

K⁺ depolarization evokes ATP, adenosine and glutamate release from glia in rat hippocampus: a microelectrode biosensor study

A Heinrich¹, RD Andó¹, G Túri², B Rózsa² and B Sperlág¹

¹*Laboratory of Molecular Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary,* ²*Femtonics Ltd, Budapest, Hungary*

Running title: K⁺ depolarization evoked gliotransmitter release

Correspondence:

Beáta Sperlág

Laboratory of Molecular Pharmacology

Institute of Experimental Medicine

Hungarian Academy of Sciences

H-1083 Budapest, Szigony u. 43., Hungary

Tel: +36-1-210-9970

This article has been accepted for publication and undergone full scientific peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/j.1476-5381.2012.01932.x

Fax: +36-1-210-9423

E-mail: sperlagh@koki.hu

Summary

Background and purpose. This study was undertaken to characterize the K⁺ depolarization-evoked ATP, adenosine and glutamate outflow in the *in vitro* rat hippocampal slice.

Experimental approach. We utilised the microelectrode biosensor technique and extracellular electrophysiological recording for the real-time monitoring of the efflux of ATP, adenosine and glutamate.

Key results. ATP, adenosine and glutamate sensors exhibited transient and reversible current during 25 mM K⁺ depolarization, with distinct kinetics. The ecto-ATPase inhibitor diethyl-β-γ-dibromomethylene-D-adenosine-5'-triphosphate trisodium salt hydrate (ARL67156) enhanced the extracellular level of ATP and inhibited the prolonged adenosine efflux suggesting that generation of adenosine may arise from the extracellular breakdown of ATP.

Stimulation-evoked ATP, adenosine and glutamate efflux was inhibited by tetrodotoxin, while Ca²⁺-free medium abolished ATP and adenosine efflux. Extracellular elevation of ATP and adenosine were decreased in the presence of NMDA receptor antagonists D(-)-2-amino-5-phosphonopentanoic acid (D-AP-5) and ifenprodil, whereas non-NMDA receptor blockade by CNQX inhibited glutamate but not ATP and adenosine efflux. The gliotoxin fluoroacetate and P2X7 receptor antagonists inhibited the K⁺-evoked ATP, adenosine and glutamate efflux, while carbenoxolone in low concentration and probenecid decreased only the adenosine efflux.

Conclusions and Implications. Our results demonstrate activity-dependent gliotransmitter release in the hippocampus in response to ongoing neuronal activity. ATP and glutamate is

released by P2X7 receptor activation into extracellular space. Although the efflux of adenosine did arise from released ATP, it might also be released directly via pannexin hemichannels.

Keywords: ATP, glutamate, adenosine, release, pannexin, connexin, hippocampus, K⁺ depolarization, glia

Abbreviations: aCSF, artificial cerebrospinal fluid; ADO, adenosine; ARL67156, 6-N,N-Diethyl- β - γ -dibromomethylene-D-adenosine-5'-triphosphate trisodium salt hydrate; AZ10606120 dihydrochloride, N-[2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinoliny]-2-tricyclodec-1-ylacetamide dihydrochloride; D-AP-5, D(-)-2-amino-5-phosphonopentanoic acid; dipyridamole, 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido pyrimidine; BBG, Brilliant blue G; CBX, carbenoxolone; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione-disodium; fEPSP, field excitatory postsynaptic potentials; FAc, fluoroacetic acid sodium salt; GLU, glutamate; INO, inosine; L-trans-2,4-PDC, L-trans-Pyrrolidine-2,4-dicarboxylic acid; SD, spreading depression; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor, TTX, tetrodotoxin; VNUT, vesicular nucleotide transporter.

Introduction

ATP and its extracellular breakdown product, adenosine are important signaling molecules in the CNS under physiological and pathological conditions. ATP, activating ionotropic P2X (P2X1-7) and metabotropic P2Y (P2Y1,2,4,6,11,12,13,14) receptors acts as a fast transmitter and as a modulator of synaptic activity in several regions of the brain, including the hippocampus (North and Verkhratsky, 2006; Pankratov *et al.*, 2006). Moreover, it also participates in long term-synaptic plasticity and regulates the survival of neurons and the following repair process under pathophysiological conditions, such as ischemia and inflammation (Cunha and Ribeiro, 2000; Abbracchio *et al.*, 2009). Adenosine, activating metabotropic adenosine receptors (A_1 , A_{2A} , A_{2B} , A_3), has also a well-established role in the hippocampus, as a pre- and postsynaptic depressant of excitatory transmission gaining significance under physiological conditions, metabolic stress and seizures (Cunha, 2001).

Accordingly, convincing evidence demonstrate the activity-dependent release of both ATP and adenosine from the hippocampus under stimuli, mimicking physiological neuronal activity such as electrical stimulation (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996; Pankratov *et al.*, 2006) or pathological challenge, such as combined oxygen-glucose deprivation (Juranyi *et al.*, 1999; Frenguelli *et al.*, 2007). However, due to their ubiquitous nature, the endogenous source of purines for synaptic modulation remained enigmatic under these conditions.

Recent studies highlighted that glial cells, in particular astrocytes, are active players in information processing in the brain and periphery (Araque *et al.*, 2001; Haydon, 2001; Fellin and Carmignoto, 2004) and both adenine nucleotides and nucleosides as well as glutamate play a key role in the glia-neuron cross talk (Nedergaard, 1994; Parpura *et al.*, 1994; Fields and Burnstock, 2006; Halassa *et al.*, 2009). Thus, ATP and glutamate coordinately activate astrocytes, through

the mobilization of their internal Ca^{2+} , which in turn triggers the release of several neuroactive molecules from astrocytes including ATP and glutamate themselves. These 'gliotransmitters' signal either to astrocytes, where they generate Ca^{2+} waves, or to neurons, where they modulate synaptic transmission and neuronal excitability (Cotrina *et al.*, 1998; Guthrie *et al.*, 1999; Fellin *et al.*, 2009). In the hippocampus, stimulation of Schaffer collaterals leads to the glutamatergic activation of AMPA receptors located on astrocytes, which in turn releases ATP from astrocytes. This ATP, by itself, or by its metabolic degradation to adenosine elicits activity-dependent heterosynaptic depression of neighboring pathways via activation of P2Y and A_1 receptors respectively (Koizumi *et al.*, 2003; Zhang *et al.*, 2003; Pascual *et al.*, 2005; Serrano *et al.*, 2006), which may lead to increased synaptic inhibition within intact hippocampal circuits (Bowser and Khakh, 2004). Although the astrocytic source of ATP for synaptic modulation has been demonstrated by a transgenic mouse model of the astrocyte-specific, inducible expression of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) instrumental for the release of gliotransmitters (Pascual *et al.*, 2005), the physiological and pathological trigger for the release of ATP from astrocytes and its mechanism is still unknown.

Being a highly charged molecule, ATP by itself cannot permeate the cell membrane. Nevertheless, in previous studies, several release mechanisms has been identified for both ATP and adenosine. ATP is co- stored in synaptic vesicles, and its classical, Ca^{2+} -dependent vesicular release has been demonstrated in a number of central synapses by both electrophysiological and neurochemical detection (Sperlagh and Vizi, 2000; Pankratov *et al.*, 2006). Vesicular exocytosis of ATP has also been demonstrated from astrocytes (Coco *et al.*, 2003; Pangrsic *et al.*, 2007). In addition, other alternative routes of ATP release have also been identified, such as P2X7 receptor channels (Ballerini *et al.*, 1996; Pellegatti *et al.*, 2005; Suadicani *et al.*, 2006) gap junction hemichannels (e.g. Arcuino *et al.*, 2002; Stout *et al.*, 2002; Bao *et al.*, 2004; Pearson *et al.*, 2005;

Anselmi *et al.*, 2008), lysosome exocytosis (Zhang *et al.*, 2007), volume sensitive anion channels (Darby *et al.*, 2003; Okada *et al.*, 2004) and the cystic fibrosis transmembrane conductance regulator protein (CFTR) (Kanno and Nishizaki, 2011). However, these latter mechanisms have primarily described in non-neuronal or simplified experimental systems, such as cultured neurons and astrocytes, and less information is available, whether these pathways also serve as a conduit of ATP release for purines involved in the modulation of synaptic signaling.

For adenosine, there are also a number of different mechanisms whereby it could accumulate in the extracellular space (Latini and Pedata, 2001; Wall and Dale, 2008). Firstly, it can be generated from the released ATP through the actions of extracellular nucleotide catabolizing enzymes, i.e. E-NTPDases, E-NPPases, alkaline phosphatases and ecto5'-nucleotidase. Secondly, it can also be released by its own right, via equilibrative or non-equilibrative transporters. Finally, although direct evidence is lacking, several studies support that adenosine can also be released in a vesicular fashion (Wall and Dale, 2008).

Despite that chemical signaling has a key role in neural function, only a few techniques are available to directly measure the concentrations of neurotransmitters and modulators in the extracellular space. Among them, the recently developed microelectrode biosensor technique allows the detection of the efflux of endogenous neurotransmitters with better resolution than conventional neurochemical methods (Llaudet *et al.*, 2003; Llaudet *et al.*, 2005; Tian *et al.*, 2009), and also circumvent the limitations of widely used electrophysiological techniques, which indirectly detect the released transmitters. Using these multi-enzymatic microelectrode biosensors, it is possible to measure the concentration of different neurotransmitters and modulators with high temporal and spatial resolution and to detect paracrine and other non-classical release mechanisms as well (Dale *et al.*, 2005).

In this study we characterized the efflux the ATP, adenosine and glutamate in the *in vitro* rat hippocampus by the microelectrode biosensor technique. To activate the intact glia-neuron ensembles of the hippocampal slice (Sul *et al.*, 2004), K⁺ depolarization was applied and we demonstrated and characterized depolarization evoked release of gliotransmitters in the hippocampal slice.

Methods

All studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory animals and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary). Animals were kept under standard laboratory conditions (12h light/12h dark cycle) with food and water *ad libitum*.

All efforts were made to minimize animal suffering and reduce the number of animal used.

Slice preparation

Young adult male *Wistar* rats (4 weeks of age, 85-110 g) were euthanized by cervical dislocation.

After decapitation, the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 11 mmol/L Mg²⁺, wherein 400 µm coronal slices were prepared by a microtome with vibrating blade (Microm HM 650 V, Microm International GmbH, Walldorf, Germany) as previously described (Dale *et al.*, 2000). Slices were placed in an incubation chamber comprising a nylon mesh within a beaker of continuously circulating, oxygenated (95% O₂/5% CO₂) standard aCSF (1.3 mmol/L Mg²⁺) and kept at room temperature for at least 1 h before use. Standard aCSF contained (in mmol/L): NaCl, 127; KCl 1.9; CaCl₂, 2.4; NaHCO₃ 26;

KH₂PO₄, 1.2; D-glucose, 10; MgSO₄ 1.3; pH 7.4 and was gassed with 95% O₂/5% CO₂. Glycerol (2 mmol/L) essential for ATP biosensor operation was added to the aCSF (see below).

Extracellular recording

A single slice was transferred to a recording chamber, fully submerged in aCSF and perfused at 6 ml/min (34-36°C). Field excitatory postsynaptic potentials (fEPSPs) were extracellularly recorded in AC mode using a ISO-80 isolated bio-amplifier (World Precision Instruments Inc, Sarasota, FL, USA), with aCSF-filled glass microelectrode (1B150F-4, < 2 MΩ; WPI), from the *stratum radiatum* of CA1 region in response to stimulation (at 3V- 7V, 0.1ms, 15s intervals; parallel bipolar electrodes; FHC Inc, Bowdoin, ME, USA) of the Schaffer collateral-commissural fiber pathway. Extracellular DC recordings were performed with the Multiclamp 700A (Molecular Devices Inc, Sunnyvale, CA, USA) amplifier in DC mode using borosilicate glass pipettes (GC120F-10, 4-7 MΩ, Harvard Apparatus, Holliston, MA, USA) filled with aCSF. Electrical signal from the electrode were acquired at 10 kHz (sampling frequency), using the pCLAMP 9 software package (Molecular Devices Inc, Sunnyvale, CA, USA) and displayed on the laboratory computer. Subtraction of the 50 Hz noise and successive Gauss filtering (2.5 kHz) were applied offline (MES, Femtonics Ltd). Signal analysis was performed using the Curve Analysis software package (Femtonics Ltd., Budapest, Hungary). Extracellular recordings were performed simultaneously with ATP and null sensor measurements in a set of experiments.

