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Activation of microglial P2Y12 receptor is required for outward potassium currents in response to neuronal injury

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Abstract

Microglia, the resident immune cells in the central nervous system, constantly survey the surrounding neural parenchyma and promptly respond to brain injury. Activation of purinergic receptors such as P2Y12 receptors (P2Y12R) in microglia has been implicated in chemotaxis towards ATP that is released by injured neurons and astrocytes. Activation of microglial P2Y12R elicits outward potassium current that is associated with microglial chemotaxis in response to injury. This study aimed at investigating the identity of the potassium channel implicated in microglial P2Y12R-mediated chemotaxis following neuronal injury and understanding the purinergic signaling pathway coupled to the channel. Using a combination of two-photon imaging, electrophysiology and genetic tools, we found the ATP-induced outward current to be largely dependent on P2Y12R activation and mediated by G-proteins. Similarly, P2Y12R-coupled outward current was also evoked in response to laser-induced single neuron injury. This current was abolished in microglia obtained from mice lacking P2Y12R. Dissecting the properties of the P2Y12R-mediated current using a pharmacological approach revealed that both the ATP and neuronal injury-induced outward current in microglia was sensitive to quinine (1 mM) and bupivacaine (400 μM), but not TEA (10 mM) and 4-AP (5 mM). These results suggest that the quinine/bupivacaine-sensitive potassium channels are the functional effectors of the P2Y12R–mediated signaling in microglia activation following neuronal injury.

Graphic Abstract

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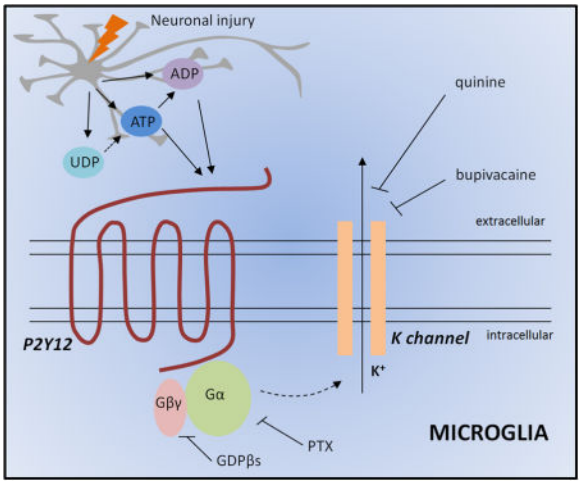
Schematic representation showing that the ATP, ADP and UDP released following neuronal injury directly /indirectly acts on microglial P2Y12 receptor, a G-protein coupled receptor linked to a potassium channel, eliciting an outward potassium current. Inhibitors of G-proteins blocked the P2Y12 receptor mediated outward potassium current. Moreover, this outward current was found to be sensitive to certain potassium channel blockers such as quinine and bupivacaine, while being insensitive to TEA and 4-AP. arrow-headed lines and bar-headed lines to indicate activation and inhibition, respectively. Dotted lines represent hypothetical associations.



Keywords

Microglia; P2Y12 receptor; ATP; K^+ channels

Introduction

Microglia, the resident immune cells in the central nervous system (CNS), plays an important role in healthy and disease brain. The microglial processes are highly motile, constantly surveying the surrounding neural parenchyma and promptly respond to brain injury (Davalos et al., 2005, Nimmerjahn et al., 2005). Evidence indicates that the microglial processes are preferentially directed towards synapses, where they may be involved in monitoring and regulating neuronal activity (Wake et al., 2009, Tremblay et al., 2010). However, in response to injury, the microglial process motility becomes targeted towards the site of insult. This site-directed movement or chemotaxis of microglial processes was shown to be in response to ATP released by damaged neurons and astrocytes (Davalos et al., 2005, Pascual et al., 2012).

Accumulating body of literature implicates ATP-induced activation of purinergic receptors in microglial chemotaxis towards the injury site. Among the purinergic receptors, P2Y12 receptor (P2Y12R) subtype is exclusively expressed in microglia in the CNS (Sasaki *et al*., 2003) and is essential for microglial chemotactic response to injury (Haynes et al., 2006). Unlike the other metabotropic P2Y receptors which transduce their signals through Gq-phospholipase C pathways (Burnstock, 2008), the P2Y12R couples to Gi-adenylyl cyclase pathways through which it mediates the morphological and dynamic responses of microglia

to extracellular nucleotides (Hollopeter et al., 2001, Zhang et al., 2001). ATP/ADP-induced activation of P2Y12R through its signaling elicits an outward potassium current in microglia. Blocking this current with quinine, a nonselective potassium channel antagonist, abolished chemotaxis to ATP, implying that this current is important for ATP/ADP-mediated microglia motility (Wu et al., 2007). In spite of the advances mentioned above, the purinergic signaling pathway coupled to the potassium channel is poorly documented (Schwab, 2001, Schilling and Eder, 2007) and the potassium channel associated with P2Y12-mediated microglial chemotaxis remains unidentified.

Microglial P2Y12R plays a pivotal role in neuropathological conditions. Our recent study showed that mice deficient in P2Y12R experienced worsened seizure outcome after kainic acid injections, suggesting a neuroprotective role for microglial P2Y12R in epilepsy (Eyo et al., 2014). P2Y12R in spinal microglia was also shown to be involved in the pathogenesis of neuropathic pain after peripheral nerve injury (Tozaki-Saitoh et al., 2008) and ischemic stroke (Webster et al., 2013). Therefore, understanding microglial P2Y12R signaling will provide novel candidates for therapeutic interventions in pathologies implicating microglia P2Y12R. In this study, we investigate the identity of the K^+ channel coupled to P2Y12R signaling, as well as the intracellular pathway mediating the response. We found the ATP-induced outward current to be largely dependent on P2Y12R activation and mediated by G-proteins. In addition, P2Y12R-coupled outward potassium current was also activated in response to laser-induced single neuron injury.

Materials and Methods

Animals

Both male and female C57BL/6N mice were used in accordance with the institutional guidelines, as approved by the Animal Care and Use Committee at Rutgers University. Homozygous GFP reporter mice ($CX3CR1^{GFP/GFP}$) expressing GFP under the control of the fractalkine receptor (CX3CR1) promoter (Jung et al., 2000) were obtained from Jackson Laboratory. The $CX3CR1^{GFP/-}$ mice were used throughout the study to identify miocroglia in brain slices. For simplicity, we named $CX3CR1^{GFP/-}$ mice as wild-type in this study. P2Y12R knockout (P2Y12 KO) mice were obtained from Dr. Michael Dailey at the University of Iowa. The $CX3CR1^{GFP/-}$ P2Y12 KO was obtained by mating the above-mentioned mouse lines.

