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Functional Characterization of $P2Y_1$ Versus P2X Receptors in RBA-2 Astrocytes: Elucidate the Roles of ATP Release and Protein Kinase C

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Abstract A physiological concentration of extracellular ATP stimulated biphasic Ca^{2+} signal, and the Ca^{2+} transient was decreased and the Ca^{2+} sustain was eliminated immediately after removal of ATP and Ca^{2+} in RBA-2 astrocytes. Reintroduction of Ca^{2+} induced Ca^{2+} sustain. Stimulation of $P2Y_1$ receptors with 2-methylthioadenosine 5′-diphosphate (2MeSADP) also induced a biphasic Ca^{2+} signaling and the Ca^{2+} sustains were eliminated using Ca^{2+}-free buffer. The 2MeSADP-mediated biphasic Ca^{2+} signals were inhibited by phospholipase C (PLC) inhibitor U73122, and completely blocked by $P2Y_1$ selective antagonist MRS2179 and protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) whereas enhanced by PKC inhibitors GF109203X and Go6979. Inhibition of capacitative Ca^{2+} entry (CCE) decreased the Ca^{2+}-induced Ca^{2+} entry; nevertheless, ATP further enhanced the Ca^{2+}-induced Ca^{2+} entry in the intracellular Ca^{2+} store-emptied and CCE-inhibited cells indicating that ATP stimulated Ca^{2+} entry via CCE and ionotropic P2X receptors. Furthermore, the 2MeSADP-induced Ca^{2+} sustain was eliminated by apyrase but potentiated by $P2X_4$ allosteric effector ivermectin (IVM). The agonist ADPβS stimulated a lesser $P2Y_1$-mediated Ca^{2+} signal and caused a two-fold increase in ATP release but that were not affected by IVM whereas inhibited by PMA, PLC inhibitor ET-18-OCH_3 and phospholipase D (PLD) inhibitor D609, and enhanced by removal of intra- or extracellular Ca^{2+}. Taken together, the $P2Y_1$-mediated Ca^{2+} sustain was at least in part via P2X receptors activated by the $P2Y_1$-induced ATP release, and PKC played a pivotal role in desensitization of $P2Y_1$ receptors in RBA-2 astrocytes. J. Cell. Biochem. 104: 554–567, 2008.
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Key words: $P2Y_1$ receptor; ATP release; Ca^{2+} signal; sustain; protein kinase C

Astrocytes are important in regulation of neurotransmission through responses to neurotransmitter stimulation and release of gliotransmitters [Newman, 2003]. ATP was known to be one of the major neurotransmitters and gliotransmitters, therefore plays an important role in regulating neuron–glia interaction. Multiple P2 receptors were found to be coexpressed on astrocytes; nevertheless, the physiological functions and interaction of these receptors remain unclear. Elevating of intracellular Ca^{2+} concentration has been considered as a sign of astrocyte activation. It is generally believed that Ca^{2+} responses play pivotal roles in regulations of many aspects of physiological functions [Fellin and Carmignoto, 2004]. Local astrocytic Ca^{2+} response was induced by neurotransmitters released from synaptic terminals during neurotransmission and the synapse-surrounding astrocytes would in turn, influence the synapse through gliotransmitters [Cunha and Ribeiro, 2000; Zhang et al., 2003; Panatier et al., 2006]. Additionally, global astrocytic Ca^{2+} response, which was thought to be responsible for long range neural transmission, could be elicited by high intensity of neuronal activity [Fellin and Carmignoto, 2004] or astrocyte network [Guthrie et al., 1999]. For example, astrocyte could exert tonic suppression on excitatory synaptic transmission by releasing ATP [Koizumi et al., 2003; Bowser and Khakh, 1999] or mediate synchrony of neuronal network by releasing glutamate [Fellin et al., 2004]. Moreover, it has been proven recently that vasomotor activity in the brain might

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not be directly affected by neurons [Parri and Crunelli, 2003] but regulated by gliotransmitter [Metea and Newman, 2006] or by neurotransmitter-induced astrocyte-released prostaglandin E2 [Zonta et al., 2003] and 20-HETE [Mulligan and MacVicar, 2004]. Taken together, these studies revealed that astrocytes were involved in the regulation of neuronal activity and network by releasing gliotransmitters.

ATP has been recognized as an important neuro- and gliotransmitter to mediate neuron–astrocyte interaction in the nervous system [Burnstock, 2006]. Purinergic receptors have been classified into two families: P2Y metabotropic and P2X ionotropic receptors. P2X consists of 1–7 subtypes, P2Y consists of 1, 2, 4, 6, 11, 12 six subtypes [Burnstock, 1997; Fredholm et al., 1997; North, 2002] and two more P2Y receptors, $P2Y_{13}$ and $P2Y_{14}$ receptors, have been identified respectively [Zhang et al., 2002; Lee et al., 2003]. Expression of multiple P2 receptor subtypes was found in rat primary astrocyte cultures [Fumagalli et al., 2003; Dixon et al., 2004]. In addition, a single astrocyte might express more than one P2Y receptor subtypes, $P2Y_1$ and $P2Y_2$ or $P2Y_4$ [Jimenez et al., 2000] or one P2Y and one P2X receptors, $P2Y_1$ and $P2X_3$ [Franke et al., 2004]. However, the exact mechanism involved in regulation of multiple P2 receptor-mediated Ca^{2+} signal and physiological function of the coexpressed P2 receptors of astrocytes remains unclear.

Intense immunological staining of $P2Y_1$ has been found in neuron and glia of the brain [Morán-Jiménez and Matute, 2000]. Although astrocytes do not have action potential to mediate fast neurotransmission, they are able to transmit their signaling through Ca^{2+}. Activation of P2Y receptor has been shown to couple with phosphatidylinositol specific phospholipase C (PLC), leading to rapid increase in intracellular Ca^{2+} concentration (Ca^{2+} transient) via Ca^{2+} release from intracellular store [Neary et al., 1991; Centemeri et al., 1997]. In addition, activation of P2Y receptors also activated extracellular signal-regulated protein kinase distinct of PLC pathway [Neary et al., 1999]. Activation of $P2Y_1$ might associate with astrocyte proliferation [Franke et al., 2003] and mediate antioxidative cytoprotective roles [Shinozaki et al., 2005]. It has been demonstrated that ATP activated $P2Y_1$ receptor on astrocytes and inhibited interneuron concomitantly, then further inhibited neurotransmission in the striatum [Bowser and Khakh, 2004]. Therefore elucidation of the $P2Y_1$ receptor signaling and examination of its relationship with other P2 receptor will certainly help us to understand the regulations of neurotransmission.

In addition, Ca^{2+} signaling of astrocytes was known to be potentiated and prolonged as Ca^{2+} oscillation. However, the regulatory mechanism of Ca^{2+} oscillation remains unclear. Recently, it is proposed that it may mediate through stimulation-induced ATP release and then retroactively act on the same cell through activation of P2Y receptors. Although activation of $P2Y_1$ or $P2Y_2$ alone was enough to initiate Ca^{2+} oscillation, it required two subtypes, $P2Y_1$ and $P2Y_2$ receptors, to reach the maximal Ca^{2+} oscillation [Gallagher and Salter, 2003].

$P2X_4$ receptors were cloned of complementary DNA from rat brain and expression in xenopus oocytes gives an ATP-activated cation-selective channel highly permeable to Ca^{2+} [Soto et al., 1996], and later identified as one of the fast ATP gated ion channel [Khakh et al., 1999a]. The mRNA distribution was found throughout the brain [Buell et al., 1996]. Coassemble $P2X_4$ and $P2X_6$ was identified as a heteromeric $P2X_{4+6}$ channel with unique functional properties in the CNS [Lê et al., 1998]. Desensitization of ion currents of $P2X_4$ receptors has been well characterized [North, 2002; Fountain and North, 2006]. Modulation of $P2X_4$ receptor density was identified through cycling into and out of plasma membranes [Bobanovic et al., 2002]. Nevertheless, the Ca^{2+} signaling of $P2X_4$ receptors in native astrocytes remains unknown.

Ivermectin (IVM) was found to potentiate the currents evoked by 100 μM ATP at homomeric expressed $P2X_4$ and possibly of heteromeric $P2X_4$/$P2X_6$ channels, but not of $P2X_2$, $P2X_3$, $P2X_2$/$P2X_3$, or $P2X_7$ channels [Khakh et al., 1999b]. Recently, IVM was shown to bind to the $P2X_4$ receptor at an allosteric binding site, and modulate the activity of $P2X_4$ receptors. IVM increased current amplitude by reducing channel desensitization [Priel and Silberberg, 2004] and increased the number of cell surface $P2X_4$ receptors by a clathrin/AP2-mediated mechanism [Toulmc et al., 2006]. Thus, IVM may be used as a tool to identify native $P2X_4$ receptor signaling of astrocytes.