Biosensor recording using ATP, adenosine and glutamate sensors

The principles and operation of the Pt/Ir microelectrode biosensors for the purines, ATP and adenosine, have been described previously (Llaudet *et al.*, 2003; Llaudet *et al.*, 2005). The biosensors used in this study were obtained from Sarissa Biomedical (Coventry, UK). The ATP

sensor comprised of two enzymes (glycerol kinase EC 2.7.1.3 and glycerol-3-phosphate oxidase EC 1.1.3.21) and the adenosine sensor comprised of three enzymes (adenosine deaminase EC 3.5.4.4, nucleoside phosphorylase EC 2.4.2, xanthine oxidase EC 1.1.3.22) entrapped within a matrix around a fine platinum wire of 50 μm diameter and 0.5 mm long. Electrochemical sensor can respond not only to the analyte of interest, but also to any electroactive species in the immediate environment. To check whether the sensors were responding to ATP, we used a dual recording configuration. To give a measure of net ATP concentrations, the signal from a NULL sensor (lacking enzymes, but otherwise identical) was used to measure background signals, which were then subtracted from the signals generated by the ATP biosensor. For the adenosine/inosine sensor (ADO/INO), a separate inosine-sensor (INO), lacking adenosine deaminase, is required to yield a signal specific to adenosine (Frenguelli *et al.*, 2003). Thus, the signal of adenosine sensor can be described as that from both adenosine and inosine, and the net adenosine signal is achieved by subtracting the signal of inosine sensor from this value. Glutamate release was measured using a glutamate (GLU) sensor, comprising glutamate oxidase (EC 1.4.3.11) and using NULL sensor, as background. The sensor is selective for GLU over glutamine, aspartate, dopamine and 5-HT (Dale *et al.*, 2005; Tian *et al.*, 2009). All microbiosensors were operated at 500-700mV (vs. Ag/AgCl) in a flow system for amperometric detection at 34-36°C temperature. In certain series of experiments ATP, NULL, ADO/INO and INO recordings were performed simultaneously, whereas in other series of experiments simultaneous ATP, GLU and NULL recordings were done in order to limit the maximal number of recording electrodes in the same measurement location. The time of the ATP peaks were not significantly different in the two sets of measurements ($0.145 \pm 0.131\text{s}$, $p > 0.1$, t -test), suggesting a negligible impact of the two different electrode arrangements on the measured data. Therefore data from the two sets of measurements are shown simultaneously on the same time axis.

The microelectrode biosensors were each inserted through the 400 μm thickness of the CA1 region of the hippocampal slice such that most of the sensing part of the sensors was in intimate contact with the tissue. Previous studies showed that insertion of the sensor is not detrimental to the slice, exhibiting fEPSP activity in its place (Frenguelli *et al.*, 2003). Before insertion and after removal, the sensors were calibrated with known concentrations of standards: ATP (10 $\mu\text{mol/L}$), adenosine (3 $\mu\text{mol/L}$), inosine (10 $\mu\text{mol/L}$) and glutamate (10 $\mu\text{mol/L}$), which allowed quantification of any rundown in sensor sensitivity over the duration of an experiment. Before experiments a standard calibration curve was generated, and a high linear correlation was observed between the peak value of signal and the ATP, adenosine, inosine, and glutamate concentration. To take into account that biosensor sensitivity varies during different experiments, we used the calibration of the sensor to normalize each signal. In addition, we tested all compounds for any interference with the sensitivity of the sensors, but none of them elicited any detectable change in the signal. Besides the sensors, the recording electrode was placed adjacent to the sensors. Continuous signal from the biosensor was acquired at 10 kHz using the pCLAMP 9 software package (Molecular Devices, CA, USA) and displayed on a PC. For calculation, 'net' signals for each experiment were calculated subtracting the currents of the corresponding baseline, which were expressed in absolute units of concentration ($\mu\text{mol/L}$). In case of pharmacological treatments, their effects on the peaks of the responses of the biosensors were quantified. Unless otherwise stated, in case of ATP recording, when a biphasic signal was recorded, the effects of treatments on the peak of the second phase of the response were calculated, i.e. the absolute maximum of the signal was taken into account. Calibration curves were constructed using the GraphPad software.

Experimental protocol

After insertion of biosensors, an approx. 20 min equilibration period was allowed to reach a steady-state baseline. Then, K^+ depolarization was applied by subjecting the preparations to modified aCSF containing 26.9 mmol/L K^+ (Na^+ : 102 mmol/L) for 270 sec. All drugs were applied to the perfusion solution, from 20 min before the beginning of K^+ depolarization until the end of experiments, except FAc, which was added 10 min before the onset of K^+ stimulation. Drugs (6-N,N-Diethyl- β - γ -dibromomethylene-D-adenosine-5'-triphosphate trisodium salt hydrate (ARL67156), N-[2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinoliny]-2-tricyclodec-1-ylacetamide dihydrochloride (AZ10606120), carbenoxolone (CBX), 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido pyrimidine (dipyridamole), fluoroacetic acid sodium salt (FAc), brilliant blue G (BBG), probenecid, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-AP-5), (1S*,2S*)-threo-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol hemitartrate (Ifenprodil), tetrodotoxin (TTX), L-trans-Pyrrolidine-2,4-dicarboxylic acid (L-trans-2,4-PDC) were dissolved into the fresh aCSF. Ca^{2+} -free aCSF contained 1 mmol/L EGTA in order to eliminate residual extracellular Ca^{2+} .

Statistical analysis

Data are expressed as mean \pm SEM with n = number of identical experiments. Student's t test (pairwise comparisons) and one-way analysis of variance (ANOVA) followed by the Dunnett test (multiple comparisons) were used as a statistical analysis, as appropriate. P values of less than 0.05 were considered statistically significant.

Materials

Salts used in the aCSF were obtained from Reanal ZRT., Budapest, Hungary. The other drugs used were: ATP, 6-N,N-Diethyl- β - γ -dibromomethylene-D-adenosine-5'-triphosphate trisodium salt hydrate (ARL67156), carbenoxolone (CBX), Brilliant blue G (BBG), 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido pyrimidine (dipyridamole), fluoroacetic acid sodium salt (FAc), probenecid (all from Sigma-Aldrich Chemical Co., St. Louise, MO, USA), adenosine, inosine (Merck, Darmstadt, Germany), N-[2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-5-quinoliny]-2-tricyclodec-1-ylacetamide dihydrochloride (AZ10606120), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), D-(-)-2-amino-5-phosphonopentanoic acid (D-AP-5), (1S*,2S*)-threo-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol hemitartrate (Ifenprodil), L-trans-Pyrrolidine-2,4-dicarboxylic acid (L-trans-2,4-PDC) (all from Tocris Bioscience, Ellisville, Mo, USA), tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel), L-glutamic acid (Pierce, Rockford, IL, USA). All solutions were freshly prepared on the day of use.

Results

ATP, adenosine and glutamate released under basal conditions and after K⁺ depolarization in the hippocampal CA1 area

The selectivity of the biosensors used versus interferences found in the hippocampus enabled to estimate the basal tone of ATP, adenosine and glutamate in the slice. As the biosensors have a background current, basal tone was measured with the following method: at first placing the sensors into the tissue chamber, allowing it to equilibrate for at least 20 minutes and then inserting the sensor into the slices, allowing it to equilibrate and measuring the changes and

potential differences between these equilibrium states. To convert this difference to absolute concentration of ATP, adenosine and glutamate we used null and inosine sensors as references (see Method). Under basal conditions extracellular ATP, adenosine and glutamate basal tones were closed to the detection limit (ATP: 4.1 ± 0.8 pA, $n = 10$; ADO: 5.4 ± 0.7 pA, $n = 10$; GLU: 6.4 ± 0.8 pA, $n = 10$) and have been estimated to be as low as a few nmol/L, respectively.

When slices were subjected to K^+ depolarization (25 mmol/L; 270 sec) easily detectable signals were recorded on ATP, glutamate and adenosine sensors, respectively (Fig 1). All sensors showed a rapid response; however, the adenosine sensor detected an earlier and long lasting signal than the ATP and glutamate sensor. The release of adenosine started 0.79 ± 0.01 min after the beginning of the K^+ depolarization and reached its peak at 7.31 ± 0.01 min eliciting a maximal release of 10.28 ± 1.41 $\mu\text{mol/L}$ ($n = 8$, Fig 1C). The efflux of glutamate started 2.68 ± 0.04 min after K^+ depolarization, and reached its peak at 5.62 ± 0.06 min (3.49 ± 0.84 $\mu\text{mol/L}$, $n = 8$, Fig 1B, Table 1). Two phases of ATP release were detected, the first occurring at the same time when glutamate release started (Fig 1A, B), while the onset of the second phase coincided with the maximum peak of glutamate release (Fig 1A, B). ATP efflux started 2.56 ± 0.05 min after the beginning of the K^+ stimulation and reached its absolute peak during the second phase at 8.08 ± 0.01 min (1.23 ± 0.23 $\mu\text{mol/L}$, $n = 8$, Fig 1A, Table 1). Null sensors that lacked enzymes in the polymer coating displayed only small fluctuations of the current around the baseline (not shown). Evoked signals of field excitatory postsynaptic potentials (fEPSP, in AC mode) recording from *stratum radiatum* of rat hippocampus simultaneously with the ATP sensor were recovered after the K^+ depolarization (Fig 1E), indicating that slices retained their viability during the experiments. During K^+ depolarization, the gradual loss of biological electrophysiological activity was observed, as expected. fEPSP activity is then completely recovered in the next 15 min during wash out. High K^+ -induced spreading depression (SD, Shock et al., 2007) was

detected at 3.81 ± 0.2 min ($n = 6$) after stimulation from *stratum radiatum* of area CA1 (Fig 1F). This large negative deflection on the extracellular DC potential occurred immediately after with the loss of fEPSP, but before adenosine release did not reach the maximum peak. ATP started to release before the beginning of SD, showed a small first peak at the time of SD (0.48 ± 0.16 $\mu\text{mol/L}$, $n = 8$) and continued to increase after the peak of SD. Likewise, glutamate release did not reach its plateau before the onset of SD (Fig 1B).

Inhibition of extracellular ATP metabolism increases ATP and inhibits adenosine accumulation

The presence of ectonucleotidases that can metabolize ATP to ADP, AMP and adenosine in the hippocampus is well documented (Goding, 2003; Zimmermann, 2000). These enzymes could play a role in the production of adenosine during K^+ depolarization (25 mmol/L; 270 sec). We therefore tested the contribution of the hydrolysis of ATP to extracellular adenosine accumulation by the selective ecto-ATPase inhibitor ARL67156. ARL67156 was applied in a concentration (100 $\mu\text{mol/L}$), which, according to previous studies (Frenguelli *et al.*, 2007; Sperlagh *et al.*, 2007) elicits a substantial, although not complete blockade of extracellular breakdown of ATP in the hippocampus.

Inhibition of extracellular ATP metabolism by ARL67156 (100 $\mu\text{mol/L}$) enhanced the extracellular level of ATP following K^+ depolarization (8.62 ± 0.76 $\mu\text{mol/L}$, $n = 8$, $p < 0.01$; Fig 2A). Moreover, K^+ depolarization evoked ATP efflux appeared earlier than in the absence of ectoATPase inhibitor (1.69 ± 0.012 min after the beginning of the K^+ stimulation). On the other hand, ARL67156 (100 $\mu\text{mol/L}$) had no significant effect on the glutamate efflux (2.43 ± 0.63 $\mu\text{mol/L}$, $n = 6$, $p > 0.05$; Fig 2B, Table 1). The elevation in glutamate efflux in this case started coincidentally with the elevation of ATP efflux (Fig 2B). In contrast, adenosine release during the

stimulation was inhibited in the presence of ARL67156 ($0.15 \pm 0.04 \mu\text{mol/L}$, $n = 6$, $p < 0.01$; Fig 2A). These results show that ATP efflux detected by the ATP biosensor is subject to extracellular metabolism and contributes to the generation of adenosine that is detected by the adenosine biosensor. When the slices were preperfused with ARL67156 ($100 \mu\text{mol/L}$) and the adenosine transport inhibitor dipyridamole ($50 \mu\text{mol/L}$) the adenosine efflux was partly inhibited (ADO: $2.71 \pm 0.23 \mu\text{mol/L}$, $n = 5$, $p < 0.001$ vs. ARL67156 alone, Table 1), i.e. it was higher, than detected in the presence of the ectoATPase inhibitor alone. These findings indicate a minor additional direct adenosine release in response to K^+ depolarization, which is not derived from the released ATP.

