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# Functional Expression of P2Y Receptors in WERI-Rb1 Retinoblastoma Cells

**Na-Hyun Kim[1], Kyu-Sang Park[2], Joon Hyung Sohn[3], Byung-Il Yeh[3], Chang Mann Ko[4], and In Deok Kong[2]**

*[1]Department of Basic Nursing Science and Institute for Nursing Science, Keimyung University, Daegu 704-701, Departments of [2]Physiology, [3]Biochemistry, [4]Pharmacology, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea*

**P2Y receptors are metabotropic G-protein-coupled receptors, which are involved in many important biologic functions in the central nervous system including retina. Subtypes of P2Y receptors in retinal tissue vary according to the species and the cell types. We examined the molecular and pharmacologic profiles of P2Y purinoceptors in retinoblastoma cell, which has not been identified yet. To achieve this goal, we used $Ca^{2+}$ imaging technique and western blot analysis in WERI-Rb-1 cell, a human retinoblastoma cell line. ATP (10 $\mu$M) elicited strong but transient $[Ca^{2+}]_i$ increase in a concentration-dependent manner from more than 80% of the WERI-Rb-1 cells (n=46). Orders of potency of P2Y agonists in evoking $[Ca^{2+}]_i$ transients were 2MeS-ATP > ATP >> UTP= $\alpha\beta$-MeATP, which was compatible with the subclass of $P2Y_1$ receptor. The $[Ca^{2+}]_i$ transients evoked by applications of 2MeS-ATP and/or ATP were also profoundly suppressed in the presence of $P2Y_1$ selective blocker (MRS 2179; 30 $\mu$M). $P2Y_1$ receptor expression in WERI-Rb-1 cells was also identified by using western blot. Taken together, $P2Y_1$ receptor is mainly expressed in a retinoblastoma cell, which elicits $Ca^{2+}$ release from internal $Ca^{2+}$ storage sites via the phospholipase C-mediated pathway. $P2Y_1$ receptor activation in retinoblastoma cell could be a useful model to investigate the role of purinergic $[Ca^{2+}]_i$ signaling in neural tissue as well as to find a novel therapeutic target to this lethal cancer.**

**Key Words: Purinergic receptor, Calcium, Retinoblastoma**

## INTRODUCTION

Extracellular nucleotides have been demonstrated to act as signaling molecules by activation of P2 purinoceptors. Two groups of P2 receptors have been identified: the ionotropic P2X receptors and the metabotropic P2Y receptors, which belong to the superfamily of G-protein-coupled receptors [1-3]. P2Y receptors are especially widespread in the central nervous system and play important roles in regulating the activity of a large number of physiological functions [4,5]. Up to date, eight subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) have been identified in mammals [1,5]. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors are coupled to Gq, to activate phospholipase C (PLC) leading to the formation of inositol 1,4,5-triphosphate ($IP_3$). $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ are coupled to Gi, to inhibit adenylyl cyclase (AC). $P2Y_{11}$ receptor has the unique property to couple through both Gq and Gs [4-7]. Subtype distribution varied among the different species and cell types [8] and each subtype of P2Y receptors may have different functional roles [9,10].

Retina is a part of the CNS in contrast to all other ocular tissues [11]. Expression profiles and physiological characteristics of P2Y receptors have been investigated in various retinal tissues. Several subtypes of P2Y receptors, including $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$, are expressed in retinal neurons and glial cells [8,10-13]. Particularly, $P2Y_2$ receptor mRNA has been verified in the ganglion cell layer and the inner nuclear layer of adult *Rhesis Macaques* and albino rabbits [9]. P2Y signaling in retinal tissues are involved in cellular processes such as neurotransmission, fluid secretion, differentiation, and cell death, which are important for retinal function and diseases [14-16].

Human retinoblastoma is the most common intraocular cancer of childhood, which is a malignant tumor originated from multipotential embryonic retinal cells [17]. Recently, much attention has been paid to this tumor cell since it provided a good model for studying mechanisms underlying differentiation, apoptosis, and tumorigenesis in neuronal cells [18]. So far, however, it has not been investigated about the response to extracellular ATP and purinoceptors mediating this signaling in retinoblastoma cells. In this regard, the present study was aimed at elucidating P2Y receptors in WERI-Rb-1 cells using $Ca^{2+}$ imaging technique and western blot analysis.

