Activation of P2X Receptors Induces Apoptosis in Human Retinal Pigment Epithelium

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Purpose. The retinal pigment epithelium (RPE) is considered a primary site of pathology in age-related macular degeneration (AMD), which is the most prevalent form of irreversible blindness worldwide in the elderly population. Extracellular adenosine triphosphate (ATP) acts as a key signaling molecule in numerous cellular processes, including cell death. The purpose of this study was to determine whether extracellular ATP induces apoptosis in cultured human RPE.

Methods. RPE apoptosis was evaluated by caspase-3 activation, Hoechst staining, and DNA fragmentation. Intracellular Ca^{2+} levels were determined by both a cell-based fluorometric Ca^{2+} assay and a ratiometric Ca^{2+} imaging technique. $P2X_7$ mRNA and protein expression were detected by reverse transcription-polymerase chain reaction (RT-PCR) and confocal microscopy, respectively.

Results. The authors found that both the endogenous $P2X_7$ agonist ATP and the synthetic, selective $P2X_7$ agonist 2′,3′-O-(4-benzoylbenzoyl)-ATP (BzATP) induced RPE apoptosis, which was significantly inhibited by $P2X_7$ antagonist oxidized ATP (oATP) but not by the P2 receptor antagonist suramin; both ATP and BzATP increase intracellular Ca^{2+} via extracellular Ca^{2+} influx; both ATP- and BzATP-induced Ca^{2+} responses were significantly inhibited by oATP but not by suramin; ATP-induced apoptosis was significantly inhibited or blocked by BAPTA-AM or by low or no extracellular Ca^{2+}; and $P2X_7$ receptor mRNA and protein were expressed in RPE cells.

Conclusions. These findings suggest that P2X receptors, especially $P2X_7$ receptors, contribute to ATP- and BzATP-induced Ca^{2+} signaling and apoptosis in the RPE. Abnormal Ca^{2+} homeostasis through the activation of P2X receptors could cause the dysfunction and apoptosis of RPE that underlie AMD. (*Invest Ophthalmol Vis Sci.* 2011;52:1522–1530) DOI:10.1167/iovs.10-6172

Extracellular adenosine triphosphate (ATP) acts as a key signaling molecule in numerous cellular processes and is viewed as an endogenous danger signal released in large quantities by cells after inflammation, oxidative stress, and cell injury.[1,2] It activates a class of cell-surface nucleotide receptors termed P2 receptors that are further categorized into P2Y receptors and P2X receptors.[3] P2 receptors are widely expressed in excitable and nonexcitable cells, where they play important functions.[4–8] P2Y receptor expression and function have been reported in human, rat, bovine, and rabbit retinal pigment epithelium (RPE).[9–18]

Little is known of P2X receptors in the RPE. Ryan et al.[13] suggested that in addition to P2Y receptors, cultured rat RPE cells exhibited functional P2X receptors. Dutot et al.[19] showed that ATP and a selective $P2X_7$ agonist 2′,3′-O-(4-benzoylbenzoyl)-ATP (BzATP) stimulated YO-PRO-1 dye uptake and confocal immunofluorescence microscopy detected $P2X_7$ receptor protein in a human RPE cell line, ARPE-19 cells. Among seven P2X receptors, the $P2X_7$ receptor is unique because it plays a critical role in oxidative stress, cell death, and inflammation as well as in several diseases such as Alzheimer's disease and kidney diseases.[20,21] However, the role of this receptor in the RPE is unknown. Because oxidative stress, cell death, and inflammation are implicated in age-related macular degeneration (AMD) and apoptotic RPE death underlies AMD,[22,23] we hypothesized that ATP may induce RPE apoptosis by activation of the $P2X_7$ receptor. By combining molecular, functional, and pharmacologic approaches, we show that the $P2X_7$ receptor is expressed in native and cultured human RPE and that activation of the $P2X_7$ receptor induces both Ca^{2+} signaling and apoptosis in RPE cells.

Methods

Materials

Ninety-six–well black clear-bottom plates were purchased from Fisher Scientific (Costar; Fisher Scientific, Pittsburgh, PA), and 35-mm glass bottom culture dishes were purchased from MatTek Corporation (Ashland, MA). Hoechst 33342, ATP, BzATP, brilliant blue G (BBG), KN-62, suramin, 1,2-Bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), and oxidized ATP (oATP) were purchased from Sigma-Aldrich (St. Louis, MO). Hanks' balanced salt solutions (HBSS), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), a RNA isolation reagent (Trizol), *Taq* DNA polymerase, AlexaFluor 555 goat anti-rabbit IgG, and Indo-1-AM (acetoxymethyl ester) were obtained from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-$P2X_7$ antibody was purchased from Abcam, Inc. (Cambridge, MA). Mounting medium with DAPI was purchased from Vector Laboratories (Vectashield; Vector Laboratories, Burlingame, CA). Caspase-3 assay kit was purchased from Biotium, Inc. (NucView 488; Biotium, Inc., Hayward, CA). DNase I (DNAfree) and first-strand synthesis kit (RETROscript) were purchased from Ambion (Austin, TX). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Human RPE Cell Culture

Human RPE cells were isolated from donor eyes by enzymatic digestion as previously described.[24,25] The protocol adhered to the provisions of

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the Declaration of Helsinki for the use of human tissue in research. In all experiments, parallel assays were performed on RPE cells between passages 3 and 6. RPE cells were seeded at the same time and density from the same parent cultures, then grown in phenol red-free complete DMEM/F12 for at least 4 days until 85% to 100% confluence. RPE cells were placed in serum-free media overnight before treatments.

Detection of Caspase-3 Activation

Caspase-3 activation was measured by caspase-3 substrate (NucView 488; Biotium), as described previously.[26] Fluorescence intensity of activated caspase-3 was measured by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Hoechst Fluorescence Staining

Nuclear staining was performed as a second marker of RPE apoptosis.[26] The numbers of stained RPE cells that exhibited apoptotic nuclear condensation and fragmentation were scored as apoptotic. RPE cells from at least 10 high-power microscopic fields from each group of cultures from each of three donors were counted and averaged. Data were normalized to the mean of control RPE cultures.