Differential susceptibility of ATP, adenosine and glutamate release to extracellular Ca^{2+}

Because ATP can be released in a $[\text{Ca}^{2+}]_o$ -dependent manner (Sperlagh and Vizi, 2000; Pankratov *et al.*, 2006), we asked whether the efflux of ATP during K^+ depolarization (25mmol/L ; 270 sec) was $[\text{Ca}^{2+}]_o$ -dependent. To examine the Ca^{2+} -dependency of purine release during K^+ depolarization, we omitted Ca^{2+} from the perfusion medium and 1 mmol/L EGTA was added to chelate any residual extracellular Ca^{2+} . Perfusion of Ca^{2+} -free/EGTA and 25 mmol/L K^+ containing aCSF caused a significant decrease in ATP ($0.21 \pm 0.01 \mu\text{mol/L}$, $n = 6$, $p < 0.01$; Table 1, Fig 3) and adenosine ($0.22 \pm 0.02 \mu\text{mol/L}$, $n = 6$, $p < 0.01$; Table 1, Fig 3) efflux during K^+ depolarization. On the other hand, glutamate efflux did not significantly change in response to stimulation ($3.64 \pm 0.15 \mu\text{mol/L}$, $n = 6$, $p > 0.05$; Table 1, Fig 3), when rat hippocampal slices were perfused with Ca^{2+} -free/EGTA aCSF solution.

Involvement of ongoing neuronal activity in ATP, adenosine and glutamate release

To test the involvement of the sodium-channel mediated axon potential propagation, the effect of TTX (3 $\mu\text{mol/L}$) was examined. Inhibition of the voltage-dependent Na^+ channels by TTX inhibited ATP and adenosine accumulation following K^+ depolarization (ATP: $0.07 \pm 0.05 \mu\text{mol/L}$; $n = 6$, ADO: $0.08 \pm 0.04 \mu\text{mol/L}$, $n = 6$, $p < 0.01$; Table 1, Fig 3). Similarly, glutamate release was reduced in the presence of TTX after high K^+ stimulation ($0.12 \pm 0.03 \mu\text{mol/L}$, $n = 6$, $p < 0.01$; Table 1, Fig 3). The Na^+ -channel blocker also caused a complete inhibition of the fEPSP activity (data not shown). These findings indicate that ATP, adenosine and glutamate released in response to K^+ stimulation is associated with ongoing axonal activity. Next, we investigated the involvement of glutamatergic excitatory transmission in K^+ depolarization evoked ATP, glutamate and adenosine efflux by testing the effect of glutamate receptor antagonists. When the slices were perfused with the non-NMDA glutamate receptor antagonist CNQX (10 $\mu\text{mol/L}$), it almost abolished glutamate efflux, but did not significantly change ATP and adenosine efflux in response to 25 mmol/L K^+ stimulation (Table 1) (GLU: $0.09 \pm 0.06 \mu\text{mol/L}$, $n = 5$, $p < 0.01$; ATP: $1.17 \pm 0.12 \mu\text{mol/L}$, $n = 5$, $p > 0.05$; ADO: $8.43 \pm 0.53 \mu\text{mol/L}$, $n = 5$, $p > 0.05$). The NMDA receptor antagonist D-AP-5 (10 $\mu\text{mol/L}$) and the NR2B-selective NMDA receptor antagonist ifenprodil (10 $\mu\text{mol/L}$) was applied before and during K^+ depolarization (25 mmol/L ; 270 sec). Elevation of extracellular ATP level was significantly decreased in the presence of D-AP-5 ($0.21 \pm 0.03 \mu\text{mol/L}$, $n=5$, $p < 0.01$; Fig 4) and ifenprodil ($0.13 \pm 0.06 \mu\text{mol/L}$, $n=5$, $p < 0.01$; Table 1). Accumulation of adenosine was also inhibited by D-AP-5 ($0.11 \pm 0.01 \mu\text{mol/L}$, $n=5$, $p < 0.01$; Fig 4) and by ifenprodil ($0.1 \pm 0.02 \mu\text{mol/L}$, $n=5$, $p < 0.01$; Table 1) under these conditions.

ATP, adenosine and glutamate is released from glial cells in response to K^+ depolarization in CA1 area

Next, we investigated, whether the source of detected extracellular purines and glutamate is glial or neuronal. Previous reports showed that fluoroacetate (FAc), a mitochondrial gliotoxin selectively impairs the oxidative metabolism of glial cells (Clarke, 1991; Canals *et al.*, 2008). The specificity of FAc in the concentration used is due to its selective uptake by acetate transporter present only in glial cells. In addition we used a relatively short time for FAc perfusion period to further ensure glia-selectivity (N. Hájos, personal communication). FAc perfusion (1 mmol/L, $n=6$) was added 10 min before the beginning of K^+ depolarization. Inhibition of oxidative metabolism in glial cells resulted in almost complete inhibition of the detected increase in the second phase of the efflux of ATP (ATP: $0.21 \pm 0.14 \mu\text{mol/L}$, $n = 8$, $p<0.01$; Fig 5A), whereas the first phase of the ATP efflux did not significantly change (ATP: $0.48 \pm 0.16 \mu\text{mol/L}$, $n = 8$, and $0.54 \pm 0.20 \mu\text{mol/L}$, $n = 8$, in the absence and presence of FAc, respectively, $p>0.05$; Fig 5A). Glutamate efflux was also inhibited by the FAc perfusion (GLU: $0.11 \pm 0.07 \mu\text{mol/L}$, $n = 7$, $p<0.01$, Fig 5B). Adenosine accumulation was decreased by FAc treatment, although was still detectable and appeared later than in the absence of FAc (ADO: $3.08 \pm 0.89 \mu\text{mol/L}$, $n = 8$, $p<0.01$, Fig 5C). The extracellular level of adenosine detected in the presence of FAc (1 mmol/L) was further inhibited in the presence of TTX ($3 \mu\text{mol/L}$; $0.2 \pm 0.1 \mu\text{mol/L}$, $n = 6$, $p<0.05$ vs. FAc alone; Fig. 3B). The electrical stimulation could evoke fEPSPs in the presence of FAc and the signal remained largely unchanged before and after stimulation, indicating that the treatment mostly affected the glial population (Fig 5D). Nevertheless, a slight reduction of the amplitude of the response could be observed (Fig 5D) when compared to signals recorded from control slices (Fig 1E), which might reflect the contribution of glia to the modulation of fEPSP activity or a minor effect of FAc on neuronal survival. These results show that glial and not neuronal cells are the main source of extracellular purines and glutamate under

our experimental conditions and this transmitter efflux might affect measured fEPSP amplitudes in the CA1 area (Fig 5).

Mechanism of hippocampal ATP, adenosine and glutamate release

In the next experiments inhibitors of ion channels, known to mediate the release of ATP and/or glutamate were tested in order to identify the underlying mechanism of their transmembrane efflux under our experimental conditions. At first we examined the role of P2X7 receptor ion channels (P2X7R) (Pellegatti *et al.*, 2005; Anselmi *et al.*, 2008), which are expressed in the hippocampus (Sperlágh *et al.*, 2002) and which are known to mediate the release of glutamate and ATP from nerve terminals (Alloisio *et al.*, 2008; Marcoli *et al.*, 2008), as well as from cultured astrocytes (Ballerini *et al.*, 1996; Duan *et al.*, 2003). To investigate the involvement of P2X7R in ATP, adenosine and glutamate efflux from the hippocampus, the effects of BBG (0.1 $\mu\text{mol/L}$) and AZ10606120 (0.1 $\mu\text{mol/L}$), antagonists of P2X7R were tested. Extracellular elevation of ATP was inhibited in the presence of BBG ($0.11 \pm 0.14 \mu\text{mol/L}$, $n=5$, $p<0.01$; Fig. 6) and AZ10606120 ($0.17 \pm 0.02 \mu\text{mol/L}$, $n=6$, $p<0.01$; Fig. 6) after depolarization. On the other hand BBG had partial ($4.60 \pm 0.74 \mu\text{mol/L}$, $n = 5$, $p<0.01$; Fig 7), whereas AZ10606120 ($0.12 \pm 0.05 \mu\text{mol/L}$, $n = 6$, $p<0.01$; Fig 7) had complete inhibitory effect on adenosine release in response to K^+ stimulation. The efflux of glutamate was also inhibited by a P2X7R-selective concentration of BBG (100 nM, $0.029 \pm 0.06 \mu\text{mol/L}$, $n = 7$, $p<0.01$, Table 1). Next, the effect of carbenoxolone (CBX) was tested, which is known as a wide spectrum gap junction inhibitor (Bruzzone *et al.*, 2005), but also inhibits P2X7R (Suadicani *et al.*, 2006). High concentration of carbenoxolone (100 $\mu\text{mol/L}$) completely inhibited the ATP, adenosine and glutamate efflux in response to 25 mmol/L K^+ stimulation (Fig 3) (ATP: $0.21 \pm 0.02 \mu\text{mol/L}$, $n = 6$, $p<0.01$; ADO: $0.22 \pm 0.02 \mu\text{mol/L}$, $n = 6$, $p<0.01$; GLU: $0.013 \pm 0.02 \mu\text{mol/L}$, $n = 6$, $p<0.01$). Because it is

known that CBX in low concentration inhibits only the Panx1-evoked current and P2X7R co-immunoprecipitate with Panx1 (Pelegrin and Surprenant, 2006) we also examined a lower, pannexin selective concentration of CBX (20 $\mu\text{mol/L}$). This treatment completely abolished the adenosine efflux during K^+ depolarization ($1.02 \pm 0.26 \mu\text{mol/L}$, $n = 5$, $p < 0.01$; Fig 7). Administration of probenecid (150 $\mu\text{mol/L}$), another pannexin inhibitor also caused a remarkable decrease in the extracellular concentration of adenosine ($0.2 \pm 0.01 \mu\text{mol/L}$, $n = 7$, $p < 0.01$; Fig. 7). In contrast, the extracellular level of ATP was significantly increased in the presence of low concentration CBX (20 $\mu\text{mol/L}$; $2.22 \pm 0.37 \mu\text{mol/L}$, $n = 5$, $p < 0.01$; Fig. 6) and in the presence of probenecid (150 $\mu\text{mol/L}$; $2.70 \pm 0.11 \mu\text{mol/L}$, $n = 7$, $p < 0.01$; Fig. 6). These data demonstrate that the mechanism of the efflux of ATP and adenosine is partly different: although the efflux of adenosine did arise from previously released ATP, through the actions of ectonucleotidase, it might also be released directly via pannexin hemichannels. In contrast, ATP is released through P2X7 receptors into the extracellular space, before its rapid breakdown to adenosine. As for the underlying mechanism of glutamate efflux, because it was Ca^{2+} -independent the participation of excitatory amino acid transporters (EAAT1-5), present on neuronal and glial membrane was taken into account, and L-trans-2,4-PDC, the wide spectrum EAAT inhibitor was examined. L-trans-2,4-PDC significantly decreased glutamate efflux evoked by K^+ depolarization ($1.24 \pm 0.66 \mu\text{mol/L}$, $n = 7$, $p < 0.05$, Table 1).

Discussion and Conclusions

In our experiments amperometric biosensors were utilized for the direct, real-time measurement of extracellular purine and glutamate levels in the hippocampus under basal conditions and in

response to K^+ depolarization. Under unstimulated conditions, only a low basal ATP, adenosine and glutamate tone were detected, which is in rough agreement with other previous studies in the hippocampus (Frenguelli *et al.*, 2007) and cerebellum (Wall *et al.*, 2007; Wall and Dale, 2007).

K^+ depolarization (25 mmol/L), a widely used depolarizing stimulus used to study purine release (cf. Latini and Pedata, 2001), elicited activity-dependent ATP, adenosine and glutamate efflux, with distinct kinetics and mechanisms. Although the physiological relevance of this kind stimulation remains to be investigated, high $[K^+]_o$ levels are detected during high frequency neuronal discharge and prolonged elevation of extracellular K^+ is also implicated in pathological conditions, such as hypoxia and ischemia (Olsen and Sontheimer, 2008). The efflux of all three compounds as well as the recorded fEPSP activity was sensitive to sodium channel inhibition by TTX. Because *in situ* astrocytes and other glial cells do not exhibit sodium channel activity and therefore resistant to the action of TTX, we can conclude that the primary signal which leads to the accumulation of ATP, adenosine and glutamate is most likely axonal activity under our experimental conditions. An alternative, although less likely possibility is that K^+ depolarization primarily evokes gliotransmitter release in a neuronal activity-independent manner, which gives rise to a subsequent action potential-dependent neuronal transmitter release.

