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Pannexin-1-mediated ATP release from area CA3 drives mGlu5-dependent neuronal oscillations

Jan Lopatář, Nicholas Dale, Bruno G. Frenguelli*

School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

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ABSTRACT

The activation of Group I metabotropic glutamate receptors (GI mGluRs) in the hippocampus results in the appearance of persistent bursts of synchronised neuronal activity. In response to other stimuli, such activity is known to cause the release of the purines ATP and its neuroactive metabolite, adenosine. We have thus investigated the potential release and role of the purines during GI mGluR-induced oscillations in rat hippocampal areas CA3 and CA1 using pharmacological techniques and microelectrode biosensors for ATP and adenosine. The GI mGluR agonist DHPG induced both persistent oscillations in neuronal activity and the release of adenosine in areas CA1 and CA3. In contrast, the DHPG-induced release of ATP was only observed in area CA3. Whilst adenosine acting at adenosine A_1 receptors suppressed DHPG-induced burst activity, the activation of mGlu5 and $P2Y_1$ ATP receptors were necessary for the induction of DHPG-induced oscillations. Selective inhibition of pannexin-1 hemichannels with a low concentration of carbenoxolone (10 μM) or probenecid (1 mM) did not affect adenosine release in area CA3, but prevented both ATP release in area CA3 and DHPG-induced bursting. These data reveal key aspects of GI mGluR-dependent neuronal activity that are subject to bidirectional regulation by ATP and adenosine in the initiation and pacing of burst firing, respectively, and which have implications for the role of GI mGluRs in seizure activity and neurodevelopmental disorders.

1. Introduction

Group I metabotropic glutamate receptor (GI mGluR) activation is implicated in various synchronised neuronal processes ranging from perception-pertinent 40 Hz oscillations (Whittington et al., 1995) to hypersynchronous neuronal activity as seen in seizures (Moldrich et al., 2003). Indeed, activation of GI mGluRs has been shown to produce epileptiform activity in hippocampal slices (Sayin and Rutecki, 2003; Taylor et al., 1995), seizures *in vivo* (Sacaan and Schoepp, 1992) and can lead to protein synthesis-dependent long-term modification of network excitability and the subsequent transition from inter-ictal activity to seizure-like events (Wong et al., 2004). Furthermore, enhanced GI mGluR activity, in particular of mGlu5, is responsible for increased cortical excitability and burst frequency in mouse models of Fragile X syndrome (Hays et al., 2011), a developmental disorder characterised by intellectual disability, autism spectrum disorder and epilepsy (Kidd et al., 2014).

Epileptic seizures in humans (During and Spencer, 1992) and epileptiform activity *in vivo* (Schrader et al., 1980) and *in vitro* (Etherington et al., 2009) are accompanied by the release of the neuromodulator adenosine which, via the inhibitory actions of adenosine A_1 receptors, attenuates the intensity and duration of seizures (Dale and Frenguelli, 2009). Adenosine can be released *per se* or as a product of extracellular ATP metabolism (Zimmermann et al., 2012). ATP and other modulators can be released as a consequence of Ca^{2+} oscillations which can be triggered by GI mGluR activation. These Ca^{2+} oscillations have been observed both in neurones (Jaffe and Brown, 1994) and astrocytes (Pasti et al., 1997).

We have recently assessed the role of ATP and its P2 receptors in the Mg^{2+}-free model of electrically-induced epileptiform activity and observed only a minor excitatory role of P2 receptors in this phenomenon. This conclusion was supported by the failure to observe ATP release during epileptiform activity using microelectrode biosensors (Lopatar et al., 2011). Given the ability for GI mGluR activation to provoke seizure activity and trigger Ca^{2+} oscillations, which in turn can cause ATP release, we investigated the potential for purinergic regulation of GI mGluR-induced bursting in

* Corresponding author. Tel.: +44 (0) 2476 150591; fax: +44 (0) 2476 523701.
E-mail address: b.g.frenguelli@warwick.ac.uk (B.G. Frenguelli).

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rat hippocampal slices using pharmacological approaches and real-time detection of purine release using microelectrode biosensors.

