

## Why does carbon dioxide produce analgesia?

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### Abstract

Carbon dioxide (CO<sub>2</sub>) has been used to euthanize laboratory animals because of its rapid analgesic and sedative effects. However, the mechanism of these effects is unclear. To investigate the anesthetic effect of CO<sub>2</sub>, we used isolated spinal cords of neonatal rats as an alternative to living animals. In this preparation, stimulation of the dorsal root mainly evokes two types of reflex potentials, a monosynaptic reflex potential (MSR) and a slow ventral root potential (sVRP), at the ipsilateral ventral root. The sVRP was considered to reflect nociceptive responses because the order of inhibitory potency of analgesics for sVRP was quite similar to that for capsaicin-induced nociceptive responses *in vivo*. Acute hypercapnic acidosis (20% CO<sub>2</sub>, pH 6.7) depressed both reflex potentials in the isolated spinal cord. This depression was reversible and partly inhibited by a selective adenosine A<sub>1</sub> receptor antagonist. Accumulation of extracellular adenosine and inhibition of adenosine kinase activity were observed during hypercapnic acidosis. These results indicate that hypercapnic acidosis promptly depresses spinal nociceptive transmission through the activation of adenosine A<sub>1</sub> receptors. It is suggested that the accumulation of extracellular adenosine results from the inhibition of adenosine kinase during hypercapnic acidosis in the spinal cord of the neonatal rat.

**Keywords:** carbon dioxide, hypercapnia, adenosine, spinal cord, neonatal rat

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### Introduction

Exposure to carbon dioxide (CO<sub>2</sub>) is widely used for euthanasia of laboratory animals. There are a number of merits for using CO<sub>2</sub> to kill animals, e.g. its immediate analgesic and sedative effect, cheapness, safety, ease of use and so on. On the other hand, there are also some concerns about side effects of CO<sub>2</sub> and thus for how CO<sub>2</sub> should be administered (Conlee *et al.*, 2005). The most important question concerning the usage of CO<sub>2</sub> is why CO<sub>2</sub> produces analgesia.

The isolated spinal cord of the neonatal rat is useful for studying the spinal action of analgesics *in vitro* (Konishi and Otsuka, 1974). Stimulation of the dorsal root evokes reflex potentials at the corresponding ipsilateral ventral root. An early part of the reflex potential is a monosynaptic reflex potential (MSR), which is followed by a slow ventral root potential (sVRP). The sVRP is considered to reflect the nociceptive reflex at the spinal level (Faber *et al.*, 1997). Therefore, it is expected that it could be useful for investigating analgesics as an alternative to living animals.

First, in our experiments, the effects of analgesics, morphine and  $\alpha_2$ -adrenoceptor agonists, were

examined to compare their potency between the isolated spinal cord *in vitro* and neonatal rats *in vivo*. To quantify the nociceptive behavior of immature rats, we used the elegant method developed by Kubota *et al.* (1996), which is suitable for objectively and easily investigating the effects of analgesics in small animals. Subsequently, we examined the effects of CO<sub>2</sub> on the isolated spinal cord *in vitro*.

### Materials and methods

All experiments were approved by the Institutional 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, 0-5 days old) were used. Neonatal rats were deeply anaesthetized with diethyl ether and then decapitated.

The isolated spinal cord was prepared as previously described (Otsuguro *et al.*, 2006a). The preparation was superfused with artificial cerebrospinal fluid (ACSF) consisting of the following (mM): NaCl 138, NaHCO<sub>3</sub> 21, NaH<sub>2</sub>PO<sub>4</sub> 0.6, CaCl<sub>2</sub> 1.25, KCl 3.5, MgCl<sub>2</sub> 2.0, glucose 10. The ACSF was gassed

