 27, 000–000 (2013). www.fasebj.org

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LYSOSOMES ARE RESPONSIBLE FOR degrading much of the lipid and protein waste material within a cell, and the pH of the lysosomal lumen can influence the rate of processing. As most lysosomal enzymes act optimally over a narrow range of acidic pH values, lysosomal alkalinization can compromise enzyme efficiency, leading to accumulation of partially degraded waste material and impairment of the autophagic process (1–3). While efficient degradation of autophagosomes is necessary for all cells, some cells carry the additional degradative burden that comes from processing phagocytosed material. In this regard, the retinal pigmented epithelium (RPE) serves as a model; its role in the daily phagocytosis and degradation of lipid-rich photoreceptor outer segments (POSs) makes it particularly sensitive to elevations in lysosomal pH (4). As in other postmitotic cells, residual material resulting from impaired clearance slowly accumulates in and around RPE cells throughout life. However, treatment with the lysosomotropic agent chloroquine (CHQ) rapidly elevates lysosomal pH and accelerates the accumulation of waste material. For example, rodents treated with CHQ show a buildup of partially degraded material both inside RPE cells and outside in Bruch’s membrane (5, 6). Given the contribution that this accumulated intracellular and extracellular waste material may have to disease, understanding how lysosomal pH is regulated may be of considerable importance.
Lysosomal pH levels are set by the summed contribution of vHATpase pumps, anion and cation channels, proton channels and exchangers, NADPH oxidases, and numerous other transporters (7). As maintaining a low pH is energetically demanding, this pH level is likely adjusted to best match the degradative burden. Regulation of this pH is predicted to be particularly complex in cells such as the RPE, where lysosomal pH influences both autophagy and heterophagy. For example, the pH of RPE cells, and the corresponding rates of lysosomal degradation, can be modified by a variety of receptors, including β-adrenergic, adenosine A2A, and dopamine D5 receptors (8, 9). While control of lysosomal pH by a variety of plasma membrane receptors provides an opportunity for enhanced responsiveness, overstimulation of receptors may lead to dysregulation of lysosomal pH levels. In this regard, the actions of the P2X7 receptor (P2X7R) may be significant. As an ionotropic cation channel, activation can lead to complex changes in cell function, resulting either in pathological or physiological functions (10, 11). While excessive stimulation of the P2X7R can be lethal to many cells, including the RPE (12), its continued presence on many postmitotic cells indicates its influence is largely nonlethal (13, 14). Of particular interest in the present context is its ability to impair autophagy in microglial cells by elevating lysosomal pH (15). Given the importance of both purinergic signaling and lysosomal pH to RPE function (8, 16), this study asked whether P2X7R stimulation elevated lysosomal pH levels in RPE cells, probed some of the consequences of this lysosomal alkalization, and examined receptor overexpression in RPE cells from a murine model of retinal degeneration.

MATERIALS AND METHODS

ARPE-19 cells

ARPE-19 cells (American Tissue Type Collection, Manassas, VA, USA) were grown to confluence in 25 cm² primary culture flasks in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium with 3 mM l-glutamine, 100 µg/ml streptomycin, and 2.5 mg/ml fungizone and/or 50 µg/ml gentamicin, and 10% FBS (all Invitrogen Corp., Carlsbad, CA, USA). Cells were incubated at 37°C in 5% CO2 and subcultured weekly with 0.05% trypsin adjusted to limit P2X7R block except where indicated.

Photoreceptor outer segment (POS) membrane preparation

Fresh bovine retinas were isolated under sterile conditions and stored at −80°C. POSs were isolated on the basis of a method of Boesze-Battaglia and Yeagle (19). Thawed retinas were agitated in 30% (w/w) buffered sucrose solution containing 5 mM HEPES, pH 7.4; 65 mM NaCl; and 2 mM MgCl₂, followed by centrifugation in a Sorvall SS-34 rotor at 7000 rpm, 4°C; Thermo Scientific). The supernatant was diluted in 2 vol of 10 mM HEPES at pH 7.4 and further centrifuged (Sorval SS-34 rotor, 20 min, 17500 rpm, 4°C). The resulting pellet was homogenized and layered on top of a discontinuous sucrose density gradient of 36, 32, and 26%, and POS membranes were harvested from the 26/32% sucrose solution interface. POSs prepared this way were washed in 3 vol of 0.02 M Tris buffer, pH 7.4 (Sorval SS-34 rotor, 10 min, 13,000 rpm, 4°C). The pellet was resuspended in 2.5% (w/w) buffered sucrose solution (Na₂HPO₄, 10 mM; NaH₂PO₄, 10 mM; and NaCl, 0.1 mM) and stored at −80°C until use.

