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RESEARCH PAPER

Roles of purines in synaptic modulation evoked by hypercapnia in isolated spinal cord of neonatal rat *in vitro*

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Background and purpose: The purine compounds, adenosine 5′-triphosphate (ATP) and adenosine, are known to accumulate in the extracellular space and to elicit various cellular responses during hypoxia/ischemia, whereas the roles of purines during hypercapnia are poorly understood. In this study, we examined the effects of various drugs affecting purine turnover on the responses to hypercapnia in the spinal cord.
Experimental approach: Electrically evoked reflex potentials were measured in an *in vitro* preparation of the isolated spinal cord of the neonatal rat by extracellular recording. Extracellular adenosine concentrations were assayed by high performance liquid chromatography (HPLC) methods.
Key results: Hypercapnia (20% CO_2) depressed the reflex potentials, which were partially reversed by an adenosine A_1 receptor antagonist, 8-cyclopentyl theophylline, but not by a P2 receptor antagonist, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid. Exogenous adenosine and ATP also depressed the reflex potentials via adenosine A_1 receptors. The hypercapnia-evoked depression was not reversed by inhibitors of gap junction hemichannels, anion channels, $P2X_7$ receptors or equilibrative nucleoside transporters, all of which might be involved in purine efflux pathways. The adenosine accumulation evoked by hypercapnia was not inhibited by tetrodotoxin, ethylene glycol-bis(β-amino ethyl ether) tetraacetic acid (EGTA) or an ecto-ATPase inhibitor, ARL 67156. Homocysteine thiolactone, used to trap intracellular adenosine, significantly reduced extracellular adenosine accumulation during hypercapnia.
Conclusions and implications: These results suggest that hypercapnia released adenosine itself from intracellular sources, using pathways different from the conventional exocytotic mechanism, and that this adenosine depressed spinal synaptic transmission via adenosine A_1 receptors.
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Keywords: hypercapnia; reflex potential; adenosine; A_1 receptors; ATP; spinal cord

Abbreviations: ACSF, artificial cerebrospinal fluid; ATP, adenosine 5′-triphosphate; BBG, brilliant blue G; CBX, carbenoxolone; CPT, 8-cyclopentyltheophylline; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; EGTA, ethylene glycol-bis(β-amino ethyl ether) tetraacetic acid; ENT, equilibrative nucleoside transporter; HCY, homocysteine thiolactone; HPLC, high performance liquid chromatography; MSR, monosynaptic reflex potential; NBTI, S-(4-nitrobenzyl)-6-thioinosine; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; sVRP, slow ventral root potential; TTX, tetrodotoxin

Introduction

The purine compounds, adenosine and ATP, are important modulators in the central nervous system (CNS). P2 receptors for ATP are mainly divided into ligand-gated P2X receptors and G protein-coupled P2Y receptors, while adenosine receptors consist of four subtypes (A_1, A_{2a}, A_{2b} and A_3) which are all G-protein-coupled receptors (Burnstock, 2007). In addition to P2 receptor activation, ATP can activate adenosine receptors after rapid conversion to adenosine (Dunwiddie *et al.*, 1997; Matsuoka and Ohkubo, 2004). The balance of adenosine and ATP tone provides fine-tuning neuromodulation in the CNS (Sebastião and Ribeiro, 2000; Sperlágh *et al.*, 2007). Reflex potentials are depressed by adenosine A_1 receptor agonists in the *in vitro* isolated spinal cord of the neonatal rat (Nakamura *et al.*, 1997) and spinal neuronal activities are also modulated by P2 receptors expressed in neurons and glial cells under physiological and pathophysiological conditions (Franke *et al.*, 2006; Nakatsuka and Gu, 2006; Trang *et al.*, 2006).

In a previous report (Otsuguro *et al.*, 2006b), we found that hypercapnia evoked adenosine A_1 receptor antagonist-sensitive and antagonist-insensitive depression of the reflex

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potentials, and we proposed that hypercapnia caused adenosine to accumulate via the inhibition of adenosine kinase activity in the isolated spinal cord of the neonatal rat. However, the mechanisms underlying adenosine A_1 receptor antagonist-insensitive depression remained unclear. In rat hippocampal slices, the hypercapnia-evoked depression of synaptic transmission is partially mediated via adenosine A_1 receptors and that A_1 receptor antagonist-insensitive depression is abolished by pyridoxal-phosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS), a non-selective P2 receptor antagonist, suggesting the involvement of ATP in the responses to hypercapnia (Dulla *et al.*, 2005). In the spinal cord, however, it remains unknown whether the activation of P2 receptors contributes to the effects of hypercapnia.

The cellular origin and membrane pathways of purine efflux in the CNS during hypercapnia are still unclear. On the other hand, adenosine accumulation has been extensively examined in response to hypoxia/ischemia (see Latini and Pedata, 2001; Pearson *et al.*, 2003; Rossi *et al.*, 2007). The inhibitory effect of adenosine on neuronal activity via adenosine A_1 receptors is thought to alleviate excitotoxicity during hypoxia/ischemia (Wardas, 2002). In the rat spinal cord, hypoxia depresses synaptic transmissions via adenosine A_1 receptors (Lloyd *et al.*, 1988; Park *et al.*, 2002). In the rat hippocampus, adenosine accumulation during hypoxia/ischemia results from the release of adenosine *per se*, but not via the extracellular degradation of ATP (Frenguelli *et al.*, 2007). Adenosine accumulation is evoked by hypoxia/ischemia in a tetrodotoxin (TTX)-resistant and extracellular Ca^{2+}-independent fashion (Dale *et al.*, 2000; Frenguelli *et al.*, 2007), suggesting that it is not caused by conventional exocytosis. ATP has also been shown to be released during ischemia via processes independent of adenosine (Frenguelli *et al.*, 2007). There are several purine efflux pathways (Volterra and Meldolesi, 2005) such as gap junction hemichannels (Kang *et al.*, 2008; Lin *et al.*, 2008), anion channels (Anderson *et al.*, 2004), $P2X_7$ receptors (Suadicani *et al.*, 2006) and the equilibrative nucleoside transporters (ENTs) (King *et al.*, 2006), but the precise mechanisms of purine accumulation under hypoxic/ischemic conditions are still a matter of debate (Frenguelli *et al.*, 2007; Martín *et al.*, 2007).

In order to investigate the possible mechanisms involved in synaptic depression during hypercapnia, we first examined the effects of several purine compounds on the reflex potentials and then the effects of various drugs affecting purine turnover on the reflex potentials and extracellular adenosine concentration in the isolated spinal cord of the neonatal rat. We found that activation of adenosine A_1 receptors, but not of P2 receptors, was responsible for the acute synaptic depression during hypercapnia. Hypercapnia induced the release of adenosine itself from intracellular sources, by a process that was different from the conventional exocytosis.