High extracellular potassium is also well known to induce spreading depression (SD), a phenomenon, which plays important role in the pathogenesis of migraine, stroke and traumatic brain injury (Lauritzen *et al.*, 2011). Because SD per se elicits ATP release in the brain (Frenguelli *et al.*, 2007; Schock *et al.*, 2007), ATP efflux detected in our study might also be the consequence of SD. Indeed, as it is shown on extracellular DC recording (Fig 1F), SD developed after K^+ depolarization in our experiments as well, although the initial rise in the level of ATP appeared earlier than SD. Therefore it appears that the first early phase of the ATP elevation was largely independent from the development of SD, whereas the second, larger elevation could be

related to the SD. Supporting this idea, treatments, which inhibited the second phase of the efflux of ATP, are known to inhibit or postpone SD as well (e.g. TTX, NMDA receptor antagonists) (Somjen *et al.*, 2001).

It has been firmly established that ATP is the constituent of synaptic vesicles (Gualix *et al.*, 1999; Sperlagh and Vizi, 2000; Pankratov *et al.*, 2006), and astrocyte lysosomes (Zhang *et al.*, 2007) and is taken up to vesicles by a recently identified specific vesicular nucleotide transporter (VNUT) (Sawada *et al.*, 2008). Removal of extracellular Ca^{2+} from the perfusion medium inhibited both ATP and adenosine signals detected upon K^+ depolarization, which implies that ATP is released by vesicular exocytosis or it is released in response to compound, which enters the extracellular space by vesicular exocytosis. Depolarization-induced Ca^{2+} -dependent ATP efflux has already reported previously from the hippocampus (e.g. Cunha *et al.*, 1996), and our observation adds further support to these findings with enhanced temporal and spatial resolution demonstrating Ca^{2+} -dependent extracellular ATP accumulation in the low micromolar level, which is adequate for the activation of most of P2 receptors. Therefore this release might underlie ATP mediated synaptic transmission (Pankratov *et al.*, 1998; 2006, Mori *et al.*, 2001) or synaptic modulation e.g. (Sperlagh *et al.*, 2002; Rodrigues *et al.*, 2005) detected in this region. Moreover, ATP levels found in the effluent probably underestimate the concentration of ATP at the release sites because of the fast metabolism of ATP in the extracellular space detected by the biosensors (see below). Adenosine release was also found to be dependent on extracellular Ca^{2+} in response to electrical stimulation in previous studies (Latini *et al.*, 1997; Wall *et al.*, 2007; Wall and Dale, 2007), whereas other stimuli, such as combined oxygen glucose deprivation (Frenguelli *et al.*, 2007) leads to Ca^{2+} independent release.

In contrast, glutamate accumulation in response to K^+ depolarization failed to decrease under Ca^{2+} free conditions. Although glutamate, being the major excitatory transmitter of the CNS is

known to release during neuronal activity in a Ca^{2+} -dependent way, in neurochemical studies depolarisation-induced glutamate release has been found to be partly or completely independent on extracellular Ca^{2+} in different studies (Bernath, 1992).

Interestingly, the appearance, peak and decay of ATP, adenosine and glutamate responses were not identical using the same stimulation paradigm. Because all three sensors responded equally rapidly to external application of the compounds during the calibration, these differences cannot be explained by the different responding time of sensors. Instead, they could reflect interrelationship between the released compounds. Thus, adenosine accumulation could be the consequence of ATP efflux. Indeed, elevation of ATP levels has started earlier and was substantially higher in the presence of the ectoATPase inhibitor ARL67156, indicating the rapid and efficient metabolism of extracellular ATP detected by the ATP biosensor, which is concordant with earlier (Dunwiddie *et al.*, 1997; Cunha *et al.*, 1998) and more recent (Frenguelli *et al.*, 2007) assumptions. In the same time, ARL67156 virtually prevented adenosine accumulation following K^+ depolarization indicating that the majority of adenosine accumulation detected by the adenosine sensor is derived from the breakdown of extracellular ATP. In contrast, Frenguelli *et al.*, (2007) reported that ARL67156 did not affect adenosine accumulation in response to ischemic like conditions in the hippocampus. However, K^+ depolarization and combined oxygen glucose deprivation probably releases purines from different pools and might also affect the activity of ectonucleotidases. Thus it appears that the extracellular metabolism of released ATP and its contribution to adenosine efflux is different, depending on the used stimulation (Cunha *et al.*, 1996), and is higher, when more physiological stimuli are applied (Latini and Pedata, 2001). Nevertheless, our results also indicate an additional direct release of adenosine, which is independent from the extracellular catabolism of ATP. We detected this additional adenosine efflux when the breakdown of ATP was inhibited by ARL67156, but the

further re-uptake of adenosine was also prevented by dipyridamole. We should also assume, however, that due to the incomplete blockade of the ectoATPases by ARL67156, a minor adenosine production from the released ATP might also contribute to this residual elevation.

On the other hand, it seems less likely that ATP efflux is the consequence of glutamate efflux detected by the biosensor, because the latter was completely inhibited by CNQX, the blocker of non-NMDA receptors, which did not affect ATP efflux. Moreover, an early elevation of ADO signal preceding the rise of GLU efflux could also be observed in response to high K^+ in the absence of any drug treatment, which also supports this assumption. In contrast, ATP and adenosine release was subject to inhibition of NMDA-type glutamate receptors by D-AP-5 and ifenprodil, which implicate that ATP is released in response to the activation of NR2B subunit containing NMDA receptors. This observation is concordant with findings showing that NMDA receptor activation releases purines, including ATP from brain slices (Pedata *et al.*, 1991; Craig and White, 1993), and from cultured astrocytes (Queiroz *et al.*, 1997). However, we were unable to dissect this “trigger” glutamate efflux within the resolution of the technique. Although NMDA receptors co-localize with AMPA receptors at the majority of central synapses, they are also targeted to extrasynaptic sites in the hippocampus (Traynelis *et al.*, 2010), where AMPA receptors are not present. In particular, NR2B subunit containing NMDA receptors have been reported to localize to extrasynaptic sites and participate in distinct forms of synaptic plasticity that do not require the activation of synaptic glutamate receptors (Tovar and Westbrook, 1999; Massey *et al.*, 2004). Therefore, it seems likely that those NMDA receptors, which mediate the release of ATP are extrasynaptic or glial and are not identical to NMDA receptors, which co-localize with AMPA receptors in excitatory synapses and which probably mediate glutamate efflux detected by the biosensor.

However, because NR2B subunit containing NMDA receptors are also expressed on astrocytes (Lee et al., 2010), both neurons and astrocytes should be taken into account as a potential source of ATP efflux.

In order to further identify the source of ATP, adenosine and glutamate released in response to K^+ depolarization, the widely used glia selective toxin FAc (Szerb and Issekutz, 1987; Berg-Johnsen *et al.*, 1993) was utilized. To ensure its glia selectivity, a relatively short perfusion time was applied, which inhibited the efflux of ATP and glutamate, whilst fEPSP activity was largely preserved. Although we cannot exclude a minor effect of FAc on neurons, the majority of ATP and glutamate accumulation detected by the biosensor is most likely originated from glial cells. Hence, the K^+ depolarization paradigm used in our study seems to be a signal whereby the neuron-glia network of the hippocampus could be challenged and simultaneous glutamate and ATP efflux could be evoked from glial cells in response to ongoing neuronal activity. Although this ATP and glutamate accumulation is not identical with synaptic neurotransmitter release responsible for fEPSP activity, it may contribute to its modulation, as the fEPSP signal was slightly attenuated in the presence of FAc. One should consider here that in case of fEPSP recording, a focal stimulation of the Schaffer collateral-commissural fiber pathway was applied, whereas elevated extracellular K^+ level affected the whole hippocampal slice. These two stimulations do not necessarily elicit identical release pattern: according to the present findings, gliotransmitter release is dominant during K^+ depolarization but contribute to evoked fEPSPs only in a minor extent. As for the accumulation of adenosine, the early elevation in its level, presumably stemming from extracellular interconversion of released ATP was almost completely inhibited in the presence FAc. On the other hand, a late elevation of adenosine signal was still detected in the presence of the gliotoxin, which might represent a direct neuronal adenosine

efflux. Supporting this assumption, the residual efflux of adenosine in the presence of FAc was fully sensitive to TTX.

In previous studies the following mechanisms have been identified for ATP to enter the extracellular space: (1) exocytotic release, which can occur both from neurons (Sperlágh and Vizi, 2000; Pankratov *et al.*, 2006) and astrocytes (Coco *et al.*, 2003; Pangrsic *et al.*, 2007; Zhang *et al.*, 2007) (2) P2X7 receptor (Ballerini *et al.*, 1996; Suadicani *et al.*, 2006) (3) connexin (Cotrina *et al.*, 1998; Stout *et al.*, 2002) and (4) pannexin mediated release (Bao *et al.*, 2004; Huang *et al.*, 2007), which have been described primarily in non neuronal cells including astrocytes and (5) volume regulated anion-channel mediated release (Darby *et al.*, 2003; Okada *et al.*, 2004), which also have been identified in non-neuronal cells (6) CFTR mediated release, which has been demonstrated in DRG neurons (Kanno and Nishizaki, 2011) and (7) cytolytic release.

Because both ATP and adenosine efflux under K^+ depolarization was Ca^{2+} - dependent, and TTX sensitive, which is inconsistent with gross cellular damage, the cytolytic origin of ATP and adenosine accumulation does not appear very likely. Also, because volume regulated anion channels are primarily activated under hypoosmotic conditions, such as swelling, which did not occur under our conditions, the involvement of volume regulated anion-channels in the transmitter efflux measured in the present study is less likely.

Instead, P2X7 receptors, which are expressed in the hippocampus (Sperlágh *et al.*, 2002) and could mediate the release of both ATP (Ballerini *et al.*, 1996) and glutamate (Sperlágh *et al.*, 2002; Marcoli *et al.*, 2008) were taken into consideration. BBG, which is a selective antagonist of P2X7 receptors in the concentration used (Jiang *et al.*, 2000), almost completely inhibited elevation of ATP and glutamate levels in response to high K^+ stimulation and adenosine accumulation was also significantly decreased. These findings imply that P2X7 receptors are also

activated in response to depolarization and participate in the efflux of ATP and glutamate. Activation of P2X7 receptors could elicit both vesicular and non-vesicular transmitter release, the latter is probably mediated by the receptor ion channel complex itself (Marcoli et al., 2008; Alloisio et al., 2008; Duan et al., 2003) or by the reversal of transporters (Sperlágh et al., 2002). The findings that glutamate, but not ATP release has been proved to be independent on $[Ca^{2+}]_o$ implies that P2X7 receptor mediated glutamate release is non-vesicular under the conditions of the present study, whereas P2X7 receptor mediated ATP efflux could be either vesicular or non-vesicular. In support for this assumption, glutamate efflux detected in our experiments was sensitive to L-trans-2,4-PDC, the wide spectrum EAAT inhibitor, implicating the reversal of the excitatory amino acid transporters as its underlying mechanism.

On the other hand, carbenoxolone, which is known as a wide-spectrum gap junction hemichannel inhibitor, but also inhibits P2X7 receptors (Suadicani *et al.*, 2006), in high concentration profoundly inhibited ATP, adenosine and glutamate accumulation upon K^+ depolarization, which raise the possibility that hemichannels participate in their release mechanisms. However, given the low specificity of carbenoxolone and other available gap junction hemichannel inhibitors, we favor the explanation that carbenoxolone prevented transmitter efflux by its inhibitory action on P2X7 receptors. Connexins could be functional in the hemichannel form (reviewed in Stout et al., 2004), and can conduct the release of ATP and glutamate from astrocytes (Stout et al., 2002; Ye et al., 2003; Iglesias et al., 2009), however, they are activated typically at low $[Ca^{2+}]_o$ (Pfahnl and Dahl 1999; Valiunas and Weingart 2000), i.e. different conditions from the present study, although there are exceptions about this rule (e.g. Pearson et al., 2005).

Further pharmacological characterization, using pannexin selective agents (low concentration of CBX (Davidson and Baumgarten, 1988; Bruzzone *et al.*, 2005) and probenecid (Silverman *et al.*, 2008)) revealed that in contrast to the efflux of ATP, adenosine accumulation was sensitive to

the action of pannexin-selective inhibitors as well. These data argues for the heterogeneous origin of extracellular adenosine in our study, which is partly released by pannexins. Pannexins can be activated under physiological calcium concentrations (Bruzzone *et al.*, 2003) and under conditions, which are known as triggers of adenosine release, like epileptiform activity (Thompson *et al.*, 2008), metabolic distress and ischemia (Latini and Pedata, 2001; Thompson and MacVicar, 2008). Although adenosine concentration in the nerve terminals is in the low micromolar level, we applied a relatively strong depolarizing stimulus, which could result in an increased ATP consumption and a consequent increase in intraterminal adenosine level. This elevated adenosine level, in turn, might provide a driving force for a channel mediated release. Because pannexins are expressed in both neurons and glia in the CNS (Thompson and MacVicar, 2008), the source of this release could be either neuronal or glial, consistently with the partial inhibition of adenosine efflux by FAc. Alternatively, pannexin1 channels are gated by P2X7 receptors (Pelegri and Surprenant, 2006) and thereby both participate in the release mechanism. We also have to note that given the low specificity of the available inhibitors of pannexin channels, we cannot exclude other explanations either.