Quantification of cellular autofluorescence

ARPE-19 cells were grown to confluence in clear 6-well plates (BD Biosciences, San Jose, CA, USA). On experimental day 0, the culture medium was removed from 3 wells and replaced either with medium or with 2 ml POS in culture medium (10⁶/ml) for 2 h (pulse); the cells were washed thoroughly with isotonic solution to remove noninternalized POSs, followed by 2 h chase in DMEM/F12. POSs are localized within the lysosomes after the 2 h chase period, so that drug treatments primarily affect lysosomal degradation and not binding or internalization (8). Subsequently, the medium was removed, and the cells were incubated for 20 h in test solutions of 10 µM CHQ + POS, or 10 µM CHQ + POS + Coomassie brilliant blue G (BBG). This pulse/chase series was repeated every 2–3 d for 1 wk. For flow cytometric quantification of lipofuscin-like autofluorescence, cells were washed, detached with 0.25% trypsin in EDTA, and analyzed on a flow cytometer (FACS Calibur; BD Biosciences) using the FITC channel (λₑx 488 nm; λₑm 530 nm). A gate was set to exclude cell debris and cell clusters.

Boron dipyrromethene (Bodipy) FL pepstatin A and Bodipy C-11 assays

To quantify the availability of the cathepsin D active site, ARPE-19 cells were grown on black-walled, clear-bottomed, 96-well plates until confluent, then incubated for 1 h in Mg²⁺-free isotonic solution with or without 100 µM 2’(3’)-O- (4-benzoylbenzoyl)adenosine-5’-triphosphate tri( triethylammonium) (BrATP). After incubation, cells were washed, followed by a 30-min incubation in 10 µM Bodipy FL pepsta-
tin A (Invitrogen) probe at 37°C in the dark. After a final wash, fluorescence was compared with the Fluoroskan plate reader (Thermo Scientific), at $\lambda_{ex}$ 485 nm/$\lambda_{em}$ 527 nm. Autofluorescence was subtracted from the fluorescence levels.

Analogous steps were taken to detect lipid oxidation with fluorescent dye Bodipy-C11. ARPE-19 cells were fed 1 x 10⁶ POS/ml for 2 h (pulse), then kept in medium alone for 2 h (chase), followed by 20 h incubation in medium containing 10 μM CHQ, CHQ + 10 μM SKF12197, or CHQ + 1 μM BBG. After washing, cells were loaded with 10 μM Bodipy-C11 dye (Invitrogen) for 30 min at 37°C and rinsed; the plate was read at $\lambda_{ex}$ 485 nm/$\lambda_{em}$ 527 nm.

### Immunoblotting

ARPE-19 cells were lysed in cold RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and protease inhibitor cocktail) and centrifuged at 13,000 g for 10 min at 4°C. Protein concentrations were determined using the BCA kit (Pierce, Rockford, IL, USA). Protein lysates were loaded in each lane in sample buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.05 M Tris-HCl, pH 6.8), separated on SDS–PAGE (Bio-Rad, Hercules, CA, USA), and transferred to polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA, USA). For identification of the LC3B protein, 20 µg protein was run on a 4–15% gel, blots were blocked with 5% nonfat milk in phosphate-buffered saline (PBS) and incubated overnight with rabbit anti-LC3B (1:1000; Cell Signaling Technology, Danvers, MA, USA). Mouse anti-GAPDH was used as a control for normalizing (1:1000; Cell Signaling Technology). Visualization of the primary antibody was performed by incubating membranes with the corresponding peroxidase-conjugated secondary antibody (1:3000; GE Healthcare, Waukesha, WI, USA) for 1 h at 25°C. p62 was detected with mouse antibody at 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and processed in an analogous way. Blots were developed by enhanced chemiluminescence and captured on an ImageQuant LAS 4000 image reader (both GE Healthcare).

