HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:

Neuropharmacology. 2015 January ; 88: 74–81. doi:10.1016/j.neuropharm.2014.09.015.

Purinergic receptor activation facilitates astrocytic $GABA_B$ receptor calcium signalling

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Abstract

Gamma-aminobutyric acid B receptors ($GABA_BRs$) are heterodimeric G-protein coupled receptors, which mediate slow synaptic inhibition in the brain. Emerging evidence suggests astrocytes also express $GABA_BRs$, although their physiological significance remains unknown. To begin addressing this issue, we have used imaging and biochemical analysis to examine the role $GABA_BRs$ play in regulating astrocytic Ca^{2+} signalling. Using live imaging of cultured cortical astrocytes loaded with calcium indicator Fluo-4/AM, we found that astrocytic $GABA_BRs$ are able to induce astrocytic calcium transients only if they are pre-activated by P2 purinoceptors (P2YRs). The $GABA_BR$-mediated calcium transients were attenuated by the removal of extracellular calcium. Furthermore, P2YRs enhance the phosphorylation of astrocytic $GABA_BR$ R2 subunits on both serine 783 (S783) and serine 892 (S892), two phosphorylation sites that are well known to regulate the activity and the cell surface stability of $GABA_BRs$. Collectively these results suggest that P2YR mediated signaling is an important determinant of $GABA_BR$ activity and phosphorylation in astrocytes.

1. Introduction

Astrocytes, the most abundant cell type in the central nervous system (CNS), are accepted to play essential roles in brain function by supporting neuronal viability and vascular integrity (Attwell et al., 2010). In addition, astrocytes release glutamate, D-serine and adenosine triphosphate (ATP), a process that has been termed gliotransmission which regulates neuronal excitability and synaptic transmission (Haydon and Carmignoto, 2006). Whilst astrocytes are not electrically active, their properties are subject to regulation via dynamic changes in intracellular Ca^{2+} signalling, events that are believed to play a critical role in

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coordination of astrocyte communication and gliotransmission (Haydon and Carmignoto, 2006; Wang et al., 2009). Astrocytes express a plethora of neurotransmitter receptors, including those activated by adenosine, ATP, glutamate and GABA (Haydon and Carmignoto, 2006). Whilst the roles glutamatergic receptors and purinoreceptors play in regulating astrocyte activity have been addressed (Cornell-Bell et al., 1990; Fumagalli et al., 2003; James and Butt, 2002), the role GABA receptors play in these processes are not as well understood.

$GABA_BRs$ are G-protein coupled receptors that mediate slow and prolonged inhibitory signalling in the brain via the activation of Gi/o type G-proteins leading to inhibition of adenylyl cyclase (AC). Structurally, $GABA_BRs$ are obligate heterodimers composed from R1 and R2 subunits (Bowery et al., 2002; Couve et al., 2000). The effector coupling and stability of $GABA_BRs$ are subject to modulation via the phosphorylation of serine residues 783 and 892 within $GABA_BR$ R2 subunit. (Couve et al., 2002; Kuramoto et al., 2007). Significantly, phosphorylation of S783 is regulated via the activation of N-Methyl-D-aspartate receptors (NMDAR), and this process plays a key role in determining neuronal morphology, in addition to cognitive behaviours (Terunuma et al., 2014; Terunuma et al., 2010b). In addition to neurons, $GABA_BR$ subunits are expressed in astrocytes and other types of glia (Charles et al., 2003; Lee et al., 2011; Oka et al., 2006). However, the role $GABA_BRs$ play in regulating astrocyte activity remains largely speculative.

In this study, we examined the mechanisms regulating $GABA_BR$ signalling in astrocytes. Our experiments reveal that $GABA_BR$ receptors induce Ca^{2+} transient in astrocytes but only after pre-activation of P2 purinoceptors. In parallel with this, we demonstrated that purinoceptors enhance the phosphorylation of S783 and S892 in the R2, events that are accepted to increase $GABA_BR$ activity. Therefore, our results reveal an unexpected role for purinoceptors in facilitating astrocytic $GABA_BR$ signalling.

2. Material and methods

2.1 Cultured astrocytes

Cerebral cortical astrocytes from P0-1 C57/Bl6 mice were cultured as described previously (Mungenast, 2011; Zhang et al., 2004). Dissected cortex were treated with 0.25 % tripsin, triturated in minimum essential medium (MEM) and transferred into flasks. They were grown to confluence at 37 °C in a humidified 5 % CO_2 atmosphere. After 7-10 days, flasks were washed with cold Earle's balanced salt solution (EBSS), and fed with cold modified MEM before shaking at 260 rpm for 3 days. Remaining adherent cells were dissociated by using 0.1 % tripsin, and plated onto coverslips. Cells were used after 4-6 days in culture (Zhang et al., 2004). All procedures have been approved by Tufts University's Institutional Animal Care of Use Committee (IACUC).