Methods

Preparations and electrophysiology

All experiments were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Both male and female neonatal rats (Wistar, 0–4 days old) were used in this experiment.

Neonatal rats were anaesthetized with diethyl ether and decapitated, and then the spinal cords were isolated. The recording of spinal reflex potentials from isolated spinal cords was performed as previously described (Otsuguro *et al.*, 2006a). Briefly, the hemisected spinal cord was superfused with artificial cerebrospinal fluid (ACSF) at a flow rate of about 2.5 mL·min^{-1} at 27 ± 2°C. The composition of ACSF was as follows (mmol·L^{-1}): NaCl 138; $NaHCO_3$ 21; NaH_2PO_4 0.6; $CaCl_2$ 1.25; KCl 3.5; $MgCl_2$ 2.0; glucose 10; gassed with 95% O_2 and 5% CO_2; pH~7.3. Hypercapnic ACSF and low oxygen ACSF were prepared with gas at 80% O_2 and 20% CO_2 (pH~6.7) and at 80% O_2, 5% CO_2 and 15% N_2 (pH~7.2), respectively. None of the drugs used changed the pH of normal or hypercapnic ACSF. Suction electrodes were used for extracellular recording from the ventral root. Electrical stimulation (20–40V, 200 μs) was applied to the dorsal lumbar roots (L3-L5) to evoke a monosynaptic reflex potential (MSR) and a slow ventral root potential (sVRP) at the ipsilateral ventral roots. The magnitudes of MSR and sVRP were estimated as the peak amplitude (mV) and the integral of depolarization (mV S) above the resting potential respectively. The time course of the magnitude of each response was expressed as a percentage of the mean of the first three control responses. The inhibitory effects of hypercapnia on spinal reflex potentials were evaluated with the mean of three responses during hypercapnia, which were expressed as a percentage of the mean of three responses just before treatment. As previously reported (Otsuguro *et al.*, 2006b), the extent of depression by hypercapnia was reproducible in the same preparation although it varied greatly from preparation to preparation. In order to examine the effects of drugs on the hypercapnia-evoked depression, hypercapnic ACSF was applied for 10 min repeatedly, at intervals of more than 30 min and the second or third exposure was performed in the presence of the drug after pretreatment for at least 20 min.

Measurement of adenosine concentration

The adenosine concentration was measured as described previously (Otsuguro *et al.*, 2006b). Briefly, the isolated spinal cord was cut into several pieces and incubated in normal ACSF (1 mL) for 10 min at 35°C, and ACSF was collected as a control. After the external solution was changed to hypercapnic ACSF (1 mL), the tissues were incubated for a further 10 min, and ACSF was collected as a sample. In some experiments, tissues were preincubated with drugs for at least 10 min before exposure to hypercapnia. The adenosine concentration was determined by HPLC according to the methods of Kawamoto *et al.* (1998) with some modifications. Each sample (250 μL) was mixed with 90 μL of 0.1 mol·L^{-1} citrate-phosphate buffer (pH 4.0), 10 μL of 40% chloroacetaldehyde and 25 μL of 4 μmol·L^{-1} α,β-methylene ADP (an internal standard), and then incubated at 80°C for 40 min. The concentrations of ethenoadenosine derivatives were measured by reverse-phase HPLC with an ODS column (Cosmosil $5C_{18}$-MS, 4.6 × 150 mm, Nacalai Tesque Inc., Kyoto, Japan) and a fluorescence detector (FP-540D, Nihon-Koden, Tokyo,

Japan). The mobile phase buffer consisted of 100 mmol·L^{-1} KH_2PO_4, 5 mmol·L^{-1} tetrabutylammonium bromide and 2.0% CH_3CN (pH 3.3 with H_3PO_4). Adenosine accumulation for 10 min was expressed as a change in extracellular adenosine concentration (ΔAdenosine) per milligram of tissue wet weight.

Data analysis

Results are expressed as mean ± SEM (n = number of observations). The IC_{50} value was calculated by fitting the data to a sigmoidal logistic curve using the program Origin (ver. 7.5J, OriginLab, Northampton, MA, USA). Statistical comparisons between two groups were performed by the paired or unpaired Student's t-test. For multiple comparisons, ANOVA followed by Dunnett's test was used. A P value of less than 0.05 was considered significant.

Drugs

Adenosine, ATP disodium Salt, brilliant blue G (BBG), capsaicin, carbenoxolone (CBX) disodium salt, 8-cyclopentyltheophylline (CPT), 6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5- triphosphate trisodium salt (ARL 67156), dipyridamole, L-homocysteine thiolactone (HCY) hydrochloride, S-(4-nitrobenzyl)-6-thioinosine (NBTI), PPADS tetrasodium salt and 2′,3′-*O*-(2,4,6-trinitrophenyl) ATP monolithium trisodium salt (TNP-ATP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TTX was from Wako Pure Chemical Ind. (Osaka, Japan). 4,4′-Diisothiocyano-2,2′-stilbenedisulphonic acid (DIDS) disodium salt was from Dojindo Lab. (Kumamoto, Japan). Receptor/channel nomenclature follows that recommended by Alexander *et al*. (2008).

Results

Involvement of adenosine A_1 receptors but not P2 receptors in depression of spinal reflex potentials during hypercapnia

Electrical stimulation of the dorsal root evoked an MSR followed by an sVRP at the ipsilateral ventral root. Exposure (10 min) of the isolated spinal cord to hypercapnia reversibly depressed both reflex potentials evoked every 2 min (Figure 1). CPT (3 μmol·L^{-1}), an adenosine A_1 receptor antagonist, had no effect on the basal sVRP (100.1 ± 3.2% of control, n = 5) or MSR (101.9 ± 2.9% of control, n = 5). The depression of sVRP and MSR in response to hypercapnia was partly but significantly reversed by CPT (Figure 1A,B), indicating the partial contribution of adenosine to the hypercapnia-evoked depression as reported previously (Otsuguro *et al*., 2006b). On the other hand, PPADS (20 μmol·L^{-1}), a non-selective P2 receptor antagonist, had little or no effect on basal reflex potentials (sVRP: 89.1 ± 7.4% of control, n = 5, MSR: 94.3 ± 3.5% of control, n = 5) and the hypercapnia-evoked depression (Figure 1C,D).