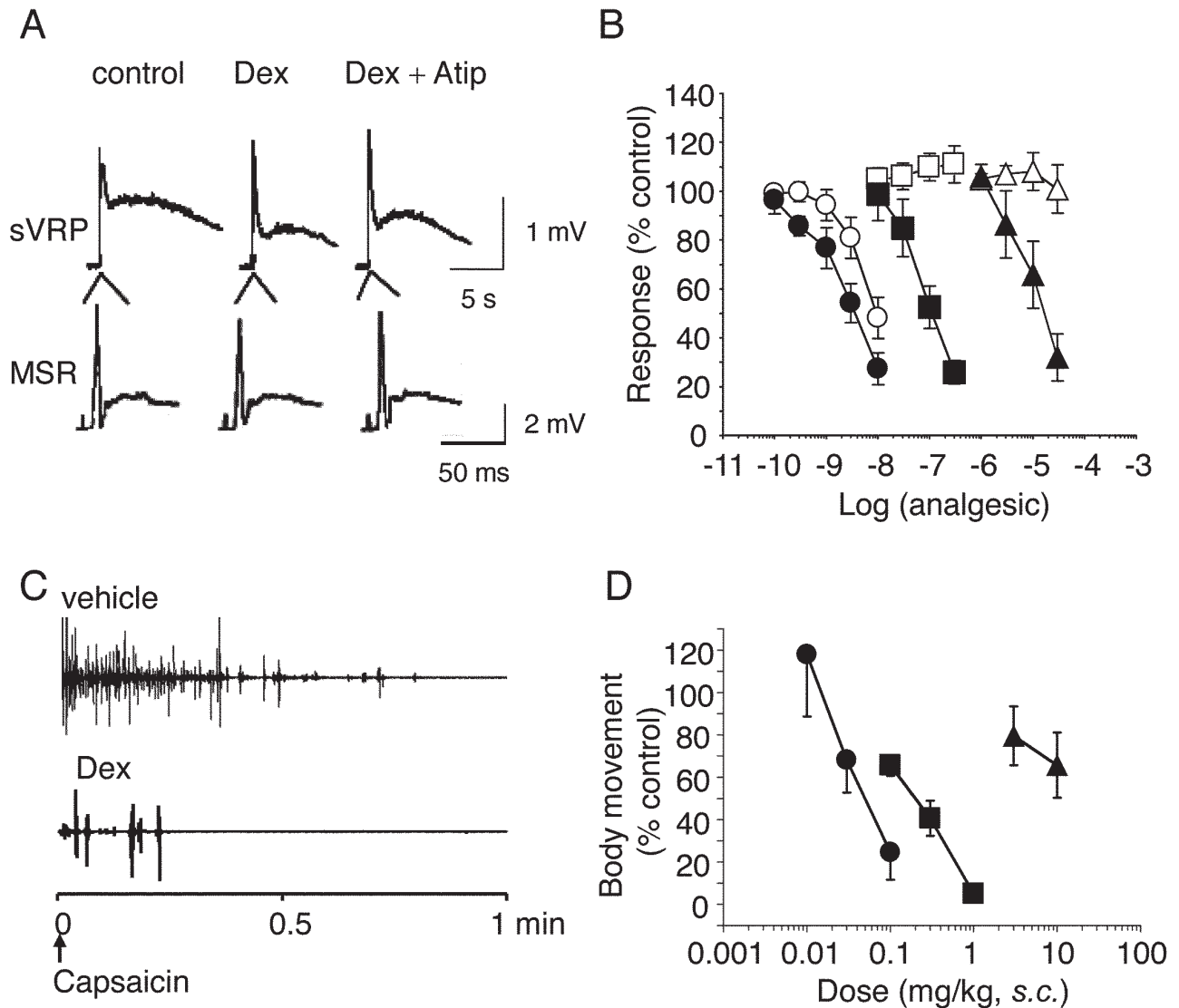


Fig. 1. Effects of analgesics in neonatal rats *in vitro* and *in vivo*. (A) Representative traces of spinal reflex potentials (sVRP and MSR) in the absence (control) and presence of 3 nM dexmedetomidine (Dex) and 1 μM atipamezole (Dex + Atip). (B) Concentration-response curves of dexmedetomidine (circle), morphine (square) and xylazine (triangle) for effects on MSR (open) and sVRP (closed). The magnitudes of MSR and sVRP are expressed as the relative peak amplitude and area under the curve, respectively. The mean ± S.E.M. (n=4-7). (C) The representative signal traces recorded from an audio amplifier. The body movement of a neonatal rat put on an audio speaker generated a voltage change in the speaker coil, which was transferred to the amplifier. Then the signal was converted to square pulses, which were counted for 1 min. The body movement was evoked by a bolus injection of capsaicin 30 min after treatment with saline (vehicle) or with dexmedetomidine (Dex). (D) Dose-response curves of dexmedetomidine (circle), morphine (square) and xylazine (triangle) for effects on the capsaicin-induced body movements of neonatal rats. The mean ± S.E.M. (n=6). Taken from Otsuguro *et al.* (2005).