2.2 Cell surface biotinylation assay

Labelling of surface proteins for steady-state assays were performed as reported previously in cultured cortical neurones (Fairfax et al., 2004).

2.3 Cyclic AMP (cAMP) assay

The measurement of cAMP in cultured astrocytes was performed using ELISA based kit (Cell Biolabs).

2.4 Confocal calcium imaging in cultured astrocytes

For calcium imaging in cultured astrocytes, cells were plated on glass cover slips. The measurement of intracellular [Ca^{2+}] was performed using the acetoxymethyl-ester form of the fluorescent dye Fluo-4 (Fluo-4/AM; Invitrogen) as described previously (D'Ascenzo et al., 2007; Xie et al., 2010). The dye was dissolved in dimethyl sulfoxide (DMSO) (5 mg/ml), and this stock solution was stored at −20 °C. Before the experiments, the stock solution was diluted in Normal Hippocampal Saline (NHS; in mM: 140 NaCl, 5 KCl, 10 D-glucose, 2 $CaCl_2$, 2 $MgSO_4$, 10 HEPES, 6 Sucrose, pH 7.35), containing 0.15% pluronic F-127 (Sigma-Aldrich) as described previously (D'Ascenzo et al., 2007). The working concentration of dye was 2 μg/ml. The cultured cells were incubated for 20 min in the dye-containing NHS, washed two times with NHS, and then incubated for another 30 min in a dye-free solution allowing time for hydrolysis of the ester dye. The imaging procedure took place at room temperature. Fluorescence was excited at 488 nm. ATP (100 μM, Sigma-Aldrich), baclofen (100 μM, Tocris) and other drugs for stimulation were applied through a perfusion system equipped with a pinch valve to control the duration of application (Xie et al., 2010). Fluorescence intensity was measured from individual astrocytes as the average intensity of fluorescence in a region of interest corresponding to the cell soma using Metamorph software package. The fluorescent signal at a given time point was expressed as $\Delta F/F_0 = (F_1 - F_0)/F_0$, where F_0 and F_1 are the value of the fluorescence in astrocytes at rest and at the given time point, respectively (D'Ascenzo et al., 2007).

2.5 Western blot

Cultured cortical astrocytes were first lysed in 1 % SDS, 50 mM NaF, 1 mM EDTA, then diluted with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 μg/mL leupeptin, 1 μg/mL aprotinin, 10 μg/mL antipain and 250 μg/mL 4-(2-Aminoethl) benzenesulfonyl fluoride hydrochloride, to reduce concentration of SDS to 0.1%. Soluble material was then subjected to immunoblotting with antibodies against $GABA_BRs$, phospho-S783/phospho-S892 or pan-R1 and R2 antibodies that recognize C-terminal epitopes in the respective proteins have been described previously (Terunuma et al., 2014). The phosphorylation and expression of AMPK was examined as described in (Terunuma et al., 2010b). Membrane were then probed with HRP-conjugated secondary antibodies and detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). The luminescence images were captured by Luminescent image analyser (LAS3000, Fujifilm) and the intensity of bands were measured by Image J. Data were analysed using GraphPad Prism and statistical significance were determined using on-way ANOVA or paired *t*-test.

3. Results

3.1 Functional $GABA_BRs$ are express on the surface of cultured cortical astrocytes

To initiate our studies, we examined $GABA_BR$ expression in cultured astrocytes prepared from P0-1 mouse cerebral cortex using immunoblotting coupled with biotinylation. Western blotting of total lysates suggested that astroctyes express multiple isoforms of the R1 and R2 subunits (Fig. 1A), and biotinylation confirmed that astroctyes express both R1 and R2 subunits on their plasma membranes (Fig. 1A). In order to identify the functionality of astrocytic $GABA_BRs$, we assessed the effects of $GABA_BR$ agonist baclofen on adenosine-3′-5′-cyclic monophosphate (cAMP) accumlutaion. Exposure of astrocytes to forskolin, an AC activator, significantly increased cAMP levels (10 μM: 227 ± 31.78, $p = 0.0008$), an effect that was reduced by baclofen (100 μM: 146 ± 13.78, $p = 0.0413$, compared to 10 μM forskolin) (Fig. 1B). Collectively, these results suggest that astrocytes express functional $GABA_BRs$.

3.2 Induction of astrocytic Ca^{2+} transients by $GABA_BRs$

To determine whether astrocytic $GABA_BRs$ modulate Ca^{2+} signalling, cultures were loaded with Fluo-4/AM and dynamic changes in cytosolic Ca^{2+} levels were analysed using time-lapse confocal microscopy (Parri and Crunelli, 2003; Simard et al., 2003). Exposure of astrocytes to increasing concentrations of baclofen did not lead to any significant changes in intracellular Ca^{2+} levels (Fig. 2A). In contrast, subsequent exposure of astrocytes to the P2 purinoceptor agonist ATP rapidly increased intracellular Ca^{2+} levels, consistent with published studies (Fig. 2A and 2B) (Fischer et al., 2009; King et al., 1996; Li et al., 2003). Importantly, after exposure to ATP, evidence of small baclofen induced Ca^{2+} transients was observed and did not occlude subsequent responses to ATP (Fig. 2A and 2B). To assess whether $GABA_BR$ signalling is facilitated by pre-exposure to ATP, we used the $GABA_BR$ antagonist CGP54626. Pre-treatment of astrocytes with 1μM CGP54626 abolished baclofen-evoked Ca^{2+} transients (Fig. 2C). However, CGP54626 alone did not have any effect on ATP-dependent increases in Ca^{2+}(Fig. 2C).