ATP- and adenosine-evoked depression of spinal reflex potentials via adenosine A_1 receptors

We investigated the effects of exogenously applied adenosine and ATP on the reflex potentials. Adenosine (0.01–300 μmol·L^{-1}) or ATP (0.1–300 μmol·L^{-1}) was cumulatively applied to the preparations. Both purines depressed the reflex potentials in a concentration-dependent manner (Figure 2). The IC_{50} values are shown in Table 1. The inhibitory effect of adenosine was more potent than that of ATP, and the sVRP was more sensitive to both adenosine and ATP than MSR. CPT antagonized the depression evoked by adenosine. Interestingly, CPT also abolished the depression evoked by ATP (Figure 2 and Table 1).

We further investigated the effects of P2 receptor agonists and antagonists on the reflex potentials. Application (10 min) of ATP (10 μmol·L^{-1}) consistently depressed sVRP by about 50% (Figure 3A), but not MSR. The depression of sVRP was completely reversed by CPT (3 μmol·L^{-1}), but not by the P2 receptor antagonists, PPADS (20 μmol·L^{-1}) or TNP-ATP (10 μmol·L^{-1}). Potent P2 receptor agonists, such as UTP, α,β-methylene ATP and 2-methylthio ATP induced slight or no depression (Figure 3B). ATPγS, a relatively stable agonist, caused depression comparable with ATP, which was also abolished by CPT (Figure 3B).

Effects of inhibitors of large conductance channels on depression of reflex potentials evoked by hypercapnia

Several channels with large conductances such as gap junction hemichannels, $P2X_7$ receptors and anion channels, are possible pathways for purine efflux. We examined the effects of inhibitors of these channels on the synaptic depression evoked by hypercapnia. During pretreatment (30 min) with 100 μmol·L^{-1} CBX, a gap junction hemichannel inhibitor, basal sVRP, but not MSR, gradually declined and became constant at around 50% of the control level (Figure 4A). Similar attenuation of neuronal activity was also reported in the rat hippocampus (Frenguelli *et al*., 2007). CBX failed to decrease the inhibitory effect of the reflex potentials in response to hypercapnia (Figure 4B), but the recovery from depression of sVRP was minimal even 20 min after washout of the drug (Figure 4C). BBG (5 μmol·L^{-1}), a $P2X_7$ receptor antagonist, had no significant effect on basal reflex potentials (sVRP: 99.9 ± 9.9% of control, n = 5, MSR: 101.3 ± 2.1% of control, n = 5) and the hypercapnia-evoked depression (Figure 4D,E). DIDS (100 μmol·L^{-1}), an anion channel inhibitor, also had no significant effect on basal reflex potentials (sVRP: 95.9 ± 3.5% of control, n = 5, MSR: 96.2 ± 2.4% of control, n = 5) and the hypercapnia-evoked depression of MSR, but the inhibitory effect of hypercapnia on sVRP was significantly enhanced by DIDS.

Effect of inhibitors of ENTs on hypercapnia-evoked depression of spinal reflex potentials

Equilibrative nucleoside transporters play an important role in regulating the extracellular adenosine concentration. In the rat hippocampus, the hypercapnia-evoked depression was not attenuated by dipyridamole, a widely used ENT inhibitor (Dulla *et al*., 2005). In the present study, we used a mixture of two inhibitors, NBTI and dipyridamole, to block ETNs, as rat ENTs are relatively insensitive to dipyridamole (Yao *et al*., 1997) and the NBTI-sensitive ENT1 is abundant in the rat spinal cord (Governo *et al*., 2005). The mixture of NBTI

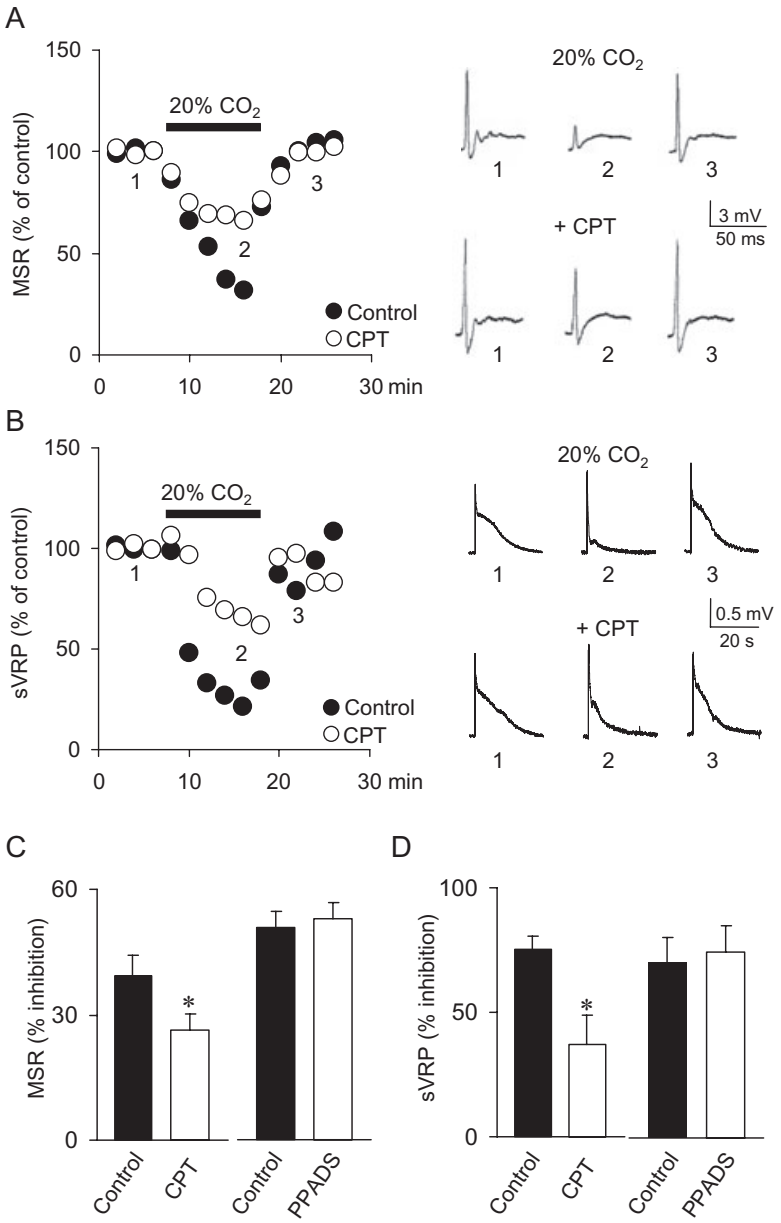

Figure 1 Effects of purinoceptor antagonists on hypercapnia-evoked depression. The depression of MSR (A) and sVRP (B) in response to hypercapnia (20% CO_2) was partly reversed by 3 μmol·L^{-1} CPT. Representative data of the time courses (left) and traces (right) before (i), during hypercapnia (ii) and after recovery from hypercapnia (iii) in the absence and presence of CPT. Summary data of effects of CPT (3 μmol·L^{-1}) and PPADS (20 μmol·L^{-1}) on the hypercapnia-evoked depression of MSR (C) and sVRP (D). Each column and error bar represents the mean ± SEM (n = 5). *P < 0.05 (paired Student's t-test). CPT, 8-cyclopentyltheophylline; MSR, monosynaptic reflex potential; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; sVRP, slow ventral root potential.