### Lactose dehydrogenase (LDH) assay

LDH released into the extracellular solution was measured as an indicator of cell membrane integrity using a coupled reaction where tetrazolium salt was reduced to the colored formazan by enzyme activity (cytotoxicity detection kit LDH; Roche Applied Science, Mannheim, Germany). Briefly, cells were incubated in 100 µl of either control or BzATP-containing solution in a 96-well plate. Reaction mixture was prepared according to the manufacturer’s instructions. BzATP was used at 50 and 100 µM for 60 min. After this incubation time, the dye absorbance was measured at 490 nm using a microplate reader. Absorbance values were converted to LDH concentration by preparing LDH standards (1-LDH; from rabbit muscle; Roche) for each experiment. The solution itself did not affect the LDH assay.

### Measurements of intracellular calcium

Initial evidence for Ca²⁺ elevation in response to P2X7R stimulation was performed using a microscope-based imaging platform. ARPE-19 cells were grown to confluence on 12-mm glass coverslips loaded with 5 µM Fura-2 AM and 2% pluronic (Invitrogen), as described previously (20). After washing, cells were mounted in a perfusion chamber and visualized using a ×40 objective on a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Cells were alternatively excited at 340 and 380 nm, and the fluorescence emitted >520 nm was imaged with a charge-coupled device camera and analyzed (all Photon Technologies International, Lawrenceville, NJ, USA). Calibration was performed separately for each ARPE-19 cell experiment using standard protocols. Cells were perfused with isotonic solution containing (in mM) 105 NaCl, 5 KCl, 6 HEPES acid, 4 Na HEPES, 5 NaHCO₃, 60 mannitol, 5 glucose, and 1.3 CaCl₂. Solutions were Mg²⁺-free except where indicated. To determine the effectiveness of antagonists, cells were grown to confluence in 96-well plates and loaded with Fura-2 AM as above. Antagonists were added at varying concentrations and fluorescence measurements were determined in a plate reader (Thermo Scientific) by alternatively exciting at 340 and 380 nm; after baseline levels were obtained, BzATP was injected to give 50 µM. The response is determined as the difference in fluorescence in the presence and absence of BzATP. Ca²⁺ levels in fresh mouse RPE cells were determined microscopically as for ARPE-19 cells, with cells incubated with 5 µM Fura-2 AM for 20 min at 25°C.

### Isolation and culture of mouse RPE cells

All mice were treated in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. C57BL6 mice were obtained from Harlan Laboratories (Indianapolis, IN, USA), reared at 5–15 lux, and euthanized with a CO₂ overdose. RPE cells were isolated as described previously (8). In brief, intact eyes were incubated in 2% dispase and 0.4 mg/ml collagenase IV for 45 min after enucleation, rinsed, and incubated for 20 min in growth medium (containing DMEM + nonessential amino acids, 3 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 mg/ml Fungizone and/or 50 µg/ml gentamicin, plus 10% FBS; all Invitrogen). In some experiments, the anterior segments and retinas were removed, and the eyecup was rinsed with Versene (Dow Chemical, Midland, MI, USA) and incubated in 0.25% trypsin for 45 min. Cells from 4 eyes were pooled, and sheets of RPE cells were separated from the choroid and triturated to single cells. Cells were used for PCR/quantitative PCR at this point. For Ca²⁺ measurements, RPE cells were seeded onto coverslips coated with 0.05% poly-l-lysine and 10 µg/ml laminin and allowed to settle for several days. ABCA4⁻/⁻ mice were a kind gift from Dr. Gabriel Travis (Jules Stein Eye Institute, University of California, Los Angeles, CA, USA). ABCA4⁻/⁻ mice were reared in parallel with C57BL6 mice.