We found that $P2Y_1$ receptor protein was identified to

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Corresponding to: In Deok Kong, Department of Physiology, Yonsei University Wonju College of Medicine, 162, Ilsan-dong, Wonju 220-701, Korea. (Tel) 82-33-741-0292, (Fax) 82-33-745-6461, (E-mail) kong@yonsei.ac.kr

**ABBREVIATIONS:** $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; AC, adenylyl cyclase; PLC, phospholipase C; $IP_3$, inositol 1,4,5-triphosphate; $\alpha\beta$-MeATP, $\alpha\beta$-methylene ATP; 2MeS-ATP, 2-methylthio ATP; ACh, acetylcholine.

be expressed in the WERI-Rb-1 cells. Furthermore, the $[Ca^{2+}]_i$ transients by application of 2-MeS-ATP and/or ATP was also strongly suppressed in the presence of $P2Y_1$ selective blocker, which means that $P2Y_1$ receptor is the main functional P2Y isotype in retinoblastoma cells.

## METHODS

### *Cell culture*

WERI-Rb-1 human retinoblastoma cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained according to the distributor's instructions [19]. Briefly, cells were transferred into 25-$cm^2$ culture flasks containing 90% RPMI 1640 medium (Cambrex, Walkersville, MD, USA) and 10% fetal bovine serum (GibcoBRL, Grand Island, NY, USA), and grown in a humidified atmosphere with 5% $CO_2$ at 37°C. Antibiotics (penicillin 100,000 unit/l and streptomycin 100 mg/l) were added to the culture medium. Cells were subcultured again every three to four days. For the experiments, cells were plated on poly-L-lysine coated glass coverslips for 1 hr before use.

### *Fluorescence imaging of $[Ca^{2+}]_i$ in single cells*

WERI-Rb-1 cells were loaded with $Ca^{2+}$-sensitive fluorescent dye, Fluo 3-AM (5 $\mu$M; Invitrogen, Eugene, OR, USA), at 37°C for 40 min in culture medium. Then, the cells were incubated for an additional 30 min in Fluo 3-AM-free physiological buffer (CaPSS, composition: 145 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, and 10 mM D-glucose; adjusted to pH 7.4 with NaOH) at room temperature (24~26°C) to remove extracellular traces of the dye and to complete de-esterification. Subsequently, the coverslips were mounted cell-side up in the free bottom of a perfusion chamber, placed on the stage of an inverted microscope (IX51, Olympus, Tokyo, Japan). Throughout the experiments, cells were continuously perfused at approximately 2 ml/min by means of a gravity-driven perfusion system with drug-free or drug-containing buffer solutions. The dye-loaded cells were excited at 488 nm by a monochromatic light source (LAMDA DG-4, Sutter, Novata, CA, USA) with 175-W xenon lamp and fluorescence images were captured at 530 nm through an objective lens (40×, UApo/340, Olympus, Tokyo, Japan) by an intensified CCD camera (Cascade, Roper, Duluth, GA, USA) controlled by a computer. Fluorescence images were acquired every 1 sec during drug applications; however, the interval was increased to every 30 sec during recovery or the wash-out period to minimize cell injury from phototoxicity. Data are expressed as $F/F_0$ by using MetaFluor 6.1 software package (Universal Imaging Corporation, Downingtown, PA, USA). $F_0$ represents the basal fluorescence value of fluo-3-loaded cells before stimulation, and $F$ represents the change in fluorescence occurring during stimulation of the cell. All fluorescence measurements were performed at room temperature.

