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# The role of intraspinal adenosine $A_1$ receptors in sympathetic regulation

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**Abstract**

Using a splanchnic nerve-spinal cord preparation in vitro, we have previously demonstrated that tonic sympathetic activity is generated from the thoracic spinal cord. Here, we sought to determine if adenosine receptors play a role in modulating this spinally generated sympathetic activity. Various adenosine analogs were applied. $N^6$-Cyclopentyladenosine (CPA, adenosine $A_1$ receptor agonist) and 5′-*N*-ethylcarboxamidoadenosine (NECA, adenosine $A_1/A_2$ receptor agonist) reduced, while $N^6$-[2-(4-aminophenyl)ethyl]adenosine (APNEA, non-selective adenosine $A_3$ receptor agonist) did not alter sympathetic activity. The inhibitory effect of CPA or NECA on sympathetic activity was reversed by 8-cyclopentyltheophylline (CPT, adenosine $A_1$ receptor antagonist) or abolished by CPT pretreatment. In the presence of 3,7-dimethyl-1-propargylxanthine (DMPX, adenosine $A_2$ receptor antagonist), sympathetic activity was still reduced by CPA or NECA. Sympathetic activities were not changed by applications of the more selective adenosine $A_2$ or $A_3$ receptor agonists or antagonists, including 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid (CGS21680), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), 2-chloro-$N^6$-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Chloro-IB-MECA), and 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191). These findings exclude a possible involvement of $A_2$ or $A_3$ receptors in sympathetic regulation at the spinal levels. Interestingly, CPT alone did not affect sympathetic activity, suggesting that adenosine $A_1$ receptors are endogenously quiescent under our experimental conditions. We conclude that intraspinal adenosine $A_1$ receptors may down-regulate sympathetic outflow and serve as a part of the scheme for neuroprotection.


*Keywords:* Autonomic nervous system; Hypertension; Neonatal, rat; Spinal cord

## 1. Introduction

Our previous studies have indicated that the generation of tonic sympathetic activity is intrinsic to the thoracic spinal cord (Su et al., 2003). Using an in vitro splanchnic nerve-spinal cord of neonatal rats, we have shown that the generation of sympathetic activity from the spinal cord requires $Ca^{2+}$-dependent synaptic transmission and is mainly driven by several amino acid neurotransmitter activities (Su, 2001). This relatively novel experimental model has provided us with an alternative way to probe various neural mechanisms underlying the sympathetic regulation at the spinal levels.

Upon many forms of insults to the central nervous system (CNS), release of adenosine can counteract the subsequent stress responses (Bantel et al., 2002; Lloyd et al., 1989; McAdoo et al., 2000). Adenosine, a product of ATP breakdown through the ecto-5′-nucleotidase cascade (Patterson et al., 2001; Salter et al., 1993), often serves as a neuroprotective agent and has been shown to affect sympathetic activities (Biaggioni et al., 1991; Coney and Marshall, 2003; Ekas et al., 1981; Fuglsang et al., 1989; Ralevic, 2000; Scislo and O'Leary, 2002; Stella et al., 1998; Tandon and Collier, 1995). Adenosine $A_1$, $A_{2A/B}$, and $A_3$ receptors have been identified. By coupling with Gi/s protein, adenosine $A_1$ and $A_3$ receptors may inhibit while adenosine $A_2$ receptors activate adenylate cyclase. In the rat spinal cord, adenosine $A_1$ and $A_2$ receptors are present (Choca et al., 1987). Activation of intraspinal adenosine $A_1$ receptor causes a cardioinhibitory effect (Koh et al., 1996), probably via the direct attenuation of excitatory inputs to sympathetic

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preganglionic neurons (Deuchars et al., 2001). Although adenosine $A_1$ and $A_2$ had opposite effects on the activity of adenylate cyclase, it was peculiar that an activation of intraspinal adenosine $A_2$ receptors produced similar responses to that of adenosine $A_1$ receptors in decreasing blood pressure and heart rate (Koh et al., 1996, 2000).

In the present studies, we aim to determine the roles of adenosine receptors in modulating the spinally generated sympathetic activity, especially to clarify a possible involvement of adenosine $A_2$ receptors in sympathetic regulation. Our results revealed that a quiescent adenosine $A_1$ receptor in the spinal cord might reduce sympathetic outflow, while adenosine $A_2$ or $A_3$ receptors were not related to sympathetic regulation at the spinal levels.

## 2. Materials and methods

### 2.1. General procedures

Neonatal Sprague–Dawley rats (postnatal days 1–7) were used in this study and all the experimental protocols were approved by institutional animal care and utilization committee (IACUC, Academia Sinica, Protocol#: PRaIBMSC2003014). The general procedures in preparing a thoracic spinal cord have been previously described (Su, 2001). Briefly, the neural tissue extending from T1 to T12 spinal segments was immersed in oxygenated icy-cold artificial cerebrospinal fluid (aCSF; in mM: 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 24 $NaHCO_3$, 0.5 $NaH_2PO_4$, 30 D-glucose, and 3 ascorbate; equilibrated with 95% $O_2$–5% $CO_2$). Under a dissecting microscope, the distal ends of the splanchnic nerves were cut at a level proximal to the celiac ganglion. A suction electrode was then placed on the nerve bundles to record compound action potentials. During experiments, this splanchnic nerve-thoracic spinal cord preparation was kept in a bath chamber that contained 30 ml freshly oxygenated aCSF and maintained at $24.5 \pm 1$ °C. Neural signals from splanchnic sympathetic activity were amplified, filtered (WPI, DAM50; bandpass: 0.1–1 kHz), and stored on a PCM-tape recorder (Neuro-Corder, DR-886). The envelope of sympathetic activity was acquired by a leaky integrator (discharging time constant: 15 ms) to display its oscillating pattern. A time-based integrator (Gould, 13-4615-70, resetting every 5 s) was used to measure the total sympathetic activity. At the end of each experiment, the background noise of neural recording was determined after raising KCl concentration in the bath solution (final concentration: 100 mM) to obtain a depolarizing-blockade of neural activities. True neural signals were obtained by subtracting the recorded signals from the background noise.

