Functional Role of Purinergic Receptor in Retinal Pigment Epithelial Cells

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감사의 글

너무나도 부족한 제가 이 결과를 얻기까지 이끌어주시고 지도해주셨던 분들께, 감사 드리고자 합니다.

본 논문이 완성되기까지 꾸준하게 격려해 주시고 지도편달을 아끼지 않으신 나상훈 교수님께 먼저 깊은 감사를 드립니다. 바쁘신 와중에도 많은 시간을 투자하셔서 많은 지식을 가르쳐 주시고 연구를 세심하게 지도해 주셨던 공인덕 교수님께 깊이 머리 숙여 감사의 말씀을 드립니다. 매 순간 격려해 주시고 용기를 주셨던 이종혁 교수님께도 감사 드립니다. 연구하는 자세와 연구에 접근하는 방법에 대해 가르쳐 주셨던 박주영 교수님, 연구에 섬세한 조언을 아끼지 않으셨던 예병일 교수님께도 감사의 말씀을 올립니다. 늦은 시간까지 실험방법을 알려주시고, 바쁜 중에도 너무도 많은 시간을 할애하여 주신 장재승 선생님께도 깊은 감사를 드립니다.

그리고 많은 기도와 끝없는 사랑으로 후원해 주신 부모님, 연구를 마칠 수 있도록 지지해준 남편 그리고 그 존재 자체로 힘이 되어준 사랑하는 아가들에게 감사를 드립니다.

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ABSTRACT

Functional Role of Purinergic Receptor in Retinal Pigment Epithelial Cells

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The RPE cell serves very important functions to support vision. RPE functions for vision are controlled by increase in intracellular-free Ca^{2+}. The ATP stimulation induces the physiologic changes in the RPE. Some of the ATP can stimulate the P2 receptors on RPE cells and lead to increase intracellular Ca^{2+} concentrations in RPE cells. The increased intracellular Ca^{2+} may open Ca^{2+} activated K^+ channel. Several previous studies of Kir 4.1 channel have reported. But the details of Kir 4.1 channel mechanism have to be completely defined. I examed Ca^{2+} activated K^+ channel and Kir 4.1 channel in ARPE cells.

I confirmed the various expression of Ca^{2+}-activated K^+ channels subunits such as small-like conductance Ca^{2+}-activated K^+ channels (SK) as well as large-like conductance in ARPE-19 cells using a combined molecular biological and electrophysiological study. The presence of Ca^{2+}-activated K^+ channel are

identified by expression profile of transcripts encoding BK and SK channel subunits (i.e., BKα1, BKβ3, BK β4, SK1, SK3 and SK4/IK). The BK, IK and SK current-voltage relationship yielded a single-channel conductance of 219.6 ± 3.26 pS, 20.24 ±0.73 and 12.55 ± 0.11 pS, respectively. I treated ATP at ARPE cells. The increases of ATP-induced intracellular-free Ca^{2+} were found to result predominantly from release of Ca^{2+} from cytosolic Ca^{2+} stores implying that these are mediated by activation of P2Y receptors. It was found that ATP-dependent increases in intracellular free Ca^{2+} activate Ca^{2+}-dependent K^{+} channels leading to increase open probabilities of their single channels using attached patches. ATP-induced transient increased of intracellular Ca^{2+} in RPE activate to BK channels and SK channels in ARPE-19 cells. SK channel was more activated than BK channel by increase of intracellular Ca^{2+}. It is probable that SK channel is physiologically more important than BK channel for homeostasis of K^{+} in RPE cells.

The application of ATP to RPE did not immediately effect on current density of Kir4.1 channel. Long term incubation(24 hours) of RPE with ATP decreased current density of Kir4.1 channel. Decreased current density of Kir4.1 channel occured via a mechanism that requires $G\alpha_{q,}$ subunit of G protein. There was no effects of DAG to change of the current density of Kir4.1 channel. Current density change was independent of PKC. Decreased current density of Kir4.1 channel by long term incubation of RPE with ATP occurred through a mechanism involving caveolin-mediated endocytosis of Kir4.1 channel in cell membrane.

Including this study, the more investigation of ion channels in the RPE will

provide further information of the physiology and pathophysiology of the RPE.

Key words : ARPE, purinergic receptor, ATP, Ca^{2+}-activated K^+ channels, Kir 4.1 channel, endocytosis, caveolin-1

I . INTRODUCTION

1.1. Retinal pigment epithelium

The RPE is a single layer of cuboidal cells, approximately 16μm in diameter. It is located between Bruch's membrane and the retina. The apical portion of the RPE borders the outer segments of photoreceptor cell and the basolateral portion of the RPE is faces with the fenestrated capillaries of the choroid (Bok 1993, 189-195, Boulton 2001, 384-389, Marmorstein 2001, 867-872, Marmorstein 1998, 1-12).

The RPE cell performs important functions for vision. The RPE cell absorbs light by melanosomes. The RPE cell forms the outer blood-retinal barrier by tight junctional complexes which lie lateral surfaces of RPE cell. The outer blood-retinal barrier prevents diffusion of metabolites between the choroid and the subretinal space. Breakdown of the blood-retinal barrier cause many retinal disorders (Gariano RF 1996, 481-490, SA 1995, 141-154).

RPE cells are in charge of phagocytic function. Light exposure of photoreceptors trigger photo-oxidative damage of proteins and phospholipids of the outer segments (Beatty 2000, 115-134). Therefore, the outer segments must be regenerated to keep the excitability of photoreceptors (Bok 1993, 189-195, Bok 1971, 664-682, Finnemann 2003, 337-342, LaVail 1976, 1071, Steinberg

1985, 327-346). The tips of the outer segments are shed from the photoreceptors and phagocytized by the RPE, and the new outer segments of photoreceptor are assembled from the connecting cilium (LaVail 1976, 1071).

Water accumulates in the subretinal space from vitreous humour and metabolic activity of photoreceptors (Hamann 2002, 395-431, MF 1990, 340-344, Moseley 1984, 145-151). Na^+-K^+ pump and secondarily the HCO_3^- transport system of RPE actively pumping water and electrolytes out of the subretinal space (Frambach DA 1985, 547-552, Hughes BA 1984, 875-899, Marmor MF 1980, 893-903, MF 1990, 340-344, S 1987, 1776-1782). The RPE has a high capacity for water transport and it transport of RPE is mostly driven by a transepithelial transport of Cl^- from the subretinal space (Rymer 2001, 1921-1929, Stamer 2003, 2803-2808, Steinberg 1985, 327-346). RPE-mediated dehydration of the subretinal space also modulates the bonding properties of the interphotoreceptor matrix. As it helps to bond the neurosensory retina with the RPE, it preserves retinal attachment. In normal conditions, water does not accumulate in the subretinal space.

The RPE helps to constitute a constant ion composition in the subretinal space (Steinberg 1983, 1315-1323, Steinberg 1973, 365-372). Na^+-K^+ ATPase is localized at the apical side of RPE cell membrane(Gundersen D 1991, 863-872) and controls the flux of Na^+ and K^+ ions across the plasma membrane. By doing so, RPE can keep the proper balance of ions in the interphotoreceptor matrix and establish membrane potentials (Tate DL 1995, 1271-1279).

1.2. Intracellular Ca^{2+} and RPE functions

RPE functions for vision are controlled by increase in intracellular-free Ca^{2+}. It is related with dark adaptation of photoreceptor activity, transepithelial transport of ions and water, phagocytosis, secretion, differentiation (Sonke wimmer 2007, 263-301).

In the photoreceptor outer segments, Ca^{2+}-dependent mechanisms occur dark adaptation of photoreceptor activity (Korenbrot 1995, 285-300, 2002, 179-203). In the dark, the subretinal Ca^{2+} concentration is increased (Gallemore 1994, 753-761) and increased subretinal Ca^{2+} source may be melanosomes of the RPE (Drager 1985, 6716-6720, Hess 1975, 471-479, Lavallee 2003, 3654-3662, Moriya 1996, 11-18, Salceda 2000, 223-229).

Increase in intracellular free Ca^{2+} stimulates Cl^- and water transport of RPE cell. The activation of apically located K^+ channels, opening of Ca^{2+}-dependent Cl^- channels in the basolateral membrane (Hartzell 2003, 453-469, Qu 2004, 371-382, Quinn 2001, 255-264, Strauss 1996, 189-200, Ueda 1994, 331-342) and modulation of the electrical driving forces (Edelman 1994, C957-C966) provoke Ca^{2+}-dependent stimulation of Cl^- transport.

In RPE cell, increased intracellular free Ca^{2+} of RPE regulates phagocytosis (Greenberger 1985, 361-372, Hall 1991, 591-598, Hess 1975, 471-479, Nakashima 1989, 149-154, Nguyen-Legros 2000, 245-313, Strauss 1998, 101-111). Stimulation by ATP or growth factors occurs to secrete of growth factor by

the RPE (Guillonneau 1997, 198–206, Mitchell 2001, 193–202, Reigada 2006, 707–720, Rosenthal 2004, 1203–1208, Slomiany 2004, 746–753). The stimulation of the RPE by ATP (Mitchell 2001, 193–202, Peterson 1997, 2324–2337, Ryan 1999, 745–759) or growth factors (Rosenthal 2004, 1203–1208) leads to increase intracellular free Ca^{2+} (Rosenthal 2005, 241–247). In RPE cell, increased intracellular free Ca^{2+} is involved in differentiation. Stimulation of the RPE by basic fibroblast growth factor (bFGF) occurs to an increase in intracellular free Ca^{2+} and to changes in the expression of the immediate early gene c-fos (Rosenthal 2005, 241–247).