### PCR

Total RNA was isolated from ARPE-19 cells and fresh mouse RPE cells using Trizol and the RNEasy mini kit (Qiagen, Valencia, CA, USA). RNA yield was determined by absorbance at 260 nm, and integrity was confirmed by gel electrophoresis. Two micrograms of total RNA was converted into cDNA using a high-capacity RNA-to-cDNA kit (no. 4387406; Applied Biosystems, Foster City, CA, USA). P2X7R primers obtained from the Primer Bank were as follows: for human material: sense, 5'-GTCCCTATCTCTCCACCTG-3'; antisense, 5'-TCCCGTTCTACGACTCAGGCT-3', 119-bp product; for mouse material: sense, 5'-TGTCCTATCTCCTCAGACTGAC-3'; antisense, 5'-ATTTCAGAAGCTGATCCACCTGG-3', 119-bp product. PCR was performed with 2 µl cDNA product, 50 nM MgCl₂, 10 µM primers, and 0.5 µl first recombinant DNA polymerase (platinum TaqDNA polymerase; Applied Biosystems) at 95°C for 15 min, followed by 35 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Firststrand DNA synthesis was emitted from the negative control. The band was visualized on a 1.5% agarose gel and photographed.
Mice were perfused with 4% paraformaldehyde in 1× PBS; postfixed for 24 h at 4°C, and stored in cryoprotectant (30% sucrose/1× PBS) at 4°C until embedded in optimal cutting temperature medium. Frozen sections were quenched in 0.1% Triton X-100/0.5% SDS, incubated in anti-rabbit P2X7 antibody (ab77413; Abcam, Cambridge, MA, USA) at 1:200 dilution in Superblock (Pierce), and then incubated in secondary goat-anti-rabbit Alexa Fluor 488 (A11008; Invitrogen) at 1:200 in Superblock. Sections were visualized on a Nikon Diaphot microscope at 488 nm using epifluorescence at the University of Pennsylvania Live Cell Imaging Center.

Quantitative PCR

Total RNA was isolated from fresh mouse RPE/choroid using the RNeasy Micro Kit (74040; Qiagen, Valencia, CA, USA). RNA from RPE/choroid of ABCA4+/− and C57BL6 mice was obtained as above. Quantitative PCR assays were performed using the real-time PCR system (7300; Applied Biosystems). Primers were as follows: mouse ecto-nucleoside triphosphate diphosphohydrolase (NTPDase1; 154 bp): forward, 5′-CATGATGATGGACACTGGACCAACTCGG-3′; and reverse, 5′-TGTCCCTATCTCTCCACGACTCAC-3′; and mouse P2X7R (119 bp): forward, 5′-TTGCTCCTATCTCTCCACGACTGA-3′; and reverse, 5′-ATTCCACACTGGCCACACTCGG-3′. All samples were normalized to mouse GAPDH expression and analyzed using the ∆∆Ct method, as done previously (17).

Data analysis

All data are expressed as means ± se. Significance was determined using a 1-way ANOVA followed by an appropriate post hoc test using Sigma Stat software (Systat Software, San Jose, CA, USA) unless otherwise noted. On occasions when data were not normally distributed, ANOVAs were performed on ranks.

RESULTS

Stimulation of the P2X7R raises lysosomal pH

As pH controls the efficiency of many degradative and processing enzymes of the lysosome, initial studies examined the direct effect of P2X7R stimulation on lysosomal pH. Receptor agonist BzATP led to a moderate but consistent elevation of lysosomal pH (Fig. 1A). While absolute levels varied across trials, the relative increases were consistent across trials. BzATP typically increased lysosomal pH by 0.3 U, although larger rises were observed. The alkalizing effect of BzATP on lysosomal pH was larger than that of the tertiary amine CHQ at 10 μM, a level previously found to impede lysosomal function in these cells (18). Application of CHQ and BzATP simultaneously did not lead to much further rise in lysosomal pH. The role of the P2X7R was confirmed with antagonists. The elevation in lysosomal pH induced by BzATP was inhibited by both A438079 and BBG (Fig. 1B), supporting a role for the P2X7R in lysosomal alkalization.