(5 μmol·L^{-1}) and dipyridamole (10 μmol·L^{-1}) caused a slight depression of basal sVRP (74.3 ± 7.7% of control, n = 5) but not MSR (99.2 ± 2.9% of control, n = 5). In the presence of ENT inhibitors, hypercapnia still depressed the reflex potentials (Figure 5A–C). Although there was no significant difference in per cent inhibition by hypercapnia in the presence and absence of ENT inhibitors (Figure 5D,E), the rate of recovery from depression of the sVRP was slower (Figure 5C). Thus the sVRP took almost 20 min to return to the same level as before hypercapnia, in the presence of ENT inhibitors. As shown in Figure 5A, CPT reversed sVRP depressed by ENT inhibitors in three of six preparations, suggesting adenosine accumulation. In the remainder, we could not accurately measure the integral of sVRP, as CPT evoked increases in spontaneous activity. In addition, CPT somewhat enhanced MSR in the presence of the ENT inhibitors. These results indicate that ENTs are important factors in decreasing extracellular adenosine under both normal and hypercapnic conditions, but they do not contribute significantly to purine efflux during hypercapnia.

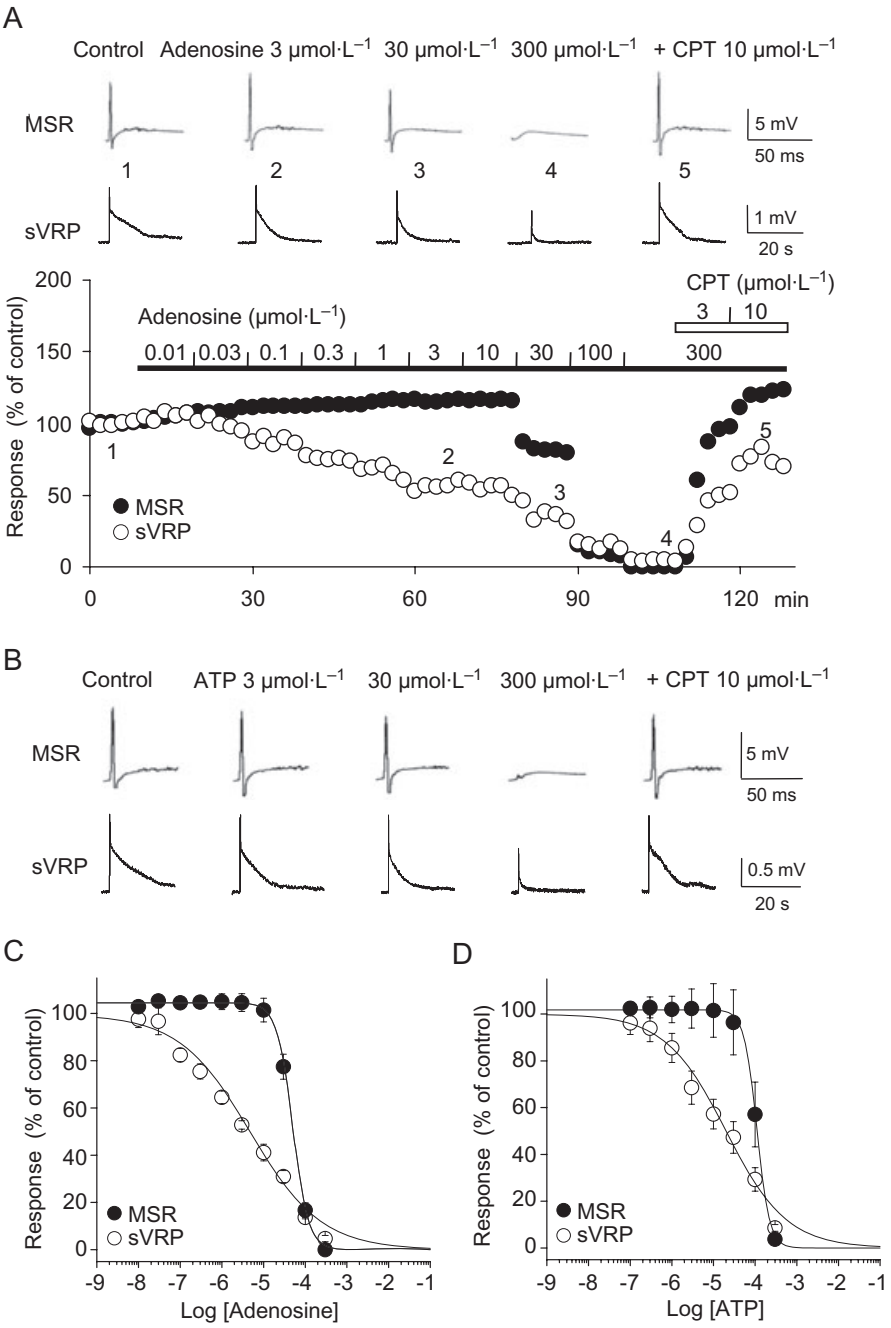

Figure 2 Depression of the reflex potentials in response to adenosine and ATP via adenosine A_1 receptors. (A) Adenosine (0.01–300 μmol·L^{-1}) was cumulatively applied to the spinal cord. Then CPT (3 and 10 μmol·L^{-1}) was added in the presence of adenosine (300 μmol·L^{-1}). The numbers in the representative traces of MSR and sVRP (upper panel) correspond to those in the lower panel. (B) Representative traces of MSR and sVRP depressed by ATP (3, 30 and 300 μmol·L^{-1}). CPT (10 μmol·L^{-1}) was added in the presence of ATP (300 μmol·L^{-1}). Concentration-response curves for MSR and sVRP in the presence of adenosine (C) and ATP (D). Each symbol and error bar represents the mean ± SEM (n = 6). ATP, adenosine 5′-triphosphate; CPT, 8-cyclopentyltheophylline; MSR, monosynaptic reflex potential; sVRP, slow ventral root potential.