In this study, we showed that a lower concentration of ATP (100 μM) stimulates a biphasic Ca^{2+} signal, transient and sustain. Pharmacological analysis indicated activation of $P2Y_1$ receptors induced a similar Ca^{2+} signal and ATP release. The Ca^{2+} transient was coupled to PLC. The Ca^{2+} sustain was dependent on Ca^{2+} entry and ATP release. We also provide evidences to show that ATP stimulated $P2X_4$ receptors to induce Ca^{2+} entry. Thus the $P2Y_1$-mediated Ca^{2+} sustain might be due to the $P2Y_1$-induced ATP release and then acts retroactively on the $P2X_4$ or $P2X_7$ receptors in these astrocytes.

MATERIALS AND METHODS

Materials

ATP bioluminescence assay kit CLS II (1699695) was purchased from Roche (Basel, Switzerland). Fura-2-AM (F-1201) was purchased from Invitrogen Ltd (Carlsbad, CA). Reverse transcriptase, Taq, aminosteroid 1-6-17b-3-methoxyestra-1,3,5 10-trien-17-yl-amino hexyl-1*H*-pyrrole-2,5-dio (U73122), phorbol 12-myristate 13-acetate (PMA), 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester), 2-(methylthio) adenosine 5′-diphosphate trisodium salt (2Me SADP), adenosine-5′-0-(2-thiodiphosphate) trilithium salt (ADPβS), 2′-deoxy-N6-methyl adenosine 3′,5′-diphosphate diammonium salt (MRS2179), 2-aminoethyl diphenyl borate (2-APB), *O*-tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate (D609), were purchased from Sigma (St. Louis, MO). Thapsigargin (TG) was purchased from Tocris Bioscience (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel) and F10 medium from Sigma.

Cell Culture

RBA-2 astrocytes were cultured in culture medium (F10 medium supplemented with 10% FBS) in a humidified atmosphere at 37°C in 95% air and 5% CO_2 [Sun et al., 1999]. In all the experiments, the cells used had been cultured for no more than 10 passages (passage 60–70).

Ca^{2+} Image Recording

RBA-2 astrocytes were seeded on 24 mm (1×10^4 cells) or 32 mm (1×10^5 cells) coverslips, and cultured in culture medium at 37°C in CO_2 incubator for 2 days. The medium was switched to serum-free F10 media and further cultured for 1–2 days. For Ca^{2+} image recording, cells were incubated in F10 media containing 1 μg/ml Fura-2/AM (Invitrogen Ltd) for 30 min in a humidified atmosphere of 95% air 5% CO_2 at 37°C. Cells were then washed twice with loading buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM Glucose, pH 7.2) and the coverslips clamped on the stage of Zeiss Axiovert 200 inverted microscope equipped with brightfield, phase fluorescent optics and filled with loading buffer. In some of the study, a Ca^{2+}-free buffer (125 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, 10 mM Glucose, 2 mM EGTA, pH 7.2) was used. Calcium imaging analysis were performed using excitation wavelengths (340/380 nm) selected by means of a computer controlled rotating filter wheel between a xenon light source and the microscope. The emission light at 510 nm was passed to an image-intensifying Photometrics CoolSNAP HQ Digital Monochrome charge-coupled device (CCD) camera system (Roper Scientific, GmbH Ottobrunn, Germany). The resulting image at each wavelength was averaged in real time, digitalized and stored in an image-processing unit. The ratio of emitted fluorescence was calculated for each frame and converted to Ca^{2+} ion concentration [Grynkiewicz et al., 1985]. The results were then calculated using a Metaflour image analysis system (Universal Imaging Corporation, Philadelphia). The experiments were performed three times with different batches of cells and results from a typical experiment with 15–20 cells of one microscopic field were shown.

RT-PCR

Total RNA was extracted from RBA-2 astrocytes by a Qiagen Kit or traditional preparation, then treated with DNase for 10 min at 37°C. First-strand cDNA was synthesized from 5 μg DNase-treated total RNA using oligo(dT) primer and SuperScript® II Reverse Transcriptase (Invitrogen Ltd) for 1 h at 42°C and 5 min at 70°C. Negative control reactions were processed in the absence of reverse transcriptase. PCR was performed in a final volume of 20 μl containing cDNA from the previous synthesis. 1 U *Taq* DNA polymerase, 3 μM of each primer,

1.25 mM dNTP and PCR was run for 30 cycles, each cycle consisting of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. For final extension, the product was kept at 72°C for 10 min and then stored at 4°C. PCR products were electrophoresed on 1% agarose gel.

Bioluminescence ATP Release Assay

RBA-2 astrocytes (5×10^4 cells per well) were seeded in the wells of a 12-well plate and cultured for 3 days. Cells were then washed with loading buffer three times and further cultured in loading buffer. ATP release into the loading buffer was determined by ATP bioluminescence assay kit CLS II (Roche, Germany) using the protocol provided by the manufacture company (Roche). In this trial, all the drugs were tested to for bioluminescence reading and to compare with the reading of ATP. The drugs that did not interfere with the measurement of ATP were selected for the assay. Thus, ADPβS and ET-18-OCH_3 were selected. Our results also revealed that 2Me SADP and U73122 interfered with bioluminescence reading of ATP (data not shown).

Data Analysis and Statistics

All the statistic results were calculated using non-paired Student's *t*-test by Microsoft Excel and graphics were drawn using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

RESULTS

ATP Stimulated a Biphasic Ca^{2+} Signaling of RBA-2 Astrocytes

Our previous studies have shown that RBA-2 astrocytes express $P2Y_1$, $P2X_4$, and $P2X_7$ but not $P2X_1$, $P2X_2$, P2X3, and $P2Y_{11}$ purinergic receptors. Prior to this study, we confirmed that RBA-2 did not express $P2X_5$ and $P2X_6$ receptors using RT-PCR and primers designed by Primer3 software (data not shown). Therefore, RBA-2 astrocytes possess two ionotropic P2X receptors, $P2X_4$ and $P2X_7$. The purpose of this study is characterization of the coactivation of $P2Y_1$ and P2X receptors by a single cell Ca^{2+} image analysis system. As shown in Figure 1A, ATP (100 μM) stimulated a biphasic Ca^{2+} signal, transient and sustain, and the Ca^{2+} sustain declined immediately after removal of extracellular Ca^{2+}, indicating that the ATP-stimulated Ca^{2+} sustain was dependent on Ca^{2+} entry. As shown in Figure 1B, ATP stimulated a much smaller Ca^{2+} transient with no Ca^{2+} sustain in the Ca^{2+}-free buffer. As compared with those measured in the Ca^{2+}-containing buffer, the peak height is approximately 50%. Reintroduction of Ca^{2+} into the Ca^{2+}-free buffer, induced a Ca^{2+} sustain. Thus, ATP activated P2Y receptors to induce Ca^{2+} transient via

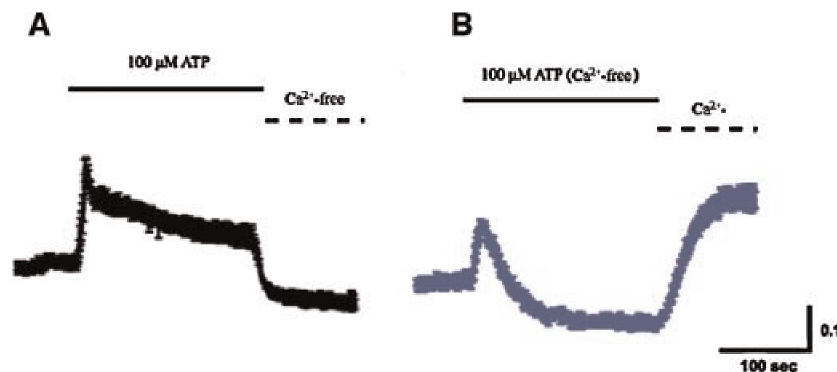

Fig. 1. ATP stimulates a biphasic Ca^{2+} signal. RBA-2 astrocytes were cultured on coverslips, loaded with fura-2-AM and rinsed. **A**: Cells were stimulated with extracellular ATP (100 μM) for the length of time indicated by the line above the figure. The extracellular ATP was removed by perfusion and replaced with a Ca^{2+}-free buffer in the presence of 2 mM EGTA and the Ca^{2+} signal recorded. **B**: Cells were stimulated with ATP in the Ca^{2+}-free buffer for the length of time indicated by the line above the figure. The ATP-containing Ca^{2+}-free buffer was removed by perfusion and replaced with the Ca^{2+} containing loading buffer. The Ca^{2+} signals were recorded using Ca^{2+} image analysis system. The average of Ca^{2+} signals from 15 to 20 cells was calculated and the trace represented the averaged results. *Y*-axis represents changes of $[Ca^{2+}]_i$ as ratio of F340/F380 and *X*-axis represents time (s). Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

release of intracellular Ca^{2+} stores and the Ca^{2+} sustain was due to Ca^{2+} entry.

