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Minor contribution of ATP P2 receptors to electrically-evoked electrographic seizure activity in hippocampal slices: Evidence from purine biosensors and P2 receptor agonists and antagonists

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ABSTRACT

While the position of adenosine as an endogenous anticonvulsant is well established, it is unclear to what extent its precursor, ATP, contributes to seizure activity via P2 receptors. In this study we have addressed this issue through the use of ATP biosensors and agonists and antagonists of ATP P2 receptors to detect the release and role of ATP, respectively, during electrically-evoked electrographic seizure-like events (eSLEs) in rat hippocampal slices. The broad-spectrum P2 receptor antagonists RB-2 and PPADS (10 μM) caused a small ~30% inhibition of eSLE duration, and a reduction in intensity. This inhibition of eSLEs was partially reproduced with the $P2X_{1,2/3,3}$ antagonist NF023 (10 μM), but not the $P2X_7$ antagonist BBG (10 μM). However, the P2X receptor agonist α,β-meATP did not enhance eSLEs, but instead reduced their duration. Furthermore, we could discern no role for $P2Y_1$ receptors in electrically-evoked eSLEs: both the $P2Y_1$ antagonist MRS2179 (10 μM) and the $P2Y_1$ receptor agonist 2-methylthioADP (10 μM) were without effect on eSLEs. Consistent with a minor role for ATP P2 receptors on eSLEs we could detect no ATP release during eSLEs, although appreciable quantities of adenosine were detected, which had a pronounced inhibitory action on eSLEs via A_1 receptors. We conclude that the role of ATP P2 receptors in modulating electrographic seizure activity is limited, at least in models such as this one requiring electrical stimulation of afferent fibres. We further conclude that the presence and action of adenosine under these conditions may primarily reflect direct release of this purine.

1. Introduction

The role of adenosine as an endogenous anticonvulsant has been known for more than two decades (Dragunow et al., 1985) and has led to the current interest in adenosine-based cell and gene therapies for the treatment of epilepsy (Boison, 2009; Naegele et al., 2010). During seizure activity in humans (During and Spencer, 1992) and in experimental animals both in vitro and in vivo (Dale and Frenguelli, 2009), adenosine is released into the extracellular space where it activates A_1 receptors (A_1R) to terminate seizures (Dunwiddie and Masino, 2001; Dale and Frenguelli, 2009). Changes in A_1R expression in human epilepsy have been reported: an increase (Angelatou et al., 1993) may be a compensatory protective mechanism, whilst their loss (Glass et al., 1996) may reflect the establishment of a chronic epileptic state. In addition, the importance of adenosine to human seizures is underscored by the use of methylxanthine antagonists of adenosine receptors such as caffeine and theophylline to augment seizure activity during electroconvulsive therapy (ECT) (Loo et al., 2010), whilst hyperventilation, which promotes seizure activity in ECT (Loo et al., 2010) and vulnerable humans (Jonas et al., 2010), may do so by reducing extracellular adenosine (Dulla et al., 2005).

Adenosine A_1 receptors are $G\alpha_{i/o}$-protein coupled receptors which exert a variety of responses, from inhibition of adenylate cyclase and activation of phospholipase C to inhibition of voltage-gated calcium channels and activation of potassium channels (Dunwiddie and Masino, 2001). In neurones the predominant actions of A_1Rs are inhibition of both synaptic transmission and neuronal excitability (Haas and Selbach, 2000). However, these inhibitory actions are not shared by the other adenosine receptors. For example, the A_{2A} and A_3Rs have been shown to exert a modest excitatory action during electrographic seizure activity (Etherington and Frenguelli, 2004).

Despite ample pharmacological, HPLC and electrochemical evidence of extracellular adenosine during seizure activity (Dale and Frenguelli, 2009), the exact source and mechanism of adenosine release during and after seizures remains unclear. Given that ATP is

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released in an activity-dependent manner (Dale and Frenguelli, 2009), extracellular adenosine may arise during seizures from the extracellular metabolism of ATP via the activity of ectonucleotidases (for review see Zimmermann, 2000).

ATP is an extracellular signalling molecule in its own right. It participates in neurotransmission, neuromodulation and gliotransmission and has been implicated in many physiological and pathophysiological conditions (Burnstock, 2007; Halassa et al., 2009). Two types of purinergic P2 receptors can be activated by ATP: (i) P2X cation-selective ion channels, and (ii) G protein-coupled P2Y receptors, some of which can also be activated by ADP. Currently there are seven P2X receptors and eight P2Y receptors and most of them are present in the hippocampus (Kukley et al., 2001; Rodrigues et al., 2005).