with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH~7.3) for control and 80% O<sub>2</sub> and 20% CO<sub>2</sub> (pH~6.7) for hypercapnic acidosis. Electrical stimulation was applied to a lumbar dorsal root and reflex potentials were recorded from a corresponding ipsilateral ventral root. The extracellular adenosine concentration was measured by making ethenoadenosine derivatives and using high-performance liquid chromatography (HPLC) according to the method described by Kawamoto *et al.* (1998). Adenosine kinase activity was assessed by measuring the *in vivo* phosphorylation of [U-<sup>14</sup>C] adenosine. The radioactivity of nucleotides bound

to ion-exchange paper disks was measured with a liquid scintillation counter according to methods described by Ives *et al.* (1969) and Lynch *et al.* (1998). Nociceptive responses of neonatal rats *in vivo* were evaluated as body movement (Kubota *et al.*, 1998).

## Results

### Depression of sVRP *in vitro* is correlated with antinociception of neonatal rats *in vivo*

Electrical stimulation of the lumbar dorsal root evoked MSR followed by sVRP (Fig. 1A). Dexmedetomidine (3 nM), an α<sub>2</sub>-adrenoceptor

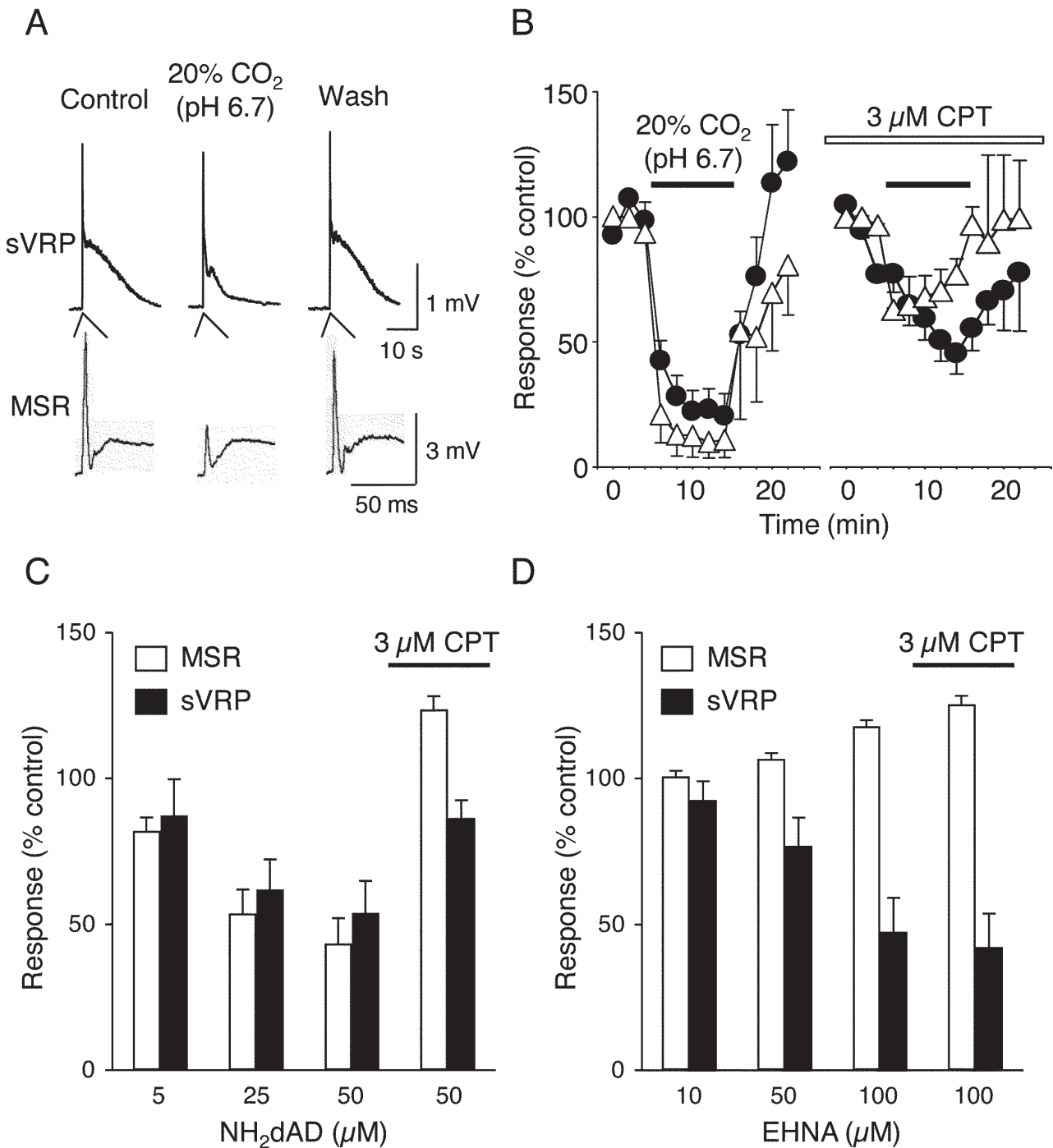