To further assess the role of ATP in promoting $GABA_BR$ activity in astrocytes, we compared the ratios of baclofen-induced Ca^{2+} transients under varying conditions (Baclofen/ATP) (Fig. 3A and 3B). These experiments confirmed that baclofen-induced Ca^{2+} transients were only seen after exposure to ATP, an effect blocked by CGP54626 (36.08 ± 9.87 % compared to ATP-baclofen, $p = 0.0015$). However these effects were seen in the majority of cultured astrocytes (>80%). The effects of CGP54626 on baclofen-dependent modulation were reversible after extensive washing with extracellular solution (109.89 ± 39.226 compared to first ATP-baclofen stimulation, $p = 0.407$) (Fig. 3B; recovery). To examine the origins of baclofen-induced Ca^{2+} transients, we used Ca^{2+} free extracellular solutions. In multiple experiments performed on distinct cultures, the removal of extracellular Ca^{2+} prevented baclofen induced Ca^{2+} transients, but not on those induced by ATP (Fig. 3C).

Collectively, these studies strongly suggest the ability of $GABA_BRs$ to modulate astrocytic Ca^{2+} signalling is facilitated by ATP.

3.3 ATP Stimulation Increases Phosphorylation of Astrocytic $GABA_BRs$

To analyse the mechanisms by which ATP regulates $GABA_BR$ activity, we examined its effects on the phosphorylation of S783 and S892 within the receptor R2 subunit, accepted substrates of 5′-AMP (AMPK) and cAMP-(PKA) dependent protein kinases, respectively (Couve et al., 2002; Terunuma et al., 2010b). To do so, we used phospho-specific antibodies against these residues, and the ratio of p-S783/R2 and p-S892/R2 was then compared between treatments (Couve et al., 2002; Kuramoto et al., 2007). Exposure of astrocytes to ATP stimulation significantly increased S783 and S892 phosphorylation in a time-dependent manner (Fig. 4A and 4B). ATP-induced phosphorylation of both residues was prevented by pre-application of the P2 purinoceptor antagonists, 300 μM Suramin and 100 μM pyridoxal phosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS) (Fig. 4C and 4D) (p-S783: $p = 0.037$; p-S892: $p = 0.018$ compared to ATP alone). Therefore, these results demonstrate that in astrocytes, P2 purinoceptors regulate the phosphorylation of S783 and S892 within the $GABA_B$ R2 subunit.

3.4 ATP-mediated S783 phosphorylation in $GABA_BR2$ is regulated by CaMKK activated AMPK

Studies in neurons suggested that S783 phosphorylation is mediated by AMPK (Kuramoto et al., 2007). The activity of AMPK is critically dependent upon phosphorylation of Threonine 172 (T172), which is facilitated by Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) upon NMDAR activation (Mairet-Coello et al., 2013; Terunuma et al., 2010b). Therefore, we examined whether CaMKK-AMPK signalling pathways also mediate S783 phosphorylation in astrocytes.

To test this, we examined the effects of ATP on phosphorylation of T172 in AMPK using phospho-specific antibodies. ATP induced a rapid increase in T172 phosphorylation (1 min: 2.0 ± 0.34, $p = 0.0214$ compared to 0 min), which returned to baseline after 10 min (10 min: 0.9494 ± 0.1760, $p = 0.3940$ compared to 0 min) (Fig. 5A). To determine if ATP-induced T172 phosphorylation is mediated by P2 purinoceptors, cultures were pre-treated with Suramin and PPADS. ATP induced phosphorylation of T172 was abolished by P2 purinoceptor antagonists (1.08 ± 0.0676, $p = 0.014$ compared to ATP alone, $p = 0.15$ compared to control). In order to assess the role CaMKK plays in ATP-induced phosphorylation of AMPK as an upstream kinase, we used the CaMKK inhibitor STO-609 (Fig. 5C). ATP dependent T172 phosphorylation was abolished by STO-609 ($p = 0.023$ compared to ATP alone) (Fig. 5C).

Whilst the direct effects AMPK plays in regulation of substrates is difficult to determine due to a lack of specific inhibitors, we found that in addition to preventing T172 phosphorylation, STO-609 also prevented ATP-dependent modulation of S783 phosphorylation ($p = 0.383$ compared to ATP alone) (Fig. 5D). Collectively, these results suggest that exposure of astrocytes to ATP leads to CaMKK dependent activation of AMPK and subsequent phosphorylation of S783.