Double Staining with Caspase-3 Substrate and Hoechst

At the end of control and experimental incubations, RPE cells were successively stained with caspase-3 substrate (NucView 488) for 30 minutes and Hoechst 33342 for 10 minutes at room temperature.[26] The caspase-3 substrate is cleaved by activated caspase-3 to release a dye that stains the cell nuclei green, whereas Hoechst 33342 stains the cell nuclei of healthy cells faintly blue and those of the apoptotic cells bluish-white.

Cell Death Detection ELISA

RPE apoptosis was also evaluated by DNA fragmentation, as measured by an ELISA kit (Cell Death Detection ELISA^{PLUS}; Roche Applied Science, Indianapolis, IN) according to procedures outlined by the manufacturer.

Cell-Based Fluorometric Ca^{2+} Assay

Intracellular Ca^{2+} levels were quantitatively determined by cell-based fluorometric Ca^{2+} assay using Indo-1-AM. RPE cells grown on 96-well culture plates were incubated with Indo-1-AM (5 μM) for 1 hour at 37°C in the dark, after which RPE cells were washed thoroughly, and control medium (HBSS/HEPES), ATP, or BzATP was added to the RPE cells. Indo-1 was excited at 355 nm, and the fluorescence emission from Indo-1 was measured at 405 nm and 485 nm with a fluorometer (FlexStation Scanning Fluorometer; Molecular Devices, Sunnyvale, CA). Fluorescence data were collected at 5-second intervals throughout the course of each experiment. Data are expressed as Indo-1 fluorescence ratios (F405/F485), which are used as a direct index of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).

Ratiometric Calcium Imaging

Intracellular Ca^{2+} levels were also determined using fluorescence microscopy. RPE cells grown on 35-mm glass-bottom culture dishes were labeled as described and then mounted on the stage of an inverted fluorescence microscope (Nikon). Cells were excited at 355 nm, and fluorescence images were collected simultaneously at the dual-emission wavelengths (405 nm and 485 nm) using an imaging apparatus (Dual-View; Optical Insights, Suwanee, GA) and imaging software (MetaFluor Ratio Imaging Software; Universal Imaging Corporation, West Chester, PA). Analysis was carried out using the MetaFluor Analyst software (Universal Imaging Corporation, PA). The fluorescence intensity ratio (F405/F485) was used as a direct index of $[Ca^{2+}]_i$.

Total RNA Isolation

Total RNA was isolated from cultured human RPE cells using a RNA isolation reagent (Trizol; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of total RNA was measured by ultraviolet spectrophotometry.

RT-PCR Analysis

RT-PCR was used to detect $P2X_7$ mRNA, as described previously.[27] PCR was performed with $P2X_7$-specific primers with the forward primer sequence 5′-GAACCAGCAGCTACTAGGGAGAAG-3′ and the reverse primer sequence 5′-TGGGCAGGTTGGCAAAGTCAGC-3′.[28] The housekeeping gene, *hypoxanthine guanine phosphoribosyl transferase 1* (*HPRT1*), served as a control. The forward primer for HPRT1 was 5′-ACCGTGTGTTAGAAAAGTAAGAAG-3′, and the reverse primer was 5′-AGGGAACTGCTGACAAAGATTC-3′.[29] The PCR products were generated by adding *Taq* DNA polymerase and cycled 40 times for $P2X_7$ or *HPRT1* (1 minute at 94°C, 0.5 minute at 64°C, 0.5 minute at 72°C), followed by a 7-minute extension at 72°C. The RT-PCR products were separated by 1.5% agarose gel electrophoresis.

Confocal Microscopy

Human RPE cells were plated onto 8-well chamber slides and grown for at least 4 days, rinsed twice in PBS, and fixed for 15 minutes in 3% paraformaldehyde in PBS. Cells were then rinsed and permeabilized for 5 minutes at room temperature in 0.2% Triton X-100/PBS. Cells were blocked in 6% BSA/10% normal goat serum in PBS for 1 hour and incubated with 8 μg/mL rabbit polyclonal anti-$P2X_7$ antibody diluted in 1% BSA in PBS overnight at 4°C. The specificity of the anti-$P2X_7$ antibody was confirmed by omitting the primary antibody. The cells were then incubated with 10 μg/mL Alexa Fluor 555 goat anti-rabbit IgG diluted in 1% BSA in PBS for 2 hours at room temperature. Finally, the cells were washed, mounted in a mounting medium with DAPI (Vectashield; Vector Laboratories), and viewed with confocal microscope (TCS SP5; Leica, Wetzlar, Germany). Digital images were collected.

Statistical Analysis

Data are expressed as mean ± SD and evaluated by Student's unpaired *t*-test or one-way analysis of variance (ANOVA), followed by a Student-Newman-Keul's post hoc test. $P < 0.05$ is considered statistically significant.

Results

ATP and BzATP Induce RPE Apoptosis

We assessed whether ATP increases RPE apoptosis. We used three apoptotic markers—activated caspase-3, nuclear condensation, and DNA fragmentation—to evaluate the effects of extracellular ATP on the multiple biochemical processes accompanying RPE apoptosis. Figure 1A shows images of RPE cells treated with ATP in the presence or absence of oATP, a $P2X_7$ antagonist, for 6 hours. ATP induced caspase-3 activation and nuclear condensation, both of which were blocked by oATP. The percentages of RPE cells with apoptotic nuclei, as judged by Hoechst 33342 staining (Fig. 1B; $P < 0.001$), and of RPE cells with activated caspase-3 (Fig. 1C; $P < 0.05$) in ATP-treated RPE cells were statistically greater than those of control RPE cell cultures. The $P2X_7$ antagonist, oATP, significantly reduced the percentages of RPE cells with apoptotic nuclei (Fig. 1B; $P < 0.001$) and activated caspase-3 (Fig. 1C; $P < 0.05$). At 24 hours, ATP significantly increased DNA fragmentation, as measured by cell death detection ELISA; this increase was completely blocked by oATP (Fig. 1D; $P < 0.001$). Each set of experiments was repeated on RPE cells isolated from three donors.