The second messengers contributing to the lysosomal alkalization following P2X7R activation were examined. The agonist BzATP was unable to elevate lysosomal pH in the absence of external calcium (Fig. 2A). However, raising intracellular calcium alone with the ionophore ionomycin was unable to elevate lysosomal pH (Fig. 2B), suggesting that calcium was necessary but not sufficient to alkalize the lysosomes. Our previous work indicated that cAMP lowers lysosomal pH in cells treated with tertiary amines, such as CHQ or tamoxifen, with the mechanism partially due to increased anion permeability of the lysosomal membrane (8, 9, 17). cAMP was also effective at lowering lysosomal pH in cells treated with BzATP (Fig. 2C). This suggests that the pathways used by cAMP to restore an acidic lysosomal pH are effective and functioning in lysosomes alkalinized following P2X7R stimulation.

P2X7R reduces lysosomal function, increases autofluorescence, and raises lipid oxidation

Most lysosomal enzymes are pH sensitive, with activity enhanced in acid environments. Although the absolute change in pH triggered by BzATP is only a few tenths of a unit, this rise is at the most sensitive portion of the pH/activity curve for many enzymes, suggesting activation of the P2X7R may slow enzyme activity and degradation. Given that a major role of RPE cells is to detoxify phagocytosed POSs, the effect of P2X7R blockade on outer segment clearance was examined. Previous work has indicated that autofluorescence at 488 nm correlates with levels of opsin on immunoblots, consistent with the autofluorescence being at least partially due to undegraded POSs (18). In the present study, ARPE-19 cells were fed with POSs for 2 h, followed by a 2-h break for internalization before drugs were applied.
At this point, the majority of outer segments were in the lysosomes, ensuring that drug actions were focused on the lysosomal actions and not the earlier steps like binding or phagocytosis (9). Treating control cells with CHQ led to a small rise in autofluorescence, likely reflecting accumulation of autophagic material. The addition of POSs and CHQ increased the autofluorescence 11-fold over baseline levels (Fig. 3A). However, treatment with the P2X7R antagonist BBG significantly reduced autofluorescence levels (Fig. 3B). Of note, because this reduction was produced by a P2X7R antagonist alone, it implies that the endogenous agonist ATP is normally released and autostimulates the P2X7R to increase autofluorescence.

Further evidence that lysosomal alkalinization by the P2X7R likely impaired lysosomal function was provided with the Bodipy-pepstatin A assay. Cathepsin D is the major lysosomal protease responsible for degrading engulfed POS proteins in RPE cells, and binding of the fluorescently tagged inhibitor pepstatin A to the cathepsin D catalytic site is pH dependent (21). Bodipy-pepstatin A fluorescence was decreased after treating cells with P2X7R agonist BzATP (Fig. 4A), consistent with the elevation in lysosomal pH produced by the receptor and with a decreased ability of cathepsin D to degrade proteins in the compromised lysosome.

POSs also contain a considerable amount of lipid in their membranes, and oxidation of these lipids has been associated with impaired function of RPE cells (22). Thus, we asked whether lysosomal pH could itself increase lipid oxidation using the Bodipy-C11 assay (23). Although the addition of outer segments did not increase the oxidation of the lipid probe, lysosomal alkalinization substantially increased lipid oxidation (Fig. 4B). Critically, treatment with P2X7R antagonist BBG reduced lipid oxidation. To support the role of lysosomal pH in lipid oxidation, the D1/D5 dopamine receptor agonist SKF81297 was tested in parallel, as it...
has been repeatedly shown to acidify compromised lysosomes (9). SKF81297 also reduced the level of lipid oxidation in these cells, strengthening the correlation between lysosomal pH and the levels of oxidized lipids. Once again, the ability of the P2X7R antagonist to reverse the effects suggests that a baseline level of P2X7R autostimulation is occurring and is revealed by the antagonist.

Changes to lysosomal pH can impair the fusion of autophagosomes with lysosomes (2, 24). Although autophagy is a stepwise process involving multiple contributors, concurrent changes to the levels of degradation product p62 and the ratio of LC3BII/LC3BI can signal altered autophagic flux (25). Stimulation of ARPE-19 cells for 15 min with BzATP increased the ratio of LC3BII/LC3BI (Fig. 5A, B), consistent with a block in autophagy flux. Likewise, stimulation of the P2X7R also decreased the level of p62, supporting impairment of autophagic flux (Fig. 5C, D).