Interestingly, a recent study detected pannexin mediated ATP release in CA3 region of the hippocampus, which was not seen in our study (Kawamura *et al.*, 2010); moreover, we observed an augmentation of the efflux of ATP in the presence of pannexin inhibitors. However, in the study of Kawamura *et al.* a different stimulus, i.e. mild hypoglycemia was used to activate pannexin-mediated ATP release. A potential explanation to the increase in ATP efflux in the presence of pannexin inhibitors is the attenuation of adenosinergic inhibitory neuromodulation on the release of ATP or glutamate in the absence of pannexin mediated adenosine release. Nevertheless, this possibility, as well as the participation of CFTR in release mechanism of ATP needs further exploration.

In conclusion, our study demonstrates the glial release of ATP, adenosine and glutamate from the hippocampus in response to depolarization of neuronal membrane. K^+ depolarization initiates sodium channel activation along the nerve terminals, which elicits the Ca^{2+} -dependent, exocytotic release of a trigger compound (probably glutamate or ATP), which leads to the subsequent efflux of ATP from glial cells. ATP is then rapidly metabolized to adenosine through the ectonucleotidase cascade. The release mechanism of ATP and glutamate from glial cells involve P2X7 receptors, whilst a part of extracellular adenosine accumulation is independent from extracellular ATP breakdown. The release mechanism demonstrated in our study might be relevant to synaptic modulation and coordination of synaptic networks carried out by ATP, the major astrocytic gliotransmitter in vivo (Halassa *et al.*, 2009).

Acknowledgments

The authors are grateful for Prof. Nicholas Dale for teaching and initial guidance in the microelectrode biosensor technique. This study was supported by project grants of the Hungarian Research and Development Fund (Grant No. NN79957), the Hungarian Medical Research Council (05-102/2009) and the Pfizer Hungary Ltd (VT44838809) to B.S. B.S. was a recipient of a Royal Society short term-fellowship.

Conflict of interest

none

References

- Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H (2009). Purinergic signalling in the nervous system: an overview. *Trends Neurosci* **32**: 19-29.
- Alloisio S, Cervetto C, Passalacqua M, Barbieri R, Maura G, Nobile M, *et al.* (2008). Functional evidence for presynaptic P2X7 receptors in adult rat cerebrocortical nerve terminals. *FEBS Lett* **582**: 3948-3953.
- Anselmi F, Hernandez VH, Crispino G, Seydel A, Ortolano S, Roper SD *et al.* (2008). ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca²⁺ signals across the inner ear. *Proc Natl Acad Sci U S A* **105**: 18770-18775.
- Araque A, Carmignoto G, Haydon PG (2001). Dynamic signaling between astrocytes and neurons. *Annu Rev Physiol* **63**: 795-813.
- Arcuino G, Lin JH, Takano T, Liu C, Jiang L, Gao Q *et al.* (2002). Intercellular calcium signaling mediated by point-source burst release of ATP. *Proc Natl Acad Sci U S A* **99**: 9840-9845.
- Ballerini P, Rathbone MP, Di Iorio P, Renzetti A, Giuliani P, D'Alimonte I *et al.* (1996). Rat astroglial P2Z (P2X7) receptors regulate intracellular calcium and purine release. *Neuroreport* **7**: 2533-2537.
- Bao L, Locovei S, Dahl G (2004). Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett* **72**: 65-68.
- Berg-Johnsen J, Paulsen RE, Fonnum F, Langmoen IA (1993). Changes in evoked potentials and amino acid content during fluorocitrate action studied in rat hippocampal cortex. *Exp Brain Res* **96**: 241-246.
- Bernath S (1992). Calcium-independent release of amino acid neurotransmitters: fact or artifact? *Prog Neurobiol* **38**: 57-91.

Bowser DN, Khakh BS (2004). ATP excites interneurons and astrocytes to increase synaptic inhibition in neuronal networks. *J Neurosci* **24**: 8606-8620.

Bruzzone R, Barbe MT, Jakob NJ, Monyer H (2005). Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in *Xenopus* oocytes. *J Neurochem* **92**: 1033-1043.

Bruzzone R, Hormuzdi SG, Barbe MT, Herb A, Monyer H (2003). Pannexins, a family of gap junction proteins expressed in brain. *Proc Natl Acad Sci U S A* **100**: 13644-13649.

Canals S, Larrosa B, Pintor J, Mena MA, Herreras O (2008). Metabolic challenge to glia activates an adenosine-mediated safety mechanism that promotes neuronal survival by delaying the onset of spreading depression waves. *J Cereb Blood Flow Metab* **28**: 1835-1844.

Clarke DD (1991). Fluoroacetate and fluorocitrate: mechanism of action. *Neurochem Res* **16**: 1055-1058.

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P *et al.* (2003). Storage and release of ATP from astrocytes in culture. *J Biol Chem* **278**: 1354-1362.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H *et al.* (1998). Connexins regulate calcium signaling by controlling ATP release. *Proc Natl Acad Sci U S A* **95**: 15735-15740.

Craig CG, White TD (1993). N-methyl-D-aspartate- and non-N-methyl-D-aspartate-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J Neurochem* **60**: 1073-1080.

Cunha RA (2001). Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. *Neurochem Int* **38**: 107-125.

Cunha RA, Ribeiro JA (2000). ATP as a presynaptic modulator. *Life Sci* **68**: 119-137.

Cunha RA, Sebastiao AM, Ribeiro JA (1998). Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors. *J Neurosci* **18**: 1987-1995.

Cunha RA, Vizi ES, Ribeiro JA, Sebastiao AM (1996). Preferential release of ATP and its extracellular catabolism as a source of adenosine upon high- but not low-frequency stimulation of rat hippocampal slices. *J Neurochem* **67**: 2180-2187.

Dale N, Hatz S, Tian F, Llaudet E (2005). Listening to the brain: microelectrode biosensors for neurochemicals. *Trends Biotechnol* **23**: 420-428.

Dale N, Pearson T, Frenguelli BG (2000). Direct measurement of adenosine release during hypoxia in the CA1 region of the rat hippocampal slice. *J Physiol* **526 Pt 1**: 143-155.

Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA (2003). ATP released from astrocytes during swelling activates chloride channels. *J Neurophysiol* **89**: 1870-1877.

Davidson JS, Baumgarten IM (1988). Glycyrrhetic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. *J Pharmacol Exp Ther* **246**: 1104-7.

Duan S, Anderson CM, Keung EC, Chen Y, Swanson, RA (2003). P2X7 receptor-mediated release of excitatory amino acids from astrocytes. *The Journal of Neuroscience* **23**: 1320-8.

Dunwiddie TV, Diao L, Proctor WR (1997). Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J Neurosci* **17**: 7673-7682.

Fellin T, Carmignoto G (2004). Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *J Physiol* **559**: 3-15.

Fellin T, Halassa MM, Terunuma M, Succol F, Takano H, Frank M *et al.* (2009). Endogenous nonneuronal modulators of synaptic transmission control cortical slow oscillations in vivo. *Proc Natl Acad Sci U S A* **106**: 15037-15042.

Fields RD, Burnstock G. (2006). Purinergic signalling in neuron-glia interactions. *Nat Rev Neurosci* **7**: 423-436.

Freguelli BG, Llaudet E, Dale N (2003). High-resolution real-time recording with microelectrode biosensors reveals novel aspects of adenosine release during hypoxia in rat hippocampal slices. *J Neurochem* **86**: 1506-1515.

Freguelli BG, Wigmore G, Llaudet E, Dale N (2007). Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *J Neurochem* **101**: 1400-1413.

Goding JW, Grobbs B, Slegers H (2003). Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim Biophys Acta* **1638**: 1-19.

Gualix J, Pintor J, Miras-Portugal MT (1999). Characterization of nucleotide transport into rat brain synaptic vesicles. *J Neurochem* **73**: 1098-1104.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999). ATP released from astrocytes mediates glial calcium waves. *J Neurosci* **19**: 520-528.

Halassa MM, Fellin T, Haydon PG (2009). Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology* **57**: 343-346.

Haydon PG (2001). GLIA: listening and talking to the synapse. *Nat Rev Neurosci* **2**: 185-193.

Huang YJ, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD (2007). The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. *Proc Natl Acad Sci U S A* **104**: 6436-6441.

Jiang LH, Mackenzie AB, North RA, Surprenant A (2000). Brilliant blue G selectively blocks ATP-gated rat P2X(7) receptors. *Mol Pharmacol* **58**: 82-88.

Juranyi Z, Sperlagh B, Vizi ES (1999). Involvement of P2 purinoceptors and the nitric oxide pathway in [³H]purine outflow evoked by short-term hypoxia and hypoglycemia in rat hippocampal slices. *Brain Res* **823**: 183-190.

Kanno T, Nishizaki T. (2011). CFTR mediates noradrenaline-induced ATP efflux from DRG neurons. *Mol Pain* **7**:72.

Kawamura M Jr, Ruskin DN, Masino SA. (2010). Metabolic autocrine regulation of neurons involves cooperation among pannexin hemichannels, adenosine receptors, and K_{ATP} channels. *J Neurosci* **30**: 3886-95.

Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K (2003). Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. *Proc Natl Acad Sci U S A* **100**: 11023-11028.

Latini S, Pedata F (2001). Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem* **79**: 463-484.

Latini S, Pedata F, Pepeu G (1997). The contribution of different types of calcium channels to electrically-evoked adenosine release from rat hippocampal slices. *Naunyn Schmiedebergs Arch Pharmacol* **355**: 250-5.

Lauritzen M, Dreier JP, Fabricius M, Hartings JA, Graf R, Strong AJ (2011). Clinical relevance of cortical spreading depression in neurological disorders: migraine, malignant stroke, subarachnoid and intracranial hemorrhage, and traumatic brain injury. *J Cereb Blood Flow Metab* **31**:17-35.

Lee MC, Ting KK, Adams S, Brew BJ, Chung R, Guillemin GJ (2010). Characterisation of the expression of NMDA receptors in human astrocytes. *PLoS One* **5**:e14123.

Llaudet E, Botting NP, Crayston JA, Dale N (2003). A three-enzyme microelectrode sensor for detecting purine release from central nervous system. *Biosens Bioelectron* **18**: 43-52.

Llaudet E, Hatz S, Droniou M, Dale N (2005). Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal Chem* **77**: 3267-3273.

Marcoli M, Cervetto C, Paluzzi P, Guarnieri S, Alloisio S, Thellung S *et al.* (2008). P2X7 pre-synaptic receptors in adult rat cerebrocortical nerve terminals: a role in ATP-induced glutamate release. *J Neurochem* **105**: 2330-42.

Massey PV, Johnson BE, Moulton PR, Auberson YP, Brown MW, Molnar E *et al.* (2004). Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J. Neurosci.* **8**:7821-8.

Mori M, Heuss C, Gahwiler BH, Gerber U (2001). Fast synaptic transmission mediated by P2X receptors in CA3 pyramidal cells of rat hippocampal slice cultures. *J Physiol* **535**: 115-123.

Nedergaard M (1994). Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* **263**: 1768-1771.

North RA, Verkhratsky A (2006). Purinergic transmission in the central nervous system. *Pflugers Arch* **452**: 479-485.

Okada SF, O'Neal WK, Huang P, Nicholas RA, Ostrowski LE, Craigen WJ *et al.* (2004). Voltage-dependent anion channel-1 (VDAC-1) contributes to ATP release and cell volume regulation in murine cells. *J Gen Physiol* **124**: 513-526.

Olsen ML, Sontheimer H (2008). Functional implications for Kir4.1 channels in glial biology: from K⁺ buffering to cell differentiation. *J Neurochem* **107**: 589-601.

Pangrsic T, Potokar M, Stenovec M, Kreft M, Fabbretti E, Nistri A *et al.* (2007). Exocytotic release of ATP from cultured astrocytes. *J Biol Chem* **282**: 28749-28758.

Pankratov Y, Castro E, Miras-Portugal MT, Krishtal O (1998). A purinergic component of the excitatory postsynaptic current mediated by P2X receptors in the CA1 neurons of the rat hippocampus. *Eur J Neurosci* **10**: 3898-3902.

Pankratov Y, Lalo U, Verkhratsky A, North RA (2006). Vesicular release of ATP at central synapses. *Pflugers Arch* **452**: 589-597.