Characterization of adenosine accumulation during hypercapnia in the spinal cord

Hypoxia is well known to cause extracellular accumulation of adenosine in the CNS. Therefore, we first examined the effect of a decrease of oxygen content from 95% to 80%, which was the equivalent oxygen content with the hypercapnic gas. Exposure of the isolated spinal cord to 80% oxygen had little effect on the adenosine concentration, but not that to hypercapnic ACSF (Figure 6A), indicating that the adenosine accumulation was caused by hypercapnia itself, not by decreased oxygen tension. TTX (100 nmol·L^{-1}) did not affect the basal adenosine level (control: 0.66 ± 0.06 pmol·mg^{-1}, TTX: 0.62 ± 0.08 pmol·mg^{-1}, n = 4) and adenosine accumulation (Figure 6B) in response to hypercapnia. The basal adenosine level was increased by removal of extracellular Ca^{2+} with 1 mmol·L^{-1} EGTA (control: 0.85 ± 0.06 pmol·mg^{-1} versus 0Ca,

Table 1 Antagonism by CPT of adenosine- and ATP-evoked depression of spinal reflex potentials

Agonist		*IC_{50} (μmol·L^{-1})*	*300 μmol·L^{-1} agonist (% of control)*		
			0 CPT	*+3 μmol·L^{-1} CPT*	*+10 μmol·L^{-1} CPT*
Adenosine	MSR	47.5 ± 4.7 (6)	0.1 ± 0.1 (6)	88.8 ± 6.4 (5)$^{\#\#}$	114.1 ± 2.0 (5)$^{\#\#}$
	sVRP	3.0 ± 3.6 (6)**	5.5 ± 1.8 (6)	45.9 ± 6.6 (5)$^{\#\#}$	83.8 ± 6.4 (5)$^{\#\#}$
ATP	MSR	98.9 ± 16.7 (6)$^{\$}$	3.7 ± 1.4 (6)	97.9 ± 3.7 (3)$^{\#\#}$	112.9 ± 32.6 (3)$^{\#\#}$
	sVRP	25.3 ± 8.8 (6)$^{\$}$,**	8.6 ± 1.4 (6)	74.5 ± 5.2 (3)$^{\#\#}$	90.7 ± 18.4 (3)$^{\#\#}$

Each value is the mean ± SEM (number of observations). $^{\$}P < 0.05$ versus adenosine (unpaired Student's *t*-test).
**$P < 0.01$ versus MSR (paired Student's *t*-test). $^{\#\#}P < 0.01$ versus 0 CPT (Dunnett's test).
ATP, adenosine 5′-triphosphate; CPT, 8-cyclopentyltheophylline; MSR, monosynaptic reflex potential; sVRP, slow ventral root potential.

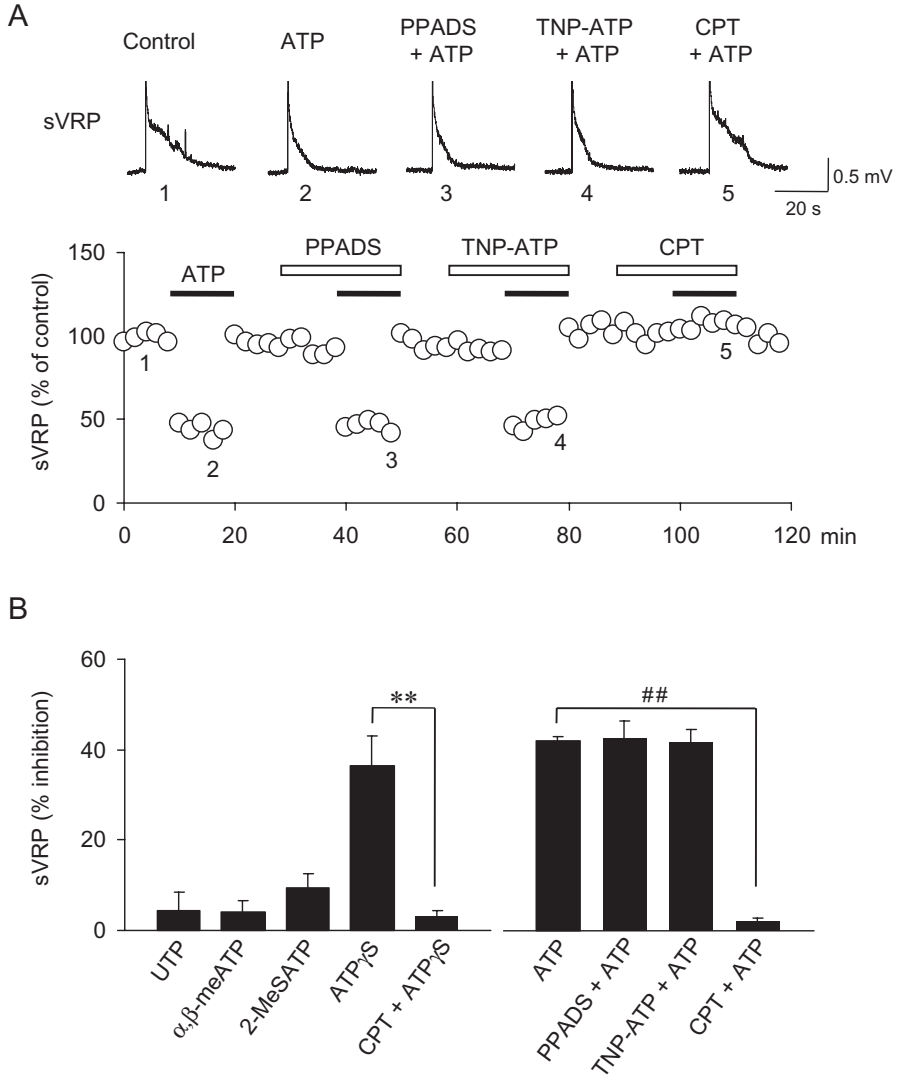

Figure 3 Effects of purinoceptor agonists and antagonists on sVRP. (A) The depression of sVRP by ATP (10 μmol·L^{-1}) in the presence of PPADS (20 μmol·L^{-1}), TNP-ATP (10 μmol·L^{-1}) or CPT (3 μmol·L^{-1}). The numbers in the representative traces of sVRP (upper panel) correspond to those in the lower panel. (B) Summary data of effects of UTP, α,β-methylene ATP (α,β-meATP), 2-methylthio ATP (2-MeSATP), ATPγS and ATP, all at 10 μmol·L^{-1} in the presence of PPADS (20 μmol·L^{-1}), TNP-ATP (10 μmol·L^{-1}) and CPT (3 μmol·L^{-1}). Each column and error bar represents the mean ± SEM (n = 4). **$P < 0.01$ (paired Student's *t*-test). $^{\#\#}P < 0.01$ (Dunnett's test). ATP, adenosine 5′-triphosphate; CPT, 8-cyclopentyltheophylline; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; sVRP, slow ventral root potential.