Presynaptic $P2X_{1,2/3,3}$ receptors act as excitatory receptors to facilitate glutamate release, whereas $P2Y_{1,2,4}$ receptors have been found to presynaptically inhibit glutamatergic neurotransmission in the hippocampus (Rodrigues et al., 2005). In addition, activation of $P2Y_1$ receptors on astrocytes causes calcium waves (Bowser and Khakh, 2007) and results in increased GABAergic synaptic inhibition in the CA1 region of the hippocampus (Bowser and Khakh, 2004).

Although the role of adenosine in epilepsy and seizures is now well established, there is a lack of evidence of the link between ATP receptors and seizure activity, despite the effects of P2 receptors on transmission described above, and despite reported changes in ATP metabolising enzymes in human epilepsy (Nagy et al., 1990; Lie et al., 1999) and P2 receptor expression in experimental models of epilepsy. For example, a decrease in $P2X_4$ receptor expression has been observed in pilocarpine-induced temporal lobe epilepsy (TLE) in rats (Dona et al., 2009) and in seizure-prone gerbils, where a decrease in $P2X_2$ receptors was also observed (Kang et al., 2003). However, an increase in $P2X_7$ receptor staining has been observed during acute and chronic phases of an *in vivo* TLE model (Dona et al., 2009). With its capability to form a large pore permitting molecules to pass of up to 900 Da, the $P2X_7$ receptor is a possible purinergic receptor candidate in the aetiology of epilepsy.

In this study we have investigated the contribution of ATP P2 receptors to electrically-evoked epileptiform activity in area CA1 of rat hippocampal slices, and attempted to detect the release of ATP with ATP microelectrode biosensors. We conclude that P2X receptors, predominately $P2X_{1,2/3,3}$, but not $P2X_7$ or $P2Y_1$ receptors, contribute a minor excitatory role during electrically-evoked electrographic seizure-like events, which is consistent with there being little evidence of appreciable ATP release. In contrast, we detect considerable adenosine release and inhibitory action via A_1Rs which suggests the potential for primarily direct activity-dependent adenosine release in this model.

2. Materials and methods

2.1. Chemicals

Salts for the aCSF (NaCl, KCl, NaH2PO4, NaHCO3, MgSO4), glucose and glycerol were obtained from Fisher (Fisher Scientific, Loughborough, Leicestershire, UK). Reactive Blue 2, Brilliant Blue G, adenosine, 8-CPT (8-Cyclopentyl-1,3-dimethylxanthine) and serotonin were purchased from Sigma (Sigma-Aldrich Company Ltd, Dorset, UK). PPADS (Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt), NF023 (8,8′-[carbonylbis(imino-3,1-phenylen ecarbonylimino)] bis-1,3,5-naphthalene-trisulphonic acid, hexasodium salt) and ARL 67156 (6-*N*,*N*-Diethyl-D-β,γ-dibromomethyleneATP trisodium salt), MRS2179 (2′-Deoxy-N6-methyladenosine 3′,5′-bisphosphate tetrasodium salt) and 2-Methylthio-ADP trisodium salt were from Tocris (Tocris Bioscience, Bristol, UK). ATP disodium salt was obtained from Roche (Roche Diagnostics Ltd., Burgess Hill, UK).

2.2. Slice preparation and maintenance

17–20 day old male Sprague–Dawley rats were killed by cervical dislocation and then decapitated in accordance with Schedule 1 of the UK Government Animals Act 1986 and Local Ethical Review guidelines. The brain was quickly removed and put into ice-cold artificial cerebrospinal fluid (aCSF) (containing, in mM: 124 NaCl, 26 $NaHCO_3$, 10 glucose, 1.3 NaH_2PO_4, 2 $CaCl_2$, 2 KCl, 1 $MgSO_4$) with additional 10 mM Mg^{2+}. 600 μm parasagittal hippocampal slices were cut on a vibratome (Microm, HM 650 V) and kept at room temperature in standard aCSF (1 mM Mg^{2+}; pH 7.4) bubbled with 95% O_2/5% CO_2 for at least 1 h before use. In our experience 600 μm slices are required to support electrographic seizure activity (Etherington and Frenguelli, 2004).