1.3. Purine receptors on RPE

ATP can be released by many different cells including RPE cells (Mitchell 2001, 193-202, Reigada 2005, C617-C624, 2005, C132-C140). ATP can act as an autocrine or paracrine extracellular messenger. (Burnstock 2004, 793-803, 2004, 31-304.). Cellular responses to ATP are mediated through purinergic receptors. Some of the ATP can stimulate the P2 receptors on RPE cells and lead to increase intracellular Ca^{2+} concentrations in RPE cells. There are multiple P2 receptors in the RPE. ATP can bind to two receptor types; the P2Y and P2X receptor. ATP-dependent signaling changes the membrane conductance and that shows the functional presence of P2X receptors(Ryan 1999, 745-759). Extracellular application of ATP can cause the activation of cation channels and lead to the depolarization of RPE cells (Sonke wimmer 2007, 263-301). The contributions of the P2Y2 receptor have been explored in PPE cells (Clare H. Mitchell 2008, 101-107). The P2Y2 receptor was initially identified in cultured human RPE(Sullivan DM 1997, 43-52). Subsequently, the $P2Y_1$ and $P2Y_{12}$ receptors were reported in ARPE-19 cells, the functional P2X receptor was reported in rat RPE (Ryan 1999, 745-759) and the functional P2Y2 and P2Y6 receptors were expressed in RPE cells (Relvas 2009, 1241-1246).

Whereas P2X receptors are a ligand-activated ion channels (North 2002, 1013-1067), P2Y receptors are G protein coupled receptors (mainly $Ga_{q/11}$ or $Ga_{i/o}$ subunits) (Burnstock 2004, 793-803, 2004, 31-304). The P2Y receptors are

heptahelical receptors and have seven hydrophobic α-helical arrays of 20-26 amino acids each of them span the plasma membrane between the extracellular amino terminus and the cytosolic carboxyl-terminal domain. Three extracellular and three intracellular 10- to 50-amino acid-long loops interconnect the putative seven transmembrane domains (Eduardo R 2003, 59-96).

Three transductional pathways are classically associated with P2Y receptor occupation:(Neer 1995, 249-257) (1) activation of phospholipase C by Gq-coupled P2Y receptors, (2) activation of adenylyl cyclase by Gs-coupled P2Y receptors, and (3) inhibition of adenylyl cyclase by Gi-coupled P2Y receptors When expressed in 1321N1 cells, the $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are preferentially coupled to the pertussis toxin-insensitive Gαq/βɣ heterotrimer, supporting the activation of phospholipase C-β. Phospholipase C hydrolyzes phosphatidylinositol 4,5-biphosphate and generates two second messengers, inositol 1,4,5-triphosphate (InsP3), which promotes the release of Ca^{2+} from intracellular stores, and diacylglycerol (DAG) which activates protein kinase C (Eduardo R 2003, 59-960,(Neer 1995, 249-257).

1.4. Ca^{2+}-activated K^+ channels on RPE

Ca^{2+}-activated K^+ channels are classified as three subgroups (Wei 2005, 463-472) : (1) SK channels with a small K^+ conductance (4-14 pS), (2) IK channels with an intermediate K^+ conductance (20-80 pS), and (3) BK or maxi K^+ channels with a large conductance (200-300 pS). SK and IK channels are gated solely by intracellular Ca^{2+}, but BK channels are gated by both intracellular Ca^{2+} and voltage. Ca^{2+}-activated K^+ channels are formed with four subunits with six transmembrane domains. The BK channel has an additional N-terminal transmembrane domain (S0) (Bond 1999, 370-378).

Application of ATP occurs to increase intracellular Ca^{2+} concentrations in RPE cells through the activation of purinergic receptors. Increase in intracellular free Ca^{2+} stimulates the apical to basolateral fluid transport (Peterson 1997, 2324-2337) that is driven by Ca^{2+}-dependent Cl^- currents and is possibly supported by Ca^{2+}-activated K^+ channels. The BK channels may serve protective mechanisms to oxidative stress in RPE cells (Sheu 2003, 1237-1244, Santarelli 2006, 329-348, Mainster 1987, 304-310, Tanito 2002, 2392-2400, Van Best 1997, 77-88). Ca^{2+}-activated K^+ channels are found close to voltage-gated Ca^{2+} channels in neurons (Gola 1993, 689-699, Grunnet 2004, 36445-36453, Marrion 1998, 900-905). In neuronal tissue, Ca^{2+}-activated K^+ channels may play a role as feedback modulators for voltage-gated Ca^{2+} channels that supply the Ca^{2+} needed for the activation of Ca^{2+}-activated K^+ channels (Fig 1). In non-neuronal tissues, Ca^{2+}-

activated K^+ channels are involved in cell cycle regulation, volume regulation and ion secretion or absorption (Begenisich 2004, 47681-47687, Feranchak 2004, 903-913, Huang 2002, 1185-1203, Joiner 2003, G185-G196, Liu 2002, 1840-1849)

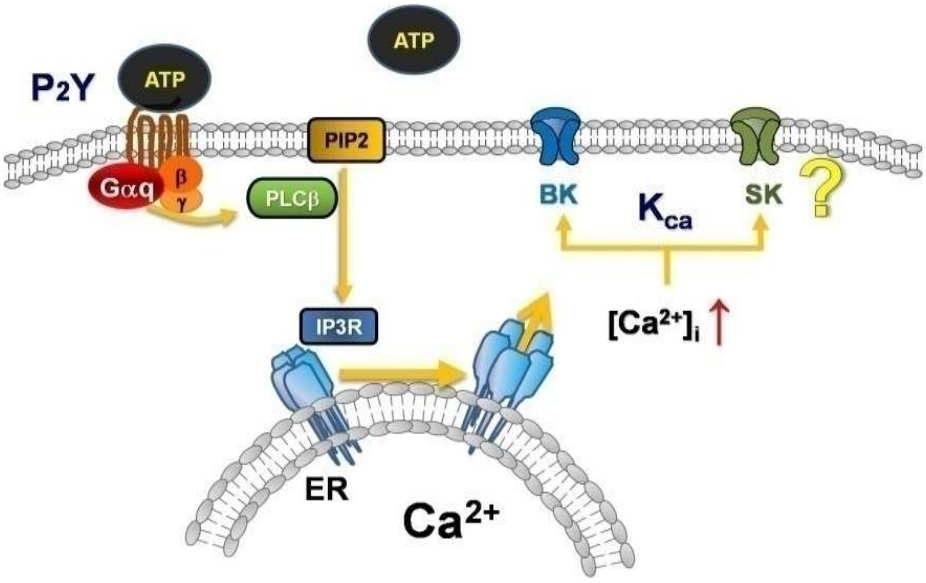

Figure 1. Possible mechanisms for Ca^{2+} activated K^{+} channels by ATP in RPE cells

1.5. Kir4.1 channel

Based on their biophysical properties and sensitivity to intracellular signals, the Kir channels are classified as seven subfamilies (Kubo 2005, 509-526). Different subtypes of Kir channel subunits were identified in the RPE (Ettaiche 2001, 118-129, Ishii 1997, 7725-7735, Kusaka 2001, 27-36, Shimura 2001, 329-346, Yang 2003, 3178-3185). RT-PCR and immunohistological studies identified the additional expression of Kir4.1 (Kusaka 1999, 373-381) in the RPE of rats (Ettaiche 2001, 118-129). Kusaka reported that Kir4.1 channels was also localized in the apical membrane by single channel recordings from the apical side of RPE cells and by immunohistochemistry (Kusaka 1999, 373-381)..

K^+ continuously enters the RPE cells through the apical membrane via the Na^+-K^+-ATPase and a Na^+-K^+-$2Cl^-$ cotransporter since very little K^+ is transported (Dornonville de la Cour 1993, 1-32), the RPE needs a way to recycle the K^+ at the apical membrane (la Cour 1986, 461-479). Kir7.1 channels and Kir4.1 channels are expressed in the apical membrane. Owing to weak inwardly rectifying properties, Kir channels are appropriate for K^+ recycling through the apical membrane. In the dark, K^+ recycling through the inward rectifier and the Na^+ recycling through the Na^+-K^+-ATPase on the apical membrane supports Cl^- transport through the cell (Bialek 1994, 401-417, La Cour 1992, 921-931). This Cl^- transport supports fluid transport in the retina to choroid through the RPE (DiMattio 1983, 409-420, Frambach 1983, F679-F685, Miller 1990, 283-300,

Tsuboi 1986, F781–F784). Kir channels in the apical membrane provide the absorption of water across the RPE. Kir channels support the reduction of the subretinal space and help to prevent retinal detachment. According to some studies, Ba^{2+}-sensitive K^{+} channel is concerned in the regulation of the RPE cell volume itself (Adorante 1995, C89–C100, Kennedy 1994, C676–C683).

As fore cited quotations, several previous studies of Kir 4.1 channel have been reported. But the details of Kir 4.1 channel mechanism have not been completely defined. The purpose of this study is to investigate whether application of ATP to RPE lead to change Kir 4.1 channel current density and which type of G protein is activated in this current density change. Furthermore, it is investigated whether this transductional pathway is through DAG, and endocytosis contributes to the change of Kir 4.1 channel current density (Fig. 2).

