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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 signalling is an important determinant of $GABA_BR$ activity and phosphorylation in astrocytes.

This article is part of the Special Issue entitled 'GABAergic Signaling in Health and Disease'.

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 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 purinoceptors 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 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, 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).

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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 subunit, 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% trypsin, 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% trypsin, 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 neurons (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 coverslips. 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-Aminoethyl) benzenesulfonyl fluoride hydrochloride, to reduce concentration of SDS to 0.1%. Soluble material was then subjected to immunoblotting with antibodies against $GABA_BR$ phospho-S783/phospho-S892 or pan-$GABA_BR$ R1 and R2 antibodies that recognise 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 one-way ANOVA or paired *t*-test.

3. Results

3.1. Functional $GABA_BRs$ are expressed 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) accumulation. 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

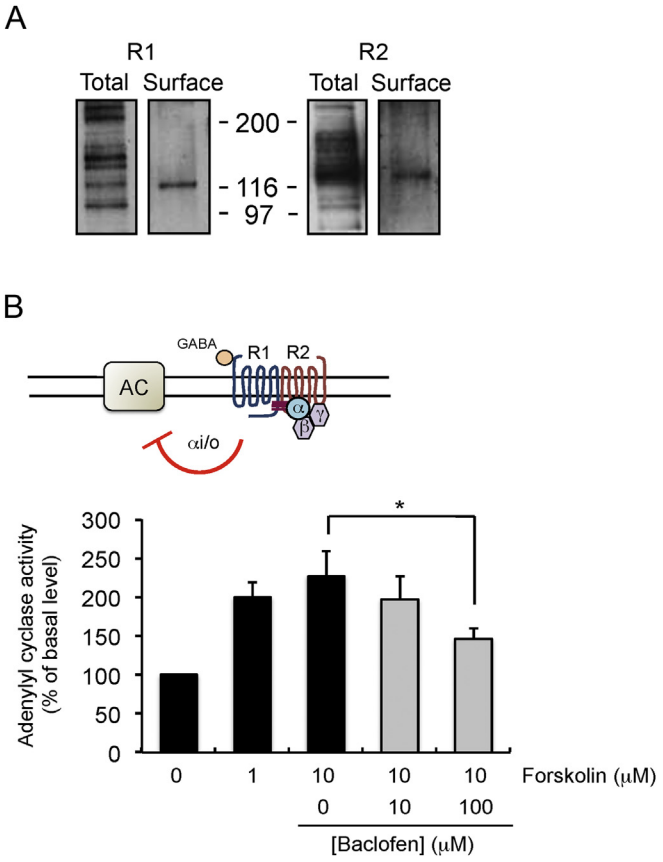

Fig. 1. Surface expression of astrocytic $GABA_BRs$ and dose-dependent inhibition of cAMP levels by baclofen. *A.* Surface biotinylation assay confirmed expression of $GABA_BRs$ on the plasma membrane of cultured cortical astrocytes. *B.* Baclofen treatment significantly reduced the production of cAMP (*$p < 0.05$, paired-*t* test, $n = 4$).