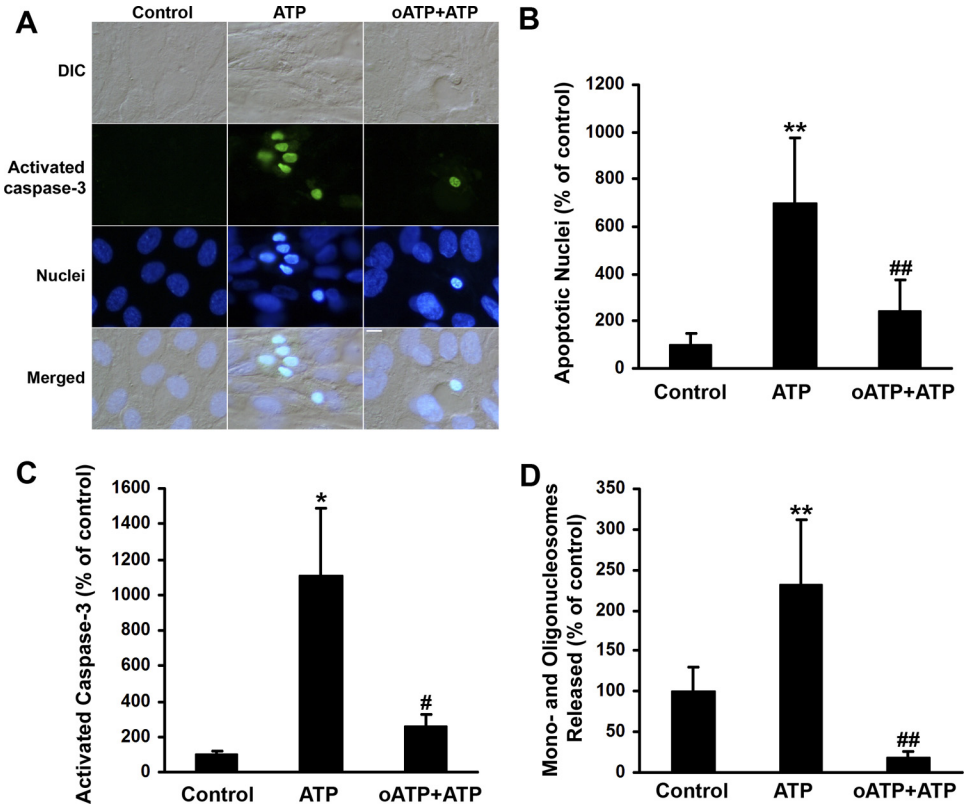

FIGURE 1. ATP induces apoptosis in human RPE cells. (**A**) RPE cells were preincubated with or without oxidized ATP (oATP; 300 μM) and then exposed to 100 μM ATP with or without oATP for 6 hours. The number of cells with activated caspase-3 and the number of apoptotic RPE cells were determined by double labeling with caspase-3 substrate and Hoechst 33342. Scale bar, 10 μm. (**B**) RPE cells were treated and stained by Hoechst 33342 as described in (**A**), and apoptotic RPE nuclei were determined. (**C**) RPE cells were treated and stained by caspase-3 substrate as described in (**A**), and activated caspase 3-positive RPE cells were determined. (**D**) RPE cells were treated as described in (**A**) for 24 hours. DNA fragmentation or released mononucleosomes and oligonucleosomes were measured by ELISA. Data are presented as mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with ATP alone.

We next tested the effects of BzATP on RPE apoptosis. BzATP is a synthetic, selective $P2X_7$ receptor agonist and is widely used in other systems.[30–32] As shown in Figure 2, BzATP significantly increased RPE apoptosis, which was completely blocked or significantly inhibited by the $P2X_7$ antagonists BBG ($P < 0.001$), KN-62 ($P < 0.001$), and oATP ($P < 0.05$), whereas suramin had no effect on BzATP-induced RPE apoptosis ($P > 0.05$), suggesting the involvement of $P2X_7$ receptors in ATP- and BzATP-induced RPE apoptosis.

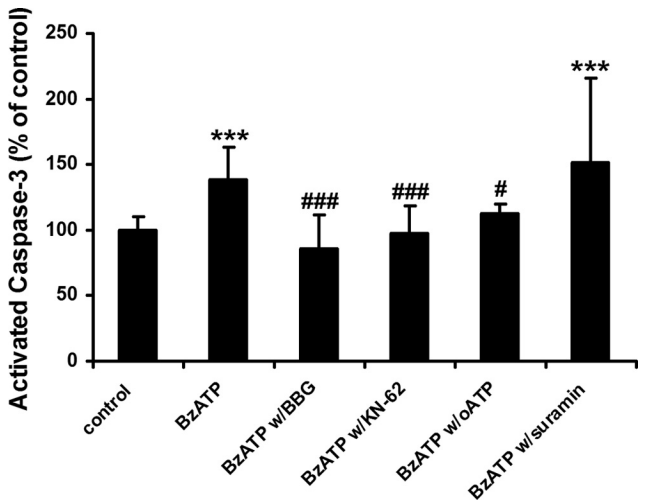

FIGURE 2. BzATP induces apoptosis in human RPE cells. RPE cells were treated with 1 mM benzoylbenzoyl adenosine triphosphate (BzATP) in the presence or in the absence of P2 receptor antagonists, brilliant blue G (BBG), KN-62, oxidized ATP (oATP), or suramin for 24 hours and then were stained by caspase-3 substrate. Activated caspase-3 fluorescence intensity was measured. Data are presented as mean ± SD. $^{***}P < 0.001$ compared with control. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared with BzATP alone.