Stimulation of the P2X7R is not lethal but leads to Ca$^{2+}$ influx

Although the P2X7R is commonly associated with cell death, its distribution throughout the nervous system and other postmitotic cells suggests its primary function is physiological (13, 26). As excessive overstimulation of the P2X7R can kill a variety of cells, including RPE cells (12), experiments were performed to examine the effect of lower levels of receptor stimulation on cell survival. Exposure of cells to the P2X7R agonist BzATP at 50 or 100 μM for 60 min did not alter levels of LDH in the extracellular bath (Fig. 6A). This demonstrates that the responses examined in this study are not associated with the cell death but instead may represent physiological responses in RPE cells.

Additional experiments were conducted to determine whether receptor stimulation could elevate cytoplasmic Ca$^{2+}$, as Fig. 2 demonstrated that extracellular Ca$^{2+}$ was necessary for the alkalization of lysosomal pH by the P2X7R. The application of agonist BzATP rapidly increased the concentration of free intracellular Ca$^{2+}$, with levels returning to near baseline on removal of BzATP (Fig. 6B). Repetitive, brief applications of BzATP led to repetitive spiking elevations in Ca$^{2+}$ (in 5 trials, the magnitude of the Ca$^{2+}$ rise increased with subsequent applications, as seen in Fig. 6B, while in 4 trials, the magnitude remained constant). Removal of extracellular Ca$^{2+}$ reduced the responses to BzATP (Fig. 6C, D), as did the addition of 0.5 mM extracellular Mg$^{2+}$ (Fig. 6E, F). P2X7R blockers BBG (Fig. 6G, H) and A438079 (Fig. 6I, J) reduced the response to BzATP. Together with the dependence on extracellular Ca$^{2+}$ and inhibition by Mg$^{2+}$, the block by A438079 and BBG is consistent with the identification of the P2X7R on RPE cells and confirms the activation of nonlethal signaling pathways by the receptor.

P2X7R expression in RPE cells and mice

To further confirm receptor involvement, the polymerase chain reaction was used to identify transcripts for the P2X7R in both the fresh mouse RPE cells (Fig. 7A) and cultured human ARPE-19 cells (Fig. 7B). The polarity of receptor expression in vivo was determined in sections from mouse RPE using immunohistochemistry. Staining for the receptor was seen on both the apical and the basolateral membranes of the mouse RPE cells (Fig. 7C). While overall staining was evenly distributed across both membranes in some sections, others showed a preponderance of basolateral staining. To confirm that mouse P2X7Rs were functional, BzATP was added to mouse RPE cells loaded with Ca$^{2+}$-dye Fura-2. The agonist triggered a rapid and reversible elevation of Ca$^{2+}$ in mouse cells (Fig. 7D). While the absolute levels of Ca$^{2+}$ were not calibrated, the response was qualitatively similar to that observed in the cultured human cells, with a large, reversible, and repeatable rise in Ca$^{2+}$ (Fig. 7E). This implies that the receptor was functioning on both fresh mouse and cultured human RPE cells.

Increase in P2X7R message in ABCA4$^{-/-}$ mice

The relative expression of P2X7R in the ABCA4$^{-/-}$ mouse model of Stargardt’s retinal degeneration was determined with qPCR. Expression of the P2X7R in fresh RPE/choroid tissue from the ABCA4$^{-/-}$ mouse was increased as compared to wild-type controls (Fig. 7F). Expression of NTPDase1 was also increased in the fresh tissue from the ABCA4$^{-/-}$ mice; as NTPDase1 can act as a marker for chronic increases in extracellular ATP (27), this suggests that stimulation of the P2X7R may be enhanced in RPE cells in this model of retinal degeneration.
DISCUSSION