Slice Preparation

Freshly isolated cortical slices were prepared from 3-6-week-old mice. Briefly, mice were anesthetized and swiftly decapitated. Brains from decapitated mice were carefully removed and placed in ice-cold oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) with the following composition (in mM): NaCl, 124; $NaHCO_3$, 25; KCl, 2.5; KH_2PO_4, 1; $CaCl_2$, 2; $MgSO_4$, 2; glucose, 10 and sucrose added to make 300-320 mOsmol. Coronal slices (300 μm) were prepared and transferred to a recovery chamber for 30 m with oxygenated ACSF with the same composition as above at room temperature before imaging or electrophysiological studies.

Two-photon Imaging

Experiments were conducted at room temperature with slices maintained in oxygenated ACSF with the same composition as above in a perfusion chamber at a flow rate of 2mL/min. It is important to note that the dynamic behavior of microglia may differ from the *in vivo* conditions due to variation in parameters such as temperature and changes in extracellular milieu. Microglia were typically imaged using a two-photon microscope (Scientifica Inc, UK) with a Ti: Sapphire laser (Mai Tai; Spectra Physics) tuned to 900nm (for GFP microglia) with a 40× water immersion lens (0.8 NA; Olympus). Fluorescence was detected using two photomultiplier tubes in whole-field detection mode and a 565 nm dichroic mirror with 525/50 nm (green channel) emission filters. The laser power was maintained at 25 mW or below. Typically, 15 consecutive z stack images were collected at 3 μm intervals every minute. To perform a general laser injury, we focused the laser 66× and parked it at ∼250 mW at 900 nm for 3 s. The lesion site was induced in a 15 × 15 pixel frame and the size of the resulting laser burn was estimated to vary between 8-15μm (25-50 pixels) in diameter. For imaging experiments, a minimum of three to four slices from different mice from the same litter were randomly selected for imaging per treatment group/condition. Images were obtained between 50-100μm from the slice surface. 45μm thick sections were made from projection z-stack images taken at 3μm intervals. Pixel size was 1024 × 1024 and field of view was 165μm × 165μm × 45μm. Images were generated using Image J and Max projection was applied to all images. For responding process velocity and number analysis either directed toward an ATP-containing pipette or a laser-induced injury, time-lapse movies were first registered using the StackReg plugin to eliminate any *x-y* drift. For responding process number analysis the number of responding processes at the final frame was manually counted. For process velocity analysis, individual processes were then tracked using the Manual Tracking plugin. Migrating processes were selected at random but only processes that were maintained through at least five frames were used. The average process velocity through the tracked period was determined and averaged from at least eight processes per experiment for three experiments.

Slice Electrophysiology

Whole cell patch-clamp recordings were made on GFP-labeled microglia from cortical slices at ∼50 μm from the slice surface. Recording electrodes (4 –5 MΩ) contained a K-based internal solution composed of (in mM): 120 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.5 EGTA, 10 Na_2 Phosphocreatine, and 10 HEPES (pH 7.2; 280 –300 mOsmol). In order to block outward potassium current we used Cs-based internal solution containing (in mM): 115 Cs-$MeSO_3$, 5 NaCl, 10 HEPES, 1 $MgCl_2$, 0.2 EGTA, and 10 Na_2 Phosphocreatine (pH 7.2; 280-300 mOsmol). Additionally, to manipulate G proteins, GDPβS (1mM) was included in the internal solution. Unless otherwise stated, the membrane potential was held at -20 mV for microglia throughout all experiments. Data were amplified and filtered at 2 kHz by a patch-clamp amplifier (Multiclamp700B), digitalized (DIGIDATA 1440A), stored, and analyzed by pCLAMP (Molecular Devices, Union City, CA). All cells showed small membrane capacitance (24.93 ± 1.2 pF, n =20), high membrane resistance (1.53 ± 0.08 GΩ, n = 20), and a more positive membrane potential (-21.9 ± 1.59 mV, n = 20) than neurons (around -70 mV). Data were discarded when the input resistance changed >20% during

recording. The voltage ramp test was performed from -100 to +20 mV in 500 ms. The ramp current/IV current is linear in resting microglia in brain slices. Rectifying currents were observed in activated microglia and were not chosen in this study. For electrophysiology, a minimum of five cells from at least three different mice from the same litter were randomly selected for recording per condition. Under conventional whole-cell patch clamp recording, the morphology of the patched microglia is not altered much in short time (within 10 min), but then becomes difficult to appreciate the cell structure after the GFP is washed out gradually. In terms of dynamics, microglia are no longer able to extend its processes towards either an ATP puff or laser burn under conventional whole-cell recording. However, microglia did retain some dynamics under perforated whole-cell recording as we reported before (Wu et al., 2007). For the reason mentioned above, the electrophysiology recordings and imaging were performed in independent experiments.

Drugs

ATP, ADP, UDP, GDPβS and bupivacaine hydrochloride were purchased from Sigma. Pertussis toxin (PTX), forskolin, tetraethylammonium (TEA), quinine, and 4-aminopyridine (4-AP) were purchased from Tocris. Stock solutions of all drugs were made in water and diluted to the appropriate working concentrations in ACSF. Drugs were applied to the slices either through bath application or using a picopump (WPI pneumatic picopump, Sarasota, FL). The diameter of the drug application pipette tip was ∼3–4 μm. The pressure (10 psi) and duration (100 ms) of the puff was controlled and the distance between the patched cell and puff pipette was kept constant (∼15 μm). This was achieved by marking the position of the two pipettes (recording and puff) on the display screen and adjusting the distance of the puff pipette until the preferred distance was reached. The holding pressure of the puff pipette was maintained at -2 psi to prevent leakage, but there may still be minimal spontaneous leakage. For experiments involving testing of antagonists on ATP-induced current, control applications of ATP were performed and then on the same cell the effect of the antagonist was tested.

Statistical Analysis

For all experiments, the number of mice used for each experimental group is stated in the corresponding figure legend. Data are presented as mean ± SEM. ANOVA with Tukey-Kramer multiple comparisons test was used to establish significance. A p value of <0.05 was considered to be significant.

Results

Characterization of microglial response to ATP, ADP and UDP

Using electrophysiological and imaging tools, we first characterized the microglial response to local application of ATP (non-selective P2X/P2Y agonist, 1mM), ADP (potent agonist of P2Y12, 1mM), and UDP (potent agonist of P2Y6, 1mM) in acute cortical slices of wild type mice. The application of all three nucleotides induced process extension (Figure 1A-C) and a rapid inward current followed by small outward potassium current (Figure 1D-F) in cortical microglia. However, puff application of ACSF did not induce an outward current or microglial chemotaxis (data not shown). ATP-induced inward currents are mediated by P2X