ATP and BzATP Increase RPE Intracellular Ca^{2+} Level

To assess the level of $P2X_7$ receptor activity, we monitored $[Ca^{2+}]_i$ using two different methods: a cell-based fluorometric Ca^{2+} assay and a Ca^{2+} imaging technique. As shown in Figure 3A, 100 μM ATP induced an increase in $[Ca^{2+}]_i$. The transient rise declined to a level that remained higher than the baseline or control $[Ca^{2+}]_i$. Stimulation with 100 μM BzATP also produced an increase in $[Ca^{2+}]_i$, but the BzATP-induced signal was lower than that caused by equimolar ATP (Figs. 3A, 3B). The ATP-induced Ca^{2+} peak or sustained Ca^{2+} level at 10 minutes was significantly higher than the control level and the baseline Ca^{2+} reading (Fig. 3B; $P < 0.001$ or $P < 0.01$). The BzATP-induced Ca^{2+} peak (Fig. 3B; $P < 0.001$) and sustained Ca^{2+} level (Fig. 3B; $P < 0.05$) were significantly higher than baseline and control Ca^{2+} levels. Using ratiometric calcium imaging, we confirmed that ATP-induced Ca^{2+} peak or Ca^{2+} level at 10 minutes is significantly greater than baseline $[Ca^{2+}]_i$ (Fig. 3C). This Ca^{2+} imaging result is representative of five independent experiments.

Both ATP and BzATP Trigger Extracellular Ca^{2+} Influx

We then asked whether the ATP- and BzATP-induced increases in intracellular Ca^{2+} were caused by the release of Ca^{2+} from intracellular stores, influx from the extracellular environment, or both. To test this, additional experiments were performed in the presence or absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, ATP or BzATP triggered a fast and sustained $[Ca^{2+}]_i$ increase (Fig. 4). In the absence of extracellular Ca^{2+}, ATP induced a much smaller $[Ca^{2+}]_i$ increase in some traces (Fig. 4; RPE cells from donor 1) or no increase at all in other traces (Fig. 4; RPE cells from donors 1 and 2), suggesting that the observed ATP-induced $[Ca^{2+}]_i$ was caused mainly by extracellular Ca^{2+} influx. In the absence of extracellular Ca^{2+},

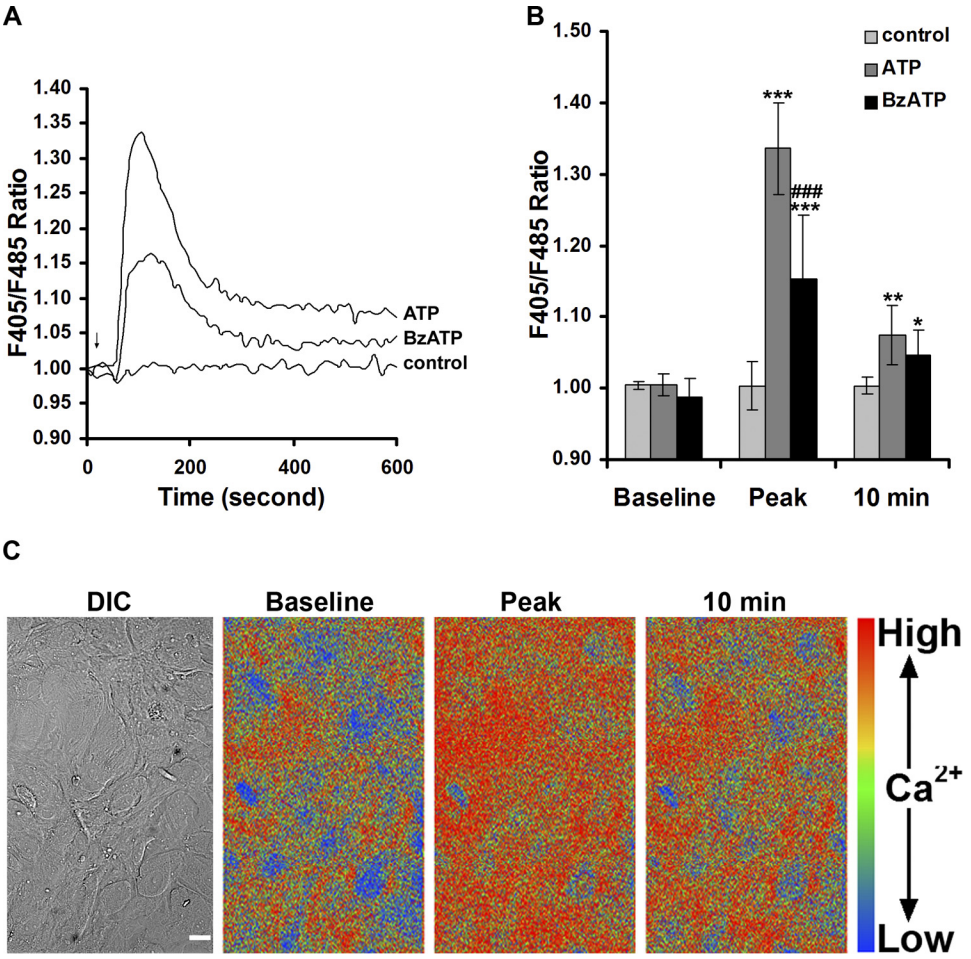

FIGURE 3. ATP and BzATP induce intracellular Ca^{2+} increase in human RPE cells. (**A**) Temporal plot of F405/F485 ratio changes recorded from Indo-1-AM–labeled RPE cells before and after 100 μM ATP or 100 μM BzATP application. Shown are the mean traces of six to nine traces for each condition from RPE cells derived from three different donors. *Arrow*: time when ATP, BzATP, or control medium (HBSS/HEPES) was added to the RPE cell cultures. (**B**) Summary of baseline Ca^{2+} (before ATP application), Ca^{2+} peak, and Ca^{2+} at 10 minutes in RPE cells derived from three different donors. Ca^{2+} concentrations were indicated by F405/F485 ratios. Data represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with its own baseline or control. $^{\#\#\#}P < 0.001$ compared with ATP alone. (**C**) Differential interference contrast (DIC) image and pseudocolor ratio (F405/F485) images of a representative field of Indo-1-AM–labeled RPE cells taken before and after ATP application. Scale bar, 20 μm.

the BzATP-induced increase in $[Ca^{2+}]_i$ was completely blocked in all traces, indicating BzATP-induced $[Ca^{2+}]_i$ is only from extracellular Ca^{2+}. In addition to the experiments shown in Figure 4, similar results were obtained from five additional independent experiments.