Stimulation of $P2Y_1$ Receptors Induce a Biphasic Ca^{2+} Signaling

To elucidate whether ATP activate $P2Y_1$ receptors, we stimulated the cells with the $P2Y_1$ selective agonist 2MeSADP. As shown in Figure 2A, single cell traces revealed that 2MeSADP (10 μM) stimulated biphasic Ca^{2+} signals, transients and sustains, the Ca^{2+} sustains were associated with irregular fluctuations of Ca^{2+} signals, and that they were completely abolished in the Ca^{2+}-free buffer indicating that Ca^{2+} sustains were mediated through Ca^{2+} entry (Fig. 2B). To further elucidate the mechanisms, we treated the cells with various antagonist and blockers. As shown in Figure 2C, the averaged trace revealed 2MeSADP-induced a biphasic signal (top trace), the Ca^{2+} sustain was eliminated in the Ca^{2+}-free buffer (middle trace), and the biphasic Ca^{2+} signaling was completely abolished by pretreating cells with $P2Y_1$ receptor selective antagonist MRS2179 (bottom trace) indicating that the initiation of the biphasic Ca^{2+} signaling was dependent on activation of $P2Y_1$ receptors. As shown in Figure 2D, the 2MeSADP-induced Ca^{2+} signal was effectively inhibited by 5 μM PLC inhibitor U73122 (middle trace) indicating that the $P2Y_1$ signaling was coupled to PLC. Interestingly, activation of protein kinase C (PKC) by 500 nM PMA completely abolished the 2MeSADP-induced Ca^{2+} signal (bottom trace) suggesting that PKC might play a pivotal role in the negative regulation of $P2Y_1$ receptors. To elucidate the involvement of PKC, we continued the study by treating the cells with PKC inhibitors. As shown in Figure 2E, general PKC inhibitor GF109203X greatly enhanced the 2MeSADP-mediated biphasic Ca^{2+} signal. In addition the Ca^{2+}-dependent inhibitor Go6979 also enhanced the Ca^{2+} signal. Thus the negative regulation of $P2Y_1$ receptors might involve

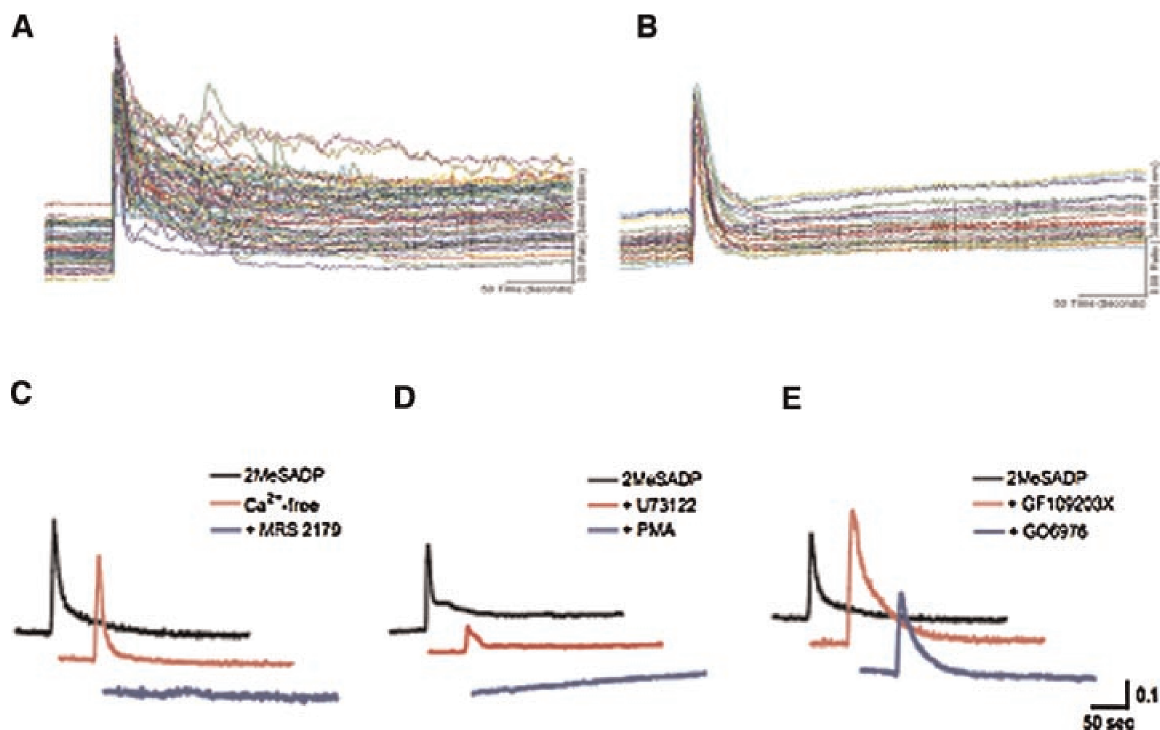

Fig. 2. Protein kinase C down-regulates the $P2Y_1$-mediated biphasic Ca^{2+} signal. RBA-2 astrocytes were cultured on coverslips, loaded with Fura-2, rinsed, cultured in (**A**) loading buffer or (**B**) Ca^{2+}-free buffer, stimulated with the $P2Y_1$ receptor selective agonist 2MeSADP (10 μM) and the traces of single cell Ca^{2+} signals were recorded by a Ca^{2+} image analysis system. The averaged traces of 2MeSADP-stimulated Ca^{2+} signals (**C**) measuring in loading buffer (top trace), in Ca^{2+}-free buffer (middle) or in the presence of $P2Y_1$ selective antagonist MRS2179 (bottom) and (**D**) in the presence of PLC inhibitor U73122 (middle trace) or PKC activator PMA (bottom trace). **E**: The averaged traces of 2MesADP-stimulated Ca^{2+} signal were conducted in the presence of PKC inhibitor GF109203 (red) or Go6979 (blue). The graphs were drawn by Prism 4. Scale bar: *X*-axis is 50 s, and *Y*-axis is 0.1 change of fluorescent ratio (F340/F380) and *X*-axis represents time (s). Graphs were drawn by Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

both Ca^{2+}-dependent and Ca^{2+}-independent types of PKC isozymes. Taken together, we demonstrated that activation of P2Y$_1$ receptor induced a biphasic Ca^{2+} signal, the Ca^{2+} transient is mediated through a PLC-sensitive mechanism, the Ca^{2+} sustain required Ca^{2+} entry and PKC played a pivotal role in the negative regulation of P2Y$_1$ receptors.

Activation of P2Y$_1$ Receptors Is Associated With Capacitative Ca^{2+} Entry (CCE)

Introduction of Ca^{2+} into Ca^{2+}-free buffer was shown to induce Ca^{2+} entry in the ATP-stimulated RBA-2 astrocytes. Thus, capacitative Ca^{2+} entry (CCE) might associate with the P2Y$_1$-mediated Ca^{2+} sustain [Putney, 1986, 1990]. RBA-2 astrocytes were pretreated with CCE inhibitor SKF96365 and then stimulated with 2MeSADP in Ca^{2+}-free loading buffer. As shown in Figure 3A, the cells were stimulated with 2MeSADP at the 50th s and then 2 mM Ca^{2+} was reintroduced into the buffer at the 300th s. Stimulation of P2Y$_1$ by 2MeSADP induced Ca^{2+} transient in the Ca^{2+}-free buffer and the reintroduction of Ca^{2+} rapidly induced a Ca^{2+} sustain (top trace). CCE inhibitor SKF 96365 did not affect the 2MeSADP stimulated Ca^{2+} transient but inhibited the Ca^{2+}-induced Ca^{2+} entry. As shown in Figure 3B, statistic analysis confirmed that pretreatment of cells with SKF96365 did not affect the P2Y$_1$-mediated Ca^{2+} transient (first peak) but decreased the Ca^{2+}-induced Ca^{2+} sustain (2nd peak). Our results revealed that CCE was at least in part associated with the P2Y$_1$-mediated Ca^{2+} sustain.

ATP Induces Ca^{2+} Entry Via P2X Receptors in RBA-2 Astrocytes

We then examined whether ATP might also stimulate Ca^{2+} entry through P2X receptors in RBA-2 astrocytes. We treated these cells with endoplasmic reticulum (ER) selective Ca^{2+} pump inhibitor, thapsigargin (TG) to empty the intracellular Ca^{2+} stores and eliminate Ca^{2+} transient. In addition, we treated the cells with 2-2-aminoethoxydiphenyl borate (2-APB) to inhibit CCE and 2-APB has been shown to be a reliable blocker for CCE [Bootman et al., 2002]. As shown in Figure 4A, introduction of 2 mM Ca^{2+} into the Ca^{2+}-free buffer in the TG-treated cells induced large and almost identical Ca^{2+} sustains in these astrocytes. We then treated the cells with both TG (1 μM) and 2-APB (40 μM). TG treatment induced a transient increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and then the $[Ca^{2+}]_i$ declined to below basal. We also performed a separate test to examine the effect of 2-APB and found that 2-APB per se did not affect $[Ca^{2+}]_i$. As shown in Figure 4B, addition of Ca^{2+} induced a much smaller but identical Ca^{2+} sustains indicating that the CCEs were inhibited by 2-APB (Fig. 4B). We then stimulated the TG- and 2-APB-treated cells with ATP (100 μM). As shown in Figure 4C, addition of Ca^{2+} and ATP induced