### *Western blot analysis*

Immunoblot technique was performed to identify proteins representing $P2Y_1$ and $P2Y_2$ receptor subtypes expressed in cell membrane using respective antibodies of each subtypes (Abcam, UK) according to the method by Xia et al. [20]. In details, a confluent monolayer of WERI-Rb-1 cells ($5\times10^6$) was isolated by 0.5% trypsin-EDTA and rinsed twice with ice cold PBS (150 mM NaCl, 4.52 mM $NaH_2PO_4$, 15.48 mM $Na_2HPO_4$, pH 7.4) and then harvested using a rubber policeman. After a brief centrifugation at 1,000×g for 10 min, the cells were resuspended in a 0.5 ml modified radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl, pH=7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, aprotinin 1 $\mu$l/ml, pepstatin 1 $\mu$g/ml. The mixture was left on ice for 30 min, with gentle shaking every 10 min. The cells were disrupted by dounce homogenization using glass douncers, and the undisrupted cells and cellular debris were removed by centrifugation at 12,000×g for 15 min at 4°C. The supernatant was then diluted fivefold with lysis buffer and subjected to high-speed centrifugation at 92,500 g in a 70.1 Ti rotor (Beckman Optima LE-80K; 30,000 rpm) for 1 hr at 4°C. The resulting pellet was resuspended in 500 $\mu$l of lysis buffer. The sample was then diluted 1 : 1 with Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) containing 20 mM DTT and incubated at 95°C for 5 min before being loaded onto a gel. Stabilized proteins were separated on a 10% Tris-HCl Ready Gel (Bio-Rad) for 90 min at 20 mM and electrophoretically transferred to polyvinylidene fluoride membranes (Pall, MI, USA) for 60 min at 225 mA. The membranes were blocked with 5% nonfat dry milk in PBST buffer (0.05% Tween-20) for 1 hr and then immediately incubated for 1 hr with anti-purinergic polyclonal antibodies (1 : 500) dissolved in TBST. The antibody used for peptide blocking was treated with 1 $\mu$g peptide/$\mu$g antibody for 1 hr at room temperature immediately before being incubated with the membrane. Dilutions of the antibodies were similar in the presence and absence of peptide. After being washed 3 times for 15 min with PBST, the membranes were incubated for 1 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoLabs, West Grove, PA). Membranes were washed and then detected using ECL Plus western blotting detection reagents (Amersham, Piscataway, NJ) following the manufacturer's instructions.

### *Solutions and drugs*

WERI-Rb-1 cells were continuously superfused by gravity at a flow rate of 2 ml/min with the bath solutions (normal PSS) or experimental solutions. Normal PSS contained 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose and was adjusted to pH 7.4 with NaOH. Adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), 2-methylthioadenosine 5'-triphosphate (2MeS-ATP), $\alpha\beta$-methylene ATP ($\alpha\beta$-MeATP), and MRS 2179 were purchased from Sigma (St. Louis, MO, USA). Fluo-3/AM was dissolved into dimethyl sulfoxide (DMSO), and all other drugs were dissolved into the distilled water as stock solutions of mM order and stored into a freezer. Each stock solution was diluted to appropriate concentrations with the normal PSS just before the start of the experiment.

### *Statistical significance*

Quantitative data are represented as the mean±S.E.M. Statistical comparisons were made by the two-tailed $t$-test and ANOVA, and when $p<0.05$, the differences were con-

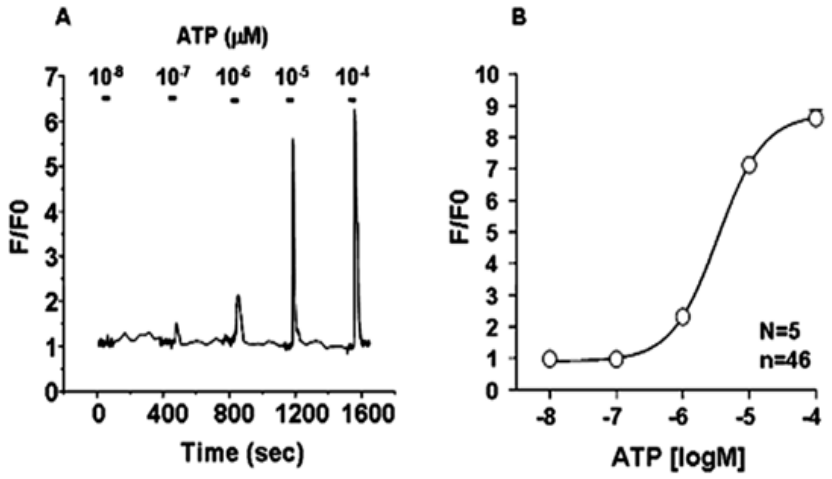


**Fig. 1.** ATP-evoked $Ca^{2+}$ signaling in WERI-Rb-1 cells. (A) Original trace of $Ca^{2+}$ responses evoked by four different concentrations of ATP in the same group of cells. (B) Dose-response curve fitting the peak responses obtained from five different groups of cells. $F_0$: basal fluorescence after Fluo 3-AM loading, F: change in fluorescence after introducing ATP, n=total cell number, N=number of experiment.

sidered to be significant.