receptors and outward currents are mediated by P2Y receptors in microglia (Haas et al., 1996, Boucsein et al., 2003). Since the reversal potential for non-selective cation channels associated with P2X receptors (inward current) is close to 0mV, a holding potential of -10mV invokes both an inward and outward current following ATP puff application (Wu et al., 2007). Analysis of the chemotactic response showed that there was no significant difference in the number of responding microglial processes, average process velocity or the peak response velocity (ATP 2.02 ± 0.109 μm/min, ADP 1.96 ± 0.111 μm/min, UDP 2.26 ± 0.127 μm/min) towards ATP, ADP or UDP puff application (Figure 1H-I). It is important to note that the chemotactic response to UDP was not noted in all instances (3 out of 5 experiments showed positive response). In the experiments with negative response, microglial processes were found crawling within the UDP filled pipette and blocking the puff application. Previous reports stated that UDP does not evoke chemotaxis but only induces phagocytosis (Koizumi et al., 2007, Neher et al., 2014). It is important to note that the earlier studies were performed in microglia cultures and hence the dynamic properties of the cultured microglia may not fully represent the *in vivo* conditions. The chemotactic response to UDP is a novel finding and needs to be further investigated. The voltage ramp test (from -100 to +20 mV, 500 ms) was performed before and following the puff application of ATP, ADP and UDP; The I/V relationship was then obtained by subtracting the first ramp current from the second one (Figure 1J). The reverse potential for the respective nucleotides were calculated from the resultant I/V curve. The reversal potential for ATP, ADP and UDP were found to be -66.06 ± 0.431 mV, -67.64 ± 0.310 mV and -60.36 ± 0.360 mV, respectively, after correction for junction potential (n=5 for each group). Here, in addition to the chemotaxis, we found that there were no significant differences in intensity or kinetics of the outward currents in response to the nucleotides (Figure 1K-L). Moreover, the current-voltage relationship for the outward current in response to the nucleotides was similar, suggesting that the same potassium channel might mediate the ATP/ADP/UDP-induced outward current.

ATP/ADP/UDP-induced microglial chemotactic response and outward currents require P2Y12 receptor activation

In order to test whether P2Y12R signaling was required for the process extension and outward potassium currents in microglial response to the nucleotides, we applied ATP, ADP and UDP (1mM) to acute cortical slices derived from P2Y12 KO mice. Both nucleotide-induced process extension (Figure 2A-C) and outward currents (Figure 2D-F) were abolished in P2Y12R deficient microglia. Surprisingly, even the application of UDP, a P2Y6 agonist, did not produce process extension or outward current in P2Y12 KO microglia. Since, it is known that ATP/ADP cannot activate P2Y6 receptors and neither can UDP act on P2Y12/13 receptors (Koizumi et al., 2007), we hypothesize that the decrease in UDP-evoked outward current in P2Y12KO may be due to the indirect effect of P2Y12 mediated signaling in P2Y6 receptor activation. Interestingly, it has been shown that P2Y12R mediated migration of microglia is regulated by other P1/P2 receptor mediated signals(Ohsawa et al., 2007, Wu et al., 2007). Another study showed that ATP-induced P2X7 receptor activation increased extracellular Ca^{2+} influx and activated phosphoinositide 3-kinase (PI3K) pathway that regulates microglial chemotaxis (Ohsawa et al., 2007). Taken together, these results suggest a synergistic effect between the different purinergic channels

cannot be excluded. Based on our results, we hypothesize that UDP could induce ATP release and hence the chemotactic response could be indirect. Firstly, in addition to the outward current, UDP also-induced an inward current which is likely mediated by P2X receptors. This inward current remained unaffected in P2Y12 KO mice. Since UDP is not known to activate P2X receptor, the results may further support the hypothesis that UDP act on microglia through indirect release of ATP. Secondly, we show that quinine also blocks the UDP-induced outward current further supporting our hypothesis. Thirdly, the absence of chemotactic response towards UDP in P2Y12 KO mice confirmed our hypothesis that UDP indirectly caused the release of ATP, and the outward current is predominantly mediated by P2Y12 receptors. Our results confirm that P2Y12R signaling is essential for ATP/ADP/UDP-mediated microglial chemotactic response and outward potassium currents (Figure 2G). This led us to further investigate the identity of the potassium channel coupled to P2Y12 receptor and investigate the underlying signaling pathway.

Potassium channels are coupled to P2Y12 receptor signaling

We applied two approaches to investigate the identity of the ion channel coupled to microglial P2Y12R activation. In our first approach, whole cell patch recordings of microglia with internal solution containing cesium ions, which is known to block potassium currents (Hagiwara et al., 1976), were performed. Predictably, the application of ATP, ADP, and UDP did not induce an outward current in the presence of cesium (Figure 3A). In our second approach, we recorded microglial currents following ATP application in the presence of broad potassium channel blockers: TEA (10 mM), quinine (1 mM), or 4-AP (5 mM) (Figure 3B). Both TEA and 4-AP were unsuccessful at blocking the outward current, while quinine effectively abolished any ATP mediated potassium outward current. In addition, quinine also inhibited ADP or UDP-induced outward currents (Figure 3D). Quinine also blocked the ATP-induced chemotactic response towards the puff pipette (data not shown, (Wu et al., 2007)). Outwardly rectifying currents of two-pore domain potassium (K2P) channels have been shown to possess similar characteristics with weak sensitivity to classical blockers of K channels, such as TEA and 4-AP, but are sensitive to quinineand bupivacaine (Girard et al., 2001, Shin et al., 2014). In line with this, low concentrations of bupivacaine (400 μM) partially blocked the ATP-induced outward current in microglia (Figure 3C). These results suggest that a K2P channel might be coupled to P2Y12 receptor and that the current mediated by this channel is quinine/bupivacaine-sensitive (Figure 3D).

Intracellular pathways coupling P2Y12 receptor to the potassium channel

We then went on to elucidate the intracellular pathway coupling ATP-induced activation of microglial P2Y12R using a pharmacological approach. P2Y12R has been found to have a direct effect on adenylyl cyclase, causing its inhibition and reduced production of cAMP (Unterberger et al., 2002, Yang et al., 2002, Witkowski et al., 2012). To this end, we recorded ATP induced currents in the presence of forskolin (10μM), a drug known to increase the intracellular levels of cAMP (Figure 4A). Interestingly, we observed no apparent changes in the ATP-induced microglial outward current (Figure 4A, B). Forskolin enhanced synaptic transmission of cortical pyramidal neurons (Wu et al., 2008) and was used as a positive control. We found that forskolin (10 μM) increased spontaneous excitatory postsynaptic currents in neurons but do not affect ATP-induced outward current

in microglia (data not shown). Therefore, these results suggest, suggesting that cAMP levels are not responsible for direct or indirect activation of the P2Y12R-associated K^+ channel (Figure 4A, B). A number of downstream targets of G-protein signaling such as protein kinase A, phospholipase C, protein kinase C and intracellular calcium, have been implicated as most crucial elements of purinergic signaling and potassium channel activation (Illes et al., 1996, Honda et al., 2001, Nasu-Tada et al., 2005, Eichhoff et al., 2011, Kataoka et al., 2011). Hence, to test whether the P2Y12R-K^+channel was coupled by G-protein signaling, we included GDPβS (1μM), in the internal solution and recorded microglia current responses to puff application of ATP (Figure 4A). Indeed, GDPβS effectively blocked the outward current (Figure 4A, B). We further tested pertussis toxin (PTX, 4μM), which is known to inactivate G_i/G_o G proteins, on ATP-induced outward currents in microglia. Consistently, PTX inhibited microglial ATP-activated outward K currents (Figure 4A, B).