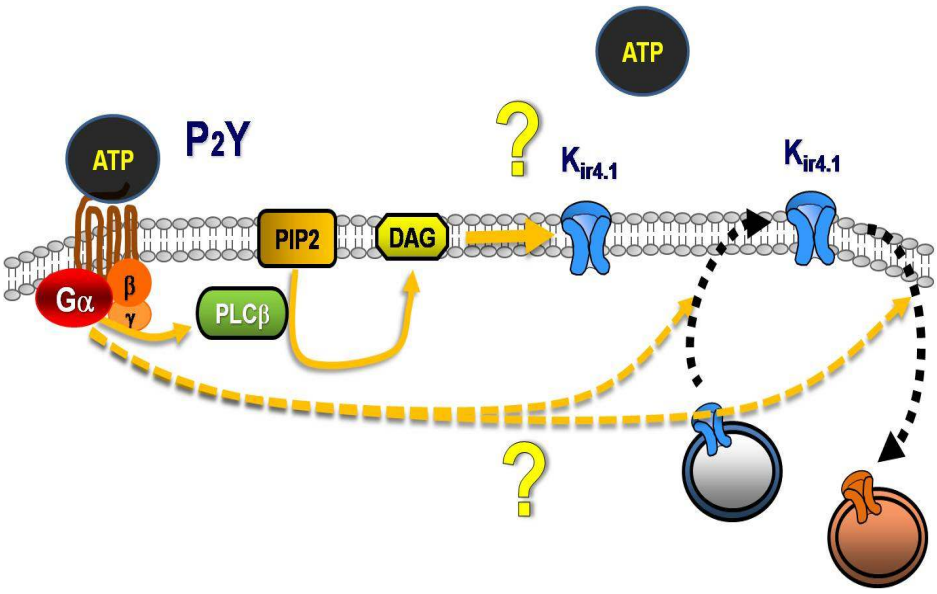

Figure 2. Possible Mechanisms for $K_{ir4.1}$ channels by ATP

Ⅱ. MATERIAL AND METHODS

2.1. ARPE-19 cell line

ARPE-19 is a spontaneously arising retinal pigment epithelia (RPE) cell line derived in 1986 by Amy Aotaki-Keen(Dunn et al. 1996, 155-69) from the normal eyes of a 19-year-old male who died from head trauma in a motor vehicle accident. The cells were subjected to selective trypsinization for the first four passages to remove superficial cells before passaging the cuboidal basal layer (ATCC, Manassas, VA). The ARPE-19 cells were cultured in Dulbecco's modified eagle medium: F-12 nutrient mixture (Invitrogen, Karlsruhe, Germany), which contained 10% fetal bovine serum (Invitrogen), 1% penicillin - streptomycin (Invitrogen). Cells were cultured in a humidified ambient atmosphere containing 5% CO_2 at 37 °C.

2.2. Transient expression of K_{ir} 4.1 channel in human embryonic kidney (HEK) 293 cells.

HEK-293 cells were transiently transfected with epitope-tagged channel constructs indicated the HA-tagged using the FuGene 6 reagent and incubated at 37°C for 24-48 hr.

2.3. RT-PCR analysis

Total RNA from ARPE-19 cells was prepared using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The complementary DNA (cDNA) was synthesized from 1ug of total RNA using 50U MuLV reverse transcriptase. The resultant PCR products were separated and visualized on a 1.5% agarose gel containing ethidium bromide (EtBr).

2.4. Western blot analysis of siRNA knockdown of endogenous caveolin-1 and CHC

For co-immunoprecipitation experiments, the medium was removed and the cells washed once with 1 x PBS. The cells were lysed with 1 x RIPA buffer, and the lysates were centrifuged at 15,000 rpm for 1 hr at 4°C. The protein concentrations in the cell lysates were determined using the BCA protein assay reagent with BSA as a standard, and then adjusted to the same concentration with buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis using 11% acrylamide gel and then processed for immunoblotting, and the levels of protein expression determined. The cellular lysates were used for co-immunoprecipitation as described (Cha, Wu, and Huang 2008, F1212-21). Briefly, the polyclonal anti-Kir4.1 antibody was separately loaded onto the Dynabead-protein A complex and slowly rotated for 2 hours. The antibody-loaded

Dynabead-protein A complex was rinsed twice, the beads were mixed with the various cell lysates, and rotated in the cold room overnight. The supernatants were discarded and the Dynabead-protein A complex was washed. Loading buffer was added and vortexed vigorously. The tubes were placed in Dynal-MPC to collect the sample buffer. The samples were heated at 55°C for 20 min for channel proteins or boiled for 5 min, and then subjected to SDS-PAGE for immunoblotting using the antibodies indicated.

2.5. Expression of siRNA

The siRNA sequences are respectively, sense and anti-sense for caveolin-1, 5'- CUAAACACCUCAACGAUGAUU and 5'- UCAUCGUUGAGGUGUUUAGUU, and for clathrin heavy chain, 5'- UCCAAUUCGAAGACCAAUU and 5'- AAUUGGUCUUCGAAUUGGA (Cha, Wu, and Huang 2008, F1212-21). The siRNAs (200 pmol) are transiently transfected into HEK-293 cells in 30 mm dishes with cDNAs as indicated using Polyfect (Qiagen Inc. Valencia CA) and studied two days later. The effects of the siRNAs are documented by demonstrating reduced protein expression by western blot.

2.6. Measurement of intracellular calcium concentration

Fluorescence microscope (Olympus, Tokyo, Japan) equipped with a ratio fluorescence system (Photon Technology International Inc., Lawrencelille, NJ) Cells were grown on a glass coverslip coated with poly-L-lysine for Ca^{2+} imaging experiments. Intracellular Ca^{2+} concentration was measured using fura-2/AM plus pluronic acid F-127 (all from invitrogen, Oregon, USA) as appropriate. Cells were loaded for 30 min at 37°C with 5 uM fura-2/AM with 0.01% pluronic F-127 in culture medium, and subsequently washed three times with the dye-free external solution. The coverslip was then transferred onto a perfusion chamber positioned on a microscope. After dye-loaded, cells were alternatively excited at 340 nm and 380 nm and the emitted light (510 nm) was captured. The intracellular Ca^{2+} concentration was derived from the ratio of the fluorescence intensities for each of the excitation wavelength (F340/F380) (Grynkiewicz, Poenie, and Tsien 1985, 3440-50).

2.7. Patch-clamp recordings

K^+ currents were recorded under the whole cell-ruptured and single channel-attached configuration of the patch clamp technique (Hamill et al. 1981, 85-100) as described previously (Cha, Wu, and Huang 2008, F1212-21, Kong et al. 2000, 331-7). Patch electrodes were fabricated from a borosilicate glass capillary

(BF150-86-15 used for single channels, BF150-117-10 for whole cell, Sutter Instrument, Novato, CA). The electrodes were coated with Sylgard 184 (Dow corning, Midland, MI), fire polished on microforge, and had resistances of 1.5~2.5 MΩ in whole cell-ruptured, 8~12 MΩ in single channels-attached and excised when filled with the internal solution described below. An Ag/AgCl pellet connected via a 0.15 M NaCl/agar bridge was used to ground the bath. For the single channel recordings, data were sampled at 1 kHz and filtered at 0.2 kHz using pulse/pulsefit (v 8.50) software (HEKA Electronik, Lambrecht, Germany). All points amplitude histograms were constructed and open probabilities were determined from 30 sec or more recordings. In the other side, for the whole cell recoding, current traces were generally low-pass filtered at 5 KHz using the Bessel filter in the clamp amplifier, digitized at 2 KHz, and stored on the computer hard drive for later analysis. Capacitance and access resistance are monitored and compensated 75% using EPC 9 patch clamp amplifier (HEKA Instruments Inc. Bellmore, NY), and the voltage protocol is a 0 mV holding potential and 400 ms steps from -100 to + 100 mV in 20 mV increments (Lazrak, Liu, and Huang 2006, 1615-20) using pulse/pulsefit (v 8.50) software (HEKA Electronik, Lambrecht, Germany). All recordings were performed at room temperature.

2.8. Solutions and chemicals

For recording Ca^{2+} activated K^{+} channels in cell attached or excised patches,

the bath solution contained (mM): 140 KCl, 1 EGTA, 0.61 $CaCl_2$ and 10 HEPES adjusted to pH 7·4 with Tris. Two concentrations of Ca^{2+} were added to bath solutions buffered by 1 mM EGTA to create Ca^{2+} activities from 10^{-7} to 10^{-6} M. To isolate K^+ current, patch pipettes were filled with an internal solution containing (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES adjusted to pH 7·4 with Tris. For identification of Ca^{2+} activated K^+ channels through symmetric voltage-dependent single channel currents, the pipette solutions were identical to the single channel bath solution, which were included 100 nM charybdotoxin to inhibit BK and/or 100nM apamin to inhibit SK. To isolate K^+ currents in the HEK-293 cells which over-expressed by Kir4.1 channels, the extracellular bath contained (mM): 145 KCl, 2 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES pH 7.4, and the pipette contained (mM): 145 KCl, 2 EDTA and 10 HEPES pH 7.4. Drugs were applied to single cells via a gravity-fed fused silica capillary tube connected to an array of polyethylene tubes. Stock solutions (10uM~100mM) were made for the following drugs: adenosine 5'-triphosphate (Sigma Chemical Co., St. Louis, MO, USA), charybdotoxin, apamin (both from Alomone Labs, Jerusalem, Israel). All drugs were stored at -20°C or -80°C and dissolved in distilled water.

2.9. Data analysis

The open probability of single channels statistical analyses were performed using multi-peaks of Gaussian distribution by Origin 7.5. Data represent means ± standard error of the mean. Values of n are the number of cells. Student's paired t tests were performed and *p* values of less than 0.05 were considered significant.

Ⅲ. RESULTS

3.1. Ca^{2+}-activated K^+ channels in ARPE-19 cells

Application of ATP caused the marked increases in the intracellular Ca^{2+} levels in ARPE-19 cell even in absence of extracellular Ca^{2+} (Fig.3). These ATP-induced Ca^{2+} transients were significantly inhibited by U73122 (1 μM), but not by U73433, an inactive analogue (1 μM) (Fig.4). As illustrated in figure 5, the presence of large conductance Ca^{2+}-activated K^+ channel (BK) are identified by expression profile of transcripts encoding BK channel subunits using RT-PCR analysis as a previous study (Wimmers et al. 2008, 2340-8, Traut et al. 2009, 28), The ARPE-19 cells expressed transcripts for various BK channel subunits (i.e., α1, β3 and β4) (Fig. 5). And also, BK channels were recorded in cell attached and inside-out patch mode, and their opening probability wase increased significantly (Fig 6-8).