3.5 ATP-mediated S783 phosphorylation in $GABA_BR2$ is through P2YRs

Astrocytes express both ionotropic P2XRs and metabotropic P2YRs (Fam et al., 2003; Fischer et al., 2009; James and Butt, 2002). To establish which P2 purinoceptor(s) mediate AMPK-dependent phosphorylation of S783, we used the P2XR agonist β,γ-methylene ATP (β,γ-meATP) (Coddou et al., 2011). βγ-meATP had no marked effects on the phosphorylation of T172 or S783, suggesting a minimal role of P2XR receptors in regulating AMPK and $GABA_BR$ phosphorylation (Fig. 6A). Next, we tested the effects of 2-Methylthio ATP (2-meSATP) and UTP which activate $P2Y_1Rs$/P2XRs and $P2Y_2$/$P2Y_4Rs$, respectively, on AMPK/$GABA_BR$ phosphorylation (Centemeri et al., 1997) (Fig. 6B-D). Both 2-meSATP and UTP significantly increased phosphorylation of S783 and T172 in AMPK (data not shown for 2-meSATP). Therefore, metabotropic P2YRs are likely to be the principle mediators of S783 phosphorylation and AMPK activation in astrocytes.

3.6 ATP-mediated S892 phosphorylation in $GABA_BR2$ is via pertussis toxin-sensitive P2YRs

We examined whether P2YRs also regulate phosphorylation of S892 in the R2 subunit, a substrate of PKA (Terunuma et al., 2010a). In contrast to S783, 2-meSATP did not significantly modify S892 phosphorylation. In epithelial cells, ATP-dependent activation of PKA is mediated by pertussis toxin (PTX)-sensitive P2YRs (Liu et al., 1998). Therefore, we incubated cultured astrocytes with 100 ng/ml PTX for 14 hrs and examined ATP-induced S892 phosphorylation. PTX pre-treatment abolished phosphorylation of this residue (Fig. 6F). Consequently, our results suggest S892 phosphorylation is regulated via PTX-sensitive P2YRs, such as $P2Y_{12}$ and $P2Y_{13}Rs$.

4. Discussion

Communication between astrocytes and their environment is largely mediated by transient elevations of intracellular Ca^{2+} in response to a variety of neurotransmitter/gliotransmitters, including ATP, glutamate, acetylcholine and norepinephrine (Haydon and Carmignoto, 2006; Wang et al., 2009). Many of these neurotransmitter events in astrocytes are mediated via GPCRs, which couple to phospholipase C to drive the production of inositol trisphosphate and the subsequent release of Ca^{2+} from the endoplasmic reticulum, in addition to the activation of AC (Petravicz et al., 2008; Volterra and Meldolesi, 2005). Astrocytes express $GABA_BRs$ (Charles et al., 2003; Oka et al., 2006), which have been suggested to play a role in regulating synaptic transmission (Ding et al., 2009; Kang et al., 1998). However, the signalling mechanisms $GABA_BRs$ use to influence astrocyte activity remain to be defined but preliminary studies suggest that they are c

In this current study, we have examined whether astrocytic $GABA_BRs$ induce Ca^{2+} transients in astrocytes. Exposure of astrocytes to the $GABA_BR$ agonist baclofen itself did not increase Ca^{2+} transients, whilst under the same conditions, ATP induced robust elevations in intracellular Ca^{2+} levels.

However, after brief exposure to ATP, baclofen-induced Ca^{2+} transients were evident in astrocytes and could be abolished by the $GABA_BR$ antagonist CGP54626. In contrast to

ATP induced Ca^{2+} transients, those resulting from the activation of $GABA_BRs$ were dependent upon extracellular Ca^{2+}. These outcomes support previous studies analysing astrocytic signalling after photothrombosis, a model of cerebral ischaemia. This injury leads to rapid release of ATP and prolonged elevations in astrocytic Ca^{2+} levels which are partly, mediated by $GABA_BRs$ (Ding et al., 2009; Rossi et al., 2007). In addition our results are consistent with studies that have shown the effects of GABA on astrocytic Ca^{2+} signalling can be partially reduced the low affinity $GABA_BR$ antagonist phaclofen (Nilsson et al., 1993).