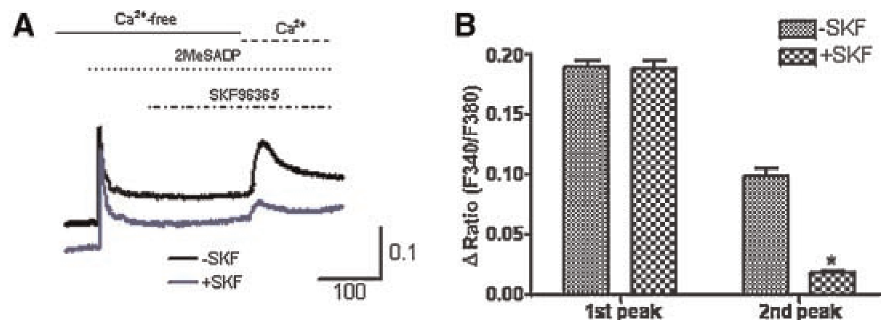

Fig. 3. Activation of P2Y$_1$ receptors induces capacitative calcium entry (CCE). **A**: RBA-2 astrocytes were cultured on coverslips, loaded with Fura-2, rinsed, cultured in Ca^{2+}-free loading buffer in the presence (bottom trace) or absence (top trace) of CCE inhibitor SKF96365. The P2Y$_1$ receptor selective agonist 2MeSADP was applied at the 50th sec (arrows) and 2 mM Ca^{2+} was added into the buffer system at the 300 s (arrow head). The Ca^{2+} signals were measured using Ca^{2+} image analysis system. The averaged traces were recorded from approximately 15–20 cells. *Y*-axis represents changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), shown as ratio of F340/F380, and *X*-axis represents time (s). **B**: Statistic analysis of the peak value of 2MeSADP-induced (1st peak) and Ca^{2+}-induced (2nd peak) increases in $[Ca^{2+}]_i$ and the data represent the mean ± SD from three separate experiments each containing 15–20 cells. Symbols * and ** indicates significant different means calculated by Student's *t*-test with $P \geq 0.05$ and 0.01, respectively. Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

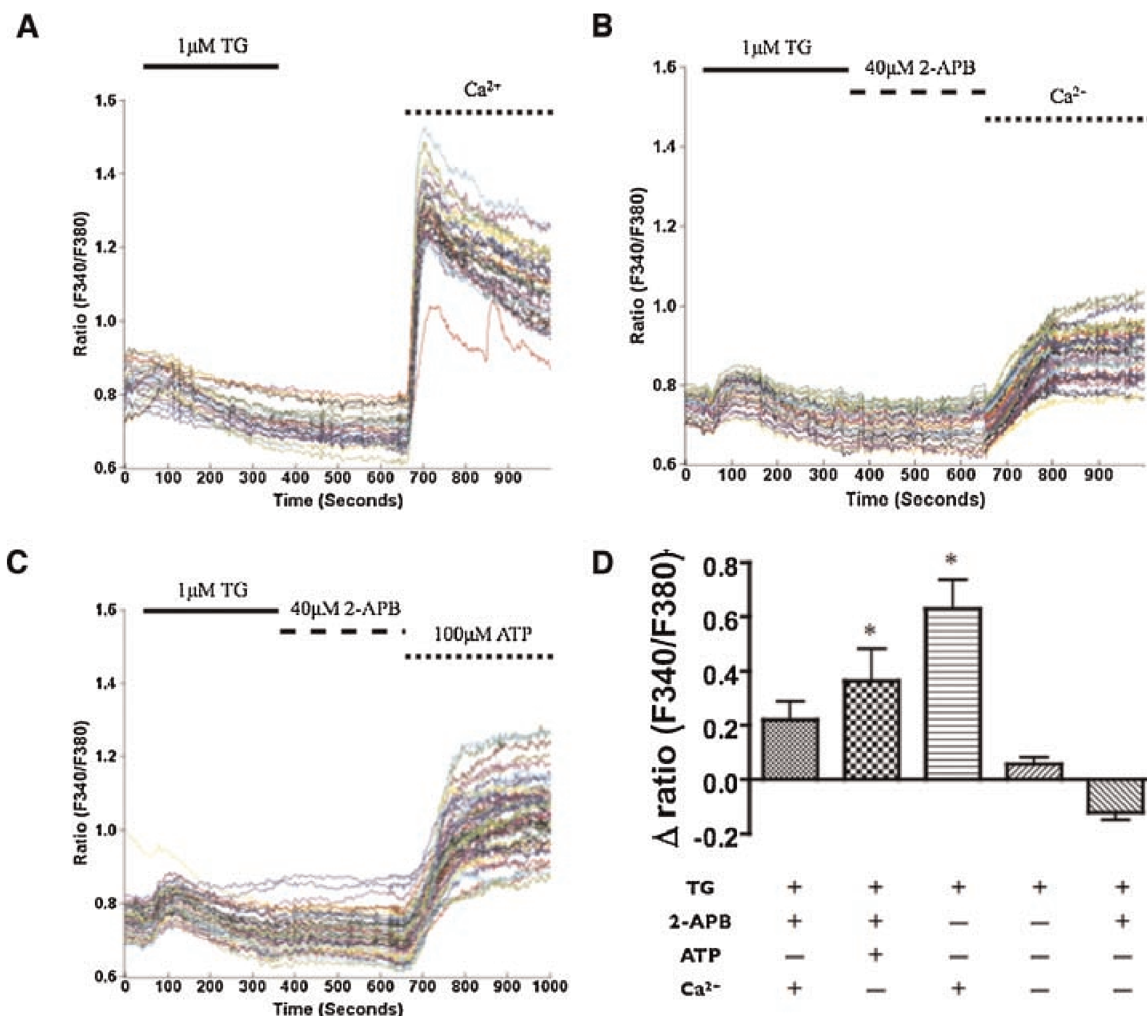

Fig. 4. ATP induces Ca^{2+} entry through CCE and P2X receptors. RBA-2 astrocytes were cultured on coverslips, loaded with Fura-2, rinsed, (**A**) incubated in Ca^{2+}-free buffer containing thapsigargin (TG) for 400 s, or (**B**) incubated in Ca^{2+}-free buffer containing thapsigargin (TG) for 350 s, replaced with Ca^{2+}-free buffer containing CCE inhibitor 2-APB and replaced with loading buffer at the 650th s or (**C**) incubated in Ca^{2+}-free buffer containing thapsigargin (TG) for 350 s, replaced with Ca^{2+}-free buffer containing CCE inhibitor 2-APB and replaced with loading buffer in the presence of ATP (100 μM) at the 650th s. The Ca^{2+} signals were measured using Ca^{2+} image analysis system. The traces represent signals from 15 to 20 cells. Y axis represents changes of $[Ca^{2+}]_i$, shown as ratio of F340/F380 and *X*-axis represents time (s). **D**: Statistic analysis of the peak values of the Ca^{2+}-induced Ca^{2+} entry from the three separated experiments and the symbol * indicates significant different means with $P \leq 0.05$, calculated by Student's *t*-test. Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

large and identical Ca^{2+} sustains in these cells. The peak levels of the Ca^{2+}- and ATP plus Ca^{2+}-induced Ca^{2+} entry of TG- and APB-treated cells were compared with the Ca^{2+}-induced Ca^{2+} of the control cells. As shown in Figure 4D in the TG and 2-APB-treated cells, Ca^{2+} plus ATP induced larger Ca^{2+} sustains as compared with those induced by Ca^{2+} only. Thus, ATP induced Ca^{2+} entry through P2X receptors. Although coassemble $P2X_4$ and $P2X_6$ was identified as a heteromeric $P2X_{4+6}$ channel in the CNS [Lê et al., 1998], RBA-2 astrocytes do not express $P2X_6$ (data not shown) but express $P2X_7$ receptors. Thus, the $P2Y_1$-mediated Ca^{2+} sustain might mediate through $P2X_4$ or $P2X_7$ receptors in these astrocytes.