In other hands, as shown in Fig 9, the presence of small conductance Ca^{2+}-activated K^+ channel(SK) was identified by expression profile of transcripts encoding SK channel subunits (i.e., SK1, SK3 and SK4/IK. Moreover, as shown in Fig 10, application of ATP led to an increase of non-BK calcium-activated potassium channel under the cell attached patches using asymmetrical K^+ gradients (5 mM / 140 mM. The open probability of SK channels was increased from 0.1 to 0.63 by the application of ATP. The single-channel conductances for

non-BK channels were 219.6 ± 3.26 pS, 20.24 ±0.73 and 12.55 ± 0.11 pS, respectively (Fig.11-12).

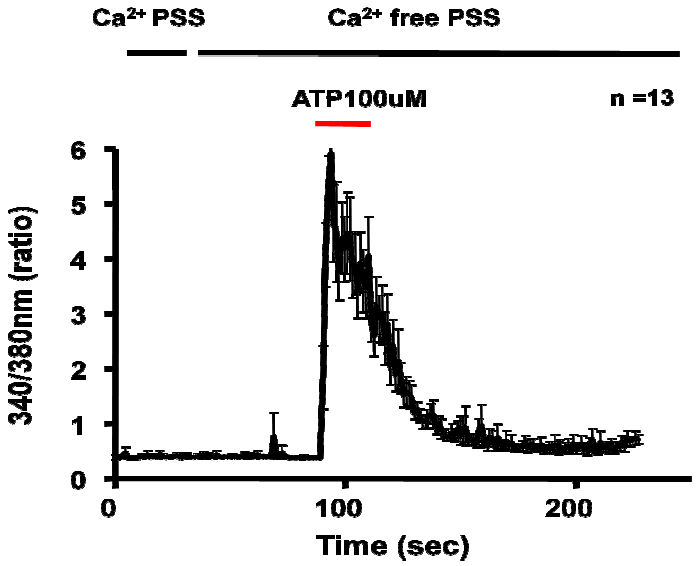

Figure 3. ATP-induced Ca^{2+} responses in ARPE-19 cells using imaging technique
Fura-2-loaded ARPE-19 cells were incubated at 37°C, and the transient elevations of $[Ca^{2+}]_i$ were monitored using the 340:380 nm ratio. Typical traces of ATP (100 μM) responses in Ca^{2+} free PSS (n = 13 cells).

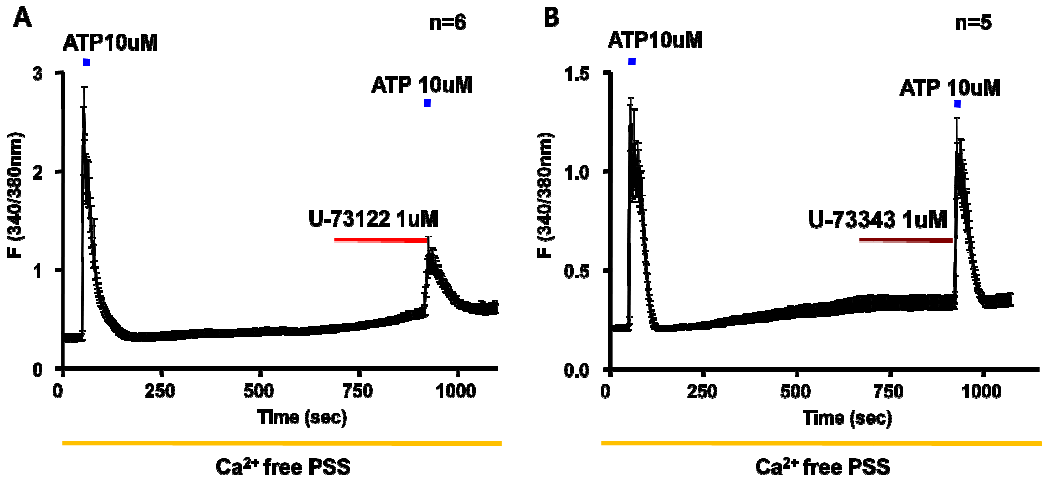

Figure 4. Role of phospholipase C in ATP-induced cytosolic calcium releases in ARPE-19 cells. Reversible ATP-induced Ca^{2+} transient was inhibited by pretreatment (3 min) of U73122. Effects of phospholipase C inhibitor U73122 (1 μM), and inactive analogue U73343 (1 μM) were investigated separately in the same ARPE-19 cells (A and B), and drug additions were as indicated by the horizontal bars.

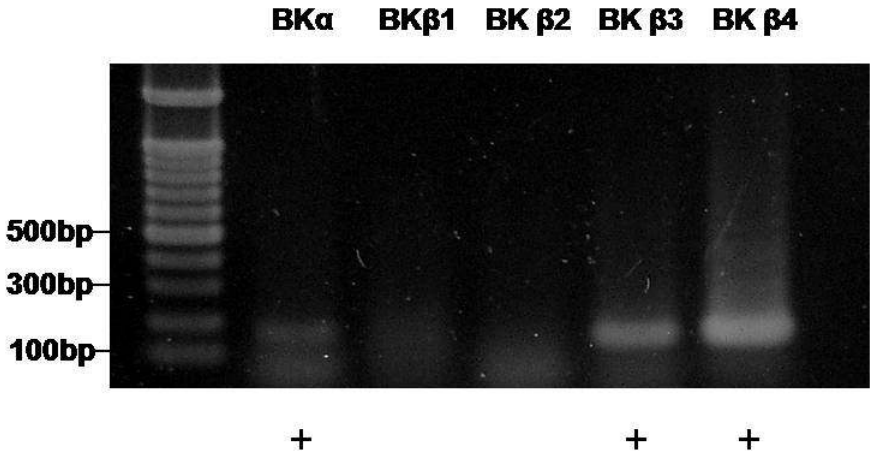

Figure 5. Various large conductance Ca^{2+}-activated K^+ channels are expressed in ARPE-19 cells. The Cultured ARPE-19 cells express mRNAs encoding BK channels as shown by RT-PCR.

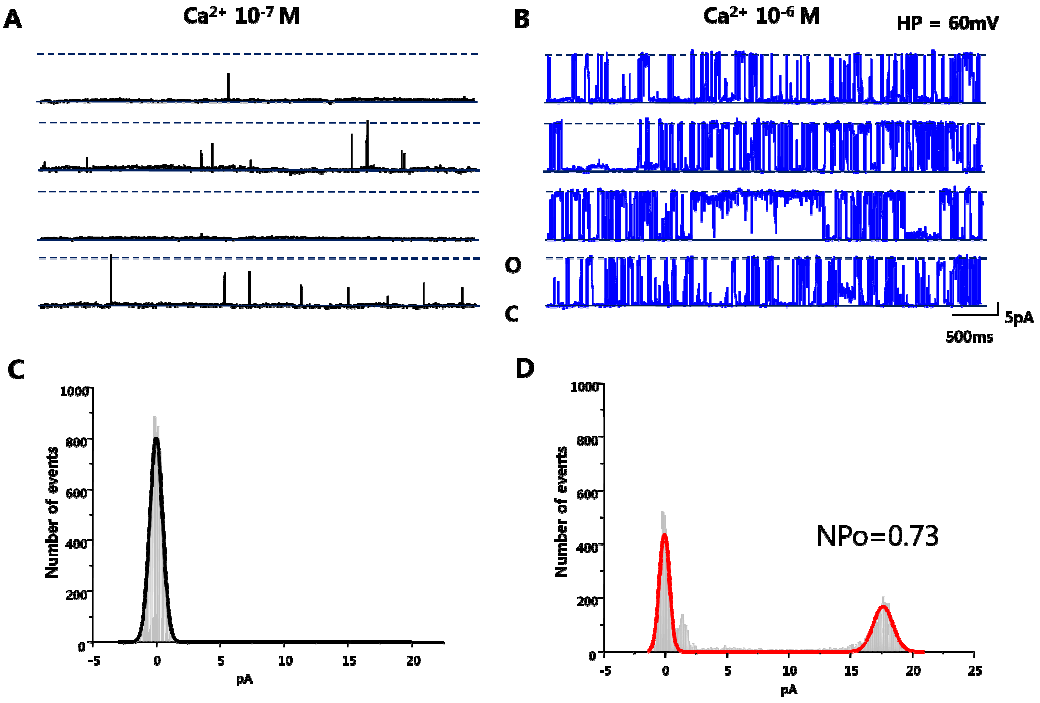

Figure 6. Ca^{2+} sensitivity on BK channels of ARPE-19 cells in inside-out patches. (A) The patches were held at 0 mV in asymmetrical K^+ concentrations (5 mM in pipette and 140 mM in bath solution). The intracellular surface of the membrane was exposed to 10^{-7} M free Ca^{2+}. The Ca^{2+} was increased to 10^{-6} M in (B). To activate BK channels, the cells recorded at a holding potential of 60 mV. Traces under the main recordings in A and B show the periods denoted by horizontal bars on an expanded time scale. (C and D) All points histograms from recordings in 10^{-7} M and 10^{-6} M Ca^{2+} on the ARPE-19 cells, respectively. Peaks at specified currents are indicated by Gaussian fit.