$GABA_BR$ activity in neurons is tightly regulated by receptor phosphorylation (Terunuma et al., 2010a). We have previously identified two phosphorylation sites for serine/threonine protein kinases that regulate $GABA_BR$ activity. S783 in the R2 subunit is phosphorylated by AMPK, an event that prevents receptor internalisation and enhanced $GABA_BR$ signalling (Kuramoto et al., 2007; Terunuma et al., 2010b). $GABA_BR$ effector coupling is subject to similar positive modulation via PKA mediated phosphorylation of the adjacent residue S892, within the R2 subunit (Couve et al., 2002). Here, we found both S783 and S892 are phosphorylated by brief ATP exposure in cultured astrocytes through the activation of P2 purinoceptors. ATP-dependent phosphorylation of S783 in R2 subunit was mediated via P2YR-CaMKK-AMPK signalling. Notably, this mechanism is similar to that employed by neurons to regulate S783 phosphorylation downstream of NMDARs (Terunuma et al., 2010b). Astrocytes express many P2YRs, which increase intracellular Ca^{2+} levels (Fischer et al., 2009). In our study using specific agonists, we found $P2Y_1R$, $P2Y_2R$ and $P2Y_4R$ are likely to mediate AMPK activation and subsequent S783 phosphorylation. ATP has also been found to activate PKA through the production of arachidonic acid, leading to activation of AC-linked GPCRs, or the activation of Gs-and Gq/11-coupled $P2Y_{11}Rs$ (Liu et al., 1998; van der Weyden et al., 2000). In astrocytes, we found that S892 phosphorylation is mediated by PTX-sensitive P2YRs. The mechanism(s) underlying the functional modulation and phosphorylation of GABABRs by P2YRs are unknown, however it may reflect synergy between G-protein signalling pathways. Significantly such cross talk has recently been demonstrated between $GABA_BRs$ and metabotropic glutamate receptors in neurons (Rives et al., 2009).

In conclusion, our study suggests $GABA_BR$ signalling in astrocytes is critically dependent upon the activation of multiple P2 purinoceptor subtypes leading to the phosphorylation of key regulatory residues in the $GABA_BR2$. This synergistic interaction between purinoceptors and $GABA_BRs$ may act as a co-incidence detector to allow the fine-tuning of astrocytic Ca^{2+} signalling.

Acknowledgement

We thank to the members of the Moss laboratory for discussions during the course of this study. MT is recipient of a National Scientist Development Grant (09SDG2260557) from the American Heart Association. SJM is supported by NIH-NINDS grants, NS051195, NS056359, NS081735, NIH-NIMH grant, MH097446, CURE and Simons Foundation. SJM serves as a consultant for SAGE therapeutics and AstraZeneca, relationships that are regulated by Tufts University and do not impact on this study.

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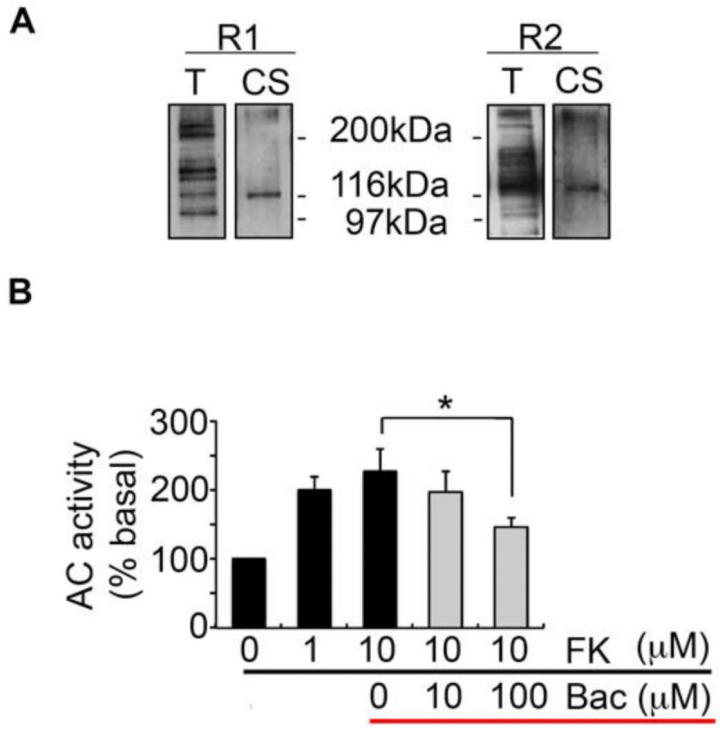

Fig. 1.

Surface expression of astrocytic $GABA_BRs$ and dose-dependent inhibition of cAMP levels by baclofen. *A*. Surface biotinylation assay confirmed expression of $GABA_BRs$ on the plasma membrane of cultured cortical astrocytes. *B*. Baclofen treatment significantly reduced the production of cAMP (*$p<0.05$, paired-*t* test, n=4).