The $P2Y_1$ Receptor-Mediated Ca^{2+} Sustain Involves Endogenous ATP Release

To further elucidate the mechanism in activation of P2X receptors in the $P2Y_1$-mediated Ca^{2+} sustain, we examine the involvement of ATP release. We treated these cells with apyrase and measured the $P2Y_1$-mediated Ca^{2+}

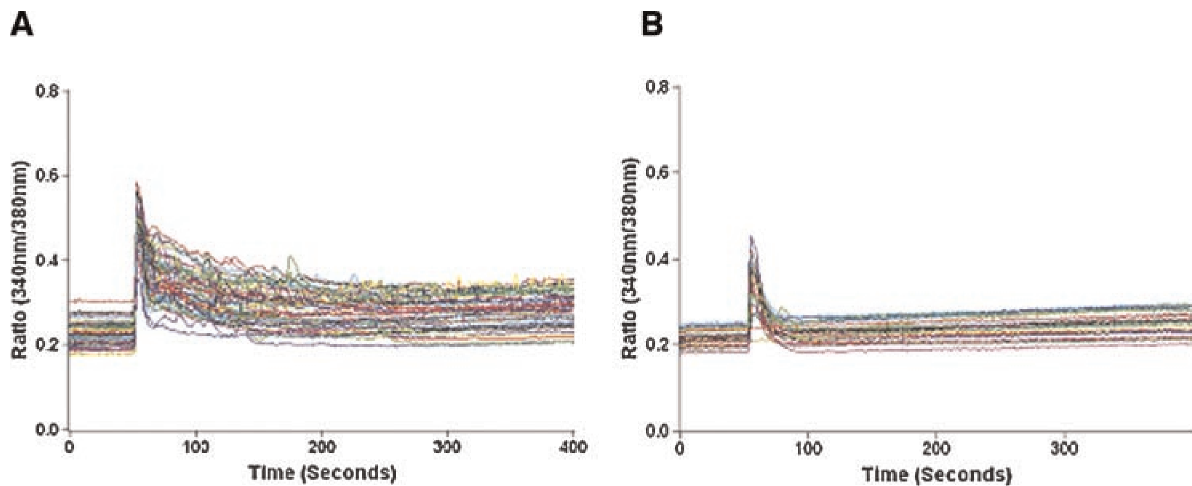

Fig. 5. The $P2Y_1$-mediated Ca^{2+} sustains requires endogenous ATP release. RBA-2 astrocytes were cultured on coverslips, loaded with Fura-2, rinsed, cultured in loading buffer and stimulated with $P2Y_1$ receptor selective agonist 2MeSADP (10 μM; **A**) in the absence or (**B**) presence of 10 U/ml apyrase. The increases in Ca^{2+} signals were measured using Ca^{2+} image analysis system. The traces represent signals from approximately 15–20 cells. *Y*-axis represents changes of $[Ca^{2+}]_i$, shown as ratio of F340/F380 and *X*-axis represents time. Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

signals. Apyrase is an ATP diphosphohydrolase and catalyses the removal of the gamma phosphate from ATP and the beta phosphate from ADP. As shown in Figure 5A, 2MeSADP stimulated Ca^{2+} transient and sustain, and apyrase abolished the Ca^{2+} sustains (Fig. 5B) whereas the Ca^{2+} transients remained. Thus Ca^{2+} sustain might mediate through ATP release stimulated Ca^{2+} entry via P2X receptors. Taken together, these results demonstrated that activation of $P2Y_1$ receptor stimulated ATP release and the released ATP might act on P2 receptors reciprocally to sustain the Ca^{2+} signaling. Because we have shown that ATP might activate P2X receptors to induce Ca^{2+} entry and RBA-2 astrocytes expresses $P2X_4$ and $P2X_7$ receptors. Thus, the endogenous released ATP might stimulate $P2X_4$ or $P2X_7$ receptors to induce Ca^{2+} sustain in these astrocytes.

Activation of $P2X_4$ Receptors Induce Ca^{2+} Entry

To measure the $P2X_4$-mediated Ca^{2+} signals, we treated the cells with a selective $P2X_4$ receptor positive allosteric effector, ivermectin (IVM). As shown in Figure 6, addition of 2MeSADP stimulated biphasic Ca^{2+} signals (Fig. 6A). As shown in Figure 6B, treating the cells with IVM (the broken line) per se did not affect the Ca^{2+} signal in any of the cells whereas addition of 2MeSADP (the solid line) into the IVM-pretreated cells induced a greater biphasic Ca^{2+} signal (Fig. 6B). In contrast, IVM has no effect on the 2MeSADP-induced Ca^{2+} signals measured when Ca^{2+} was removed from the buffer (the dot line) confirming that the Ca^{2+} sustains was due to Ca^{2+} entry (Fig. 6C). Taken together, the results indicated that IVM potentiated the $P2Y_1$-mediated Ca^{2+} signals by inducing Ca^{2+} entry via $P2X_4$ receptors. Thus, $P2X_4$ receptors can be activated by ATP and induce Ca^{2+} entry to prolong and potentate the $P2Y_1$-mediated Ca^{2+} signaling. We demonstrated for the first time that ATP stimulated distinct Ca^{2+} signals via native $P2Y_1$ and $P2X_4$ receptors. Nevertheless, we are not rule out the possibility that ATP may also activate $P2X_7$ receptor to induce Ca^{2+} entry in these astrocytes.

Stimulation of $P2Y_1$ Receptor Induce Endogenous ATP Release

To reconfirm that activation of $P2Y_1$ receptors induce ATP release in these astrocytes, we examined the $P2Y_1$-mediated ATP release by measuring ATP concentration in the conditioned media using Bioluminescence Assay (Roche). Our preliminary data indicated that 2MeSADP and U73122 interfered with ATP measurement using Bioluminescence assay. We then used another potent $P2Y_1$ receptor agonist ADPβS [Kűgelgen, 2006] in this assay. We also

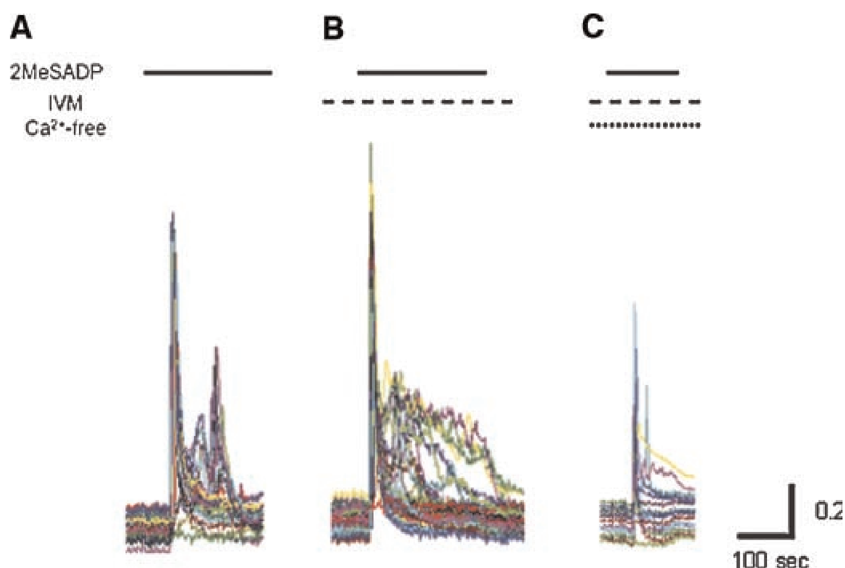

Fig. 6. Ivermectin potentiates the $P2Y_1$ receptor-mediated biphasic Ca^{2+} signals. RBA-2 astrocytes were cultured on coverslips, loaded with Fura-2, rinsed (**A**) incubated in loading buffer or (**B**) pretreated with 10 μM $P2X_4$ receptor selective positive allosteric effector ivermectin (IVM) for 10 min, and (**C**) incubated in Ca^{2+}-free buffer. The cells were then stimulated with $P2Y_1$ receptor selective agonist 2MeSADP (10 μM) and the Ca^{2+} signal from single cells recorded. The traces represent signals from approximately 15–20 cells. *Y*-axis represents changes of $[Ca^{2+}]_i$, shown as ratio of F340/F380 and *X*-axis represents time (s). Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tested other drugs for Bioluminescence intensity and those that have no interference with ATP were selected for the assay. We first examined ADPβS induced Ca^{2+} signaling via $P2Y_1$ receptors. As shown in Figure 7A, in the presence of $P2Y_1$ selective antagonist MRS2179, ADPβS failed to induce any Ca^{2+} signaling, MRS2179 was then washout and cells were stimulated with ADPβS. As shown in Figure 7A, ADPβS induced a delayed biphasic Ca^{2+} signaling. Thus ADPβS effectively activated $P2Y_1$ receptors. In addition, ADPβS induced a nearly twofold increase in ATP release, the ADPβS-stimulated release was blocked by PMA whereas enhanced by removal of extracellular Ca^{2+} and by chelating the intracellular Ca^{2+} with BAPTA, and was not affected by IVM. In addition, BAPTA per se did not affect ATP release in these cells (data not shown). Thus activation of $P2Y_1$ but not $P2X_4$ receptors induced ATP release and the release was independent of $P2Y_1$-mediated Ca^{2+} signaling. As shown in Figure 7B, PLC inhibitor ET-18-OCH3 or phospholipase D (PLD) inhibitor D609 per se did not affect ATP release but inhibited the ADPβS-induced ATP release effectively. Taken together, these results indicated that activation of $P2Y_1$ receptors stimulated ATP release through a Ca^{2+}-independent mechanism but the release was also sensitive to the subsequently activated PLC and PLD signaling in these astrocytes.