1G: 1.08 ± 0.07 pmol·mg^{-1}, n = 6, $P < 0.01$, paired Student's *t*-test) and 5 mmol·L^{-1} EGTA (control: 0.84 ± 0.07 pmol·mg^{-1} versus 0Ca, 5G: 1.05 ± 0.08 pmol·mg^{-1}, n = 4, $P < 0.05$, paired Student's *t*-test). In addition, removal of extracellular Ca^{2+} with 5 mmol·L^{-1} EGTA significantly enhanced the hypercapnia-evoked adenosine accumulation (Figure 6B).

Homocysteine thiolactone is used to trap intracellular adenosine (Lloyd *et al.*, 1993). In the rat hippocampus, it is

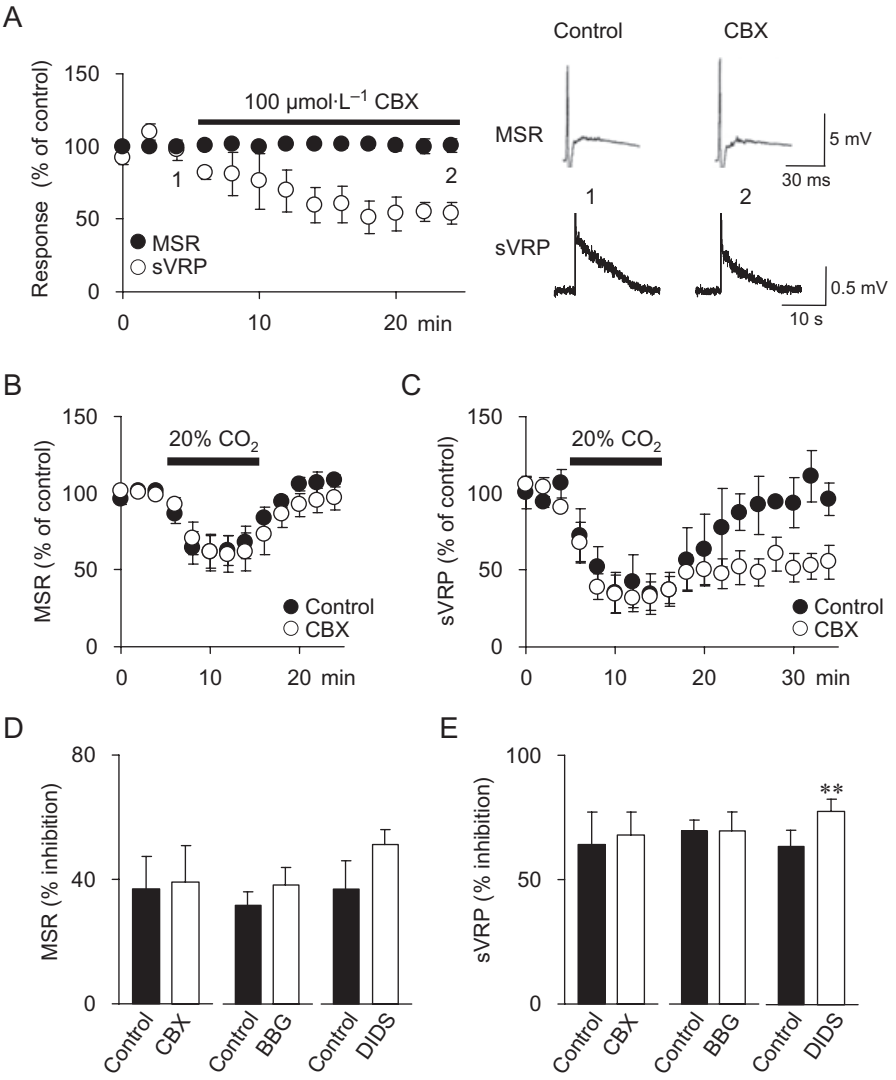

Figure 4 Effects of inhibitors of large conductance channels on hypercapnia-evoked depression. (A) The spinal cord was pretreated with CBX (100 μmol·L^{-1}) for 20 min. The numbers in the representative traces of MSR and sVRP (right panel) correspond to those in the left panel. The time course of effects of CBX on the hypercapnia-evoked depression on MSR is shown (B) and for sVRP in (C). Summary data of effects of CBX (100 μmol·L^{-1}), BBG (5 μmol·L^{-1}) and DIDS (100 μmol·L^{-1}) on the hypercapnia-evoked depression of MSR (D) and sVRP (E). Each symbol and error bar represents the mean ± SEM (n = 5). **P < 0.01 versus control (paired Student's t-test). BBG, brilliant blue G; CBX, carbenoxolone; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; MSR, monosynaptic reflex potential; sVRP, slow ventral root potential.

reported that the adenosine accumulation during ischemia is reduced by HCY, suggesting that adenosine is released from an intracellular origin (Frenguelli *et al.*, 2007). Treatment with HCY did not affect the basal adenosine level (control: 0.59 ± 0.06 pmol·mg^{-1}, HCY: 0.58 ± 0.04 pmol·mg^{-1}, n = 6) but significantly reduced adenosine accumulation during hypercapnia in the spinal cord (Figure 6C).

Another important route for adenosine accumulation is the extracellular degradation of ATP. In order to test this possibility, we examined the effect of ARL67156, an ecto-ATPase inhibitor. ARL67156 did not affect the basal adenosine level (control: 1.00 ± 0.06 pmol·mg^{-1}, ARL67156: 0.91 ± 0.05 pmol·mg^{-1}, n = 4) or the hypercapnia-evoked adenosine accumulation (Figure 6C), suggesting that extracellular degradation of ATP was not involved. To confirm the effectiveness of ARL67156 in our system, we used capsaicin to release ATP, which is known to be degraded to adenosine in the spinal cord synaptosomes (Sweeney *et al.*, 1989). As shown in Figure 6D, capsaicin (100 μmol·L^{-1}) caused adenosine accumulation, which was significantly attenuated by ARL67156.