We also tested whether the microglial P2Y12R-mediated outward current is linked to the store operated Ca^{2+} channel (SOC) (Jantaratnotai et al., 2009). Microglia in acute slices were patched with internal solution containing EGTA (5 mM) and current responses to local application of ATP were recorded (Figure 4C). We detected no significant inhibition of the outward current (Figure 4D), implying that microglia P2Y12R-mediated outward current is not dependent on intracellular calcium. Together, these results suggest that P2Y12R is coupled to G_i/G_o G-proteins and their downstream targets independent of cAMP pathway or Ca^{2+} to activate the P2Y12R-associated outward potassium currents in microglia.

Neuronal injury induced P2Y12R-mediated outward currents in microglia

We then pursued to address the physiological relevance of ATP-induced microglial outward current after brain injury. We were able to induce neuronal injury associated with microglial process chemotaxis in the brain slices using two photon laser beam (Eyo et al., 2014). Interestingly, we found that the outward current response was recorded from a microglia when the neighboring neuron was damaged with a laser (Figure 5A). The injury-induced outward microglial current was similar to the one induced by ATP was recorded in WT mice, which was absent in P2Y12 KO mice (Figure 5B). A recent study showed that irradiation-induced increase in extracellular ATP was is in the nanomolar range (Xu et al., 2015) suggesting that the lack of inward current by laser burn could be due to the insufficient ATP released following laser-induced nerve injury. Consistently, we have previously shown that at ATP at 30 μM only induced outward currents but not inward currents (Wu et al., 2007).

Next, we recorded laser burn induced currents in the presence of previously described K^+ channel blockers: TEA, 4-AP, quinine or bupivacaine (Figure 5C). Again, only quinine and bupivacaine were successful at inhibiting the outward current (Figure 5D). Similar to the ATP-induced current, the laser burn-induced current was also sensitive to GDPβS (data not shown). Also, we found that quinine could block the laser burn induced chemotactic response (data not shown). Our data also shows that the amplitude of the outward current diminishes with increasing distance from the laser burn (Figure 5E). This is in line with our previous report in which the amplitude of current decreased with ATP concentration (Wu et al., 2007). Since the ATP concentration gradient reduces with distance, the amplitude of

outward current reduces as the distance from the ATP source/injury site increases. Collectively, these data suggests that laser damage to a single neuron is sufficient to produce outward potassium currents in microglia, most likely due to release of ATP from injured neuron and activation of microglial P2Y12R-associated K^+ channel.

Discussion

Our current findings indicate that ATP-induced microglial P2Y12R activation elicits outward potassium currentsvia the opening of potassium channels. In this study, we investigate the identity of the potassium channel coupled to P2Y12R and the intracellular signaling pathway linking the receptor to the channel. Additionally, the properties of microglial outward current in response to neuronal injury was found to be similar to the one induced by ATP/ADP. Also we show that this injury-induced microglial current was mediated by P2Y12R.

K^+ channel coupled to microglial P2Y12R

P2Y12R were initially identified on platelets and are responsible for platelet activation and aggregation during the blood clotting process (Hollopeter et al., 2001). The expression of P2Y12R in microglia was first described by Sasaki et al (Sasaki et al., 2003) and has been implicated in microglia activation, migration, chemotaxis and phagocytic ability (Haynes et al., 2006, Wu et al., 2007, Orr et al., 2009, De Simone et al., 2010, Sunkaria et al., 2015). P2Y12R plays a vital role in microglia activation, as they act as the primary site at which nucleotides act to induce microglial chemotaxis in response to local CNS injury (Haynes et al., 2006, De Simone et al., 2010). It has been shown that enhanced P2Y12R-mediated purinergic signaling was responsible for microglial activation and the resultant increase in voltage-activated potassium currents in hippocampal microglia following status epilepticus (Avignone et al., 2008) and in spinal microglia following peripheral nerve injury (Gu et al., 2015). P2Y12R activation has also been shown to mediate microglial process extension and convergence towards dendrites following neuronal hyperactivity (Eyo et al., 2014, Eyo et al., 2015). Apart from chemotaxis, P2Y12-dependent purinergic signaling regulates the phagocytic ability of microglia (Michaelis et al., 2015, Preissler et al., 2015, Sunkaria et al., 2015). In platelets, G-protein gated inwardly rectifying potassium (GIRK) channels was identified to be the functional effectors downstream of P2Y12 receptor activation (Shankar et al., 2004). Interestingly, the adhesion molecule integrin-β1 is activated by P2Y12R and is critical for ATP-induced process extension (Ohsawa et al., 2010). However, P2Y12R-coupled K^+ channel and integrin-β1 activation may represent two parallel pathways coupled to P2Y12 receptors in microglia. Similarly, we have shown that P2Y12R-induced PI3K activation is critical for ATP-induced process extension but not for ATP-induced outward K^+ currents (Wu et al., 2007). Nevertheless, althoughP2Y12R has been shown to be important for a myriad of microglial functions, the effectors mediating its effects in microglia have not been entirely identified.

Coupling to ion channels is an important component of P2Y12R signal transduction, but one that has remained unknown. In studies of channel coupling by P2YR, heterologous expression in commonly transfected host cell lines such as CHO or HEK293, or in the

Xenopus oocyte is done. However, usually both the P2Y receptor and the identified ion channel under study are introduced into the cells, and even then, the final interaction and protein environment of those components may be far from that in native cell (Marcet et al., 2004). In this study, we used an electrophysiological approach to reveal the identity of the channel coupled to microglia P2Y12R. The results of our study show that ATP/ADP/UDP-induced chemotaxis and outward currents were abolished in P2Y12 KO mice, indicating that they are mediated by microglial P2Y12R signaling. In addition, we showed that the ATP-induced currents were potassium currents sensitive to quinine and bupivacaine, but insensitive to TEA and AP-4. Similarly, outwardly rectifying currents of the K2P channels show similar characteristics with weak sensitivity to classical blockers of K channels, such as TEA and 4-AP, but are sensitive to quinine or quinidine (Girard et al., 2001) and low concentrations of bupivacaine (Shin et al., 2014). Therefore, leakK2P channels may be the functional effectors of the P2Y12R–mediated signaling in microglia activation. The exact subtype of K2P channel linked to P2Y12R still remains unknown and warrants further investigation.

Intracellular signaling pathway associated with P2Y12R activation

P2Y receptors, unlike P2X receptors, are metabotropic in nature and act via G protein signaling. In platelets, P2Y12R was shown to be coupled to Gαi2, by photolabeling with radiolabeled GTP (Ohlmann et al., 1995) and was confirmed in Gαi2-deficient mouse platelets (Jantzen et al., 2001). Consistent with these studies, our results show that P2Y12R is coupled to potassium channels via a G_i/G_o-protein signaling pathway.