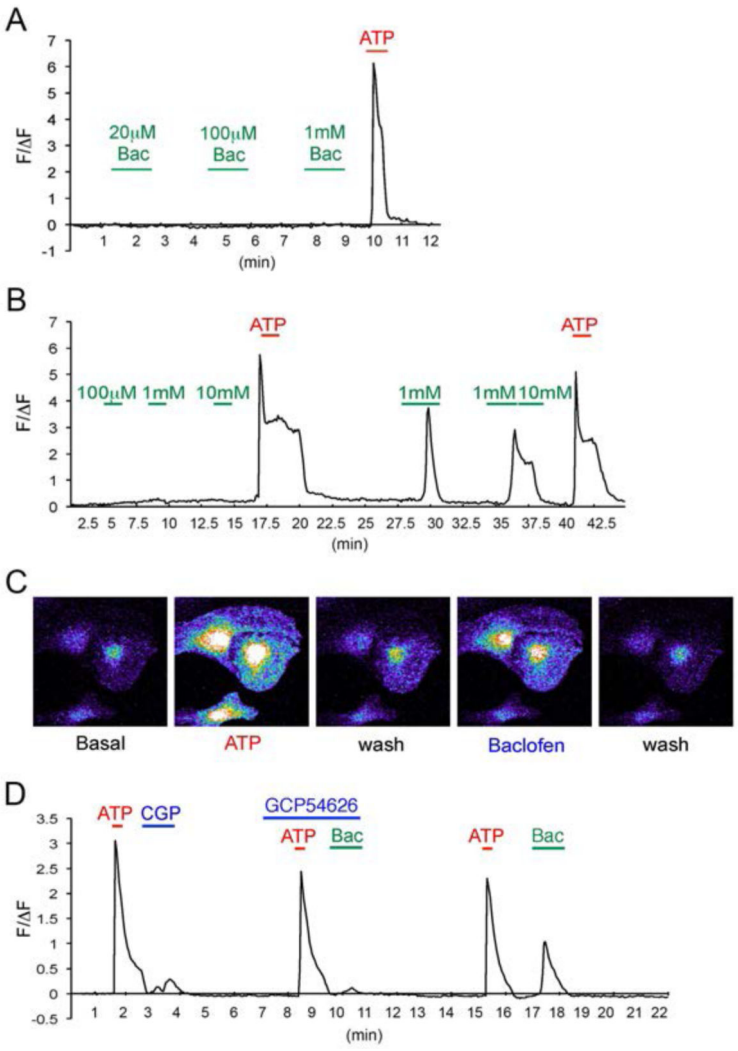

Fig. 2.

Calcium imaging in cultured astrocytes. *A*. Time course of Ca^{2+} fluorescence changes ($\Delta F/F_0$) by baclofen before and after ATP (100 μM) treatments. Data points are averages of 7 cells in the imaging field in one experiment. The horizontal bar represents the application of stimuli. Green: baclofen, Red: 100 μM ATP. *B*. An example of images of cultured cortical astrocytes loaded with the Fluo-4/AM. Cells were stimulated with 100 μM ATP for 15 sec, washed until the calcium levels are reduced to baseline, then stimulated again with 100 μM baclofen for 1 min. *C*. Representative Ca^{2+} fluorescence changes ($\Delta F/F_0$) of baclofen-induced calcium increase with/without $GABA_BR$ antagonist CGP54626 (1 μM). Data points are averages of 6 cells in the imaging field in one experiment. The horizontal bar represents the application of stimuli. Green: 100 μM baclofen (Bac), Red: 100 μM ATP, Blue: 1 μM CGP54626.

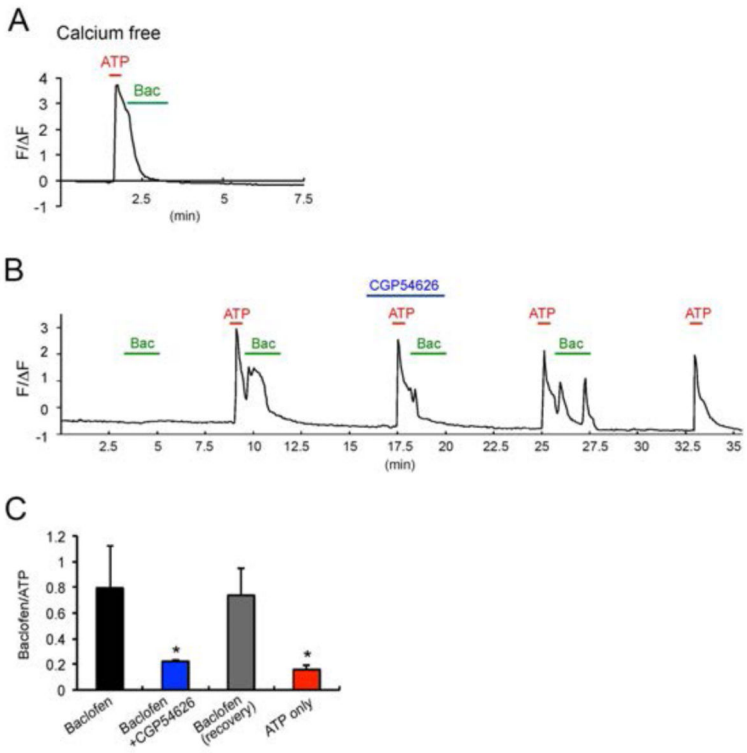

Fig. 3.