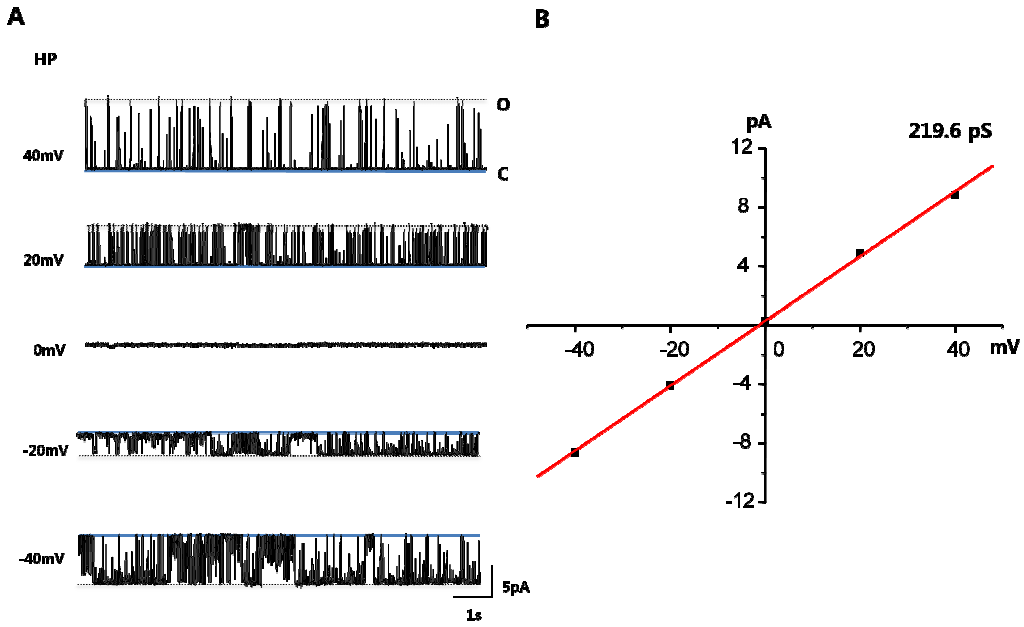

Figure 7. Identification of BK channels which are functional in inside-out recordings of ARPE-19 cells. (A) Symmetric voltage-dependent BK analysis of on-cell channel recording (from -40 mV to 40 mV pipette potential) (B) The BK current-voltage relationship yielded a single-channel conductance of 219.6 ± 3.26 pS and a reversal potential of -2mV (n = 3 recordings on individual cells). Data points represent means ± SEM (error bars only depicted when more than two values were available). All currents were recorded under symmetrical high K^+ concentration.

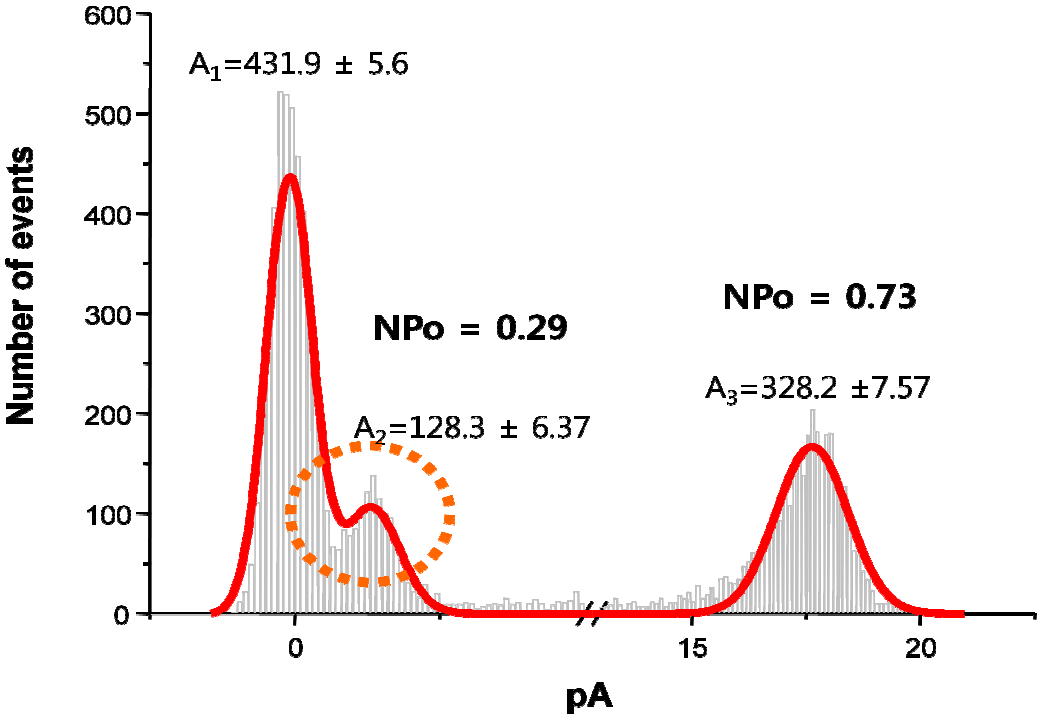

Figure 8. Two different types of Ca^{2+}-activated K^{+} channels of ARPE-19 cells in inside-out patches. All points histograms from recordings in 10^{-6} M Ca^{2+} on the ARPE-19 cells, respectively. Peaks at specified currents are indicated by Gaussian fit.

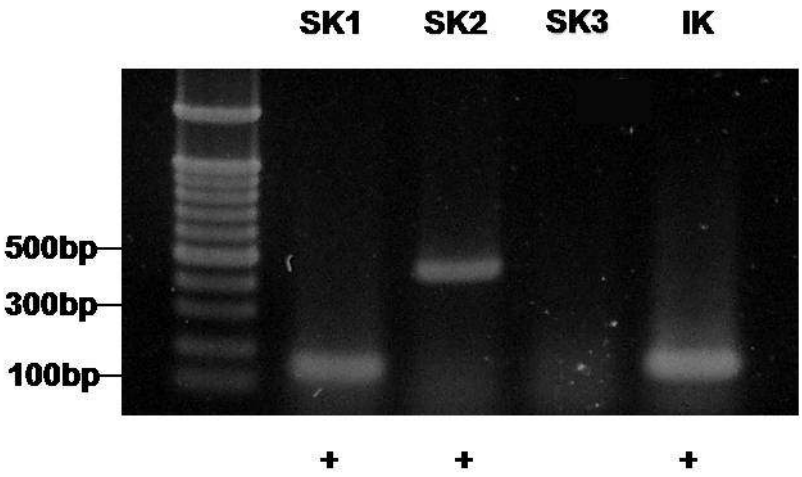

Figure 9. Various Small conductance Ca^{2+} - activated K^{+} channels are expressed in ARPE-19 cells. Cultured ARPE-19 cells express mRNAs encoding SK and IK channels as shown by RT-PCR.

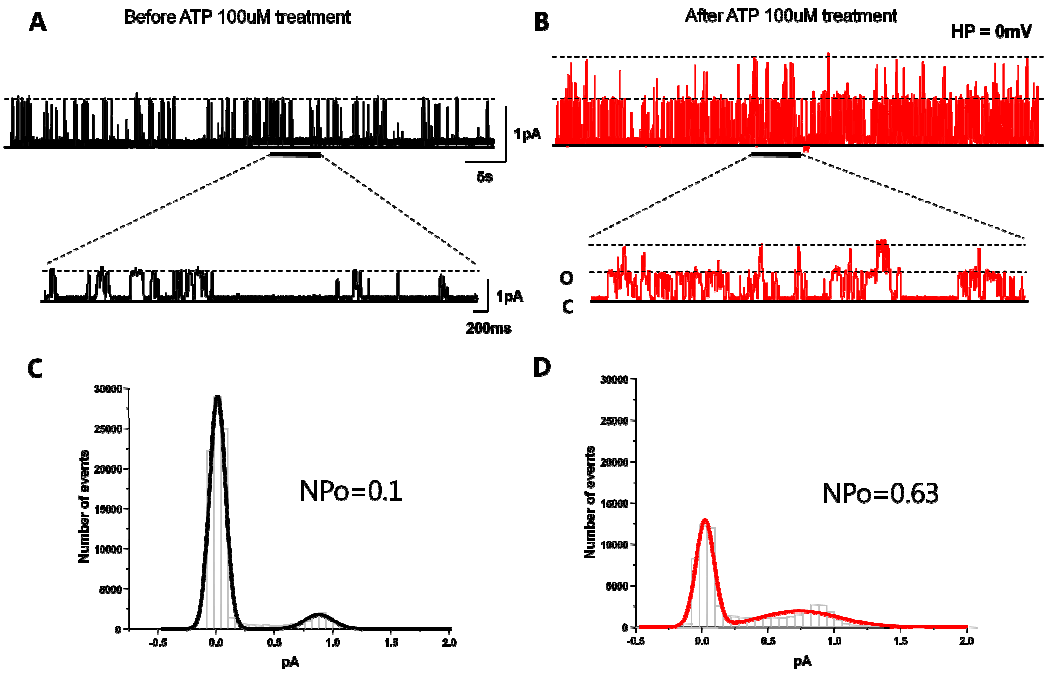

Figure 10. Effects of ATP on SK channels in ARPE-19 cell during on-cell patches. (A and B) On-cell outward patch currents of SK channels before and after 100 μM ATP treatment, the cells recorded at a holding potential of 0 mV in asymmetrical K^+ concentrations (5 mM in pipette and 140 mM in bath solution; 100 mM Chrybdotoxin in pipette; 10^{-7} M free Ca^{2+} in bath). O, channel open state; C, channel closed state. (C and D) All points amplitude histograms from recordings in A and B, respectively. Peaks at specified currents are indicated by Gaussian fit.