A majority of the P2Y receptors are linked to phospholipase C and mediate increases in inositol phosphates. It has been shown that phospholipase C-mediated increase in intracellular calcium and Akt activation is involved in ADP-induced microglial chemotaxis and may be the pathway underlying P2Y12R-mediated signaling (Irino et al., 2008). Another recent study demonstrated that activation of P2Y12R involved the phosphorylation of p38 mitogen-activated protein kinase in spinal microglia (Tatsumi et al., 2015). However, P2Y12R are often associated with reduced cyclic AMP (Harden et al., 1995), and this pathway has been extensively characterized in human platelets and in rat glioma cells (Boyer et al., 1993, Hollopeter et al., 2001, Jin et al., 2001). In line with this, another study showed that similar reduction in cyclic AMP in rat endothelial cells was mediated by P2Y12R (Simon et al., 2001). In our study, addition of forskolin to enhance the levels of cyclic AMP did not have any effect on ATP-induced P2Y12R-mediated outward current. This suggests that cAMP levels are not responsible for direct or indirect activation of the P2Y12R-associated K^+ channel. This however does not rule out the possibility that reduction in cAMP levels and coupling to potassium channels may be two independent downstream signaling pathways following microglia P2Y12R activation.

Microglial P2Y12R signaling in injury

Microglial P2Y12R has been implicated in a number of neuropathological conditions such as epilepsy (Eyo et al., 2014), neuropathic pain (Kobayashi et al., 2008, Tozaki-Saitoh et al., 2008) and ischemic stroke (Webster et al., 2013). Microglial P2YR activation is associated with neuroinflammation (Gyoneva et al., 2014) and is a hallmark response to brain injury

(Hernandez-Ontiveros et al., 2013). Following injury, neurons and astrocytes release nucleotides such as ATP/ADP and UDP, which act on purinergic receptors in microglia (Lazarowski et al., 1997, Koizumi et al., 2007). ATP/ADP is a chemoattractant, while UDP elicits a phagocytic response in microglia (Koizumi et al., 2007, Neher et al., 2014). Hence, it is suggested that the adenosine and uridene nucleotides may act on the microglial purinergic receptors in a coordinated fashion aiding in chemotaxis and phagocytosis, respectively, following injury. Two photon *in vivo* imaging studies show that optically injured site is rapidly sealed by microglia cell processes and this process extension is mediated by P2Y12R (Haynes et al., 2006, Wu et al., 2007, Eyo et al., 2014). In this study we show that focal laser burn injury of a neighboring neuron resulted in outward potassium current in microglia similar the ATP-induced microglial current. Like the ATP-induced outward current, the focal burn-induced current was inhibited by quinine but not TEA and 4-AP and was abolished in P2Y12R deficiency. Taken together our study suggests that ATP/injury-induced P2Y12R activation results in initiation of G-protein coupled cascade of events involving opening of potassium channels in microglia.

In addition to the animal models that implicate microglia P2Y12R in pathological scenarios, a recent study showed that alternatively activated human microglia expresses P2Y12R (Moore et al., 2015). In humans, molecular defects in P2Y12R have been identified in four families of patients with hemorrhagic syndromes (Cattaneo, 2005). The significance of a defect in microglial P2Y12R has not been studied in humans, but would be a worthy investigation. Given the physiological and pathological relevance of microglia P2Y12R activation, understanding the underlying signaling pathway will provide novel insights for developing therapies for the treatment of clinical conditions that implicate microglialP2Y12R.

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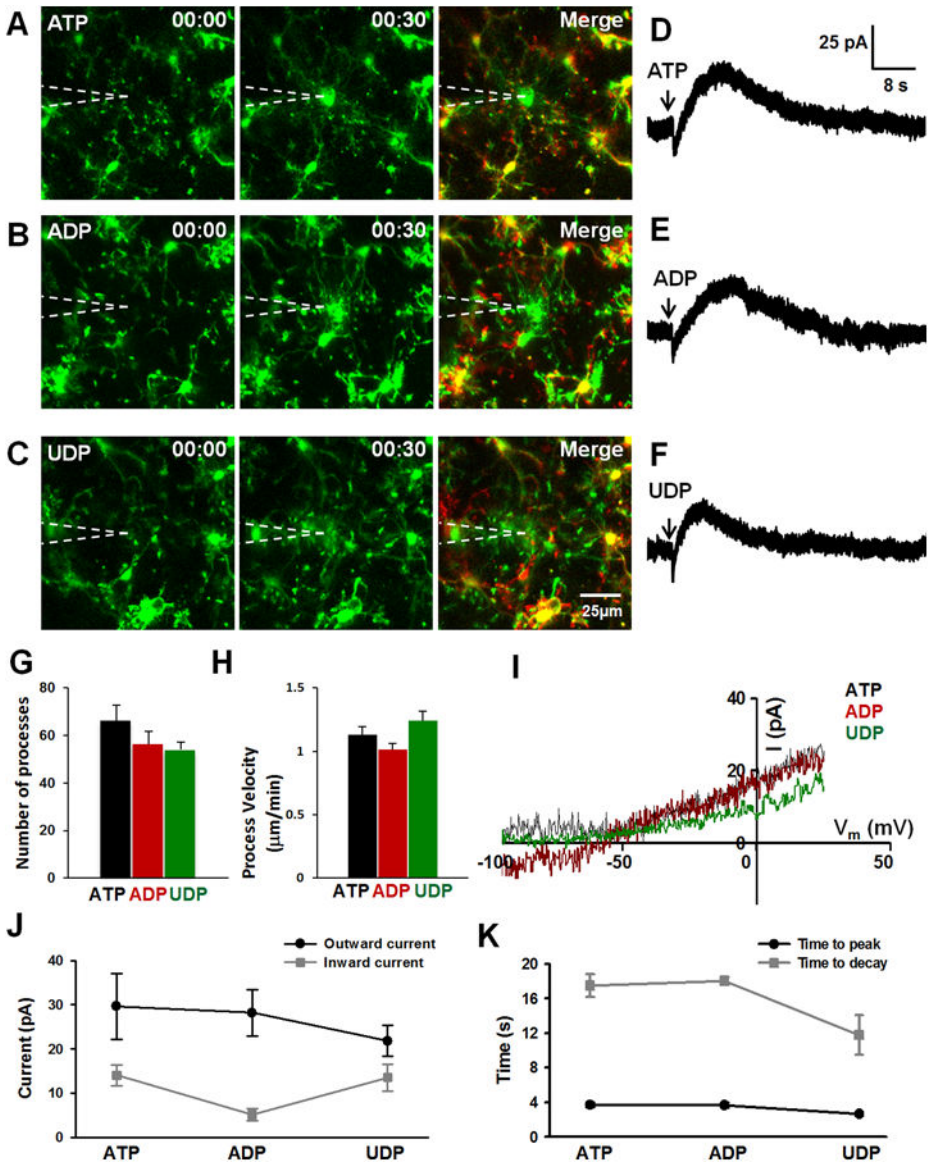