P2 Receptor Antagonists Affect ATP- or BzATP-Induced Ca^{2+} Level

We next asked whether P2 receptor antagonists block the induced Ca^{2+} rise. To this end, we preincubated RPE cells with

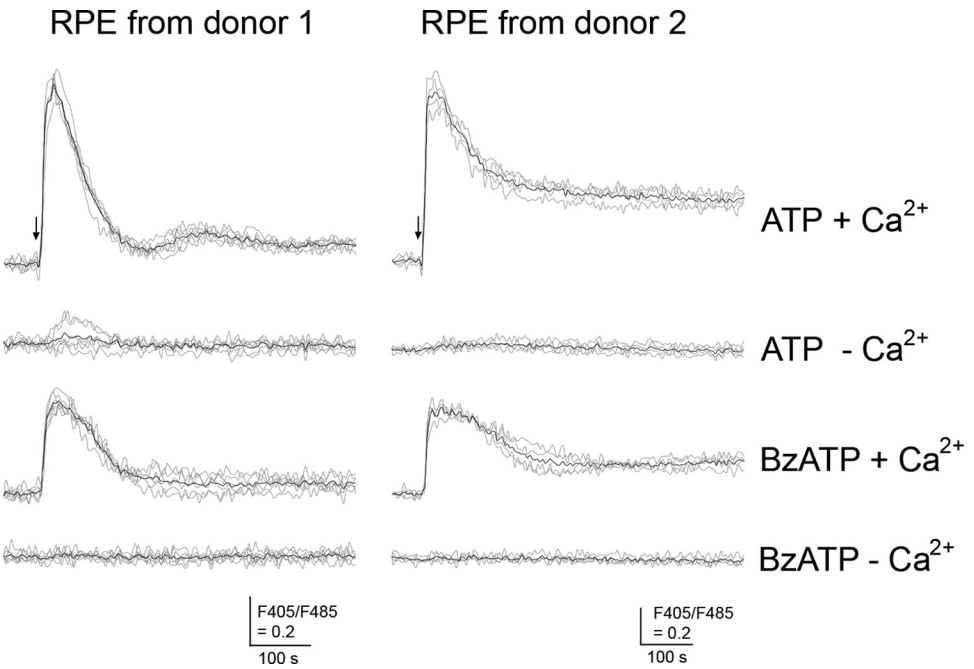

FIGURE 4. ATP and BzATP trigger extracellular Ca^{2+} influx in human RPE cells. Human RPE cells (1×10^4) were plated onto a 96-well plate, cultured, and labeled with Indo-1-AM. After washes, the RPE cells were stimulated with 100 μM ATP or 100 μM BzATP in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of extracellular Ca^{2+}. The responses were measured using a fluorometer. *Gray line*: individual traces. *Black line*: mean trace of individual traces in each condition. *Arrow*: time when ATP or BzATP was added to the RPE cell cultures. Similar results were obtained from five additional independent experiments.

oATP (300 μM), an irreversible inhibitor of the $P2X_7$ receptors,[33,34] or with suramin (50 μM), which was reported to inhibit the $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, $P2Y_1$, and $P2Y_2$ receptors at similar concentrations,[9,31,35] followed by application of ATP or BzATP. As shown in Figure 5, pretreatment of human RPE cells with oATP dramatically reduced the ATP- and BzATP-induced Ca^{2+} peak ($P < 0.001$), abolished ATP-induced sustained Ca^{2+} levels, and inhibited BzATP-induced sustained Ca^{2+} levels. In contrast, suramin did not inhibit the Ca^{2+} rise evoked by ATP or BzATP (Fig. 6).

Buffering Intracellular Ca^{2+} or Decreasing Extracellular Ca^{2+} Inhibits ATP-Induced Apoptosis

BAPTA-AM is a cell-permeable free calcium chelator that is widely used to reduce intracellular Ca^{2+} levels in many systems, including RPE cells.[9] If ATP-induced RPE apoptosis is dependent on an increase in $[Ca^{2+}]_i$, then pretreatment of RPE cells with BAPTA-AM would inhibit ATP-induced RPE apoptosis. As expected, BAPTA-AM pretreatment dose dependently inhibited ATP-induced RPE apoptosis, as measured by DNA fragmentation (Fig. 7; $P < 0.01$ and $P < 0.001$ for 5 μM and 10 μM BAPTA-AM, respectively). If the extracellular Ca^{2+} influx contributes to the ATP-induced rise in $[Ca^{2+}]_i$, then removing extracellular Ca^{2+} or reducing extracellular Ca^{2+} would be expected to block ATP-induced RPE apoptosis. As shown in Figure 7, removing extracellular Ca^{2+} ($P < 0.01$) or reducing extracellular Ca^{2+} ($P < 0.001$) from normal (1.8 mM) to low (0.3 mM) concentrations significantly inhibited or blocked ATP-induced apoptosis. These results were obtained from RPE cells derived from three different donors.

RPE Expresses $P2X_7$ receptor

Our data suggest that functionally active $P2X_7$ receptors are expressed by human RPE cells. To obtain molecular evidence for this, we performed RT-PCR experiments and confocal immunofluorescence microscopy. Figure 8A shows the results of RT-PCR of RNA extracted from native human RPE cells, cultured human RPE cells derived from three donors, and ARPE-19 cells. RT-PCR of all RPE cells generated single bands with the expected size of 476 bp, demonstrating the presence of $P2X_7$ receptor mRNA in human RPE cells. To demonstrate that this message yielded $P2X_7$ receptor protein, confocal immunofluorescence microscopy was performed. As shown in Figure 8B, $P2X_7$ receptor protein expression was confirmed.