Fig. 2. Effects of hypercapnic acidosis on the spinal cord of the neonatal rat *in vitro*. (A) Representative traces of spinal reflex potentials (sVRP and MSR) before (control), during (20% CO<sub>2</sub>, pH 6.7) and after (wash) hypercapnic acidosis. (B) Effects of CPT on hypercapnic acidosis-evoked depression of sVRP (closed circle) and MSR (open triangle). The mean±S.E.M. (n=4). (C, D) Effects of NH<sub>2</sub>dAD (C) and EHNA (D) on MSR (open columns) and sVRP (closed columns). The mean±S.E.M. (n=4-6). Taken from Otsuguro *et al.* (2006b)

agonist, strongly depressed sVRP but not MSR. This depression was reversed by atipamezole (1 nM),  $\alpha_2$ -adrenoceptor antagonist. As shown in Fig. 1B, dexmedetomidine depressed both sVRP and MSR in a concentration-dependent manner, and sVRP was more sensitive to dexmedetomidine than MSR. Xylazine, another  $\alpha_2$ -adrenoceptor agonist, and morphine also depressed sVRP, but not MSR, in a concentration-

dependent manner. The order of the potency for the depression of sVRP was dexmedetomidine > morphine > xylazine.

To investigate the analgesic effects of  $\alpha_2$ -adrenoceptor agonists and morphine in neonatal rats *in vivo*, the nociceptive response was quantified as body movement detected by an audio speaker. A bolus application of capsaicin (30 ng/body, *s.c.*) in the