Figure 1. Dynamic and electrophysiological characteristics of microglial response to ATP, ADP and UDP

Two-photon images showing local application of 1mM of ATP (**A**), ADP (**B**) and UDP (**C**) induced rapid extension of microglial processes toward the tip of puff pipette in acute cortical slices of wild type (WT) mice (A: n =5, B: n=5, C: n= 4). Two time points (0 and 30 min) are shown here. The merged image is an overlay of imaging at 0 min (red) and 30 min (green). Scale bar: 25 μm. Unless otherwise stated, merged pictures are the overlay of imaging at 0 min (red) and 30 min (green) in the following figures. Whole cell patch clamp recordings of microglia using K^+ intracellular solution (IS) showed that local application of 1mM of ATP (**D**), ADP (**E**) and UDP (**F**) induced a rapid small inward current followed by an outward current (D-F: n=5). Holding potential is -20mV. The number of responding microglial processes (**G**) and the process velocity (**H**) in response to ATP, ADP and UDP was analyzed. (**I**) A representative IV trace obtained from the difference in ramp test (from

-100 to +20 mV, 500 ms) performed before and during the ATP/ADP/UDP-induced outward current. From the IV curve, the reversal potential for ATP, ADP and UDP were found to be -66.06 ± 0.431 mV (n=5), -67.64 ± 0.310 mV (n=5) and -60.36 ± 0.360 mV (n=5), respectively. The intensity of inward/outward microglia currents (**J**) and the kinetics of the outward current (**K**) in response to the nucleotides are summarized. Error bars represent SEM.

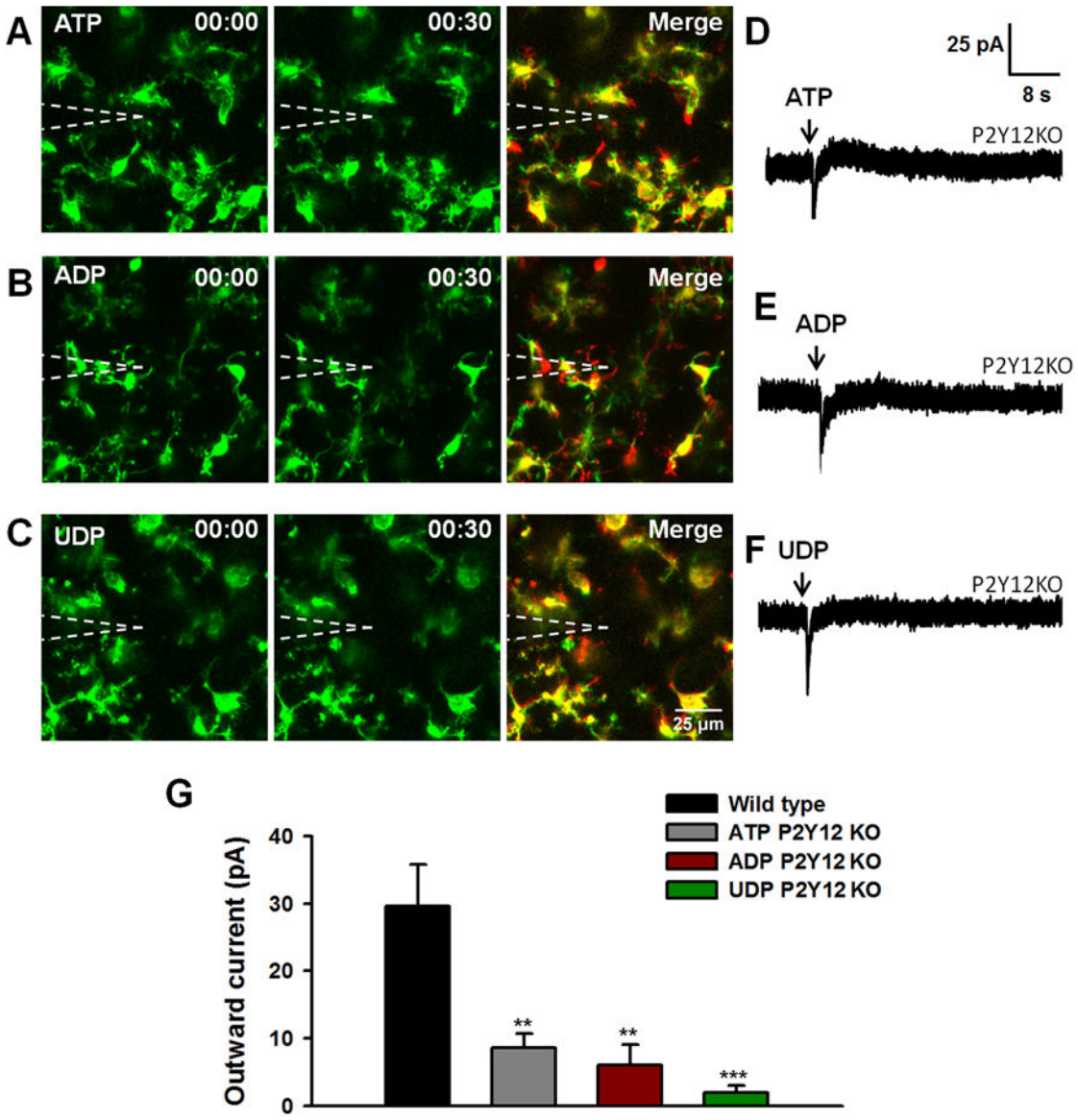

Figure 2. Microglial chemotactic response to ATP, ADP and UDP requires purinergic signaling and P2Y12 receptor

Two photon images showing local application of 1mM of ATP (A), ADP (B) and UDP (C) failed to induce robust microglial process extension toward the puff pipette in acute brain slices obtained from P2Y12 KO mice (A-C: n =3). Scale: 25 µm. In the P2Y12 KO mice, abolition in outward currents in response to 1mM of ATP (D), ADP (E) and UDP (F) was noted (D-F: n=5), suggesting an association between P2Y12 receptor mediated outward current and microglial chemotactic response. (G) The intensity of outward microglia currents in response to the nucleotides is summarized and was found to be significantly reduced in P2Y12 KO compared to WT mice. Error bars represent SEM. **P<0.01, ***P<0.001(ANOVA Tukey-Kramer multiple comparisons test).