## RESULTS

### *Changes in $[Ca^{2+}]_i$ via ATP in control WERI-Rb-1 cells*

We observed the ATP-induced $[Ca^{2+}]_i$ changes in WERI-Rb-1 cells to determine the existence of P2 purinoceptor. As shown in Fig. 1, ATP triggered $[Ca^{2+}]_i$ responses in a dose-dependent manner confirming the implication of P2 purinergic receptors. ATP (10 $\mu$M) applied for 30 sec produced a sharp $[Ca^{2+}]_i$ spike within 1~2 s on application and recovered rapidly after removal of ATP. During repeated applications with 7 min intervals, ATP-induced $[Ca^{2+}]_i$ transient was not desensitized prominently showing about 93% of the first one after the 3rd application (data not shown). We found that more than 80% of the cells examined by fluo-3 imaging exhibited $[Ca^{2+}]_i$ responses to ATP.

### *Effects of purinergic agonists on $[Ca^{2+}]_i$ response*

The characteristics of functional purinergic receptors were examined using more selective agonists to P2 receptors in WERI-Rb-1 cells. 2MeS-ATP, a selective P2Y$_1$ receptor agonist, evoked intracellular $[Ca^{2+}]_i$ transient in a concentration-dependent manner, which was more potent than ATP (Fig. 2). Interestingly, applications of 100 $\mu$M $\alpha\beta$-MeATP (P2X$_3$, P2X$_4$ agonist) and 100 $\mu$M UTP (P2Y$_2$, P2Y$_4$, P2Y$_6$ agonist) did not elicit any $[Ca^{2+}]_i$ rise, implying that P2 receptors other than P2Y$_1$ act a negligible functional role in WERI-Rb-1 cells.

### *Pharmacological characterization of ATP-induced $Ca^{2+}$ response*

The ATP (10 $\mu$M)-induced $[Ca^{2+}]_i$ transient was almost completely abolished by pretreatment with 30 $\mu$M MRS 2179, a selective P2Y$_1$ antagonist (92.5±1.6% inhibition; n=45; N=4; Fig. 3). In addition, MRS 2179 also successfully blocked the 2MeS-ATP (1 $\mu$M)-induced $[Ca^{2+}]_i$ response (n=76, N=6, Fig. 4). These evidences strongly indicate that purinergic $[Ca^{2+}]_i$ signaling is mainly mediated by P2Y$_1$ receptor in WERI-Rb-1, a human retinoblastoma cell line.

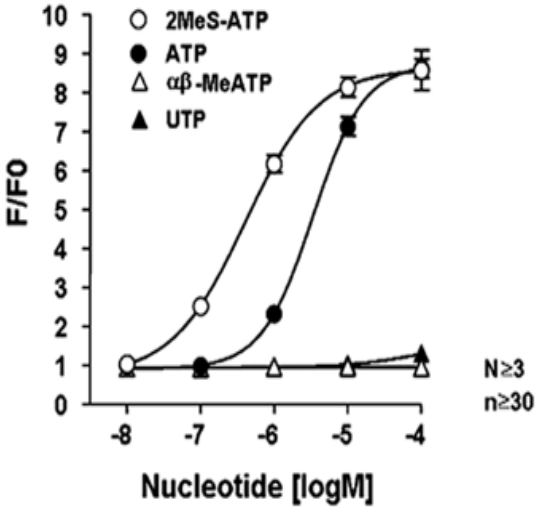


**Fig. 2.** Concentration-response curve of P2Y receptor agonists in WERI-Rb-1 cells. Each curve shows the peak calcium responses of increasing concentrations of 2MeS-ATP, a selective P2Y$_1$ agonist, $\alpha\beta$-MeATP, a P2X agonist, and UTP, a P2Y$_2$/P2Y$_4$/P2Y$_6$ agonist. Both $\alpha\beta$-MeATP and UTP did not induce calcium rise. Values are means±S.E.M. n=total cell number, N=number of experiments.