Baclofen-induced Ca^{2+} waves are ATP and extracellular calcium dependent. *A*. Representative trace of long-term imaging of baclofen-induced Ca^{2+} fluorescence changes ($\Delta F/F_0$). Data points are averages of 13 cells in the imaging field in one experiment. The horizontal bar represents the application of stimuli. Green: 100 μM baclofen, Red: 100 μM ATP, Blue: 1 μM CGP54626. *B*. Summary histograms showing that baclofen induces Ca^{2+} transients and it is reduced by pre-treatment with CGP54626 (blue). The second baclofen treatment (recovery, grey) after wash induced Ca^{2+} transient. **p<0.01, ***p<0.001, compare to first ATP-baclofen stimulation (black) n=. *C*. Representative trace of baclofen-induced Ca^{2+} fluorescence changes ($\Delta F/F_0$) after removal of extracellular calcium. Data points are averages of 8 cells in the imaging field in one experiment. The horizontal bar represents the application of stimuli. Green: 100 μM baclofen, Red: 100 μM ATP.

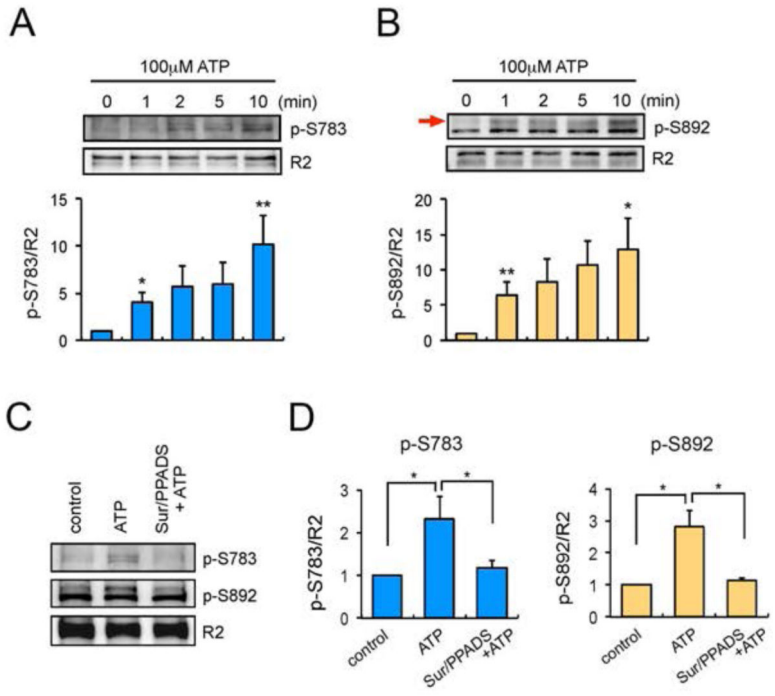

Fig. 4.

ATP stimulation increases phosphorylation of $GABA_BRs$. *A and B*. Cortical astrocytes were treated with 100 μM ATP for 0-10 min. Total lysates were subjected to SDS-PAGE and visualised by immunoblotting with anti-p-S783 antibodies (A), anti-p-S892 antibodies (B) or $GABA_BR2$ antibodies. Integrated intensities were calculated by densitometry measurements of immunoblots in Image J and were normalised to averages of R2 subunits. Bar graphs are shown as a change relative to control (0 min) and represent mean value ± SEM of 3 independent experiments. *p<0.05, **p<0.01, one-way ANOVA. The black arrow on panel B is pointing the band representing p-S892. *C*. Cortical astrocytes were treated with P2 purinoceptor antagonists Suramin (300 μM) and PPADS (100 μM) for 15 min prior to ATP (100 μM) stimulation. ATP was then applied for 10 min and the phosphorylation of S783 (p-S783) and S892 (p-S892) were visualised by immunoblotting. *D*. Normalised quantification of p-S783 (left) and p-S893 (right). Data are shown as a change relative to control and represent mean ± SEM of 5 independent experiments for p-S783 and 3 independent experiments for p-S892. *p<0.05, one-way ANOVA.