We show that the activation of GI mGluRs, and in particular mGlu5, by DHPG induced burst firing in area CA1. DHPG also caused a large increase in extracellular adenosine which was, however, independent of neuronal activity triggered by DHPG, but which exerted a powerful inhibitory influence on DHPG-induced bursting via adenosine A_1 receptors. This adenosine was released primarily as adenosine, although sub-micromolar CA3 region-specific levels of ATP likely triggered bursting via the activation of $P2Y_1$, but not $P2X_7$ receptors. ATP release was sensitive to probenecid and a low concentration of carbenoxolone indicative of ATP release via the pannexin-1 hemichannel. Our data extend our understanding of the consequence for neuronal function of GI mGluR activation by incorporating key purine steps in this process.

2. Materials and methods

2.1. Drugs and chemicals

(S)-3,5-Dihydroxyphenylglycine (DHPG) was obtained from Tocris or Ascent Scientific. 2′-Deoxy-N6-methyladenosine 3′,5'-bisphosphate tetrasodium salt (MRS 2179), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl)phenol (ZM 241385), tetrodotoxin (TTX), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine (A438079) and 6-N,N-diethyl-D-β,γ-dibromomethyleneATP (ARL 67156) were from Tocris. 8-Cyclopentyltheophylline (8-CPT), probenecid, serotonin and carbenoxolone were purchased from Sigma Aldrich. Salts and glucose for the aCSF and glycerol were purchased from Fisher Scientific. DHPG, MRS2179, TTX, MPEP, A438079, ARL 67156, carbenoxolone were all dissolved in distilled H_2O at stock concentrations at least 200 times the final concentration. Probenecid was dissolved in DMSO to give a final concentration of 0.2% DMSO and 8-CPT was dissolved in 0.1 M NaOH at a 10 mM stock concentration.

2.2. Preparation of hippocampal slices

17–23 day old male Sprague–Dawley rats were used and were killed by cervical dislocation and then decapitated in accordance with Schedule 1 of the UK Government Animals (Scientific procedures) Act 1986 and with Local Ethical Review procedures. The brain was quickly removed and put into ice-cold aCSF containing in mM: 124 NaCl, 26 $NaHCO_3$, 10 D-glucose, 1.3 NaH_2PO_4, 2 $CaCl_2$, 2 KCl and 1 $MgSO_4$ with additional 10 mM $MgCl_2$. 600 or 400 μm parasagittal hippocampal slices were cut on a vibratome (Microm, HM 650 V) and kept at 34 °C in standard aCSF (containing 1 mM Mg^{2+} solution; pH 7.4) gassed with 95% O_2/5% CO_2 for at least 1 h before use.

2.3. Electrophysiological recordings and drug application

After the recovery period, slices were submerged in a recording chamber, secured with a platinum harp with nylon threads, and were perfused with oxygenated aCSF at a flow rate of 6–7 ml/min and maintained throughout experiments at 30 °C. Extracellular field excitatory postsynaptic potentials (fEPSPs) and epileptiform activity were recorded (10 kHz sampling; 1 Hz to 3 kHz filtering) from stratum radiatum using an aCSF-filled glass microelectrode of ~1 MΩ resistance. To elicit fEPSPs as a measure of slice viability and recording stability, the Schaffer collateral-commissural pathway was stimulated at 0.067 Hz. Electrical stimulation of the Schaffer pathway at the level of stratum radiatum was provided via a bipolar stimulating electrode made from twisted Teflon-coated tungsten wire (50 μm in diameter). Stimulus parameters and acquisition and analysis of fEPSPs were under the control of LTP software (Anderson and Collingridge, 2001, 2007). Epileptiform activity was recorded and analysed using Spike 2 software. All pharmacological agents were bath-applied. Hippocampal slices were pre-incubated with drugs of interest for 15 min after which time DHPG was applied.

2.4. Electrophysiological data analyses

The fEPSP was used as an index of slice viability and recording stability and involved the measurement of the negative-going slope of the fEPSP typically over a 1 ms time range. Periodic stimulation of the Schaffer pathway was stopped prior to the induction of epileptiform activity by DHPG. The analysis of epileptiform activity was as described previously (Lopatar et al., 2011): extracellular recordings were rectified, in that negative-going activity was multiplied by −1 to give positive-going signals. For the most part epileptiform activity was quantified in terms of burst frequency (i.e. the number of bursts per minute) as bursting behaviour was a common feature of the activity induced by DHPG and readily quantifiable across slices and treatments. Bursts were defined as periods of sustained repetitive activity of at least twice the amplitude of the background noise and separated by periods of quiescence. In the case of experiments involving the adenosine A_1R antagonist 8-CPT, which provoked increased epileptiform activity, the intensity of activity was additionally measured in terms of the number of spikes in a given period and the inter-burst interval. To avoid double counting of rectified spikes a threshold inter-spike interval of 50 ms was applied. When ARL 67156 was used, the duration of bursting was measured.