DISCUSSIONS

ATP has been recognized to regulate synaptic neurotransmission through activation of purinergic receptors in astrocytes. We characterized the $P2Y_1$ and $P2X_4$ receptor-mediated Ca^{2+} signaling and elucidate the mechanisms involved in RBA-2 astrocytes known to coexpress the two P2 receptors. $P2Y_1$, $P2X_4$, and $P2X_7$ receptors were also known to be the most widely expressed purinergic receptors in many brain areas and are both activated by similar physiological ranges (μM) of extracellular ATP. Although primary cortical astrocytes were found to express all cloned P2X and P2Y except $P2X_6$ receptors, the ATP-induced Ca^{2+} signaling was identified to mediate through $P2Y_1$ and $P2X_7$ receptors [Fumagalli et al., 2003]. The $P2Y_1$-mediated Ca^{2+} signals have been characterized in many types of cells but the $P2X_4$-mediated Ca^{2+} signaling in astrocytes has never been detected due to lack of specific agonist and antagonist. Although RBA-2 astrocytes possess the receptors, our early results indicated that a higher concentration of extracellular ATP

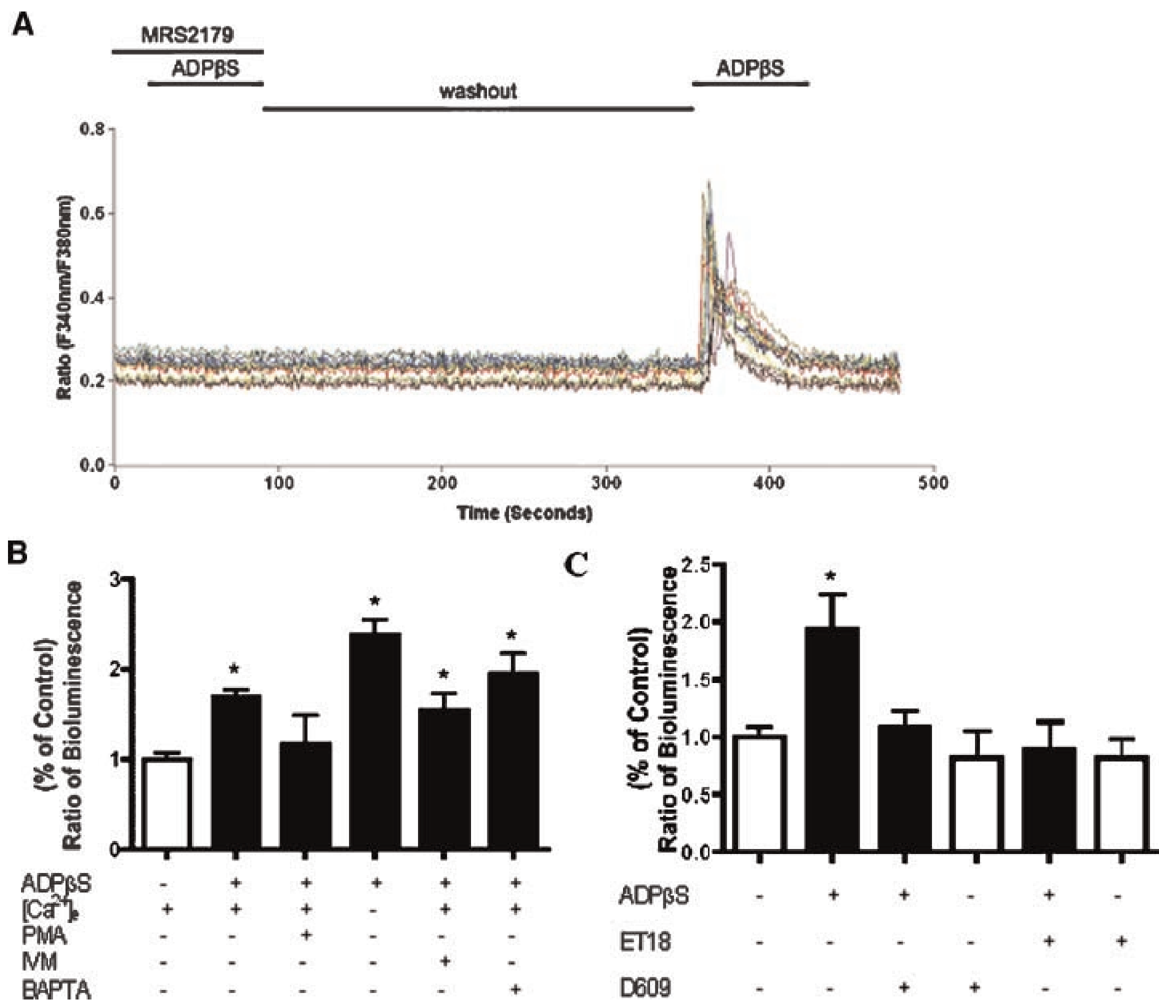

Fig. 7. Activation of P2Y$_1$ receptors induces ATP release. **A**: RBA-2 astrocytes were loaded with Fura-2, treated with P2Y$_1$ selective inhibitor MRS2179, stimulated with ADPβS, washout, stimulated with ADPβS as indicating by the lines shown above the figure. **B**: RBA-2 astrocytes (5×10^4 cells per well) were seeded in the wells of a 12-well plate and cultured for 3 days and then pretreated with either PKC activator PMA (500 nM, 15 min), P2X$_4$ receptor allosteric effector IVM (10 μM, 10 min) or intracellular Ca^{2+} chelator BAPTA (50 μM, 30 min) and stimulated with or without ADPβS in the loading buffer. In one trial the cells were incubated in the Ca^{2+}-free loading buffer (-$[Ca^{2+}]_e$) and stimulated with ADPβS. **C**: The cells were pretreated with PI-PLC inhibitor ET-18-OCH3 (ET-18) (5 μM, 5 min) or PC-PLC/PLD inhibitor D609 (20 μM, 5 min) in the loading buffer and stimulated with or without P2Y$_1$ receptor agonist ADPβS. The ATP releases into the media were then measured by Bioluminescence assay and mean ± SD calculated from three independent studies each with three measurements. The results shown are ratio of controls and the symbol * represents significant different means compared with the controls calculated by Student's *t*-test with $P \leq 0.05$. Graphs were drawn using Prism 4 software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(≥300 μM) stimulated Ca^{2+} signaling via P2X$_7$ receptors using suspended cells [Sun et al., 1999]. The higher concentration of extracellular ATP might be accumulated from a pathological or stressful condition [Dubyak and El-Moatassim, 1993; Chen et al., 2006]. Thus characterization of the Ca^{2+} signals of P2Y$_1$ and P2X$_4$ receptors are physiologically important.

Astrocytes have been shown to express P2Y$_1$ receptors [Morán-Jiménez and Matute, 2000; Zhu and Kimelberg, 2001; Fumagalli et al., 2003]. Early studies revealed that the signaling of the stable transfection human P2Y$_1$ in 1321N1 astrocytoma cells was coupled selectively to PLC [Nicholas et al., 1996]. In the present study, the 2MeSADP-mediated Ca^{2+} signals were completely blocked by the P2Y$_1$ selective antagonist MRS2179 and PKC activator PMA, and inhibited by the PLC inhibitor U73122. Similarly MRS2179 was shown to completely block the ADP-stimulated Ca^{2+} increases in C6 glioma cells [Czajkowski et al.,

2002]. Recently, PKC was shown to play an important role in the regulation of $P2Y_1$ receptor function in human platelets [Hardy et al., 2005; Mundell et al., 2006] and both novel and conventional isoforms of PKC were identified to regulate the $P2Y_1$ receptors in platelets [Mundell et al., 2006]. RBA-2 astrocytes have been shown to express at least five PKC isozymes [Hung et al., 2005]. In the present study, we found GF109203X greatly enhanced $P2Y_1$-mediated biphasic Ca^{2+} signal and Go6979 also enhanced the Ca^{2+} sustain indicating that both Ca^{2+}-dependent and Ca^{2+} independent PKC were involved. Therefore $P2Y_1$ receptors may be negatively regulated by conventional and novel PKC isozymes in these astrocytes. U73122 has been used as a selective PLC inhibitor. In the present study, pretreatment with U73122 effectively inhibited but did not block the 2MeSADP-mediated Ca^{2+} signaling. The discrepancy is not known at this moment. Although ATP/P2Y receptors have been shown to couple to PLC-Ca^{2+} signaling, another signaling pathway was identified in astrocytes [Neary et al., 1999]. Therefore, $P2Y_1$ receptors might also couple with a U73122-insensitive signaling mechanism in RBA-2 astrocytes.

Using a single cell Ca^{2+} imaging system, we demonstrated that ATP (100 μM) and the selective $P2Y_1$ receptor agonist 2MeSADP [Fam et al., 2000] induced biphasic Ca^{2+} signals, transients and Ca^{2+} sustains. The ATP-induced Ca^{2+} transients were greatly decreased in the Ca^{2+}-free buffer but not the 2MeSADP-induced Ca^{2+} transients, suggesting that ATP might stimulate other extracellular Ca^{2+}-dependent P2 receptors in RBA-2 astrocytes. In addition, the 2MeSADP-induced Ca^{2+}sustains were abolished in the Ca^{2+}-free buffer indicating that the $P2Y_1$-mediated Ca^{2+} sustains were due to Ca^{2+} entry. Although our results indicated that activation of $P2Y_1$ receptors also induced Ca^{2+} entry through CCE, ATP further induced Ca^{2+} entry in intracellular Ca^{2+} stores emptied and CCE inhibited cells. Thus, ATP might stimulate Ca^{2+} entry through P2X receptors. Our results also revealed that apyrase abolished the 2MeSADP-stimulated Ca^{2+} sustain, and ADPβS stimulated a twofold increase in ATP release into the extracellular media. Although IVM potentiated the 2MeSADP-mediated Ca^{2+} signal; nevertheless, IVM has no effect on the ADPβS-mediated ATP release. Thus, activation of $P2Y_1$ receptors, but not $P2X_4$ receptors, stimulated ATP release in these astrocytes.