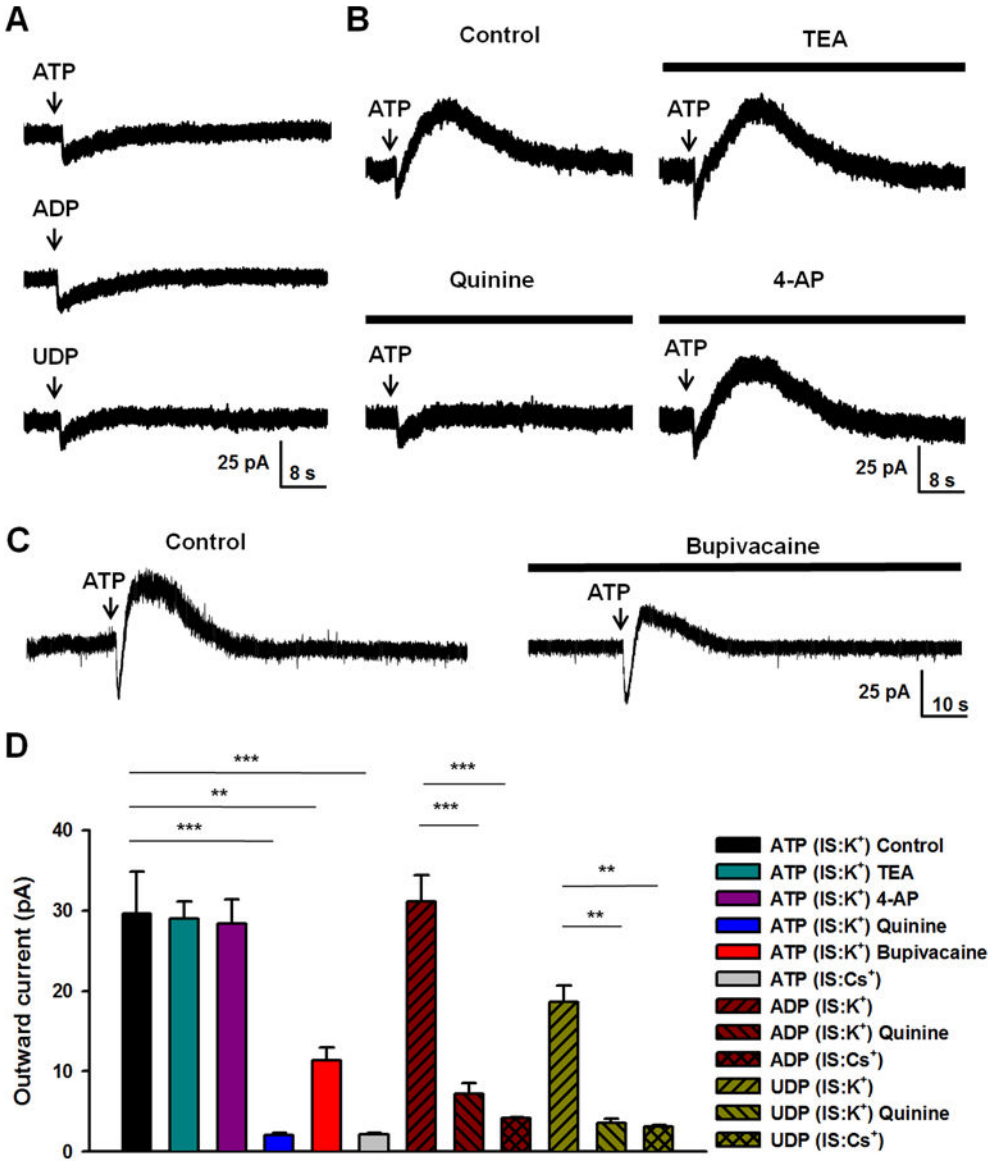

Figure 3. Potassium channels are coupled to P2Y12 receptor signaling

(A) In whole cell recordings made from microglia, ATP, ADP and UDP induced outward currents were abolished when cesium chloride was used as internal solution, suggesting that ATP/ADP/UDP-induced outward currents are mediated by potassium channels (n=5). (B) Broad spectrum potassium channel blockers such as TEA (10mM) and 4-AP (5mM) were unsuccessful at blocking the outward current, while quinine (1mM) effectively abolished any ATP-mediated potassium current (n=5 for each treatment group). (C) Low concentration of Bupivacaine (400 μM) was able to partially block the ATP-induced outward current. (D) Summarized results showing outward currents induced by ATP/ADP/UDP with K^+ or Cs^+ internal solution (IS) in the presence of broad-spectrum potassium channel blockers (such as TEA, 4-AP or quinine) or bupivacaine. (***p<0.001) (ANOVA Tukey-Kramer multiple comparisons test).