2.5. Adenosine and ATP biosensor measurements

Adenosine, ATP and null biosensors (Pt/Ir wire of 50 μm in diameter and 500 μm in length) were purchased from Sarissa Biomedical Ltd (Coventry, UK) and were inserted through the thickness of the slice in either area CA3 or area CA1 (Fig. 1A). The ATP biosensor requires glycerol (2 mM) to be present in the extracellular perfusion medium, which had no discernable effect on the fEPSP (data not shown). The biosensors contain specific enzymatic cascades able to break down ATP (Llaudet et al., 2005) or adenosine (Llaudet et al., 2003) to produce hydrogen peroxide (Fig. 1B). The hydrogen peroxide is oxidised on the surface of the polarised Pt/Ir wire to give rise to a current linearly-related to the concentration of the measured analyte. Null sensors contain no enzymes and measure only non-specific electroactive signals. Pairs of sensors (ATP and null; adenosine and null) were inserted into the stratum radiatum of the hippocampal CA1 or CA3 regions (Fig. 1A). At least one hour was given for the signal associated with sensor insertion to dissipate before experiments started. After each experiment, sensors were withdrawn from slices and calibrated with 10 μM adenosine or 10 μM ATP. The values from adenosine biosensors are given as μM′ (μM prime) to reflect that the adenosine signal is a composite signal of adenosine and its metabolites but is referred to as the adenosine signal for simplicity (Frenguelli et al., 2007). A 10 μM serotonin solution was also used to assess the patency of the electro-active interferent screening layer of the sensors. Biosensor measurements were only accepted and further processed if serotonin response did not exceed 150 pA. The current response of the simultaneously-recorded null sensors was subtracted from ATP and adenosine signal to reveal the net purine signal. Biosensor reading was taken once the release had stabilized which was usually 10–15 min after DHPG or other drug application. A one-minute rolling average of biosensor measurements around the 15th minute after drug application was taken for quantification across slices and treatments. Sensor recordings were made using Spike 2 software at a sampling rate of 1 kHz.

2.6. Malachite green phosphate assay

Slices were placed for 15 min in 500 ml phosphate-free aCSF to remove the majority of inorganic phosphate from the slices. Afterwards, slices were transferred onto a mesh support in small beakers containing 33 ml of phosphate-free aCSF where they were pre-treated with carbenoxolone and with DHPG. Ten minutes after DHPG application, slices were transferred into a 24-well plate and 0.5 ml of the appropriate solution was added. 95% O_2/5% CO_2 was blown across the surface of the wells to ensure constant oxygen supply. In order to maintain the slice viability, we restricted our incubations in the wells to 15 min after which time the solution was removed and was further used to assess inorganic phosphate using malachite green assay as described previously (Carter and Karl, 1982; Frenguelli et al., 2007; Wall et al., 2008). Probenecid, but not carbenoxolone, skewed the calibration curve and thus probenecid was not used in this assay.

2.7. Statistical analyses

Biosensor traces were smoothed (rolling average, 1s) before pooling together. All values are expressed as mean ± S.E.M. N values represent the number of slices per condition. No more than two slices were used from the same animal per condition. A one way ANOVA was used when more than two groups were to be compared. Graphs were drawn and statistical analyses were performed in OriginPro 8.5 software. Statistical significance was taken as $p < 0.05$.

3. Results

3.1. Activation of group I metabotropic glutamate receptors induces epileptiform bursting and the release of adenosine from hippocampal areas CA1 and CA3

Treatment of 600 μm hippocampal slices with the group I metabotropic glutamate receptor agonist (S)-3,5-dihydroxyphenylglycine (S-DHPG, 25–50 μM) induced repetitive discharges at a frequency of 3.7 ± 0.6 bursts/minute (bpm; n = 14, Fig. 1C) and a burst duration ranging from ~0.5–5 s, which remained constant during DHPG application (1.75 ± 0.15 s for first 5 min in DHPG vs 1.88 ± 0.22 s for the last 5 min in DHPG, n = 14; paired t-test, p = 0.33). This bursting pattern was not different from the pattern obtained using 50–100 μM (R,S)-3,5-DHPG (4.2 ± 0.8 bpm, p = 0.4, unpaired t-test, n = 11, data not shown) which