### *Functional expression of major P2Y receptor(s) in human retinoblastoma*

To further clarify the subtype of P2Y receptors in WERI-Rb-1 cells, we used western blot analysis. We tested the protein expression of P2Y$_1$ and P2Y$_2$ receptor, since it has been reported that P2Y$_2$ receptor is widely expressed in ocular tissues [9]. As shown in Fig. 5, we identified a band for P2Y$_1$ subtype at approximately 70 kDa that is compatible with the deduced size of P2Y$_1$ receptor protein in western blot analysis. Interestingly, we could not detect any band for P2Y$_2$ receptor.

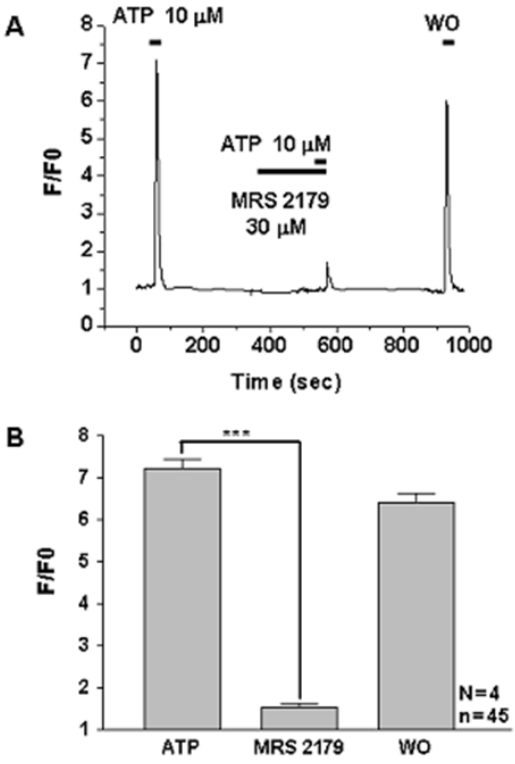


**Fig. 3.** Differential effect of P2Y agonists on $[Ca^{2+}]_i$ in WERI-Rb-1 cells. Original traces (A) and graph (B) showing calcium transient evoked by ATP (10 $\mu$M), a putative P2Y agonist, and its suppression after application of 30 $\mu$M MRS2179, a selective $P2Y_1$ antagonist. $F_0$: basal fluorescence after Fluo 3-AM loading, F: changes in fluorescence after introducing agonist or antagonist, n=total cell number, N=number of experiment. ***Denotes p<0.001.

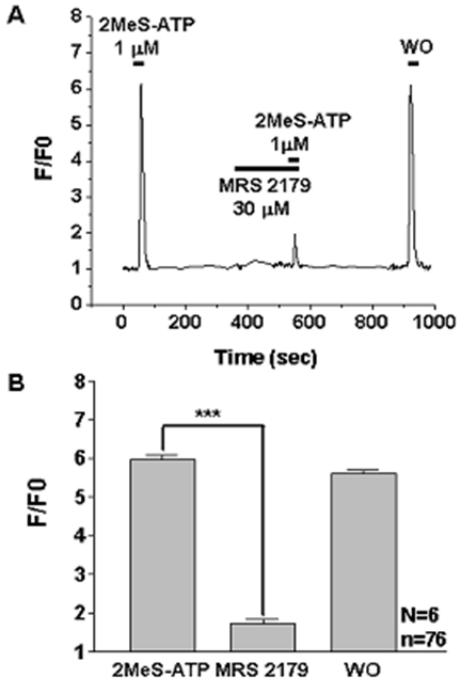


**Fig. 4.** Effect of $P2Y_1$ antagonist on 2-MeS-ATP-induced $[Ca^{2+}]_i$ changes in WERI-Rb-1 cells. Original traces (A) and graph (B) showing calcium transient evoked by 2-MeS-ATP (1 $\mu$M), a $P2Y_1$ agonist, and its suppression after application of the 30 $\mu$M MRS2179, a selective $P2Y_1$ antagonist. $F_0$: basal fluorescence after Fluo 3-AM loading, F: changes in fluorescence after introducing agonist or antagonist, n=total cell number, N=number of experiment, ***denotes p<0.001.

## DISCUSSION

In this study, we pursued the expressional and functional profiles of P2Y receptor subfamilies in WERI-Rb-1 cells, a human retinoblastoma cell line, by using $[Ca^{2+}]_i$ measurement and western blotting techniques. Results showed that $P2Y_1$ receptor subtype was functionally expressed and induced a marked increase in $[Ca^{2+}]_i$ in WERI-Rb-1 cells.