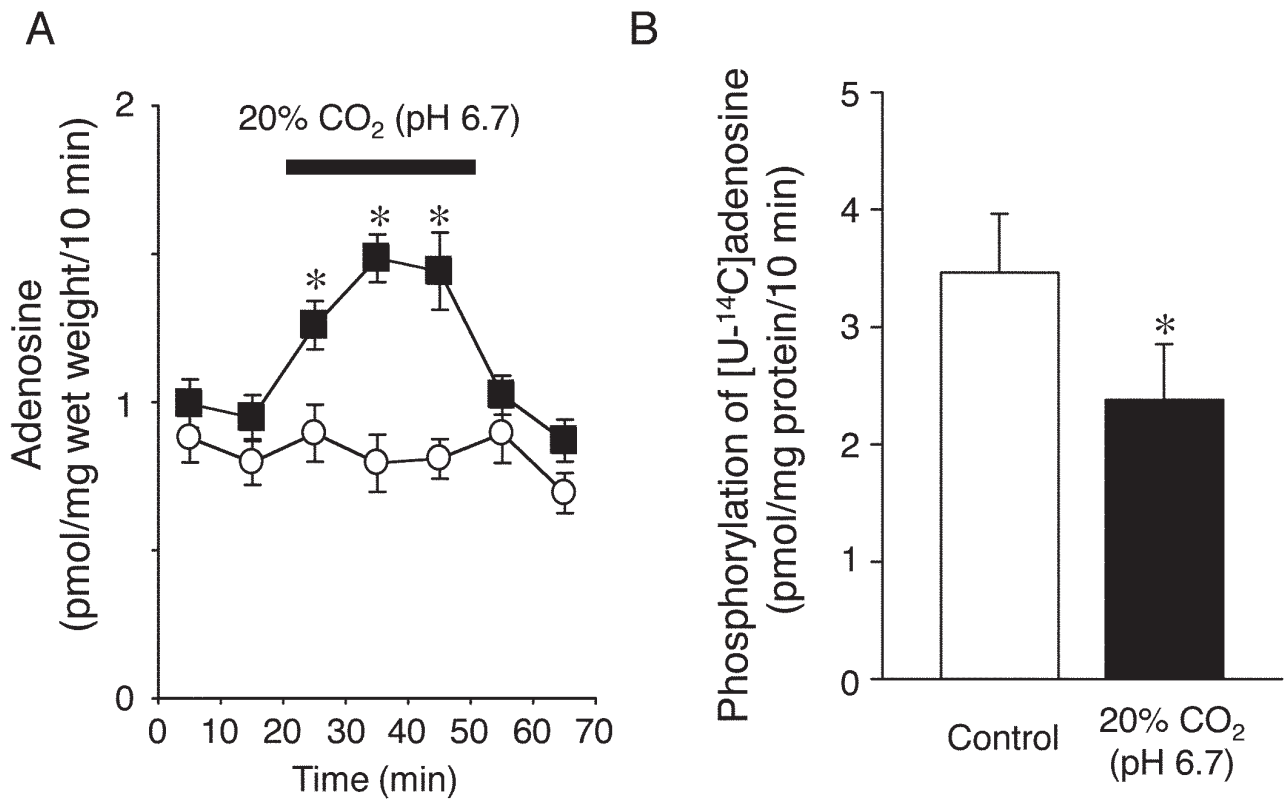


Fig. 3. Extracellular adenosine concentration and adenosine kinase activity in the spinal cord during hypercapnic acidosis. (A) Changes in the extracellular adenosine concentration during hypercapnic acidosis. Each point shows the amount of adenosine in control (open circle) or hypercapnic ACSF (closed square) in which an isolated spinal cord was incubated for 10 min. The mean±S.E.M. (n=6-8). \* $P < 0.05$  vs. control (unpaired Student's *t*-test). (B) Adenosine kinase activity during hypercapnic acidosis. The mean±S.E.M. (n=6). \* $P < 0.05$  vs. control (paired Student's *t*-test). Taken from Otsuguro *et al.* (2006b).

neonatal rats dorsum increased body movement only for 30 s. Dexmedetomidine (100  $\mu\text{g}/\text{kg}$ , *s.c.*) almost abolished the capsaicin-induced body movement (Fig. 1C). Dexmedetomidine, xylazine and morphine inhibited the capsaicin-induced body movement in a dose-dependent manner (Fig. 1D). The order of the potency for the inhibition of the capsaicin-induced body movement was dexmedetomidine > morphine > xylazine. This order for antinociception *in vivo* is similar to that for the depression of sVRP but not MSR *in vitro*.

#### Hypercapnic acidosis depresses spinal reflex potentials via adenosine A<sub>1</sub> receptors

When the concentration of CO<sub>2</sub> in ACSF was increased from 5 to 20%, the pH of ACSF decreased from 7.3 to 6.7 (hypercapnic acidosis). Brief exposure (10 min) of the isolated spinal cord preparation to hypercapnic acidosis immediately depressed both sVRP and MSR (Fig. 2A). This depression was completely recovered 10 min after normal ACSF exposure. Such acute hypercapnic acidosis did not inhibit compound action potentials of the dorsal root or depolarizing responses to exogenously-applied glutamate in this preparation (Otsuguro *et al.*, 2006b), suggesting that hypercapnic acidosis mainly

depressed synaptic transmission, but not action potential conduction or postsynaptic responses, in the spinal cord.