Parpura V, Basarsky TA, Liu F, Jęftinija K, Jęftinija S, Haydon PG (1994). Glutamate-mediated astrocyte-neuron signalling. *Nature* **369**: 744-747.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY *et al.* (2005). Astrocytic purinergic signaling coordinates synaptic networks. *Science* **310**: 113-116.

Pearson RA, Dale N, Llaudet E, Mobbs P (2005). ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. *Neuron* **46**: 731-744.

Pedata F, Pazzagli M, Pepeu G (1991). Endogenous adenosine release from hippocampal slices: excitatory amino acid agonists stimulate release, antagonists reduce the electrically-evoked release. *Naunyn Schmiedebergs Arch Pharmacol* **344**: 538-543.

Pelegri P, Surprenant A (2006). Pannexin-1 mediates large pore formation and interleukin-1 β release by the ATP-gated P2X7 receptor. *Embo J* **25**: 5071-5082.

Pellegatti P, Falzoni S, Pinton P, Rizzuto R, Di Virgilio F (2005). A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. *Mol Biol Cell* **16**: 3659-3665.

Pfahnl A, Dahl G (1999). Gating of cx46 gap junction hemichannels by calcium and voltage. *Pflugers Arch* **437**: 345-353.

Queiroz G, Gebicke-Haerter PJ, Schobert A, Starke K, von Kugelgen I (1997). Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. *Neuroscience* **78**: 1203-1208.

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA (2005). Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. *J Neurosci* **25**: 6286-6295.

Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H *et al.* (2008). Identification of a vesicular nucleotide transporter. *Proc Natl Acad Sci U S A* **105**: 5683-5686.

Schock SC, Munyao N, Yakubchik Y, Sabourin LA, Hakim AM, Ventureyra EC *et al.* (2007). Cortical spreading depression releases ATP into the extracellular space and purinergic receptor activation contributes to the induction of ischemic tolerance. *Brain Res* **1168**: 129-38.

Serrano A, Haddjeri N, Lacaille JC, Robitaille R (2006). GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. *J Neurosci* **26**: 5370-5382.

Silverman W, Locovei S, Dahl G (2008). Probenecid, a gout remedy, inhibits pannexin 1 channels. *Am J Physiol Cell Physiol* **295**: C761-767.

Somjen GG (2001). Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiol Rev.* **81**:1065-96.

Sperlgh B, Kofalvi A, Deuchars J, Atkinson L, Milligan CJ, Buckley NJ *et al.* (2002). Involvement of P2X7 receptors in the regulation of neurotransmitter release in the rat hippocampus. *J Neurochem* **81**: 1196-1211.

Sperlgh B, Vizi ES (2000). Regulation of purine release, In *Handbook of Experimental Pharmacology*, (Abbracchio MP and Williams M, eds), pp. 179-209. Springer, Berlin.

Sperlagh B, Zsilla G, Baranyi M, Illes P, Vizi ES (2007). Purinergic modulation of glutamate release under ischemic-like conditions in the hippocampus. *Neuroscience* **149**: 99-111.

Stout CE, Costantin JL, Naus CC, Charles AC (2002). Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. *J Biol Chem* **277**: 10482-10488.

Stout C, Goodenough DA, Paul DL (2004). Connexins: functions without junctions. *Curr Opin Cell Biol* **16**: 507-512.

Suadicani SO, Brosnan CF, Scemes E (2006). P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca²⁺ signaling. *J Neurosci* **26**: 1378-1385.

Sul JY, Orosz G, Givens RS, Haydon PG (2004). Astrocytic Connectivity in the Hippocampus. *Neuron Glia Biol* **1**: 3-11.

Szerb JC, Issekutz B (1987). Increase in the stimulation-induced overflow of glutamate by fluoroacetate, a selective inhibitor of the glial tricarboxylic cycle. *Brain Res* **410**: 116-120.

Thompson RJ, Jackson MF, Olah ME, Rungta RL, Hines DJ, Beazely MA *et al.* (2008). Activation of pannexin-1 hemichannels augments aberrant bursting in the hippocampus. *Science* **322**: 1555-1559.

Thompson RJ, Macvicar BA (2008). Connexin and pannexin hemichannels of neurons and astrocytes. *Channels (Austin)* **2**.

Tian F, Gourine AV, Huckstepp RT, Dale N (2009). A microelectrode biosensor for real time monitoring of L-glutamate release. *Anal Chim Acta* **645**: 86-91.

Tovar KR, Westbrook GL (2002). Mobile NMDA receptors at hippocampal synapses. *Neuron* **34**: 255-264.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK *et al.* (2010).

Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* **62**:405-96.

Valiunas V, Weingart R (2000). Electrical properties of gap junction hemichannels identified in transfected HeLa cells. *Pflugers Arch* **440**: 366-379.

Wall MJ, Atterbury A, Dale N (2007). Control of basal extracellular adenosine concentration in rat cerebellum. *J Physiol* **582**: 137-151.

Wall MJ, Dale N (2007). Auto-inhibition of rat parallel fibre-Purkinje cell synapses by activity-dependent adenosine release. *J Physiol* **581**: 553-565.

Wall M, Dale N (2008). Activity-dependent release of adenosine: a critical re-evaluation of mechanism. *Curr Neuropharmacol* **6**: 329-337.

Wieraszko A, Goldsmith G, Seyfried TN (1989). Stimulation-dependent release of adenosine triphosphate from hippocampal slices. *Brain Res* **485**: 244-250.

Zhang Z, Chen G, Zhou W, Song A, Xu T, Luo Q *et al.* (2007). Regulated ATP release from astrocytes through lysosome exocytosis. *Nat Cell Biol* **9**: 945-953.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL *et al.* (2003). ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. *Neuron* **40**: 971-982.

Zimmermann H (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch Pharmacol* **362**: 299-309.

Tables

Table 1. The effect of different treatments on the efflux of ATP, adenosine and glutamate from rat hippocampal slices.

Condition ($\mu\text{mol/L}$)	Net ATP $\mu\text{mol/L}$	n	Net Adenosine $\mu\text{mol/L}$	n	Net Glutamate $\mu\text{mol/L}$	n
Control	1.23 ± 0.23	8	10.28 ± 1.41	8	3.49 ± 0.84	8
Ca^{2+} free and EGTA	0.21 ± 0.01 p<0.01	6	0.22 ± 0.02 p<0.01	6	3.64 ± 0.15 p>0.05	6
TTX (3)	0.07 ± 0.05 p<0.01	6	0.08 ± 0.04 p<0.01	6	0.12 ± 0.03 p<0.01	6
FAc (1000)	0.21 ± 0.14 p<0.01	8	3.08 ± 0.89 p<0.01	8	0.11 ± 0.07 p<0.01	7
Carbenoxolone (100)	0.21 ± 0.02 p<0.01	6	0.22 ± 0.02 p<0.01	6	0.013 ± 0.02 p<0.01	6
Carbenoxolone (20)	2.22 ± 0.37 p<0.05	5	1.02 ± 0.26 p<0.01	5	-	-
BBG (0.1)	0.11 ± 0.14 p<0.01	5	4.60 ± 0.74 p<0.01	5	0.029 ± 0.06 p<0.01	7
Probenecid (150)	2.70 ± 0.11 p<0.05	7	0.22 ± 0.01 p<0.01	7	-	-
ARL67156 (100)	8.62 ± 0.76 p<0.01	6	0.15 ± 0.04 p<0.01	6	2.43 ± 0.63 p>0.05	6
D-AP-5 (10)	0.21 ± 0.03 p<0.01	5	0.15 ± 0.01 p<0.01	5	-	-
Ifenprodil (10)	0.13 ± 0.06 P<0.01	5	0.11 ± 0.02 P<0.01	5	-	-
CNQX (10)	1.17 ± 0.12 p>0.05	5	8.43 ± 0.53 p>0.05	5	0.09 ± 0.06 p<0.01	5
AZ10606120 (0.1)	0.17 ± 0.02 p<0.01	6	0.12 ± 0.05 p<0.01	6	-	-
Dipyridamole (50) + ARL67156(100)	-	-	2.71 ± 0.23 p<0.01	5	-	-
FAc (1000) + TTX (3)	-	-	0.22 ± 0.1 p<0.01	6	-	-
L-trans-2,4-PDC	-	-	-	-	1.24 ± 0.66 p < 0.05	7

The net ATP, adenosine and glutamate efflux was calculated according to the procedures described in Methods. Data show the mean \pm SEM with n = number of identical experiments. P values indicate significant differences from respective controls (25 mmol/L K⁺; 270 sec), calculated by one-way analysis of variance followed by Dunnett's test.

Accepted Article

Figure legends

Figure 1. A-D. Real-time detection of the release of ATP, adenosine and glutamate during K^+ (25 mmol/L; 270 sec; black bar) depolarization from the rat hippocampus *in vitro*. Two phases of ATP release were detected, as indicated by the arrows on the top. The first occurring at the same time when glutamate release started, while the onset of the second phase coincided with the maximum peak of glutamate release. B. The adenosine sensor detected an earlier and long lasting signal than the ATP and glutamate sensor. ‘Net’ signal for each experiment was calculated subtracting the currents of the corresponding baseline. Net ATP and glutamate signal were obtained after subtraction of the simultaneously-recorded null sensor, while net adenosine (ADO) signal were obtained after subtraction of the simultaneously-recorded INO sensor (D) from the ADO/INO signal (B). E. Field excitatory postsynaptic potentials (fEPSP) recorded in AC mode before (i) and following K^+ depolarization (ii). Note that full recovery of the fEPSP occurred (iii). Stimulus artifacts have been truncated. *Bottom*, Magnified examples of the fEPSP are shown at the times indicated on the continuous AC trace. F. Extracellular DC potential recorded simultaneously with the ATP sensor signal showed spreading depression (SD, negative deflection on the DC trace) following K^+ depolarization. Note that K^+ (25 mmol/L; 270 sec) depolarization evoked purine efflux occurs before the SD (indicated by a continuous vertical line). ATP, NULL, ADO/INO, INO, were recorded from one set of experiments and ATP, GLU, NULL from a separate set of experiments. All data show means (black) \pm S.E.M (in grey) of 8 independent experiments.

Figure 2. The effect of the inhibition of extracellular ATP metabolism with ARL67156 (100 μ mol/L) on ATP (A, B), adenosine (A) and glutamate (B) efflux.

The treatment enhanced ATP release (A, B) but had no significant effect glutamate release (B) following K^+ depolarization (black bar). By contrast, adenosine (ADO) efflux during the stimulation was inhibited in the presence of ARL67156 (A). Traces are means (black) \pm S.E.M (in grey) of 6 independent experiments.

Figure 3. The effects of different treatments on K^+ depolarization induced efflux of ATP, adenosine and glutamate from rat hippocampal slices.

Inhibition of the voltage-dependent Na^+ channels by TTX (3 μ mol/L) totally inhibited the ATP (A) and adenosine (B) efflux following the K^+ depolarization (25 mmol/L; 270 sec). Similarly, glutamate release was reduced in the presence of TTX after high K^+ stimulation (C). Removal of extracellular Ca^{2+} (Ca^{2+} free) differentially affected ATP, adenosine (A, B) and glutamate (C) efflux. The hemichannel blocker carbenoxolone (CBX high: 100 μ mol/L) almost completely inhibited ATP, adenosine and glutamate efflux (A, B, C). Release of ATP (A), adenosine (B) and glutamate (C) following the K^+ stimulation substantially decreased in the presence of FAc (1 mmol/L; 10 min). TTX (3 μ mol/L; 20 min) almost completely inhibited adenosine efflux in the presence of FAc (1 mmol/L; 10 min) (B). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n= 5-6; ns, $p>0.05$; **, $p<0.01$).

Figure 4. The effect of the NMDA glutamate receptor antagonist D-AP-5 on adenosine and ATP efflux evoked by K^+ depolarization.

D-AP-5 (10 μ mol/L) decreased ATP and adenosine efflux in the rat hippocampal slices. Black bars denote the period of K^+ depolarization and D-AP-5 perfusion. Individual recordings are representative of at least 5 independent experiments.

Figure 5. Effect of the mitochondrial gliotoxin fluoroacetic acid perfusion (FAC, 1 mmol/L) on ATP (A) adenosine (B) and glutamate (C) efflux evoked by K^+ depolarization. FAC was added 10 min before the beginning of K^+ depolarization (black bar). Inhibition of oxidative metabolism in glial cells resulted in almost complete inhibition of the detected increase in the efflux of ATP and glutamate. D. Recording of the evoked field excitatory postsynaptic potentials (fEPSP, in AC mode) before (i) and following K^+ depolarization (ii). Note the recovery of the fEPSP during wash out (iii). Stimulus artifacts have been truncated. *Bottom*, Magnified examples of the fEPSP (average of 5 responses) shown at the times indicated on the continuous AC trace. ATP, NULL, ADO/INO, INO, were recorded from one set of experiments and GLU, NULL from a separate set of experiments. All data show means (black) \pm S.E.M (in grey) of 6 independent experiments.