Discussion

Our data indicated that hypercapnia released adenosine itself from intracellular sources and depressed the spinal reflex potentials via adenosine A_1 receptors, a finding similar in several aspects to the responses to hypoxia reported in the brain. We have previously reported that the hypercapnia-evoked depression of reflex potentials is partially reversed by an adenosine A_1 receptor antagonist but not by adenosine A_{2A}, $GABA_A$, glycine, opioid and α_2 adrenergic receptor antagonists

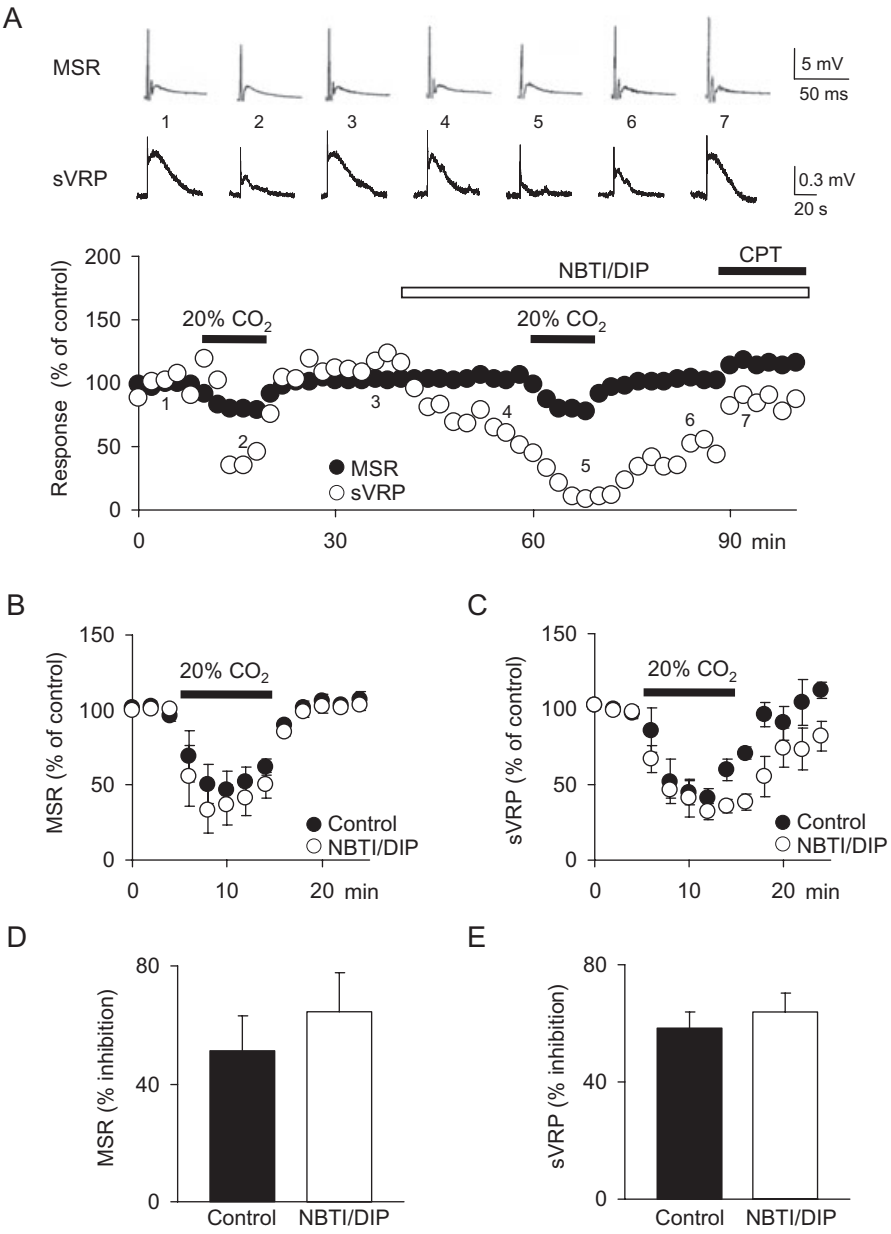

Figure 5 Effects of ENT inhibitors on hypercapnia-evoked depression. (A) The preparations were exposed to hypercapnia in the presence or absence of the mixture of NBTI (5 μmol·L^{-1}) and dipyridamole (DIP, 10 μmol·L^{-1}). Then CPT (3 μmol·L^{-1}) was added in the presence of the ENT inhibitors. The numbers in the representative traces of MSR and sVRP (upper panel) correspond to those in the lower panel. The time courses of effects of ENT inhibitors (NBTI/DIP) on the hypercapnia-evoked depression on MSR (B) and sVRP (C). Summary data of effects of ENT inhibitors (NBTI/DIP) on the hypercapnia-evoked depression of MSR (D) and sVRP (E). Each symbol and error bar represents the mean ± SEM ($n = 5$). CPT, 8-cyclopentyltheophylline; ENT, equilibrative nucleoside transporter; MSR, monosynaptic reflex potential; NBTI, S-(4-nitrobenzyl)-6-thioinosine; sVRP, slow ventral root potential.

in the neonatal rat spinal cord (Otsuguro *et al*., 2006b). In the rat hippocampus, hypercapnia also evokes synaptic depression, which was partially reversed by 1,3-dipropyl-8-cyclopentylxanthine, an adenosine A_1 receptor antagonist or PPADS, a non-selective P2 receptor antagonist, and completely abolished by the combination of these two antagonists. Therefore, it was proposed that adenosine and ATP were involved in the depression of synaptic activity induced by hypercapnia via adenosine A_1 and P2 receptors respectively (Dulla *et al*., 2005). In the present study of the spinal cord, however, the hypercapnia-evoked depression was not affected by PPADS. In addition, it is unlikely that the activation of P2 receptors elicits acute depression of reflex potentials because the potent P2 receptor agonists had little inhibitory effect on the reflex potentials. Like ATP, a stable P2 receptor agonist, ATPγS evoked the depression via adenosine A_1 but not P2 receptors. In the rodent hippocampus, it is reported that ATP causes synaptic depression by degrading to adenosine (Cunha *et al*., 1998), and ATPγS is also rapidly converted to adenosine (Dunwiddie *et al*., 1997; Masino *et al*., 2002). Taken together, these findings suggest that the adenosine A_1 receptor antagonist-insensitive depression in response to hypercapnia