In the presence of a selective adenosine A<sub>1</sub> receptor antagonist, 8-cyclopentylthiohyppiline (CPT), the hypercapnic acidosis-evoked depression of spinal reflex responses was partially reversed (Fig. 2B). Antagonists for adenosine A<sub>2A</sub>, GABA<sub>A</sub>, glycine, opioid and  $\alpha_2$ -adrenergic receptors did not affect the hypercapnic acidosis-evoked depression. These results indicated that the activation of adenosine A<sub>1</sub> receptor was involved in the effects of hypercapnic acidosis in the spinal cord.

#### Accumulation of extracellular adenosine and inhibition of adenosine kinase activity occur during acute hypercapnic acidosis

An ecto-5'-nucleotidase inhibitor failed to reduce the depression by hypercapnic acidosis, suggesting that degradation of ATP released from the spinal cord was not involved (Otsuguro *et al.*, 2006b). Adenosine kinase and deaminase are important enzymes for regulating intracellular and extracellular adenosine concentrations. If this holds in the spinal cord, the inhibition of these enzymes would be expected to cause accumulation of extracellular adenosine. Both

sVRP and MSR were depressed by 5'-amino-5'-deoxyadenosine (NH<sub>2</sub>dAD), an adenosine kinase inhibitor and this depression was reversed by CPT (Fig. 2C). On the other hand, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an adenosine deaminase inhibitor, depressed only sVRP, but this depression was insensitive to CPT (Fig. 2D). Therefore, it seems likely that adenosine kinase is predominantly involved in regulating the adenosine concentration in this preparation. We measured the extracellular concentration of adenosine and adenosine kinase activity in the isolated spinal cord of the neonatal rat during hypercapnic acidosis. As shown in Fig. 3, exposure of the isolated spinal cord of the neonatal rat to hypercapnic acidosis significantly increased the extracellular adenosine concentration. The phosphorylation rate of [U-<sup>14</sup>C]adenosine during hypercapnic acidosis was reduced by about 30% compared to the control.

### Discussion

Slow VRP has been considered to reflect nociceptive responses (Faber *et al.*, 1997). Measurement of reflex potentials in the isolated spinal cord of the neonatal rat seems to be available as an alternative method to measurement of nociceptive responses in living animals. In the present study, although the order of potency of analgesics for inhibitory effects on sVRP *in vitro* was similar to that for antinociceptive effects *in vivo*, slight differences were also observed. Dexmedetomidine was about 30 times more effective than morphine for sVRP, whereas it was only about 4 times more effective for capsaicin-induced body movement. This was probably due to much stronger activation of primary afferent nerve fiber by capsaicin *in vivo* than that by electrical stimulation *in vitro*, and/or additional mechanisms via supraspinal effects *in vivo*. If drugs mainly act on the spinal cord, then this preparation is useful for screening analgesics and investigating analgesic mechanisms in the spinal cord.

Hypercapnia (20% CO<sub>2</sub>) caused a marked decline of pH in the ACSF from 7.3 to 6.7. Brief exposure of the isolated spinal cord to hypercapnic acidosis (20% CO<sub>2</sub>, pH 6.7) immediately and reversibly depressed both sVRP and MSR. CPT, an adenosine A<sub>1</sub> receptor antagonist, partially reduced this depression, suggesting that adenosine was involved in hypercapnic acidosis-evoked depression. This hypothesis was supported by the fact that the concentration of extracellular adenosine increased during hypercapnic acidosis. It has been reported that adenosine inhibits synaptic transmission in the spinal cord via presynaptic adenosine A<sub>1</sub> receptors (Li and Perl, 1994; Nakamura *et al.*, 1997; Lao *et al.*, 2004). Extracellular adenosine accumulated during hypercapnic acidosis is considered to inhibit presynaptic transmitter release via adenosine A<sub>1</sub>

receptors in the isolated spinal cord of the neonatal rat.