It was suggested to require two P2 subtype receptors, $P2Y_1$ and $P2Y_2$ receptors, to reach the maximal Ca^{2+} oscillation [Gallagher and Salter, 2003]. In the present study, we found that IVM potentiated both Ca^{2+} transient and sustain. Thus, coactivation $P2Y_1$ and $P2X_4$ receptors enhanced the Ca^{2+} signaling in these astrocytes. Recent reports have shown that $P2X_4$ receptors were associated with pain and microglia activation in the spinal cord [Kukley et al., 2001; Tsuda et al., 2003; Inoue et al., 2004]. A striking increase in $P2X_4$ receptor expression in the spinal cord microglia after injury and inhibition of $P2X_4$ receptor expression reversed the allodynia [Tsuda et al., 2003]. Astrocytes were known to express $P2X_4$ receptors [Fumagalli et al., 2003; Kukley et al., 2001; Wang et al., 2003] but its signaling and physiological function remains to be examined. Denervation induced increases in a number of cells with $P2X_4$ receptor responses in parotid tissue suggesting that it might associate with cell proliferation [Neary et al., 1996]. Thus, astrocytic $P2X_4$ receptors may be both physiologically and pathologically important. Taken together, using single cell Ca^{2+} image system, the distinct Ca^{2+} signals of $P2Y_1$ and $P2X_4$ receptors can be recorded and differentiated, and coactivation of the two P2 receptors potentiated the Ca^{2+} signals. However, the physiological function associated with $P2X_4$ receptors in astrocytes needs to be further analyzed.

The neuronal $P2X_4$ receptors mediated fast synaptic transmission and changed ion selectivity within seconds [Khakh et al., 1999a]. The signals declined rapidly within 5–10 s at maximal ATP concentration (100 μM) [North, 2002]. IVM was found to prolong the action of $P2X_4$ receptors, thus it was used as a specific positive allosteric effector [Khakh et al., 1999b]. Single-point mutation revealed that the effect of IVM on $P2X_4$ receptor was on transmembrane domains and the nearby ectodomain region [Jelínková et al., 2006]. In addition, IVM caused an increase in the number of cell surface $P2X_4$ receptors resulting from a mechanism dependent on clathrin/AP2-mediated mechanism [Toulmc et al., 2006]. In the present study, we demonstrated that IVM per se could not induce Ca^{2+} signal, but potentiated the 2MeSADP-stimulated Ca^{2+} signal of RBA-2

astrocytes. Thus, IVM might sensitize the $P2X_4$ receptors and enhanced its response to ATP stimulation. Our results showed that stimulation of $P2Y_1$ receptors induced Ca^{2+} entry, apyrase abolished the $P2Y_1$-mediated Ca^{2+} entry and activation of $P2Y_1$ receptors induced ATP release. Thus, the $P2Y_1$-induced ATP release might activate $P2X_4$ receptors to cause Ca^{2+} entry/sustain in these astrocytes.

ATP has been shown to induce Ca^{2+} oscillation through activation of $P2Y_2$ receptors and possibly depolarized cells through activation of $P2X_4$ receptors in the human macrophages [Hanley et al., 2004]. Although coactivation of one P2Y and one P2X has been suggested, in the present study we provide evidence for the first time showing that ATP stimulated a biphasic Ca^{2+} signaling through coactivation of $P2Y_1$ receptors and $P2X_4$ receptors in astrocytes. In addition, activation of $P2Y_1$ receptors stimulated ATP release of these astrocytes. The astrocytes-released ATP has been shown to be important in promoting astrocyte survival and growth [Neary et al., 2005]. Early studies revealed that ATP-stimulated ATP release through activation of $P2Y_1$ receptors of astrocytes [Darby et al., 2003]. Similarly, we revealed that apyrase abolished the 2MeSADP-mediated Ca^{2+} sustain, and ADPβS stimulated biphasic Ca^{2+} signal and ATP release. Thus activation of $P2Y_1$ and $P2X_4$ receptors might lead to greater and longer Ca^{2+} signals and to promote growth and better survival of these astrocytes.

In the present study, the 2MeSADP-stimulated Ca^{2+} signaling (Fig. 2B) and ADPβS-stimulated ATP release (Fig. 7B) were completely blocked by PMA. ADPβS has been shown to be a $P2Y_1$ receptor agonist [Yoshioka and Nakata, 2004; Kűgelgen, 2006]. Our results revealed that MRS2179 blocked the ADPβS-stimulated Ca^{2+} signals (Fig. 7A) indicating that ADPβS activated $P2Y_1$ receptors. Thus activation of $P2Y_1$ receptors induced ATP release. We also demonstrated that the $P2Y_1$ receptor-mediated ATP release was not affected by IVM, and decreased by ET-18-OCH3 and D609 suggesting that the release might not involve $P2X_4$ receptor but was regulated by phosphatidylcholine-phospholipase C (PC-PLC) or PLD signaling. Although $P2Y_1$ receptor signaling is known to couple to PI-PLC, activation of P2Y receptors has been shown to also couple to the D609-sensitive PC-PLC or PLD pathways [Brambilla et al., 2003]. RBA-2 astrocytes have been shown to express PLD1b and PLD2 [Hung and Sun, 2002]. The $P2Y_1$ receptors signaling pathways of RBA-2 astrocytes needs to be further examined.

ATP release from astrocytes may be important for glial–glial and glial–neuronal interaction and function, and that has been shown to mediate through multiple mechanisms. In the present study, our result indicated that the $P2Y_1$-mediated ATP release in RBA-2 astrocytes was enhanced by removal of extracellular Ca^{2+} and chelating of intracellular Ca^{2+}. Early reports indicated that ATP might be released from GAP junction through a Ca^{2+}-dependent mechanism [Coco et al., 2003]. Recent evidences showed that astrocytes concentrated ATP in a granule and release through a secretion pathway [Cotrina et al., 1998] and the ATP-induced ATP release from astrocytes was not affected by chelating intracellular Ca^{2+}, or by blocking extracellular Ca^{2+} influx [Anderson et al., 2004]. The exact mechanism of $P2Y_1$-mediated ATP release in RBA-2 astrocytes is not known at this moment. Nevertheless, $P2Y_1$ and $P2X_4$ act collaterally to potentiate Ca^{2+} signaling and that may be important in astrocyte–astrocyte or astrocyte–neuron communication.

REFERENCES

Anderson CM, Bergher JP, Swanson RA. 2004. ATP-induced ATP release from astrocytes. J Neurochem 88: 246–256.

Bobanovic LK, Royle SJ, Murrell-Lagnado RD. 2002. P2X receptor trafficking in neurons is subunit specific. J Neurosci 22:4814–4824.

Bootman MD, Collins TJ, Mackenzie L, Llewelyn RH, Berridge MJ, Pepplatt CM. 2002. 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. FASEB J 16:1145–1150.

Bowser DN, Khakh BS. 2004. ATP excites interneurons and astrocytes to increase synaptic inhibition in neuronal networks. J Neurosci 24:8606–8620.

Brambilla R, Neary JT, Fumagalli M, Cottini L, Cattabebi F, Schiller PR, Abbracchio MP. 2003. P2Y receptors in brain astroglial cells: Identification of a gliotic P2Y receptor coupled to activation of a calcium-independent ras/ERK1/2 pathway. Drug Dev Res 59:161–170.

Buell GN, Lewis C, Collo G, North RA, Surprenant A. 1996. An antagonist-insensitive P2X receptor expressed in epithelia and brain. EMBO J 15:55–62.

Burnstock G. 1997. The past, present and future of purine nucleotides as signalling molecules. Neuropharmacol 36: 1127–1139.

Burnstock G. 2006. Historical review: ATP as a neurotransmitter. Trends Pharmacol Sci 27:166–176.

Centemeri C, Bolego C, Abbracchio MP, Cattabeni F, Puglisi L, Burnstock G, Nicosia S. 1997. Characterization

of the Ca^{2+} responses evoked by ATP and other nucleotides in mammalian brain astrocytes. Br J Pharmacol 121:1700–1706.

Chen HB, Chan Y-T, Hung AC, Tsai Y-C, Sun SH. 2006. Elucidation of ATP-stimulated stress protein expression of RBA-2 type-2 astrocytes: ATP potentiate HSP60 and Cu/Zn SOD expression and stimulates pI shift of peroxiredoxin II. J Cell Biochem 97:314–326.

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C. 2003. Storage and release of ATP from astrocytes in culture. J Biol Chem 278:1354–1362.

Cotrina ML, Lin JH-C, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CCG, Nedergaard M. 1998. Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95:15735–15740.

Cunha RA, Ribeiro JA. 2000. ATP as a presynaptic modulator. Life Sci 68:119–137.