The major conclusion of this study is that stimulation of the P2X7R can raise the lysosomal pH of RPE cells and impair lysosomal function. Lysosomal alkalinization by the P2X7R agonist BzATP was comparable to that triggered by retinal toxin CHQ and was inhibited by P2X7R antagonists (Fig. 1). The lysosomal alkalinization by the P2X7R was dependent on extracellular Ca\(^{2+}\), although Ca\(^{2+}\) itself was insufficient to change lysosomal pH (Fig. 2). Blockage of the P2X7R was itself able to reduce lipofuscin-like autofluorescence (Fig. 3); this is supported by the decrease in access to lysosomal protease cathepsin D with P2X7R stimulation and decreased lipid oxidation with block of the receptor (Fig. 4). P2X7R stimulation increased LC3BII/LC3BI and lowered p62, suggesting a disruption in autophagic pathways (Fig. 5). Stimulation of the P2X7R was not lethal but led to a reversible elevation in cytoplasmic Ca\(^{2+}\) (Fig. 6). The receptor was shown to be present on both apical and basolateral membranes of RPE cells in vivo, and expression was increased in the ABCA4\(^{-/-}\) mouse model of Stargardt’s retinal degeneration (Fig. 7). Together, these observations strongly implicate a role for the P2X7R in regulating lysosomal pH of RPE cells, as modeled in Fig. 8, and suggest a possible contribution of the receptor in some degenerative diseases.

The mechanism by which P2X7R stimulation alkalinizes RPE lysosomes is unknown, although several possible contributors can be identified. The response is receptor mediated and not a chemical effect of agonist BzATP, as the alkalinization was reduced by receptor antagonists. The removal of extracellular Ca\(^{2+}\) prevented the rise in lysosomal pH. The ability of P2X7R stimulation to rapidly raise intracellular Ca\(^{2+}\), and for this lysosomal alkalinization to be largely dependent on extracellular Ca\(^{2+}\) in the pH response. However, direct elevation of intracellular Ca\(^{2+}\) with ionophore ionomycin did not itself alter the pH. This suggests that a rise in intracellular Ca\(^{2+}\) is necessary for the lysosomal alkalinization by the P2X7R, but not sufficient for the response. This is consistent with the pathways involved in the disruption of autophagy by the P2X7R in macrophages (15);
by cAMP is mimicked by receptors linked to Gs, dependent on protein kinase A, and partially due to enhanced anion permeability on the lysosomal membrane (9, 17). It is unclear whether changes in cAMP contribute to the lysosomal alkalinization by the P2X7R. cAMP inhibited the expression of the P2X7R in THP-1 monocytic cells but did not alter the permeability of the channel directly (28), while stimulation of the P2X7R may decrease axonal levels of cAMP by inhibiting adenylate cyclase 5 (29). Human RPE cells express the Ca\(^{2+}\)-inhibited adenylyl cyclase isoforms 5 and 6 (30), although it is unclear whether these enzymes contribute to the change in lysosomal pH initiated by P2X7R stimulation.

While the absolute rise in lysosomal pH triggered by the P2X7R is modest in pH terms, functional implications are considerable, consistent with a recent report showing that an elevation of only 0.2 pH units is sufficient to impede phagocytosis in macrophages (31). In the current study, the decreased binding of Bodipy FL pepstatin A to cathepsin D with P2X7R stimulation supports a functional effect of the moderate lysosomal alkalinization and suggests a mechanism for impaired protein degradation. The increase in lipofuscin-like autofluorescence found with CHQ is also consistent with the rise in inadequately processed material following lysosomal alkalinization. The ability of P2X7R antagonist BBG to reduce this autofluorescence parallels the effects on autofluorescence produced by acidic nanoparticles (18) and the D1/D5 dopamine agonist SKF81297 (9), both of which acidify compromised lysosomes. The effect of BBG on autofluorescence is unique, however, in that no exogenous receptor agonist was added.

The effect of lysosomal pH on lipid oxidation is of particular interest, given the proposed role that oxidation plays in AMD (32). Bodipy-C11 is a fluorescent fatty acid analog that resides in organelle membranes, and its fluorescence changes from red to green when oxidized by oxy-radicals and peroxynitrite (23). It is only slightly more sensitive to oxidation than endogenous polyunsaturated fatty acids, making it a reliable reporter. The rise in Bodipy-C11 oxidation in cells treated with CHQ, combined with the decreased signal with acidifying agent SFK81297, supports a role of lysosomal pH in this lipid oxidation. The ability of P2X7R antagonist BBG to reduce the signal is also consistent with its effect on lysosomal pH. Again, this whether p44/42 MAPK is involved downstream in RPE cells as it is in macrophages remains to be determined.