Discussion

This study provides the first evidence that $P2X_7$ receptors are expressed in native and cultured human RPE cells and that activation of P2X receptors induces both Ca^{2+} signaling and apoptosis. Extracellular ATP can induce apoptosis through the ligation of P2X and P2Y receptors.[36] The P2X receptors, particularly $P2X_7$ receptor, have been shown to play a more important role in the induction of apoptosis than the P2Y receptors.[37,38]

P2Y Receptors in the RPE

P2Y receptors have been implicated in RPE function, and P2Y mRNA and protein have been identified in cultured human RPE cells, rabbit, rat, and monkey RPE cells.[12,16–18, 39–41] Activation of $P2Y_2$ receptors increases $[Ca^{2+}]_i$, RPE fluid transport, and retinal reattachment in rat and rabbit models of retinal detachment.[9,11,12,14,15,42] In addition to $P2Y_2$ receptors, $P2Y_1$ and $P2Y_6$ receptors were reported to regulate Ca^{2+} levels in cultured human RPE cells,[17] and stimulation of ARPE-19 cells with extracellular nucleotides induced IL-8 gene expression and protein secretion, possibly through $P2Y_2$ and $P2Y_6$ receptors.[18] Among the three functional receptors ($P2Y_1$, $P2Y_2$, and $P2Y_6$) identified in human RPE, $P2Y_2$ receptor can be activated

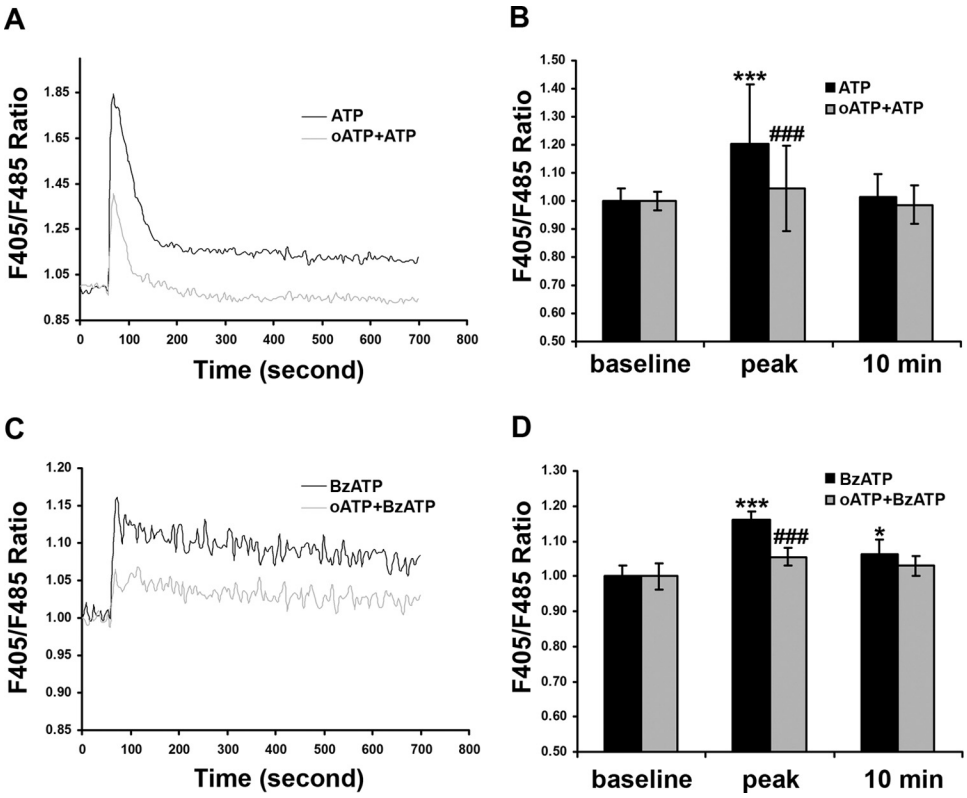

Figure 5. Effects of oxidized ATP on ATP- or BzATP-induced Ca^{2+} in human RPE cells. RPE cells were treated with or without $P2X_7$ receptor antagonist or oxidized ATP (oATP; 300 μM) and then were exposed to 100 μM ATP (**A**, **B**) or BzATP (**C**, **D**). Histograms show the effects of oATP on the ATP- or BzATP-induced Ca^{2+} peak and the Ca^{2+} at 10 minutes. Data represent the mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with baseline. $^{\#\#\#}P < 0.001$ compared with ATP or BzATP alone.

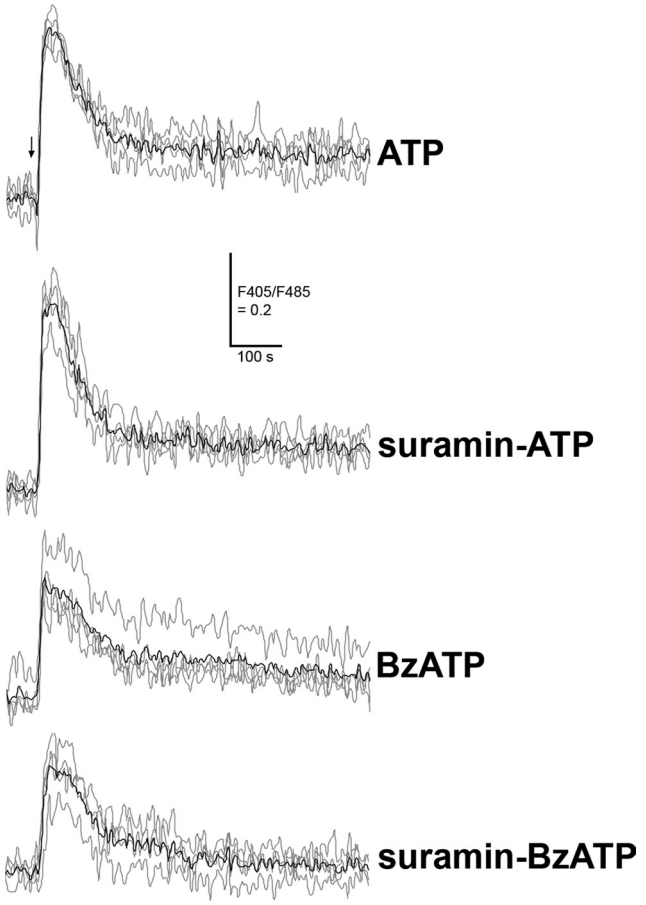

FIGURE 6. Effects of suramin on ATP- or BzATP-induced Ca^{2+} in human RPE cells. The RPE cells were pretreated with or without P2 receptor antagonist, suramin (50 μM), and then exposed to 100 μM ATP or BzATP in the presence (suramin-ATP or suramin-BzATP) or in the absence (ATP or BzATP) of suramin. The responses were measured using a fluorometer. *Gray line*: individual traces. *Black line*: mean trace of individual traces in each condition. *Arrow*: time when ATP or BzATP was added to the RPE cell cultures. Similar results were obtained from RPE cells derived from another donor.