Figure 6. The effect of P2X7 receptor antagonists and pannexin inhibitors on ATP efflux evoked by K^+ depolarization.

BBG (100 nmol/L), and AZ10606120 (100 nmol/L) inhibited ATP efflux (A, normalized signal, B (see methods). Inhibition of pannexin hemichannels by CBX (20 μ mol/L) significantly increased the extracellular level of ATP during K^+ stimulation (25 mmol/L; 270 sec; black bar), which was also pronounced in the presence of probenecid (150 μ mol/L). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n=5; **, p<0.01)

Figure 7. The effect of P2X7 receptor antagonists and pannexin inhibitors on adenosine efflux evoked by K^+ depolarization.

BBG (100 nmol/L), and AZ10606120 (100 nmol/L) inhibited adenosine efflux (A, normalized signal, B (see methods). Inhibition of pannexin hemichannels by CBX (20 μ mol/L) greatly reduced adenosine efflux during K⁺ stimulation (25 mmol/L; 270 sec; black bar). Administration of probenecid (150 μ mol/L) also profoundly decreased the extracellular level of adenosine (A, normalized signal, B (see methods)). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n=5; **, p<0.01; ns, p>0.05).

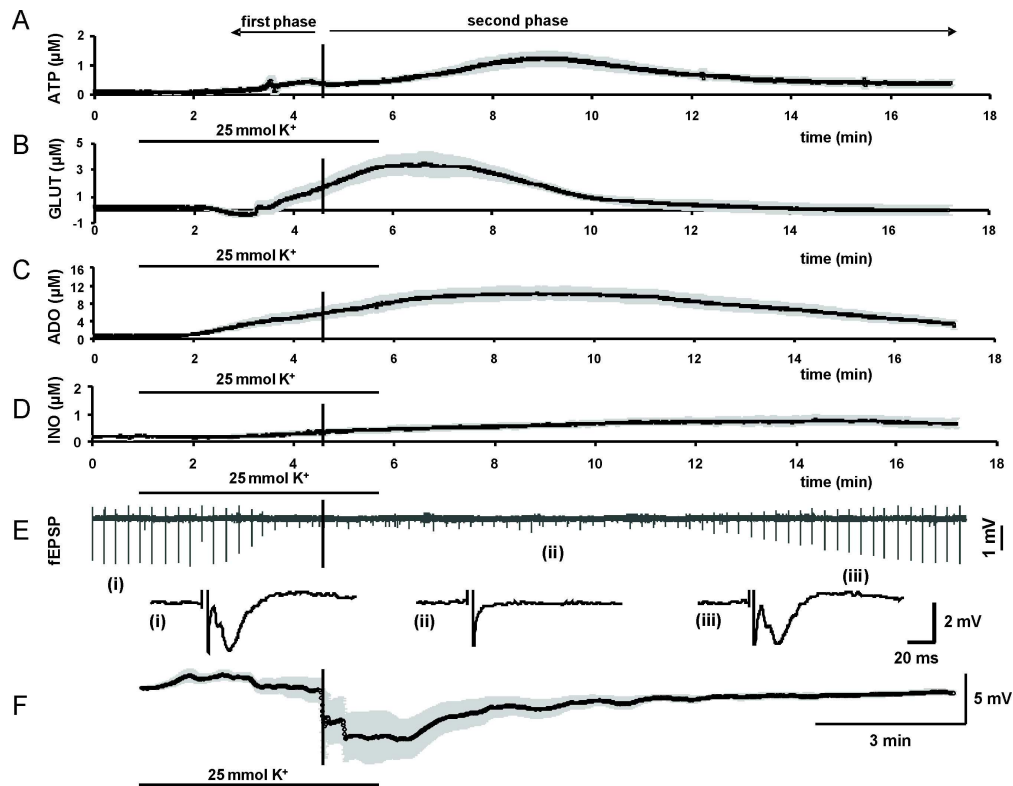


Figure 1. A-D. Real-time detection of the release of ATP, adenosine and glutamate during K⁺ (25 mmol/L; 270 sec; black bar) depolarization from the rat hippocampus *in vitro*. Two phases of ATP release were detected, as indicated by the arrows on the top. The first occurring at the same time when glutamate release started, while the onset of the second phase coincided with the maximum peak of glutamate release. B. The adenosine sensor detected an earlier and long lasting signal than the ATP and glutamate sensor. 'Net' signal for each experiment was calculated subtracting the currents of the corresponding baseline. Net ATP and glutamate signal were obtained after subtraction of the simultaneously-recorded null sensor, while net adenosine (ADO) signal were obtained after subtraction of the simultaneously-recorded INO sensor (D) from the ADO/INO signal (B). E. Field excitatory postsynaptic potentials (fEPSP) recorded in AC mode before (i) and following K⁺ depolarization (ii). Note that full recovery of the fEPSP occurred (iii). Stimulus artifacts have been truncated. Bottom, Magnified examples of the fEPSP are shown at the times indicated on the continuous AC trace. F. Extracellular DC potential recorded simultaneously with the ATP sensor signal showed spreading depression (SD, negative deflection on the DC trace) following K⁺ depolarization. Note that K⁺ (25 mmol/L; 270 sec) depolarization evoked purine efflux occurs before the SD (indicated by a continuous vertical line). ATP, NULL, ADO/INO, INO, were recorded from one set of experiments and ATP, NULL, ADO/INO, INO, were recorded from a separate set of experiments. All data show means (black) \pm S.E.M (in grey) of 8 independent experiments.

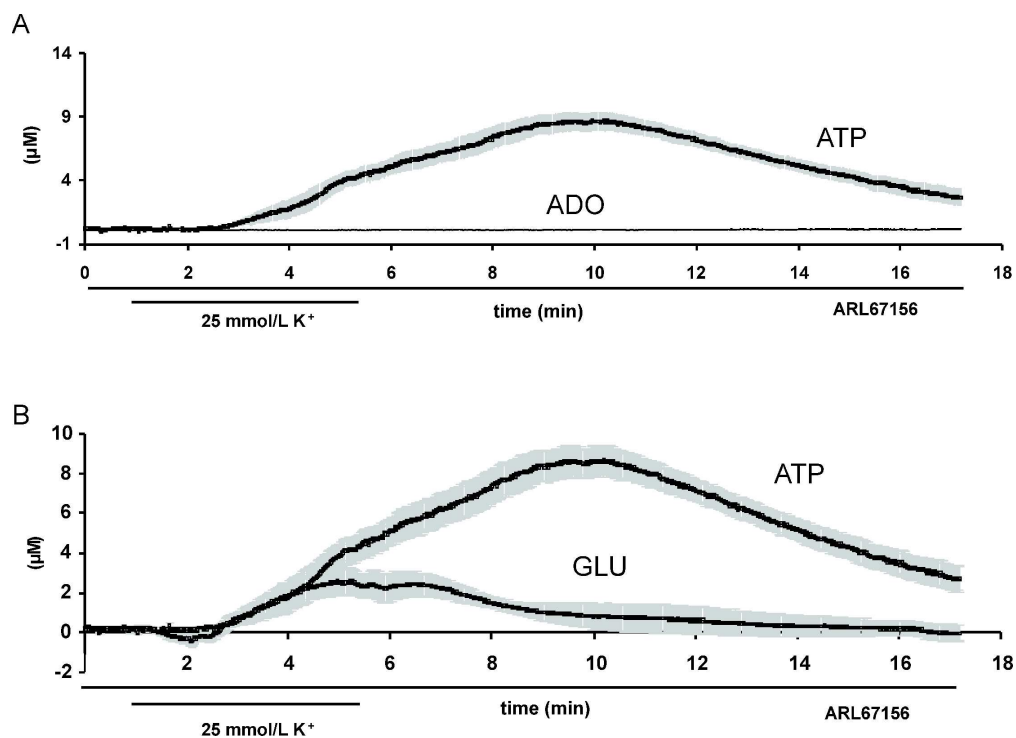


Figure 2. The effect of the inhibition of extracellular ATP metabolism with ARL67156 (100 µmol/L) on ATP (A, B), adenosine (A) and glutamate (B) efflux.

The treatment enhanced ATP release (A, B) but had no significant effect glutamate release (B) following K⁺ depolarization (black bar). By contrast, adenosine (ADO) efflux during the stimulation was inhibited in the presence of ARL67156 (A). Traces are means (black) ± S.E.M (in grey) of 6 independent experiments.

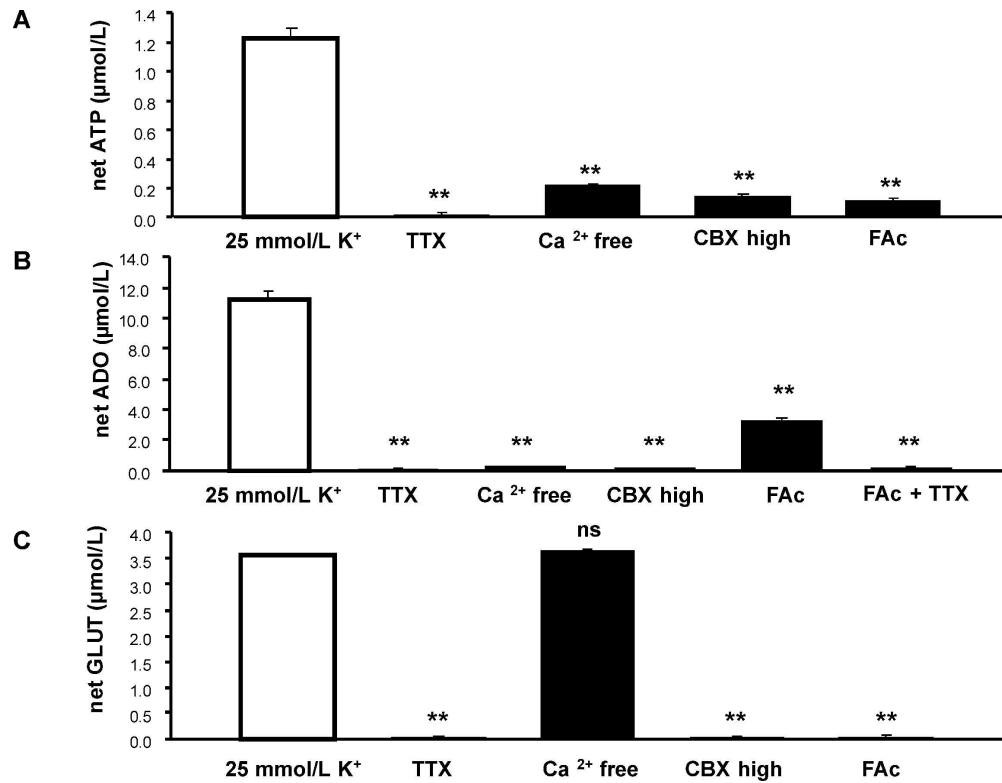


Figure 3. The effects of different treatments on K⁺ depolarization induced efflux of ATP, adenosine and glutamate from rat hippocampal slices. Inhibition of the voltage-dependent Na⁺ channels by TTX (3 µmol/L) totally inhibited the ATP (A) and adenosine (B) efflux following the K⁺ depolarization (25 mmol/L; 270 sec). Similarly, glutamate release was reduced in the presence of TTX after high K⁺ stimulation (C). Removal of extracellular Ca²⁺ (Ca²⁺ free) differentially affected ATP, adenosine (A, B) and glutamate (C) efflux. The hemichannel blocker carbenoxolone (CBX high: 100 µmol/L) almost completely inhibited ATP, adenosine and glutamate efflux (A, B, C). Release of ATP (A), adenosine (B) and glutamate (C) following the K⁺ stimulation substantially decreased in the presence of FAc (1 mmol/L; 10 min). TTX (3 µmol/L; 20 min) almost completely inhibited adenosine efflux in the presence of FAc (1 mmol/L; 10 min) (B). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n= 5-6; ns, p>0.05; **, p<0.01).