Increase in $[Ca^{2+}]_i$ is a regulatory signal for many normal developmental events such as fertilization, embryogenesis, cell proliferation, and even for cell death [21,22]. In addition, it may also be involved in various pathophysiologic changes [8,23]. Muscarinic and purinergic receptors are well known to raise $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from intracellular stores [24]. We, previously, demonstrated that the $M_3$ and $M_5$ muscarinic subtypes were expressed and mobilized $Ca^{2+}$ via PLC-$IP_3$-dependent pathway in retinoblastoma cells [25]. The involvement of PLC in the $Ca^{2+}$ responses to acetylcholine (ACh) and ATP were demonstrated by the inhibitory effect of U-73122. U-73343, the succinimide analog of U-73122, showed no significant inhibitory effect on the ACh response [25].

In the present study, we tried to identify the subtypes responsible for ATP-induced $[Ca^{2+}]_i$ transient by pharmacological approach and western blot analysis. As earlier mentioned, WERi-Rb-1 cells expressed at least 7 different P2Y receptor subtypes at the mRNA level. All of these subtypes are metabotropic G-protein-coupled receptors. However, MRS 2179, a selective $P2Y_1$ blocker, markedly suppressed the ATP-induced calcium transients by 92.5±1.6% of ATP effect. $P2Y_1$ agonist (2MeS-ATP; 1 $\mu$M) also increased $[Ca^{2+}]_i$ up to 85.9±3.1% of 10 $\mu$M ATP-induced response. There was not any $[Ca^{2+}]_i$ change by the application of 100 $\mu$M $\alpha\beta$-MeATP ($P2X_3$ and $P2X_4$ agonist) and 100 $\mu$M UTP

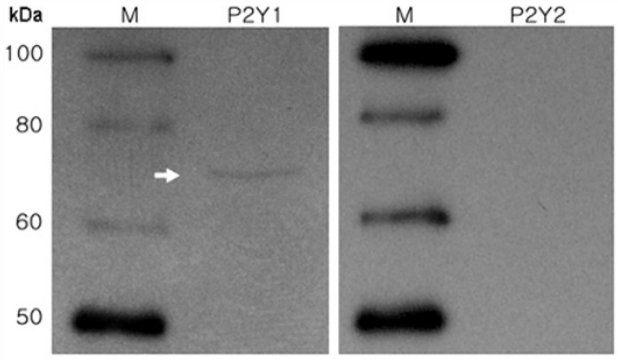


**Fig. 5.** Immunoblots of P2Y receptor subtypes in WERI-Rb-1 cells. $P2Y_1$ purinoceptor was identified by the band at around 70 kDa, which is compatible with its deduced protein size.

($P2Y_2$, $P2Y_4$, $P2Y_6$ agonist) (Fig. 2). $P2Y_1$ receptor protein was also identified by western blot. From these results, $P2Y_1$ receptor subtype was dominantly expressed and functionally involved in calcium mobilization in WERI-Rb-1 cells.

$P2Y_2$ receptors have been identified in a wide variety of ocular cell types, particularly, in retina [26], retinal pigment epithelium [27-29], cornea [30,31], conjunctiva [32], lens [33], and ciliary epithelia [9,34]. $P2Y_2$ receptor signaling and its function are apparent in the eye [35], and regulates multiple cellular functions in ocular physiology by calcium mobilization [9] including ion transport and fluid absorption [27,29], and mucin discharge [36]. Those synthetic $P2Y_2$ receptor agonists INS37217 and INS 365 are currently being adopted in clinical applications for the treatment of retinal detachment and dry eye disease, respectively [37-40]. In this study, however, we could not detect the $P2Y_2$ receptor protein by using western blotting technique, which is consistent with scarce $[Ca^{2+}]_i$ response to $P2Y_2$ agonist in retinoblastoma cells.

$P2Y_1$ receptor has been reported to be expressed in the retina of rat [8,11,12], developing chick embryo [13], and human glial cells [10]. However, little information is available to understand the role of $P2Y_1$ activation not only for physiologic action but also for tumorigenesis in retinoblastoma cells. Moreover, it could be worthy to elucidate the expressional changes in P2Y subtypes during malignant transformation. Further studies about P2Y receptors may provide a clue to understand the roles of purinergic signaling in the regulation of cellular functions of retina as well as the process of malignant transformation in retinoblastoma.

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