In the spinal cord, an ecto-5'-nucleotidase inhibitor had no effect on hypercapnic acidosis-evoked depression of the spinal reflex potentials (Otsuguro *et al.*, 2006b), suggesting that adenosine *per se*, but not ATP, was released during hypercapnic acidosis. Elevation of the intracellular adenosine concentration can cause the release of adenosine to the extracellular space. Adenosine kinase is a key enzyme for regulating adenosine levels in the rat spinal cord (Golembiowska *et al.*, 1996). In our experiments, an adenosine kinase inhibitor mimicked the inhibitory effects of hypercapnic acidosis, and adenosine kinase activity decreased during the hypercapnic acidosis. These results suggest that the inhibition of adenosine kinase activity is involved in the hypercapnic acidosis-evoked depression of the spinal reflex potentials in neonatal rats. It is still unclear how hypercapnic acidosis suppresses the activity of adenosine kinase. In addition, there are other mechanisms for the hypercapnic acidosis-evoked depression because an adenosine A<sub>1</sub> receptor antagonist failed to abolish the depression. The isolated spinal cord of the neonatal rat may also be useful as an alternative method to living animals for further investigation of pathological conditions during hypercapnic acidosis in the spinal cord.

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### References

- Conlee K.M., Stephens M.L., Rowan A.N. and King L.A. (2005) Carbon dioxide for euthanasia: concerns regarding pain and distress, with special reference to mice and rats, *Lab. Anim.*, 39, 137-161.
- Faber E.S.L., Chambers J.P., Brugger F. and Evans R.H. (1997) Depression of A and C fibre-evoked segmental reflexes by morphine and clonidine in the *in vitro* spinal cord of the neonatal rat, *Br. J. Pharmacol.*, 120, 1390-1396.
- Golembiowska K., White T.D. and Sawynok J. (1996) Modulation of adenosine release from rat spinal cord by adenosine deaminase and adenosine kinase inhibitors, *Brain Res.*, 699, 315-320.
- Ives D.H., Durham J.P. and Tucker V.S. (1969) Rapid determination of nucleotide kinase and nucleotidase activities with tritium-labeled substrates, *Anal. Biochem.*, 28, 192-205.
- Kawamoto Y., Shinozuka K., Kunitomo M. and Haginaka J. (1998) Determination of ATP and its metabolites released from rat caudal artery by isocratic ion-pair reversed-phase high-performance liquid chromatography, *Anal. Biochem.*, 262, 33-38.

- Kubota K., Takahashi T., Yanagisawa M., Fujibayashi K. and Saito K. (1996) A novel method for detecting the analgesic effect of drugs by quantifying body movement after noxious stimulation in neonatal rat, *J. Pharmacol. Toxicol. Methods*, 40, 33-37.
- Konishi S. and Otsuka M. (1974) Electrophysiology of mammalian spinal cord in vitro, *Nature*, 252, 733-734.
- Lao L.J., Kawasaki Y., Yang K., Fujita T. and Kumamoto E. (2004) Modulation by adenosine of A $\delta$  and C primary-afferent glutamatergic transmission in adult rat substantia gelatinosa neurons, *Neuroscience*, 125, 221-231.
- Lynch J.J. III, Alexander K.M., Jarvis M.F. and Kowaluk E.A. (1998) Inhibition of adenosine kinase during oxygen-glucose deprivation in rat cortical neuronal cultures, *Neurosci. Lett.*, 252, 207-210.
- Li J. and Perl E.R. (1994) Adenosine inhibition of synaptic transmission in the substantia gelatinosa, *J. Neurophysiol.*, 72, 1611-1621.
- Nakamura I., Ohta Y. and Kemmotsu O. (1997) Characterization of adenosine receptors mediating spinal sensory transmission related to nociceptive information in the rat. *Anesthesiology*, 87, 577-584.
- Otsuguro K., Yasutake S., Ohta T. and Ito S. (2005) Effects of opioid receptor and  $\alpha_2$ -adrenoceptor agonists on slow ventral root potentials and on capsaicin and formalin tests in neonatal rats, *Dev. Brain Res.*, 158, 50-58.
- Otsuguro K., Ohta T. and Ito S. (2006a) Zinc modulates primary afferent fiber-evoked responses of ventral roots in neonatal rat spinal cord *in vitro*, *Neuroscience*, 138, 281-291.
- Otsuguro K., Yamaji Y., Ban M., Ohta T. and Ito S. (2006b) Involvement of adenosine in depression of synaptic transmission during hypercapnia in isolated spinal cord of neonatal rats, *J. Physiol.*, 574, 835-847.