The ability of cAMP to reacidify lysosomes following P2X7R stimulation suggests that the cAMP response is independent of the method used to raise the lysosomal pH. Elevation of cAMP has lowered the lysosomal pH in cells exposed to tertiary amines CHQ and tamoxifen and the retinoid A2E and in RPE cells of mice known to have alkalinized lysosomes and elevated pH. Elevation of cAMP has lowered the lysosomal pH in independent of the method used to raise the lysosomal pH. P2X7R stimulation suggests that the cAMP response is independent downstream in RPE cells.

Figure 7. Expression of P2X7R and increase in mouse model of retinal degeneration. A, B) PCR indicating the presence of the P2X7R in fresh mouse RPE cells (A) and in ARPE-19 (B) cells. C) P2X7R was expressed on both apical (a) and basolateral (b) membranes of RPE cells from a 15-mo-old C57BL6 mouse. Scale bar = 5 μm. D) Primary cultures of mouse RPE cells respond to 50 μM BzATP with a similar elevation of Ca\(^{2+}\), as determined from the uncalibrated ratio of light excited at 340/380 nm in cells loaded with Fura-2. Note the increased duration of BzATP exposure and expanded timescale. E) Mean rise in 340/380 ratio from mouse RPE cells as in D, normalized to control; n = 22. *P < 0.05, paired Student’s t test. F) Expression of P2X7R and NTPDase1 (marker of extracellular ATP levels; ref. 24) were increased in RPE/choroid cells from ABCA4+/− mice vs. C57BL6 (n=11). *P < 0.05.

Figure 8. Schematic model highlighting possible consequences of lysosomal alkalinization by the P2X7R on RPE cells. Stimulation of the P2X7R by endogenous agonist ATP elevates lysosomal pH via a Ca\(^{2+}\)-dependent process. The rise in lysosomal pH leads an increase in lipid oxidation (orange) and impairs the degradation of phagocytosed POSs and autophagic vesicles (AP). Impaired lysosomal function may lead to the accumulation of intracellular and extracellular material. For clarity, the P2X7R is shown on only one face of the membrane but is present on both apical and basolateral surfaces.
change was observed by the antagonist alone, in the absence of an added agonist. As the addition of externally oxidized lipids to RPE cells is itself sufficient to reduce the action of lysosomal enzymes (33), this oxidation may magnify the impaired degradation and increased autofluorescence found with P2X7R activation.

Although the major contributions of the P2X7R to RPE cells are likely to be beneficial under physiological conditions, overstimulation of the receptor is consistent with some of the pathologies found in chronic diseases. The P2X7R was initially associated with cell death in cells (34), and overstimulation of the receptor in primary human RPE cultures can induce apoptosis (12), but the identification of the receptor on neurons implies that the overriding role of the receptor on postmitotic cells is not lethal (14, 26, 35). Of particular relevance is the observation that P2X7Rs modulate the visual signal in the retina (36). While the P2X7R may contribute to the eventual death of RPE cells under conditions where extracellular ATP is substantially elevated for sustained periods, high levels of ectoATPases will limit agonist levels in all but the most extreme conditions (37, 38). RPE cells are, in fact, remarkably resilient, and survive under very challenging conditions, suggesting that the activation of the P2X7R is normally kept tightly controlled. ATP released locally through pannexin channels adjacent to the P2X7R can provide some degree of selectivity over other P2X and P2Y receptors, although coactivation of these other receptors by endogenous signaling networks cannot be ruled out.

While the death of RPE cells is fortunately rare, we are increasingly aware of how their dysfunction can lead to disease. Increased expression of P2X7Rs and of extracellular ATP marker NTPDase1 in RPE cells from ABCA4−/− mice as compared to age-matched controls implicates the receptor in the retinal degeneration found in these mice, although the magnitude of any causal role is hard to predict. While RPE cells rarely die in this mouse model, their lysosomal pH is substantially elevated, and their RPE is full of improperly digested material of photoreceptor origin (8, 39). Whether excessive stimulation of the P2X7Rs contributes to the lysosomal alkalinization and lipofuscin formation in ABCA4−/− mice is presently unresolved.

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P2X7R RAISES LYSOSOMAL PH IN RPE CELLS

REFERENCES


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