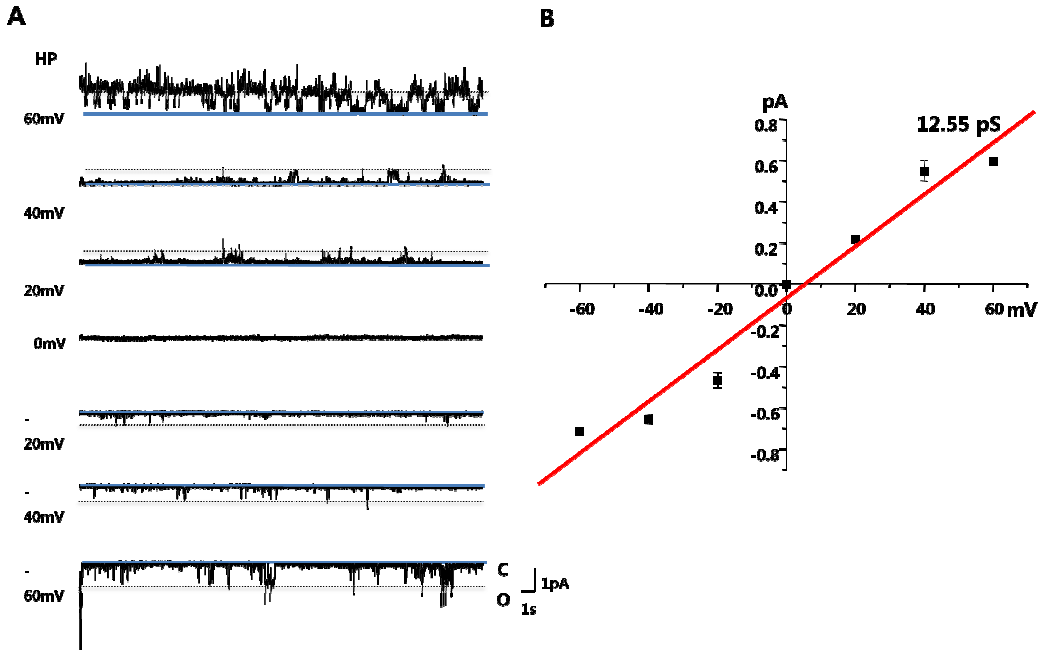

Figure 11. Identification of SK channels which are functional in inside-out recordings of ARPE-19 cells. (A) Symmetric voltage-dependent SK analysis of on-cell channel recording (from -60 mV to 60 mV pipette potential) after chrybdotoxin (100 nM) added to the pipette. (B) The SK current-voltage relationship yielded a single-channel conductance of 12.55 ± 0.11 pS and a reversal potential of 5 mV (n = 3 recordings on individual cells). Data points represent means ± SEM (error bars only depicted when more than two values were available). All currents were recorded under symmetrical high K^+ concentration

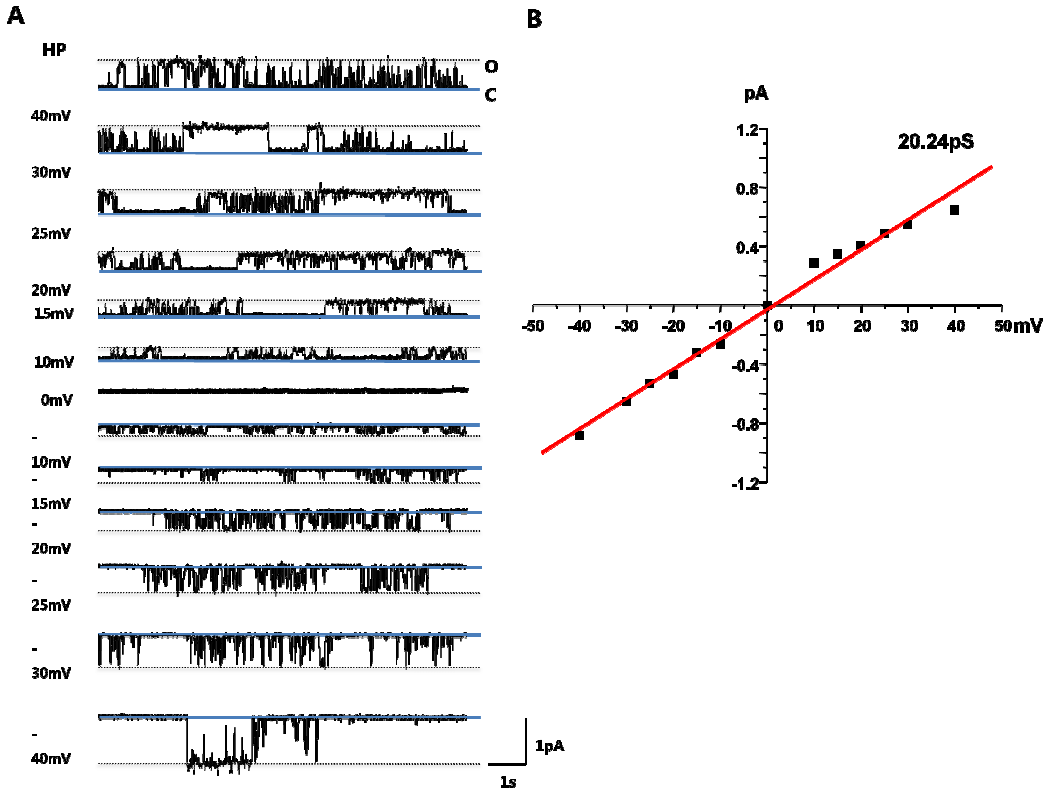

Figure 12. Identification of IK channels which are functional in inside-out recordings of ARPE-19 cells. (A) Symmetric voltage-dependent IK analysis of on-cell channel recording (from -40 mV to 40 mV pipette potential) after chrybdotoxin (100 nM) and apamin (100 nM) added to the pipette. (B) The IK current-voltage relationship yielded a single-channel conductance of 20.24 ±0.73 pS and a reversal potential of 2 mV (n = 3 recordings on individual cells). Data points represent means ± SEM (error bars only depicted when more than two values were available). All currents were recorded under symmetrical high K^+ concentration.

3.2. Inward rectifying K^+ channel 4.1 (Kir 4.1).

It was not easy to isolate pure currents of Kir4.1 channels from Kir 7.1 in ARPE-19 cells, HEK-293 cells in which transiently expressed Kir4.1 were used in these experiments. Although there were no acute effects of ATP-induced changes upon current density of Kir4.1 channel (data not shown), the current density of Kir4.1 channel was decreased 64% from 716.4 ± 66.94 pA/pF to 255.7 ± 53.44 pA/pF by incubation of ATP for 24hours (Fig. 13). Various subunits of the inward rectifying K^+ channels are regulated by G protein-coupled receptors that alter the open probability over short time frames through PIP_2 metabolism or interactions among proteins (Lopes et al. 2005, 117-29, Rohacs et al. 2003, 745-50).

Purinergic receptors can signal via members of the $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ families of G protein α subunits. In order to determine which types of G protein α subunit can be responsible, co-expressed cells were used in this study with constitutively active mutant forms of several G protein subunits with Kir4.1 in HEK-293 cells. The subunits tested were $G\alpha_q$ ($G\alpha_{qQ209L}$), $G\alpha_{i2}$ ($G\alpha_{iQ204L}$), $G\alpha_{13}$ ($G\alpha_{13Q226L}$), and $G\alpha_s$ ($G\alpha_{sQ227L}$). Co-expression of Kir4.1 with the G protein α_{iQ204L}, $\alpha_{13Q226L}$, or α_{sQ227L} subunits did not reduce their current density, but only form was statistically different from control group; where it was reduced by 52% by $G\alpha_{qQ209L}$(Kir4.1 2,500 ± 400 pA/pF, vs Kir4.1 + $G\alpha_{qQ204L}$ 1,200 ± 200 pA/pF, $p< 0.05$) (Fig. 14). In general, the downstream of phospholipase C occurred on two

different pathways which are both/either via inositol triphosphate (IP_3) or diacylglycerol. Thus, to test for an effect of protein kinase C which is a kinase downstream of $G\alpha_q$, I treated HEK-293 cells expressing Kir4.1 alone or Kir4.1 with $G\alpha_{qQ204L}$ (constitutively active mutant forms of $G\alpha_q$) with oleoyl-acetyl-glycerol (OAG, 10 μM) a PKC activator. While there were no effects of OAG to the current density of cells only expressing Kir4.1, the current density of Kir4.1 was affected by $G\alpha_{qQ204L}$, as shown in Fig 15. These results indicate that the incubation with ATP affects the current density of Kir4.1 via a mechanism that requires $G\alpha_q$, but that is independent of PKC.

I finally examined whether the reduction in cell surface abundance of Kir4.1 by the ATP via $G\alpha_q$ occurs through a mechanism involving clathrin or caveolin-mediated trafficking. When knocked down in endogenous caveolin-1 or clathrin heavy chain using siRNA in HEK-293 cells co-expressing Kir4.1 and $G\alpha_{qQ209L}$, or vector, the $G\alpha_{qQ209L}$ construct reduced current density by approximately 50% in cells transfected with the control oligonucleotides (Fig. 16). The effect of $G\alpha_{qQ209L}$ was blocked by siRNAs directed against caveolin-1 (Fig. 16A), but it was not done by clathrin heavy chain (CHC) (Fig. 16B). In the cells with the siRNA directed against caveolin- 1 (Fig. 16A), the Kir4.1 current density appeared greater than in the absence of the siRNA suggesting that Kir4.1 may undergo baseline caveolin-1-dependent internalization. Knockdown of endogenous caveolin-1 and CHC was demonstrated in the western blot analysis (Fig. 16C). Thus, the ATP might inactivate Kir4.1 via a signaling pathway that requires Gαq and caveolin leading to reduced cell surface expression.