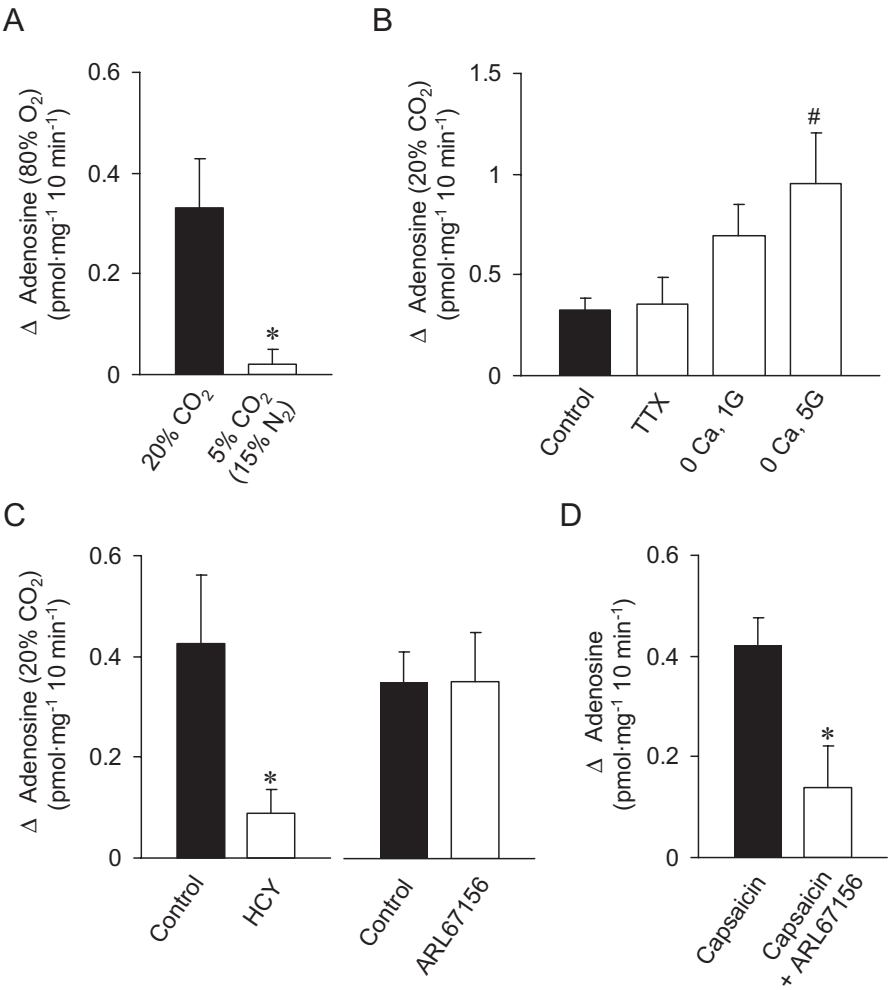

Figure 6 Characterization of adenosine accumulation during hypercapnia. (A) Accumulation of adenosine during hypercapnic (20% CO_2; 80% O_2, $n = 6$) and low O_2+normocapnic conditions (5% CO_2 : 15% N_2; 80% O_2, $n = 6$). (B) Hypercapnia-evoked adenosine accumulation (control, $n = 5$) in the presence of TTX (100 nmol·L^{-1}, $n = 4$) or after removal of extracellular Ca^{2+} with 1 mmol·L^{-1} EGTA (0 Ca, 1G, $n = 6$) and 5 mmol·L^{-1} EGTA (0 Ca, 5G, $n = 4$). (C) Hypercapnia-evoked adenosine accumulation (control, $n = 6$ for HCY; $n = 5$ for ARL67156) in the presence of HCY (1 mmol·L^{-1}, $n = 6$) or ARL67156 (50 μmol·L^{-1}, $n = 4$). (D) Capsaicin-evoked adenosine accumulation (100 μmol·L^{-1} capsaicin, $n = 5$) in the presence of ARL67156 (50 μmol·L^{-1}, $n = 7$). Each column and error bar represents the mean ± SEM. $^{*}P < 0.05$ (unpaired Student's *t*-test), $^{\#}P < 0.05$ versus control (Dunnett's test). EGTA, ethylene glycol-bis(β-amino ethyl ether) tetraacetic acid; HCY, homocysteine thiolactone; TTX, tetrodotoxin.

is not due to the activation of P2 receptors in the rat spinal cord. The effects of hypercapnia on neuronal activity seem to vary with the region of the CNS studied (Dale, 2006).

Extracellular degradation of ATP to adenosine is mediated by a cascade of ecto-enzymes (Matsuoka and Ohkubo, 2004). In the present study, ARL 67156, an ecto-ATPase inhibitor, failed to reduce adenosine accumulation during hypercapnia, which was consistent with our previous finding that α,β-methylene ADP, another ecto-5′-nucleotidase inhibitor, did not affect the hypercapnia-evoked depression (Otsuguro *et al.*, 2006b). In the rat hippocampus, hypercapnia-evoked adenosine accumulation is not affected by inhibitors for these ecto-enzymes (Dulla *et al.*, 2005). On the other hand, in the rat spinal cord, adenosine accumulation during hypercapnia was attenuated by HCY, which is able to trap, and thus decrease, intracellular adenosine (Lloyd *et al.*, 1993). These results suggest that hypercapnia-evoked adenosine accumulation results from the release of adenosine itself and not from the extracellular degradation of ATP. These data support our previous hypothesis that hypercapnia inhibits adenosine kinase activity and increases the intracellular level of adenosine, which is in turn released into the extracellular space.

There are several possible mechanisms for purine efflux across the plasma membrane, including via exocytosis, transporters and large conductance channels (Volterra and Meldolesi, 2005; Franke and Illes, 2006). Hypoxia/ischemia is well known to evoke adenosine accumulation resulting in synaptic depression in the hippocampus. Hypoxia/ischemia-evoked adenosine accumulation is TTX-resistant and enhanced by removal of extracellular Ca^{2+}, which differs from the conventional exocytotic pathway (Dale *et al.*, 2000; Frenguelli *et al.*, 2007). In addition, adenosine accumulation and synaptic depression during hypoxia/ischemia are insensitive to inhibitors of ENTs, gap junction hemichannels and $P2X_7$ receptors (Frenguelli *et al.*, 2007; Martín *et al.*, 2007). In the present study, similar results were obtained with adenosine accumu-

lation and synaptic depression in response to hypercapnia in the spinal cord. In addition, the hypercapnia-evoked depression was not blocked by DIDS, an anion channel inhibitor, which is reported to inhibit ATP-induced ATP release in mouse cortical astrocytes (Anderson *et al*., 2004). These results suggest that these channels and transporters are not involved in the responses to hypercapnia in the spinal cord. However, we cannot exclude the possibility of the involvement of some ENT isoforms which are relatively insensitive to both NBTI and dipyridamole. The most likely candidate may be ENT4, which is widely expressed in the CNS, including the spinal cord (Engel *et al*., 2004), and is activated at acidic pH (Barnes *et al*., 2006). The ATP-binding cassette proteins have been associated with efflux of ATP and cyclic AMP (Volterra and Meldolesi, 2005; Gödecke, 2008), but there is no evidence that such proteins can also carry adenosine. Further experiments are needed to reveal the mechanisms of purine efflux during hypercapnia.

In conclusion, adenosine A_1 receptors play an important role in the hypercapnia-evoked depression of the reflex potentials in the neonatal rat spinal cord. These receptors are activated by adenosine of intracellular origin which is released during hypercapnia.

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Conflict of interest

None.

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