by ATP. Suramin is known to block $P2Y_2$ receptors in different cell types, including RPE cells.[9,31,35] However, we found that pretreatment of RPE cells with suramin did not significantly inhibit the ATP-induced increase in intracellular Ca^{2+} levels (Fig. 6). P2X receptors are ATP-gated ion channels that are permeable to Ca^{2+}, and the influx of extracellular Ca^{2+} contributes to the initial Ca^{2+} peak when P2X receptors are activated.[20,43] P2Y receptors are G-protein–coupled receptors, and Ca^{2+} released from intracellular stores contributes to the initial Ca^{2+} increase when P2Y receptors are activated.[17] Therefore, removal of extracellular Ca^{2+} can help determine whether ATP acts on P2X receptors, P2Y receptors, or both. We show here that, in the absence of extracellular Ca^{2+}, ATP-induced Ca^{2+} responses were almost completely blocked (Fig. 4), suggesting that P2X receptors rather than P2Y receptors contribute to the ATP-induced Ca^{2+} responses in human RPE cells under our experimental conditions.

Harada et al.[37] reported that stimulation of $P2Y_2$ or $P2Y_4$ receptors, or both, induced cell proliferation, whereas stimulation of $P2X_7$ receptors induced cell apoptosis in glomerular mesangial cells. Based on this, we suggest that the relative expression of P2X and P2Y receptors by RPE cells could determine cell fate: proliferation or apoptosis in response to extracellular ATP. Furthermore, local concentrations of extracellular ATP are important in determining cell death. We observed that 100 μM ATP increased human RPE apoptosis as judged by three different approaches (Fig. 1). Consistent with our results, Sugiyama et al.[32] showed that ATP at 100 μm, but not 30 μM, significantly increased rat retinal neuron death. Thus, ATP may activate P2Y receptors and $P2X_{1-6}$ receptors at lower concentrations ($<$100 μM) and $P2X_7$ receptors at higher concentrations ($\geq$100 μM) in RPE cells.

P2X Receptors in the RPE

P2X receptors have two transmembrane domains and can form trimeric channels by polymerization of their subunits homomerically or heteromerically. Functional P2X receptors include six homomeric channels and seven heteromeric channels.[35,44] The ability of P2X receptors to act as direct conduits for Ca^{2+} influx or indirect activators of voltage-gated Ca^{2+} channels underlies their important roles in Ca^{2+}-based signaling responses.[44]

ATP was found to be released by RPE cells[10,16,45–47] and by neural retina[48,49] and to be capable of acting on P2X receptors in the RPE cells in an autocrine or a paracrine manner. The study by Sullivan et al.[12] supports the presence of functional P2X receptors in the RPE cells. Sullivan et al.[12] applied 100 μM ATP to cultured human RPE cells and found that ATP induced an initial Ca^{2+} peak and sustained rise in $[Ca^{2+}]_i$. In the absence of extracellular Ca^{2+}, the ATP-induced Ca^{2+} peak was reduced, and the sustained $[Ca^{2+}]_i$ increase was abolished.[12] Ryan et al.[13] presented evidence that cultured rat RPE cells exhibited functional P2Y and P2X receptors and showed that ATP-induced increases in $[Ca^{2+}]_i$ did not depend on extracellular Ca^{2+} influx. The discrepancies between studies could be explained by cell strains or cell lines, cell sensitivity, cell proliferation state, ATP concentration, and exposure time to ATP. Dutot et al.[19] showed that YO-PRO-1 dye uptake was increased in ATP- and BzATP-stimulated ARPE-19 cells and $P2X_7$ receptor protein was detected in ARPE-19 cells. In this

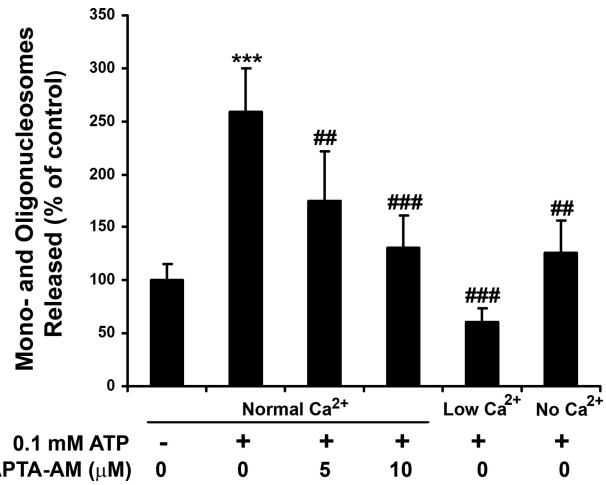

FIGURE 7. Effects of BAPTA-AM and decreasing extracellular Ca^{2+} on ATP-induced apoptosis in human RPE cells. The RPE cells were pretreated with or without a cell-permeable intracellular Ca^{2+} chelator, BAPTA-AM (5 μM, 10 μM) for 30 minutes and then were exposed to 100 μM ATP for 24 hours in the absence and presence of BAPTA-AM in normal extracellular Ca^{2+} (1.8 mM). RPE cells were also exposed to 100 μM ATP for 24 hours in low extracellular Ca^{2+} (0.3 mM) or in the nominal absence of extracellular Ca^{2+}. DNA fragmentation or released mononucleosomes and oligonucleosomes were measured by ELISA. Data are presented as mean ± SD. $^{***}P < 0.001$ compared with control (unstimulated cells in normal extracellular Ca^{2+}). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with ATP-stimulated cells in normal extracellular Ca^{2+}.