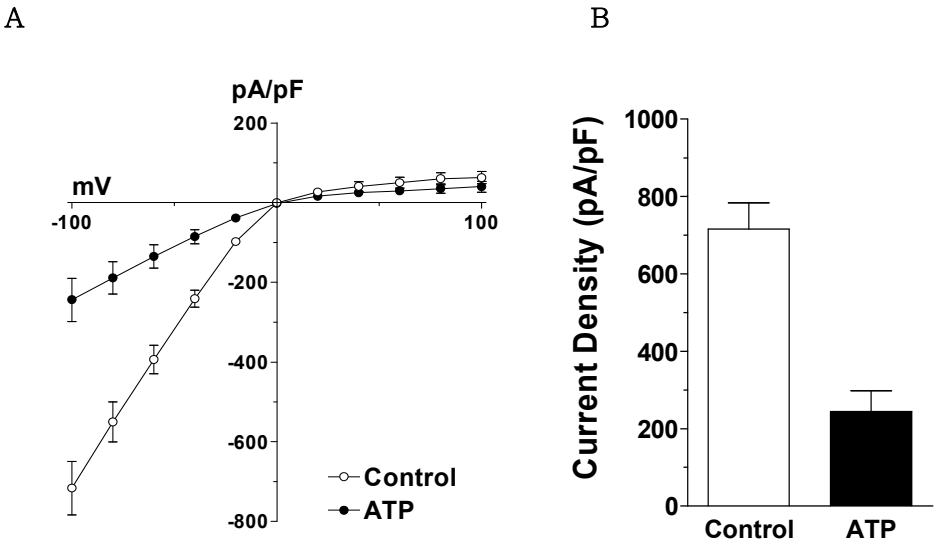

Figure 13. Decrease of inward rectifier K+ channel 4.1 (K_{ir} 4.1) current density by ATP treatment. (A) Effects of adenosine tri-phosphate on K_{ir} 4.1. HEK cells transiently transfected with the HA-tagged Kir4.1pCruz were incubated in media containing 100uM ATP for 24 hours before ruptured whole cell recording. The K_{ir} 4.1 current density (current at -100 mV normalized to the cell surface area; pA/pF, means ± SE, n=7) was evoked by test pulses from -100 to -100 mV with -20 mV increments for 500 ms. (B) Bar graphs of current density (pA/pF at -100 mV) from *A*.

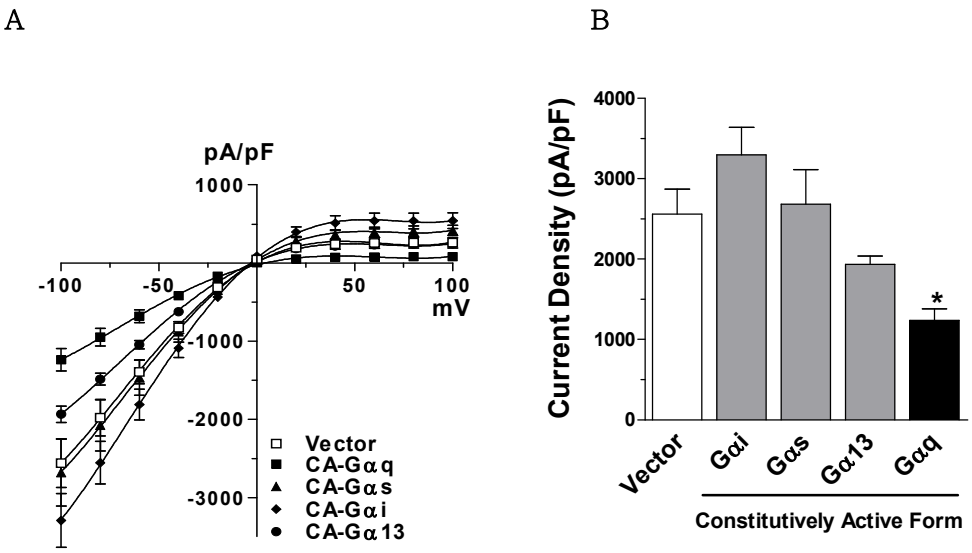

Figure 14. G protein signaling and Kir4.1 cell surface expression and activity. cDNAs coding for Kir4.1 and vector or GTPase-deficient G protein α subunit mutants (GαiQ204L, GαsQ227L, Gα13Q226L or GαqQ209L) were expressed transiently in HEK-293 cells. (A) K_{ir} 4.1 Current density in HEK-293 cells expressing Kir4.1 and vector or the G protein α subunit constructs shown (current at -100 mV normalized to the cell surface area; pA/pF, means ± SE, n=8), which was evoked by test pulses from -100 to -100 mV with -20 mV increments for 500 ms. (B) Bar graphs of current density (pA/pF at -100 mV) from *A*. * indicates $p < 0.05$ compared to Kir4.1 (Vector) alone (ANOVA).

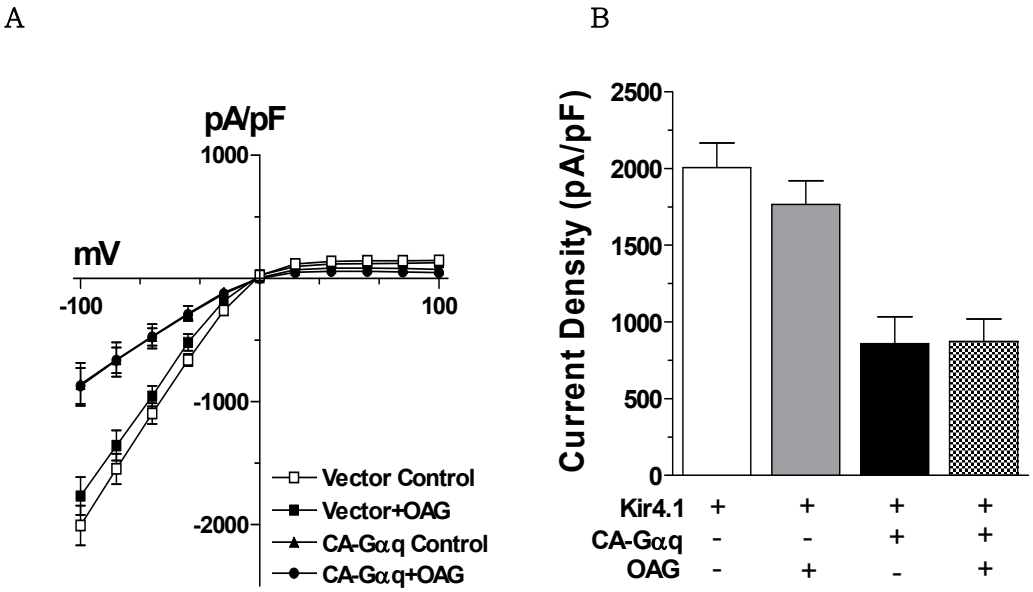

Figure 15. The effect of protein kinase C activation on K_{ir}4.1 channel current density. HEK-293 cells were transiently transfected with Kir4.1 and vector or GαqQ204L as shown, and treated with OAG 10 μM for 1 hr before rupture for current density measurements (n = 10, ± SEM). (A) K_{ir} 4.1 current density (current at -100 mV normalized to the cell surface area; pA/pF, means ± SEM, n=10) was evoked by test pulses from -100 to -100 mV with -20 mV increments for 500 ms. (B) Bar graphs of current density (pA/pF at -100 mV) from *A*.

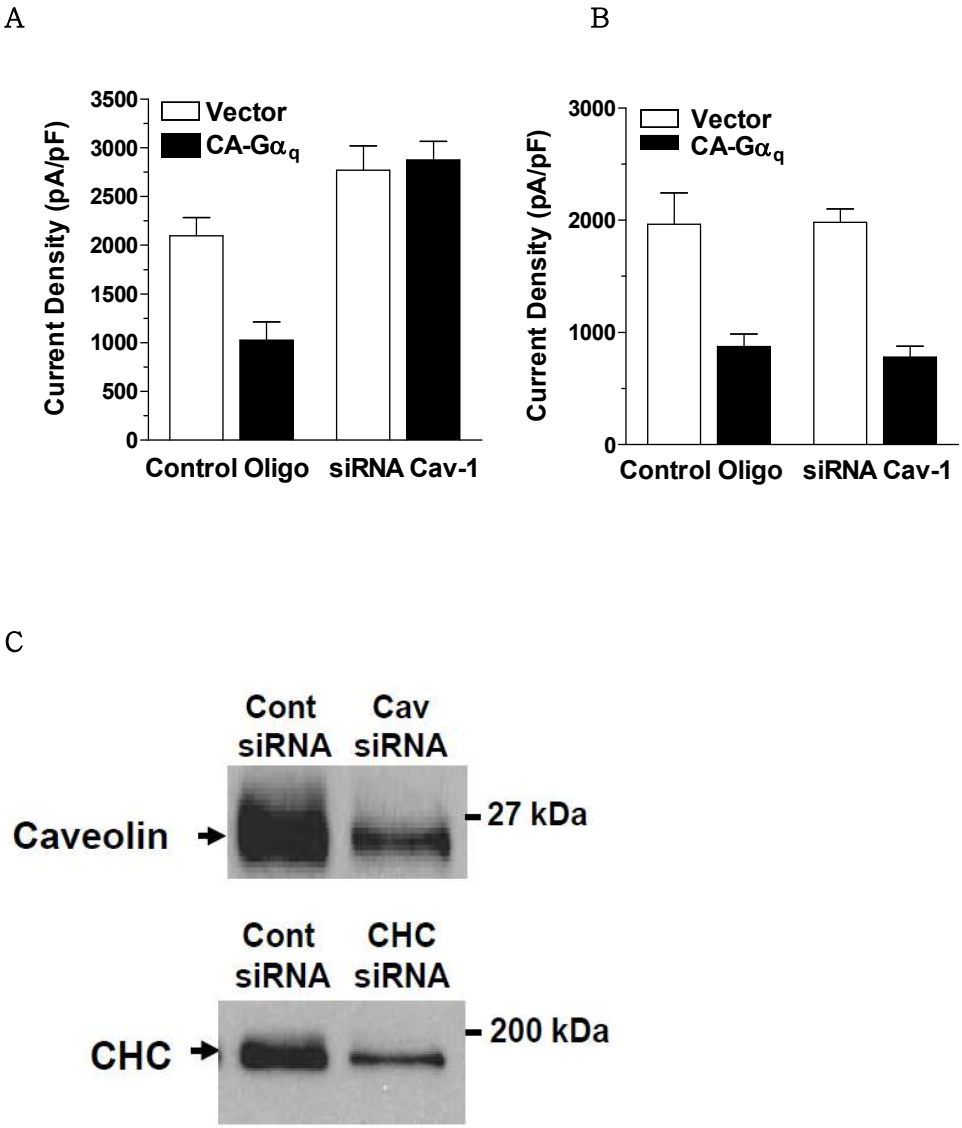

Figure 16. Caveolin versus clathrin-dependent current density of Kir4.1 channels. Cells transiently expressing Kir4.1 alone or Kir4.1 and GαqQ209L were transfected with either a control oligonucleotide or siRNAs directed against caveolin-1 (A, n = 8) or the heavy chain of clathrin (B, n = 8) as indicated. Bars represent mean current density ± SEM (* $p < 0.05$ compared to Kir4.1 alone, ANOVA). Successful knock-down of caveolin-1 (A) and clathrin heavy chain is shown by western blot in panel C.