Czajkowski R, Lei L, Sabala P, Barańska J. 2002. ADP-evoked phospholipase C stimulation and adenylyl cyclase inhibition in glioma C6 cells occur through two distinct nucleotide receptors, P2Y1 and P2 Y12. FEBS Lett 513: 179–183.

Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA. 2003. ATP released from astrocytes during swelling activates chloride channels. J Neurophysiol 89:1870–1877.

Dixon SJ, Yu R, Panupinthu N, Wilson JX. 2004. Activation of P2 nucleotide receptors stimulates acid efflux from astrocytes. Glia 47:367–376.

Dubyak GR, El-Moatassim A. 1993. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol 265:C577–606.

Fam SR, Gallagher CJ, Salter MW. 2000. P2Y1 purinoceptor-mediated Ca^{2+} signaling and Ca^{2+} wave propagation in dorsal spinal cord astrocytes. J Neurosci 20:2800–2808.

Fellin T, Carmignoto G. 2004. Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. J Physiol 559:3–15.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. 2004. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron 43:729–743.

Fountain SJ, North RA. 2006. A C-terminal lysine that controls human P2X4 receptor desensitization. J Biol Chem 281:15044–15049.

Franke H, Kittner H, Grosche J, Illes P. 2003. Enhanced P2Y receptor expression in the brain after sensitisation with d-amphetamine. Psychopharmacol 167:187–194.

Franke H, Krügel U, Grosche J, Heine C, Härtig W, Allgaier C, Illes P. 2004. P2Y receptor expression on astrocytes in the nucleus accumbens on rats. Neurosci 127:431–441.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U, Williams M. 1997. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci 18:79–82.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonté C, Matteoli M, Verderio C, Abbracchio MP. 2003. Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. Glia 43:218–230.

Gallagher CJ, Salter MW. 2003. Differential properties of astrocyte calcium waves mediated by P2Y1 and P2Y2 receptors. J Neurosci 23:6728–6739.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca21 indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. 1999. ATP released from astrocytes mediates glial calcium waves. J Neurosci 19: 520–528.

Hanley PJ, Musset B, Renigunta V, Limberg SH, Dalpke AH, Sus R, Heeg KM, Preisig-Müller R, Daut J. 2004. Extracellular ATP induces oscillations of intracellular Ca^{2+} and membrane potential and promotes transcription of IL-6 in macrophages. Proc Natl Acad Sci USA 101:9479–9484.

Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mund SJ. 2005. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Mech Blood 105:3552–3560.

Hung AC, Sun SH. 2002. Characterization of the P2X7 receptor-mediated phosphatidylcholine-phospholipase D activities in a rat brain-derived type-2 astrocyte cell line, RBA-2. Cell Signal 14:83–92.

Hung AC, Chu Y-J, Lin Y-H, Weng J-Y, Chen HB, Au Y-C, Sun SH. 2005. Roles of protein kinase C in regulation of P2X7 receptor-mediated calcium signalling of cultured type-2 astrocyte cell line, RBA-2. Cell Signal 17:1384–1396.

Inoue K, Tsuda M, Koizumi S. 2004. ATP- and adenosine-mediated signaling in the central nervous system: Chronic pain and microglia: Involvement of the ATP receptor P2X4. J Pharmacol Sci 94:112–114.

Jelínková I, Yan Z, Liang Z, Moobat S, Teisinger J, Stojilkovic SS, Zemková H. 2006. Identification of $P2X_4$ receptor-specific residues contributing to the ivermectin effects on channel deactivation. Biochem Biophy Res Commun 349:619–623.

Jimenez AI, Castro E, Communi D, Boeynaems JM, Delicado EG, Miras-Portugal MT. 2000. Coexpression of several types of metabotropic nucleotide receptors in single cerebellar astrocytes. J Neurochem 75:2071–2079.

Khakh BS, Bao XR, Labarca C, Lester HA. 1999a. Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. Nat Neurosci 2:322–330.

Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA. 1999b. Allosteric control of gating and kinetics at P2X4 receptor channels. J Neurosci 19:7289–7299.

Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K. 2003. Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. Proc Natl Acad Sci USA 100:11023–11028.

Kűgelgen I. 2006. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacol Therapeu 110: 415–432.

Kukley M, Barden JA, Steinhauser C, Jabs R. 2001. Distribution of P2X receptors on astrocytes in juvenile rat hippocampus. Glia 36:11–21.

Lê K-T, Babinski K, Séguéla P. 1998. Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. J Neurosci 18:7152–7159.

Lee BC, Cheng T, Adams GB, Attar EC, Miura N, Lee SB, Saito Y, Olszak I, Dombkowski D, Olson DP, Hancock J,

Choi PS, Haber DA, Luster AD, Scadden DT. 2003. P2Y-like receptor, GPR105(P2Y14), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells. Genes Dev 7:1592–1604.

Metea MR, Newman EA. 2006. Glial cells dilate and constrict blood vessels: A mechanism of neurovascular coupling. J Neurosci 26:2862–2870.

Morán-Jiménez M-J, Matute C. 2000. Immunohistochemical localization of the P2Y purinergic receptor in neurons and glial cells of the central nervous system. Mol Brain Res 78:50–58.

Mulligan SJ, MacVicar BA. 2004. Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. Nature 431:195–199.

Mundell SJ, Jones ML, Hardy AR, Barton JF, Beaucourt SM, Conley PB, Poole AW. 2006. Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. Mol Pharmacol 70:1132–1142.

Neary JT, Laskey R, van Breemen C, Bilcharska J, Norenberg LOB, Norenberg MD. 1991. ATP-evoked calcium signal stimulates protein phosphorylation/dephosphorelyation in astrocytes. Brain Res 566:89–94.

Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G. 1996. Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19:13–18.

Neary JT, Kang Y, Bu Y, Akong K, Peters CM. 1999. Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: Involvement of a calcium-independent protein kinase C, extracellular signaling-regulated protein kinase pathway distinct from phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci 19: 4211–4220.

Neary JT, Kang Y, Tran M, Feld J. 2005. Traumatic injury activates protein kinase B/Akt in cultured astrocytes: Role of extracellular ATP and P2 purinergic receptors. J Neurotrauma 22:491–500.

Newman EA. 2003. New roles for astrocytes: Regulation of synaptic transmission. Trends Neurosci 26:536–542.

Nicholas RA, Lazarowski ER, Watt WC, Li Q, Boyer J, Harden TK. 1996. Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. J Auton Pharmacol 16:319–323.

North RA. 2002. Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067.

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH. 2006. Glia-derived D-serine controls NMDA receptor activity and synaptic memory. Cell 125:775–784.

Parri R, Crunelli V. 2003. An astrocyte bridge from synapse to blood flow. Nat Neurosci 6:5–6.

Priel A, Silberberg SD. 2004. Mechanism of ivermectin facilitation of human P2X4 receptor channels. J Gen Physiol 123:281–293.

Putney JW, Jr. 1986. A model for receptor-regulated calcium entry. Cell Calcium 7:1–12.

Putney JW, Jr. 1990. Capacitative calcium entry revisited. Cell Calcium 11:611–624.

Shinozaki Y, Koizumi S, Ishida S, Sawada J, Ohno Y, Inoue K. 2005. Cytoprotection against oxidative stress-induced damage of astrocytes by extracellular ATP via P2Y1 receptors. Glia 49:288–300.

Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, Stühmer W. 1996. P2 X4: An ATP-activated ionotropic receptor cloned from rat brain. Proc Natl Acad Sci USA 93:3684–3688.

Sun SH, Lin L-B, Hung AC, Kuo J-S. 1999. ATP-stimulated Ca^{2+} influx and phospholipase D activities of a rat brain-derived type-2 astrocyte cell line, RBA-2, are mediated through P2X7 receptors. J Neurochem 73:334–343.

Toulmc E, Soto F, Garret M, Boué-Grabot E. 2006. Functional properties of internalization-deficient P2X4 receptors reveal a novel mechanism of ligand-gated channel facilitation by ivermectin. Mol Pharmacol 69:576–587.

Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter MW, Inoue K. 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778–783.

Wang CM, Chang YY, Sun SH. 2003. Activation of P2X7 purinoceptor stimulated TGF-β1 mRNA expression via PKC/MAPK pathway in the RBA-2 type-2 astrocytes. Cell Signal 15:1129–1137.

Yoshioka K, Nakata H. 2004. ATP- and adenosine-mediated signaling in the central nervous system: Purinergic receptor complex:generating adenine nucleotide-sensitive adenosine receptors. J Pharmacol Sci 94:88–94.

Zhang F, Luo L, Gustafson E, Palmer K, Qiao X, Fan XG, Yang S, Laz TM, Bayne M, Monsma F, Jr. 2002. P2 Y13: Identification and characterization of a novel Gαi-coupled ADP receptor from human and mouse. J Pharm Exp Ther 301:705–713.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S. 2003. ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971–982.

Zhu Y, Kimelberg HK. 2001. Developmental expression of metabotropic P2Y1 and P2Y2 receptors in freshly isolated astrocytes from rat hippocampus. J Neurochem 77:530–541.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G. 2003. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6:43–50.