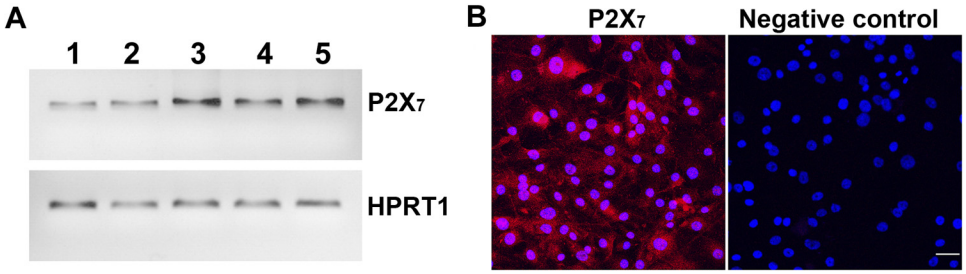

FIGURE 8. $P2X_7$ receptor expression in human RPE cells. (**A**) RT-PCR analysis. Total RNAs isolated from native human RPE (*lane 1*), cultured human RPE cells derived from three donors (*lanes 2–4*), and ARPE-19 cells (*lane 5*) were treated with DNase I and reverse transcribed. PCR was performed using a primer set specific for $P2X_7$ receptor or *HPRT1*, which served as an endogenous control. (**B**) Immunofluorescence labeling of $P2X_7$ in human RPE cells. Immunofluorescence labeling omitting primary antibody serves as a negative control. Scale bar, 40 μm.

study, we detected not only $P2X_7$ receptor protein but also $P2X_7$ receptor mRNA in human RPE cells. Our functional data indicate that P2X receptors contribute to both ATP- and BzATP-induced Ca^{2+} increases and apoptosis in human RPE cells because the BzATP- and ATP-triggered Ca^{2+} responses were abolished or largely blocked after the removal of extracellular Ca^{2+}. Our findings that the reduction or removal of extracellular Ca^{2+} or the buffering of intracellular Ca^{2+} with BAPTA-AM significantly inhibited or blocked ATP-induced apoptosis suggest that RPE apoptosis is triggered by the ATP-induced rise in $[Ca^{2+}]_i$ (Fig. 4). However, ATP-induced apoptosis seems to be lower at low extracellular Ca^{2+} than in the nominal absence of extracellular Ca^{2+} (Fig. 7), indicating that some extracellular Ca^{2+} may be required for RPE cell survival. Our pharmacologic data further support the notion that the $P2X_7$ receptors contribute to the responses of RPE cells to ATP because ATP-induced Ca^{2+} influx and apoptosis were blocked by oATP, and the selective $P2X_7$ agonist BzATP induced a Ca^{2+} influx that was significantly inhibited by oATP. Furthermore, BzATP-induced RPE apoptosis was blocked or significantly inhibited by $P2X_7$ receptor antagonists BBG, KN-62, and oATP. However, Ca^{2+} influx evoked by ATP was higher than that by equimolar BzATP, indicating that in addition to $P2X_7$, other P2X receptors might be present because BzATP is known as a much better $P2X_7$ agonist than ATP.[43] Both ATP and BzATP-triggered Ca^{2+} influx were insensitive to suramin, suggesting that $P2X_4$ and $P2X_7$ receptors may contribute to the induced Ca^{2+} influx given that $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_5$ receptors, but not $P2X_4$ and $P2X_7$ receptors, were found to be sensitive to suramin,[35,50] and the $P2X_6$ receptor cannot form a homomeric channel by itself.[35,44] Further studies are needed to test whether $P2X_4$ homomeric channels, $P2X_4$/$P2X_7$ heteromeric channels, or both are expressed in the RPE. This is important because $P2X_4$ and $P2X_7$ receptors are coexpressed in immune cells and epithelial cells, and heteromerization can change both the functional and pharmacological properties of P2X receptors.[51,52] Future studies to determine whether knockdown of $P2X_7$ reduces ATP-induced RPE Ca^{2+} responses and apoptosis may further support our findings. Taken together, our results support the idea that P2X, especially $P2X_7$, receptors mediate ATP-induced Ca^{2+} signaling and apoptosis in human RPE.

Pathophysiological Implications

$P2X_7$ receptor requires a relatively high ATP concentration to be activated, with a 50% effective concentration (EC_{50}) of approximately 0.1 to 1 mM compared with other P2X receptors with EC_{50} of approximately 1 to 10 μM.[35,53] Given that extracellular ATP is normally in the low micromolar range, the activation of $P2X_7$ may be not favored under physiological conditions.

We show here that activation of the P2X receptors, especially the $P2X_7$ receptor, increases both fast and sustained Ca^{2+} levels within RPE cells and induces RPE apoptosis. We have demonstrated previously that proinflammatory cytokines induce reactive oxygen species in human RPE cells[25] and that oxidative stress increases mononuclear phagocyte-induced mouse RPE apoptosis, especially when mononuclear phagocytes were activated by IFN-γ and superoxide dismutase 2 (SOD2) expression was reduced by partial knockout of the *SOD2* gene.[26] Proinflammatory cytokines and ATP can be released at sites of inflammation and can upregulate $P2X_7$ receptor expression in human monocytes, astrocytes, and epithelial cells.[34,54–57] Thus, it is possible that proinflammatory cytokines and ATP released during pathologic conditions may increase $P2X_7$ expression in the RPE and thus increase the vulnerability of RPE cells to extracellular ATP-induced apoptosis.

$P2X_7$ receptor is also involved in inflammation and oxidative stress in many cell types.[20,58–60] Cell death, inflammation, and oxidative stress are implicated in AMD.[22,23,61,62] Therefore, defining the role of $P2X_7$ receptor in the RPE under physiological and pathophysiological conditions could have important implications for the pathogenesis of AMD. Selectively interfering with P2X receptor expression and activation could generate new preventions and therapies for AMD.

In summary, the present study provides the first evidence of functional $P2X_7$ receptor expressed in human RPE and demonstrates that activation of P2X receptors, especially $P2X_7$ receptor, induces Ca^{2+} signaling and RPE apoptosis. It is tempting to speculate that the $P2X_7$ receptor identified here could impact RPE function physiologically and pathologically.

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