2.3. Experimental procedure

A hippocampal slice was submerged in a recording chamber and perfused with aCSF at a flow rate of 6–7 mL/min and at 30 °C. The slice was supported on a nylon mesh to ensure perfusion of both the upper and lower surfaces to maximise delivery of oxygen and glucose, which was facilitated by the high flow rate. This ensured that the slices were not hypoxic and show comparable levels of adenosine tone as 400 μm slices (Etherington et al., 2009). The Schaffer collateral-commissural pathway was stimulated in stratum radiatum with a custom-made bipolar Teflon-coated tungsten stimulating electrode (100 μm overall diameter). Field excitatory post-synaptic potentials (fEPSPs) were recorded every 15 s (basal stimulation) from area CA1, with an aCSF-filled glass microelectrode of ~1 MΩ resistance. Paired-pulse facilitation was evoked by delivering two pulses separated by 50 ms.

Once stable fEPSPs were obtained, which typically measured ~50% of the maximal fEPSP slope, reflecting around 125–150 μA of stimulation current, extracellular Mg^{2+} was washed out. After 30 min in nominally Mg^{2+}-free aCSF, three electrographic seizure-like events (eSLEs) were evoked by means of brief high-frequency stimulation (HFS; 60 Hz, 2 s) of the Schaffer pathway as previously described (Etherington and Frenguelli, 2004; Etherington et al., 2009). eSLEs were induced once fEPSPs had recovered from the post-ictal depression caused by the previous eSLEs, which was approximately 10 min. After the fEPSP recovered from the third eSLE, a drug was washed in. Slices were usually incubated with drug for 30 min. After this period another set of three eSLEs were elicited. Periodic stimulation at 15 s intervals was delivered throughout the experiments (except those involving the application of 0 mM Mg^{2+}/6 mM K^+) to monitor the effects of SLE and drugs on basal transmission but had no influence on eSLEs activity.

2.4. Real-time detection of adenosine/ATP

Sarissaprobe™-ATP or Sarissaprobe™-adenosine microelectrode biosensors (0.5 mm length, 50 μm diameter) were purchased from Sarissa-Biomedical Ltd (Coventry, UK). Sensors were inserted quasi-perpendicular through the thickness of the slice in stratum radiatum of the CA1 region. The principle behind the operation of the ATP and adenosine biosensors have been described previously (Llaudet et al., 2003, 2005), as has their use in hippocampal slices (Frenguelli et al., 2003, 2007). Briefly, when ATP or adenosine is released, a cascade of enzymes entrapped in the matrix of the sensor breaks down ATP or adenosine (Ado) to release hydrogen peroxide, which is then detected as a current on a platinum wire polarised to 600 mV. The current is linearly-related to the concentration of the purine over an extensive range – tens of nM to tens of μM. The release of purines was recorded during eSLEs with pairs of sensors (ATP-null, Ado-null or ATP-Ado). The Ado sensors are sensitive to adenosine and its metabolites which are primarily inosine and hypoxanthine. No subtraction of the inosine/hypoxanthine signal was attempted, which would have required an additional sensor lacking adenosine deaminase, the first enzyme in the cascade. As such, the values reported for the adenosine signal reflect the concentration of adenosine and its metabolites and we refer to it as μM' to reflect this (Frenguelli et al., 2007). Where ATP biosensors were used, 2 mM glycerol was added to the aCSF as ATP sensors require glycerol as a co-substrate (Llaudet et al., 2005). ATP sensors were briefly immersed in pure glycerol before the insertion of sensors in the tissue. After insertion of the sensors slices were left to recover for 1 h, which allowed signals associated with their insertion to dissipate. At the end of experiments, sensors were withdrawn from slices and calibrated, which consisted of bath-application of 10 μM adenosine or ATP. Furthermore, 5-HT (10 μM) was applied to test the patency of the selectivity layer of the sensor which excludes the majority of non-specific electroactive interferents. Data from sensors was only accepted if the signal evoked by 5-HT was less than 100 pA.

2.5. Assessment of electrographic epileptiform activity

Provided the first three (control) electrographic seizure-like events (eSLEs) were alike and were at least 10 s long, which happened in the majority of cases, the experiment proceeded, which involved either time controls for the effects of drugs, or for the drugs themselves. An eSLE was considered to have terminated when two successive spikes were more than 1 s apart. eSLE duration was calculated by pooling together the average durations of the three eSLEs in control conditions and the durations of the subsequent three eSLEs in the presence or absence of a drug. In order to evaluate the intensity of eSLEs, continuous recording of eSLEs were first rectified, to detect positive and negative going spikes, and the number of ictal spikes was counted during both the early tonic and later clonic part of an eSLE. A spike was taken into account provided its amplitude was at least 0.1 mV. A 50 ms minimal