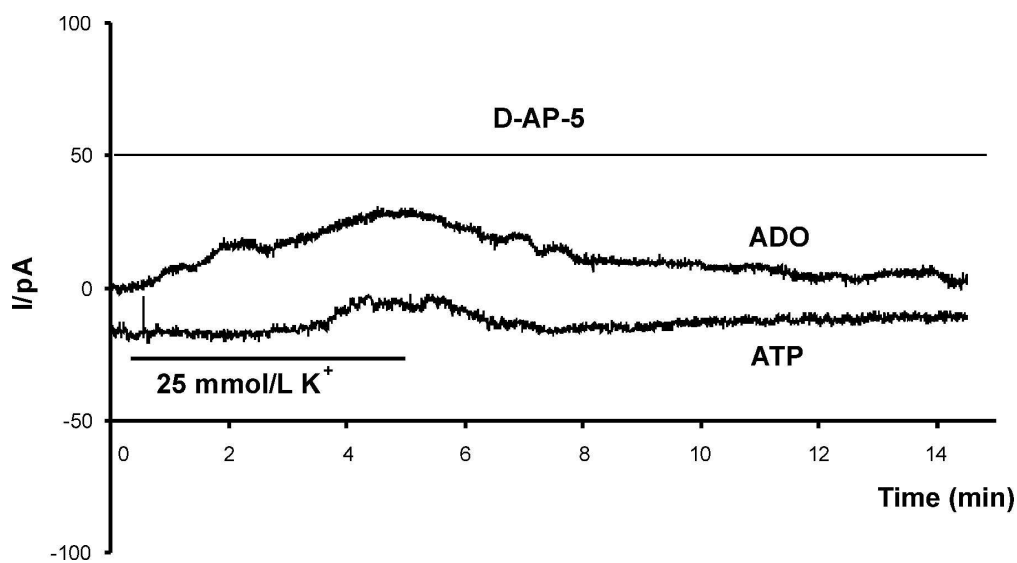


Figure 4. The effect of the NMDA glutamate receptor antagonist D-AP-5 on adenosine and ATP efflux evoked by K⁺ depolarization.

D-AP-5 (10 μ mol/L) decreased ATP and adenosine efflux in the rat hippocampal slices. Black bars denote the period of K⁺ depolarization and D-AP-5 perfusion. Individual recordings are representative of at least 5 independent experiments.

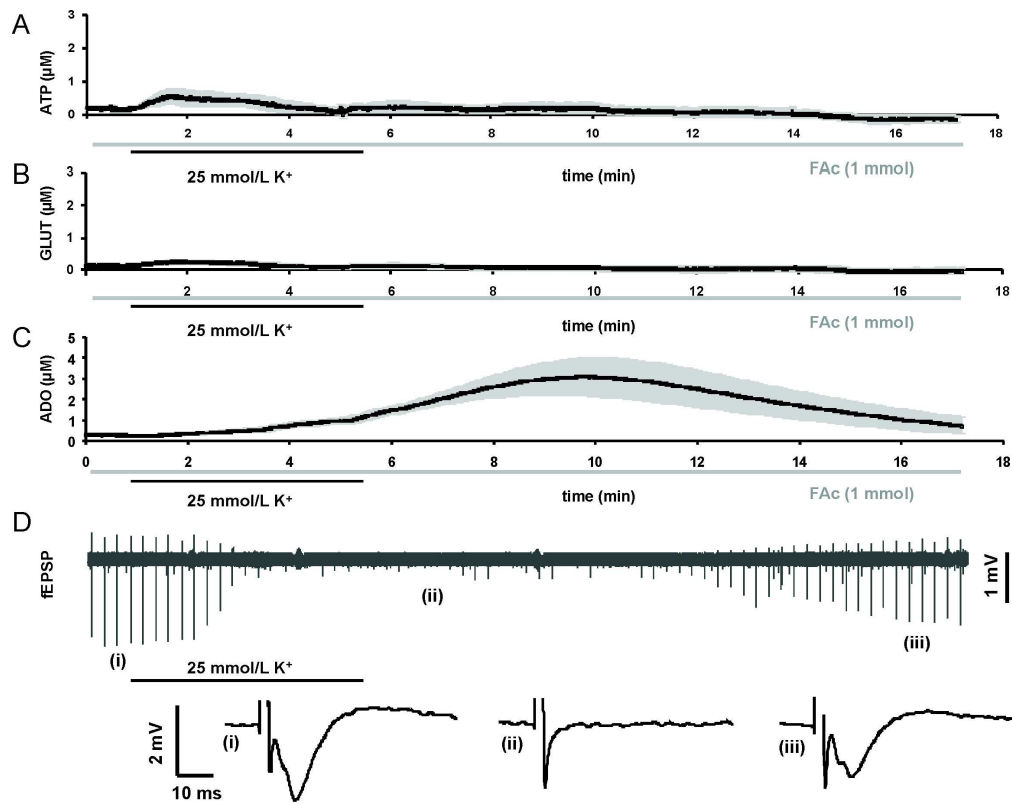


Figure 5. Effect of the mitochondrial gliotoxin fluoroacetic acid perfusion (FAC, 1 mmol/L) on ATP (A) adenosine (B) and glutamate (C) efflux evoked by K⁺ depolarization. FAC was added 10 min before the beginning of K⁺ depolarization (black bar). Inhibition of oxidative metabolism in glial cells resulted in almost complete inhibition of the detected increase in the efflux of ATP and glutamate. D. Recording of the evoked field excitatory postsynaptic potentials (fEPSP, in AC mode) before (i) and following K⁺ depolarization (ii). Note the recovery of the fEPSP during wash out (iii). Stimulus artifacts have been truncated. Bottom, Magnified examples of the fEPSP (average of 5 responses) shown at the times indicated on the continuous AC trace. ATP, NULL, ADO/INO, INO, were recorded from one set of experiments and GLU, NULL from a separate set of experiments. All data show means (black) \pm S.E.M (in grey) of 6 independent experiments.

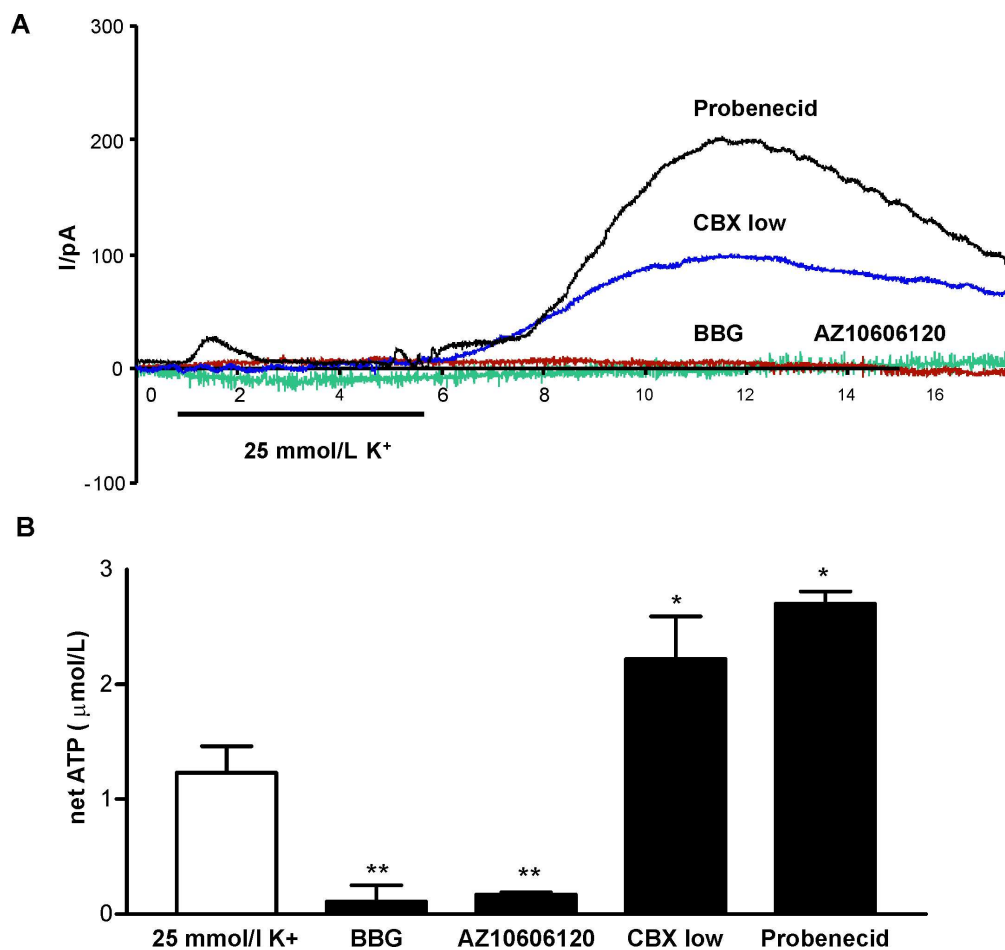


Figure 6. The effect of P2X7 receptor antagonists and pannexin inhibitors on ATP efflux evoked by K⁺ depolarization.

BBG (100 nmol/L), and AZ10606120 (100 nmol/L) inhibited ATP efflux (A, normalized signal, B (see methods)). Inhibition of pannexin hemichannels by CBX (20 μmol/L) significantly increased the extracellular level of ATP during K⁺ stimulation (25 mmol/L; 270 sec; black bar), which was also pronounced in the presence of probenecid (150 μmol/L). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n=5; **, p<0.01)

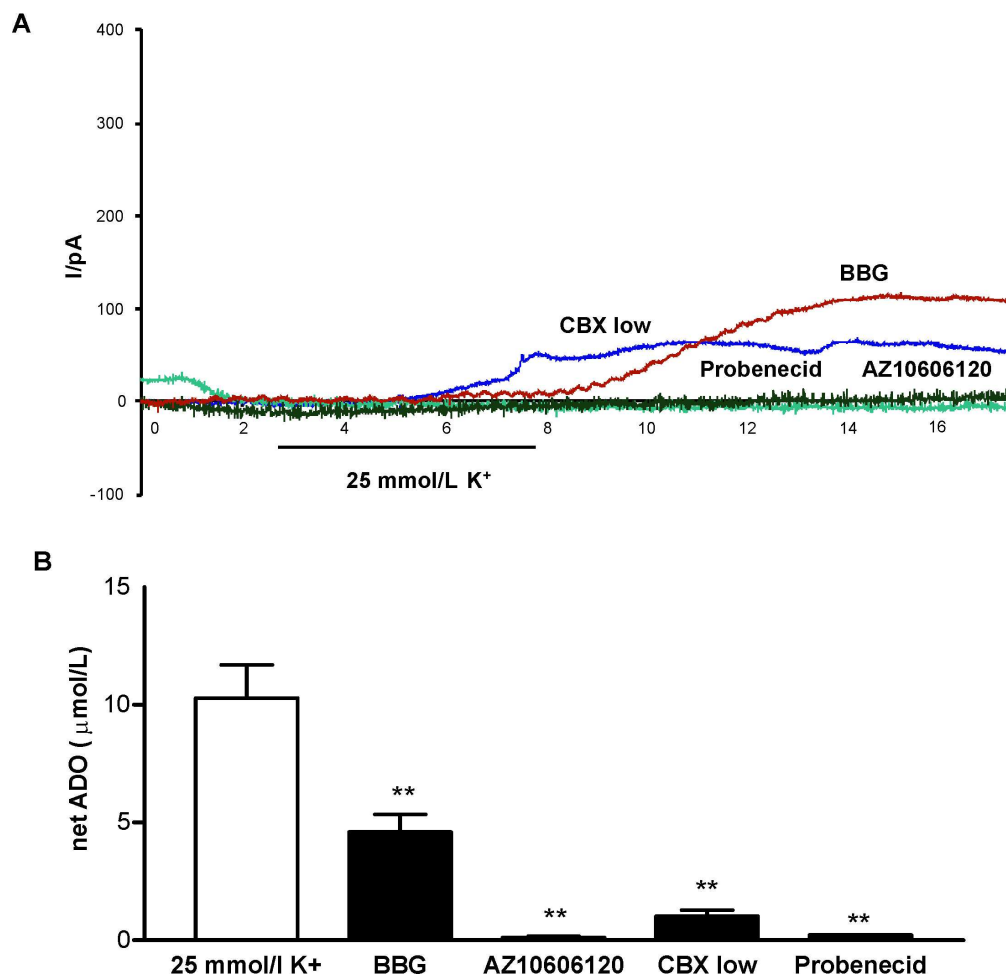


Figure 7. The effect of P2X7 receptor antagonists and pannexin inhibitors on adenosine efflux evoked by K⁺ depolarization.

BBG (100 nmol/L), and AZ10606120 (100 nmol/L) inhibited adenosine efflux (A, normalized signal, B (see methods)). Inhibition of pannexin hemichannels by CBX (20 μmol/L) greatly reduced adenosine efflux during K⁺ stimulation (25 mmol/L; 270 sec; black bar). Administration of probenecid (150 μmol/L) also profoundly decreased the extracellular level of adenosine (A, normalized signal, B (see methods)). Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test. (n=5; **, p<0.01; ns, p>0.05).