Ⅳ. DISCUSSION

The RPE cell serves very important functions to support vision. Normal ion channel function of the RPE can play a role in the retinal degenerations. For example, L-type Ca^{2+} channels can carry out the autocrine stimulatory pathway of VEGF secretion. And also the ion channels and transporters might play key roles in the initiation of choroidal neovascularization in age related macular degeneration (Strauss 2003, 3926, Sonke wimmer 2007, 263-301). Malfunction of ion channels such as loss of function or decreased number of channels in the cell membrane can cause channelopathies (Celesia 2001, 2-18, Hubner 2002, 2435-2445, Jentsch 2004, 1039-1047, Lehmann-Horn 1999, 1317-1372, Sonke wimmer 2007, 263-301 , Striessnig 2004, 1341-1346). Channelopathies in the RPE cells could have some deteriorative effects on the retina, cause to lead retinal degenerations such as retinitis pigmentosa, Best's vitelliform macular dystrophy (Cross 1974, 46-50, Godel 1986, 1-31, Sonke wimmer 2007, 263-301 , Weingeist 1982, 1108-1114).

It is well known that two types of Ca^{2+}-activated K^+ channels exist in many cells. SK channels are gated solely by intracellular Ca^{2+}, but BK channels are gated by both intracellular Ca^{2+} concentration and voltage. Ca^{2+}-activated K^+ channels are involved in volume regulation in RPE cells in response to hypotonic stress, BK currents are increased by hypoosmotic stress (Sheu 2004, 563-575). Application of ATP has been shown to increase intracellular Ca^{2+} concentrations

in RPE cells through the activation of purinergic receptors. The increased intracellular Ca^{2+} may open Ca^{2+}-activated K^+ channel. Recent studies have suggested that SK channels in many tissues are activated by purinergic stimulation of P2Y receptors. Coupling between P2Y receptors and SK channel activation occurs via G-protein-dependent activation of phopholipase C and production of IP_3. Localized Ca^{2+} release due to IP_3 receptor-operated stores appears to be the prime stimulus for SK activation. In this study, we found that the activation of purinergic receptor lead to increase not only the open probability of BK channels, but also the one of SK channels (Fig 8. 10). Activation of SK channels leads to outward current and reduced membrane excitability. The present study indicates that the SK channels can be activated via P2Y under the more physiologic conditions. Even transient, localized Ca^{2+} release events may increase the activation of SK channels to and allow the cell to achieve functional increases in SK without large amplitude or sustained Ca^{2+} transients. Because RPE is non-neuronal tissue, SK channel is physiologically more important than BK channel for homeostasis of K^+. The findings of the present study provide an important new dimension to the regulation of SK channels in the RPE cells. It is the first report about SK channels in the RPE cells.

Inward rectifier K^+ (Kir) channels maintain K^+ inward current and bring the membrane potential towards the K^+ equilibrium potential. Kir channels are composed of four pore-forming subunits with two transmembrane domains, a pore loop and cytoplasmic N- and C-termini (Bichet 2003, 957-967). A block of the channel pore by internal Mg^{2+} and cytoplasmic polyamines causes inward

rectification at positive voltages (Lu 2004, 103-129, Nichols 1997, 171-191). Kir channels are activated on hyperpolarization.

In the present study we found that Kir4.1 cell surface abundance was modified after 24 hr exposure of extracellular ATP (Fig 13). The activity of ion channels at the cell surface is a function of the number of channels and the opening probability (Po). Many members of the Kir family of K channels are regulated by G protein-coupled receptors that alter the Po over short time frames through PIP2 metabolism or protein-protein interactions. But we did not have any shot-term effect on the Kir4.1 current density (data not shown). Decreased current density of Kir4.1 channel occured via a mechanism that requires $G\alpha_{q,}$ subunit of G protein, and there were no effects of DAG to change of the current density of Kir4.1 channel. Importantly, current density change was independent of PKC (Fig 14, 15). And also, decreased current density of Kir4.1 channel by long term incubation with ATP occurred through a mechanism involving caveolin-mediated endocytosis (Fig 16). These results suggest that purinergic stimulation could affect the potassium secretion via Kir4.1 channel found in the RPE cells

The studies of ion channel in RPE will enhance the understanding of ocular diseases. Furthermore, the overall function of purinergic signaling should be revealed in more detail under the physiological and many pathophysiological conditions.

Ⅴ. CONCLUSION

ATP has an important regulatory role both in calcium-activated potassium channels and Kir4.1 channels. Purinergic activation increased the the open probability of SK channels as well as one of the BK channels, decreased the current density of Kir4.1 through a mechanism involving caveolin-mediated endocytosis

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Ⅶ. ABSTRACT IN KOREAN

망막색소상피세포에서 퓨린 수용체의 기능적 역할

망막색소상피세포에서 분비되는 ATP 는 자가분비작용으로 세포 내 칼슘이온 농도를 증가시켜서 세포 기능에 중요한 여러 이온 채널에 영향을 준다. 망막색소상피세포와 같은 비신경조직에 분포하는 포타슘 채널은 세포막전위를 유지시키고, 세포의 부피를 조절하며, 이온을 이동시키며, 성장인자 등의 분비 조절에 관여하는 등 세포의 생리기능에 중요한 역할을 담당한다. 그러나 현재 ARPE-19 세포에서의 포타슘 채널에 대한 ATP 의 영향에 대해서는 자세히 알려져 있지 않다. 단지 Ca^{2+}-dependent K^+ channels 중 BK 의 존재만이 보고되어 ATP-induced $[Ca^{2+}]_i$ 증가를 통해 그 활성이 이루어 질 것으로 제안된다. 또한 inward rectifying K^+ channel 4.1 에 대한 ATP 의 영향은 아직 밝혀지지 않은 실정이다. 따라서 본 연구에서는 ATP 가 Ca^{2+}-dependent K^+ channels 과 inward rectifying K^+ channel 4.1 (Kir4.1) 에 어떠한 영향을 주는지 알아보고자 한다. 분자생물학적 및 전기생리학적으로 ARPE-19 cell 에서 Ca^{2+}-activated K^+ channels 의 존재를 확인하였다. 본 연구에서 분자생물학적으로 확인된 Ca^{2+}-activated K^+ channels 의 subunit 은 BKα1, BKβ3, BK β4, SK1, SK3, SK4(IK)였다. BK, IK 그리고 SK channel 의 single-channel conductance 는 각각 219.6 ± 3.26 pS, 20.24 ±0.73 pS 그리고 12.55 ± 0.11 pS 이었다. ATP 에 의해 P2Y 수용체가 활성화되면 주로 세포내 칼슘 보관소에서 칼슘이온이 유리되어 세포내 자유칼슘이온 농도가

증가하였다. ATP 에 의해서 세포내 칼슘 이온이 증가하면 Ca^{2+}-activated K^+ channels 의 open probabilities 는 증가하는 것이 관찰되었다. Open probability 가 증가하는 정도로 볼 때 small conductance 를 갖는 SK 나 IK channel 이 large conductance 를 갖는 BK channel 보다 더 많이 활성화되므로, RPE cell 에서는 small conductance 를 갖는 SK, IK channel 이 세포생리학적으로 더 중요한 것으로 보인다. RPE 세포에 ATP 를 투여하였을 때 Kir4.1 channel 에는 즉각적인 변화가 관찰되지는 않았다. 그러나 24 시간 동안 ATP 를 투여하면서 세포를 배양한 결과 Ca^{2+}-activate K^+ channel 과는 달리, Kir4.1 channel 의 경우에는 P2Y 를 경유하는 ATP 의 작용으로 인해 current density 가 감소하는 것이 관찰되었다. 이러한 current density 감소의 결과는 G protein α subunits 중 $G\alpha_q$ 를 통해 나타난다. 일반적인 $G\alpha_q$ 활성은 inositol triphosphate (IP_3) 와 diacylglycerol (DAG) 를 경유하는 두가지 기전이 있으며, 본 연구에서 감소된 current density of Kir4.1 는 DAG 보다는 IP_3 를 경유 하는 것으로 판단된 추가적으로 $G\alpha_q$ 를 통한 current density 는 cell surface expression 에 의존적으로 변화되는데, 그 주요한 영향은 caveolin-1 을 통한 endocytosis 에 의한 것으로 나타났다.

핵심되는 말: 망막색소상피세포, 퓨린수용체, ATP, Ca^{2+}-activated K^+ channels, Kir 4.1 channel, endocytosis, caveolin-1