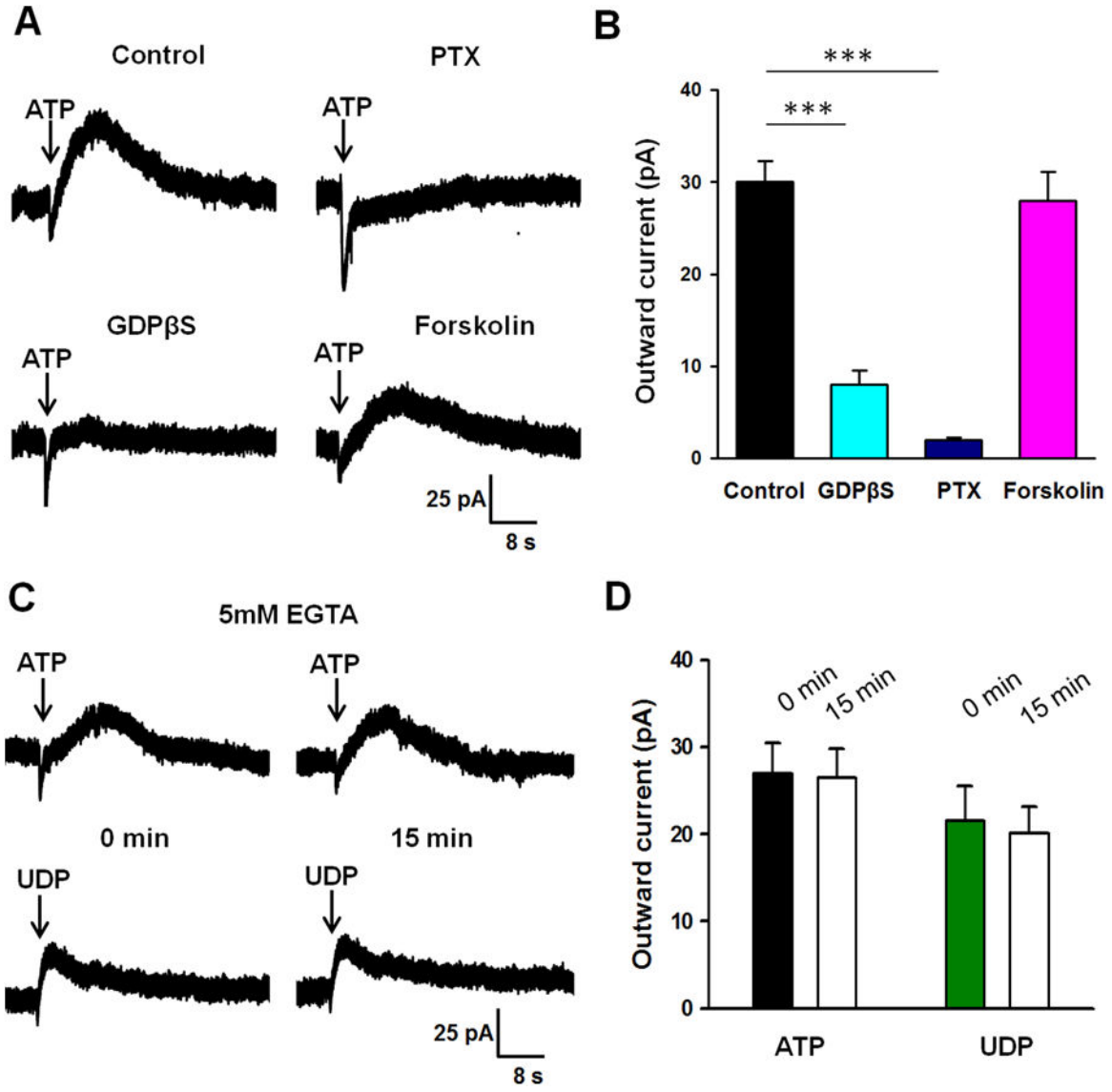

Figure 4. Analysis of the intracellular pathways coupling P2Y12 receptor to the potassium channel

(A) Perfusion of forskolin did not alter the ATP-induced outward currents, whereas inclusion of G-protein inhibitors, PTX or GDPβS in the internal solution abolished ATP-induced outward currents in microglia, suggesting that G-proteins are involved in the coupling of P2Y12 receptor and the potassium channel (n=5). (B) Summarized results showing the effect of PTX or GDPβS in significantly reducing ATP-induced outward currents. ***p< 0.001. (C) Microglia in acute cortical slices were patched with internal solution containing EGTA (5mM) and responses to local application of ATP and UDP showed no inhibition of the outward current, implying that it is not dependent on intracellular Ca^{2+} (ATP: n=5; UDP: n=3). (D) Summarized data depicting outward currents in response to ATP/UDP in the presence of EGTA (5mM) in the internal solution is shown. No significant differences between groups. All error bars represent SEM; ANOVA Tukey-Kramer multiple comparisons test.

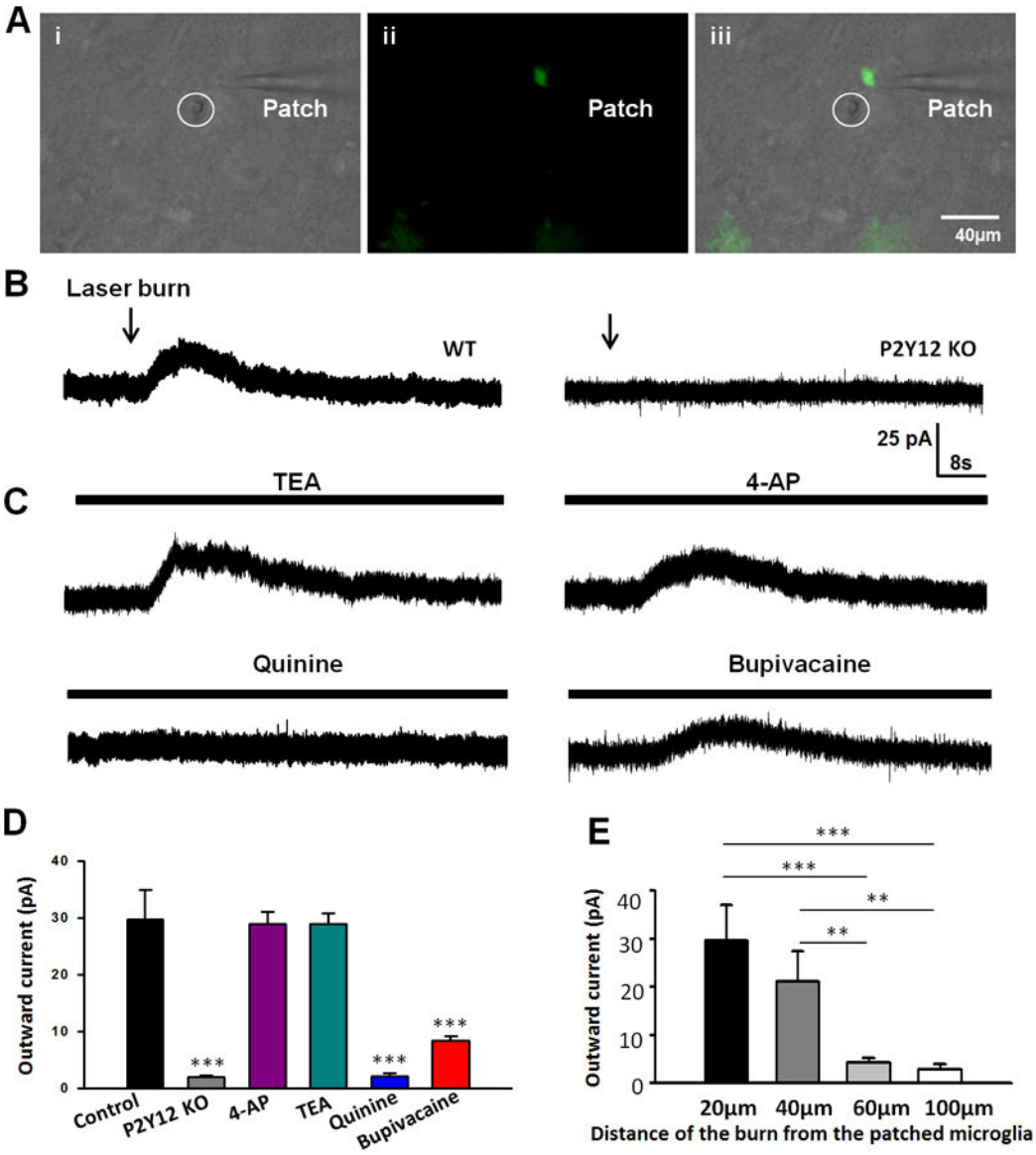

Figure 5. Laser burn injury induced ATP-like outward currents in microglia

(A) Whole-cell patch clamp recordings of microglia identified by GFP labeling. Patch refers to the patch pipette and the white circle encloses the laser burn site where a single neuron is damaged. Scale bar: 40 μm (B) Laser burn induced outward currents in microglia resembled the ATP-induced outward currents in WT mice. However, laser-burn induced outward current was absent in P2Y12 KO (n=8 for each group). (C) Like the ATP-induced outward current, the laser burn-induced current was sensitive to quinine and bupivacaine, but not TEA and 4-AP (n=5 for each group). (D) Summarized data represents the laser burn induced outward current in the presence of broad-spectrum potassium blockers or bupivacaine. (E) Data shows that the amplitude of the outward current diminishes with increasing distance between the burn site and patched microglia. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (n=3 for

each group). All error bars represent SEM. ANOVA Tukey-Kramer multiple comparisons test.