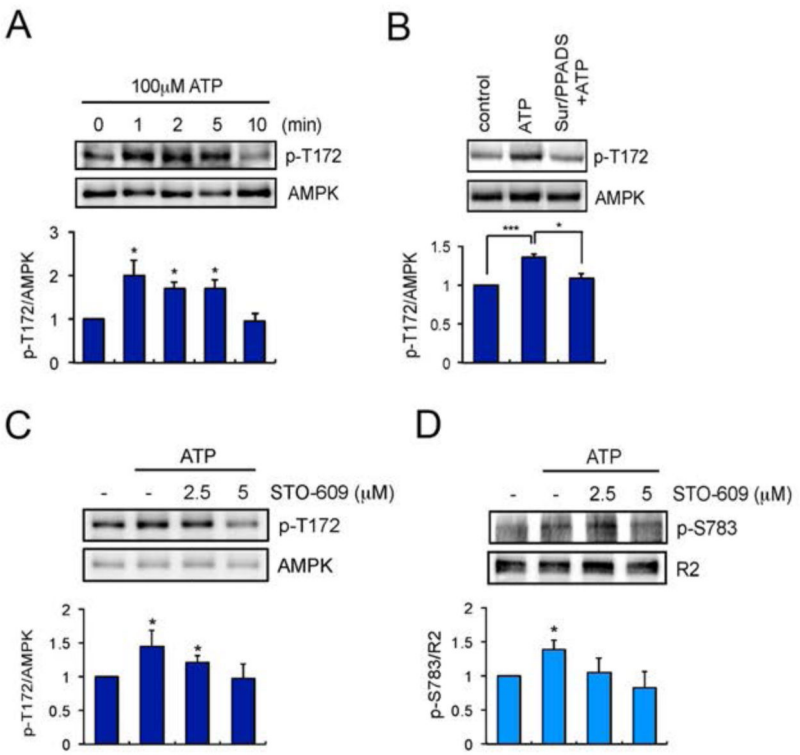

Fig. 5.

ATP stimulation increases the phosphorylation of AMPK. *A*. Cortical astrocytes were treated with 100 μM ATP for 0-10 min. Total lysates were subjected to SDS-PAGE and visualised by immunoblotting with anti-p-T172 antibodies or AMPK antibodies. Bar graphs are shown as a change relative to control (0 min) and represent mean value ± SEM of 3 independent experiments. *p<0.05, one-way ANOVA. *B*. Cortical astrocytes were treated with P2 purinoceptor antagonists Suramin (300 μM) and PPADS (100 μM) for 15 min prior to ATP (100 μM) stimulation. ATP was then applied for 2 min and the phosphorylation of T172 (p-T172) were visualised by immunoblotting. Data are shown as a change relative to control and represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, one-way ANOVA. *C and D*. Increased concentration of STO-609 (0-5 μM) inhibited ATP-induced phosphorylation of T172 in the AMPK and S783 in the $GABA_BR2$ subunits. Data are shown as a change relative to control (-) and represent mean ± SEM of 3 independent experiments. *p<0.05, **p<0.01, one-way ANOVA and paired *t*-test.

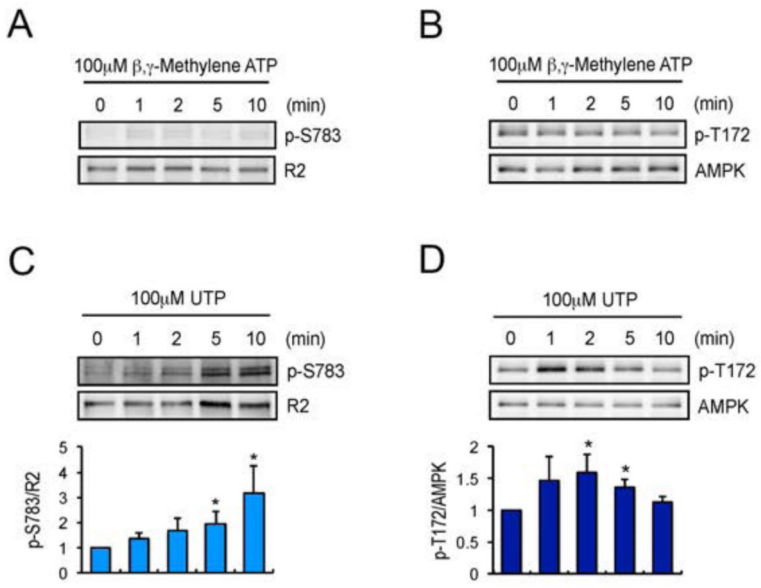

Fig. 6.

P2YR agonists increase the phosphorylation of $GABA_BR2$ and AMPK. *A*. Cortical astrocytes were treated with P2XR agonist β,γ-meATP for 0-10 min. Total lysates were subjected to SDS-PAGE and visualised by immunoblotting with anti-p-S783, anti-$GABA_BR2$, anti-p-T172 or anti-AMPK antibodies. *B and C*. Astrocytes were treated with P2YR agonist UTP for 0-10 min. Total lysates were subjected to SDS-PAGE and visualised by immunoblotting with anti-$GABA_BR2$ antibodies (B), or AMPK antibodies (C). Integrated intensities of phosphorylation were calculated by densitometry measurements of immunoblots in Image J and were normalised to averages of total proteins. Bar graphs are shown as a change relative to control (0 min) and represent mean value ± SEM of 4 independent experiments. *p<0.05, one-way ANOVA. *D and E*. Phosphorylation of S783 (D) and S892 (E) in R2 subunits were determined by P2YR agonist 2-meSATP. Bar graphs are shown as a change relative to control (0 min) and represent mean value ± SEM of 3 independent experiments. *p<0.05, one-way ANOVA. *F*. PTX inhibits ATP-mediated S892 phosphorylation in R2 subunit. PTX was applied for 14 hr before 10 min ATP (100 μM) stimulation. Bar graphs are shown as a change relative to control (-) and represent mean value ± SEM of 3 independent experiments. *p<0.05, ***p<0.001, one-way ANOVA.