### 2.2. Drugs and applications

Four adenosine analogs purchased from Sigma were used as agonists: $N^6$-Cyclopentyladenosine (CPA, adenosine $A_1$ receptor agonist), 5′-*N*-ethylcarboxamidoadenosine (NECA, adenosine $A_1/A_2$ receptor agonist (Choca et al., 1987)), 2-chloro-$N^6$-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Chloro-IB-MECA, adenosine $A_3$ receptor agonist), and $N^6$-[2-(4-aminophenyl)ethyl]adenosine (APNEA, adenosine $A_3$ receptor agonist). The more selective adenosine $A_2$ receptor agonist, 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS21680), and its antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), were purchased from Tocris. Another three drugs purchased from Sigma were used as antagonists: 8-cyclopentyltheophylline (CPT, adenosine $A_1$ receptor antagonist), 3,7-dimethyl-1-propargylxanthine (DMPX, adenosine $A_2$ receptor antagonist), and 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191, adenosine $A_3$ receptor antagonist). To prepare concentrated solutions, drugs were dissolved in water (in mM: 3 CPA, 5 NECA, 1 CGS21680, 1 APNEA, 0.5 CPT, and 2 DMPX) or DMSO (in mM: 0.1 Chloro-IB-MECA, 1 ZM241385, and 1 MRS1191). Concentrated solutions were stored at −20 °C and thawed in room temperature prior to applications. A final concentration of drugs in micromolar ranges was achieved by adding an aliquot of concentrated solution directly into the bath chamber. If effective, changes in sympathetic activity were discernible within ~3 min. Following each drug application, a period of 15 min was allowed for equilibrium. The dose–response tests were conducted by cumulatively raising drug concentrations in the bath solution. In some experiments, the preparation was incubated in 1 μM CPT or 8 μM DMPX for 15 min before CPA or NECA was added into the bath solution. Such a pretreatment procedure was used to further verify the specificity of agonistic effects.

### 2.3. Data analysis

The height of time-based integration of sympathetic activity under control condition was taken as 100% activity. Drug effects on sympathetic activity were then calculated as percent changes from the control activity. The effectiveness of drugs on changing sympathetic activity in a dose-dependent manner was evaluated first by analysis of variance (ANOVA) followed by the post hoc multiple comparison, using adjusted *t*-tests with *P*-values corrected by Bonferroni procedure. A *P*-value less than 0.05 was considered significant. All values are presented as means ± S.E.M.

## 3. Results

### 3.1. Inhibition of spinal sympathetic activity by CPA or NECA, but not by CGS21680, APNEA, or chloro-IB-MECA

Three adenosine analogs, including CPA, NECA, and APNEA, were applied in our earlier trials to test which types

of adenosine receptors could affect the sympathetic activity that was spontaneously generated by the spinal cord of neonatal rats in vitro. The selective adenosine $A_1$ receptor agonist, CPA, has been shown to inhibit synaptic transmission in cortical neurons at a concentration $\leq 10$ μM (Brand et al., 2000). Fig. 1 shows an example that bath application of CPA (0.5–8 μM) reduced sympathetic activity in a dose-dependent manner. Fig. 2 summarizes the effects induced by applications of CPA, NECA, or APNEA on total sympathetic activities. At a concentration as low as 0.5 μM, CPA slightly reduced sympathetic activity to $70 \pm 10\%$ of the control activity ($P = 0.149$; $n = 8$). The reduction of sympathetic activity became statistically significant in the presence of 1 μM CPA ($P < 0.001$). As concentrations of CPA increased from 1 to 8 μM, sympathetic activity further decreased and reached its minimum at 2–4 μM CPA. In the presence of 8 μM CPA, only $26 \pm 5\%$ ($n = 8$) sympathetic activity remained.

The adenosine $A_3$ receptor agonist, APNEA, at a concentration $\leq 0.3$ μM has been shown to potentiate high-threshold calcium current in hippocampal CA3 pyramidal neurons (Fleming and Mogul, 1997). Here, using the same time course of drug applications as in the CPA tests, we investigated whether 0.5–8 μM APNEA would affect the sympathetic activity. In contrast to the inhibitory effects of CPA on the sympathetic activity, applications of APNEA up to 8 μM did not cause discernible effects (Fig. 2). Table 1 summarizes the effects of all the drugs tested in the present studies that fail to cause significant changes in sympathetic activities.

NECA has been used as a mixed adenosine receptor agonist that acts on both $A_1$ and $A_2$ subtypes of adenosine receptors (Bruns et al., 1986; Mauborgne et al., 2002). At a concentration of 10 nM or $\sim$ 10 μM, NECA either caused a membrane depolarization or reduced noradrenergic inhibitory postsynaptic potentials in the submucosal neurons of guinea pig by acting via the postsynaptic adenosine $A_2$ and presynaptic adenosine $A_1$ receptors, respectively (Barajas-Lopez et al., 1991). Herein, bath application of 0.5–8 μM NECA reduced sympathetic activity in a dose-dependent manner (Fig. 2). Compared to the effects of CPA, NECA appeared to be less potent in reducing sympathetic activity. NECA at a concentration of 0.5–2 μM did not reduce sympathetic activity significantly. When the concentration was increased to 8 μM, NECA reduced sympathetic activity to $39 \pm 8\%$ of control activity. The Bonferroni test revealed

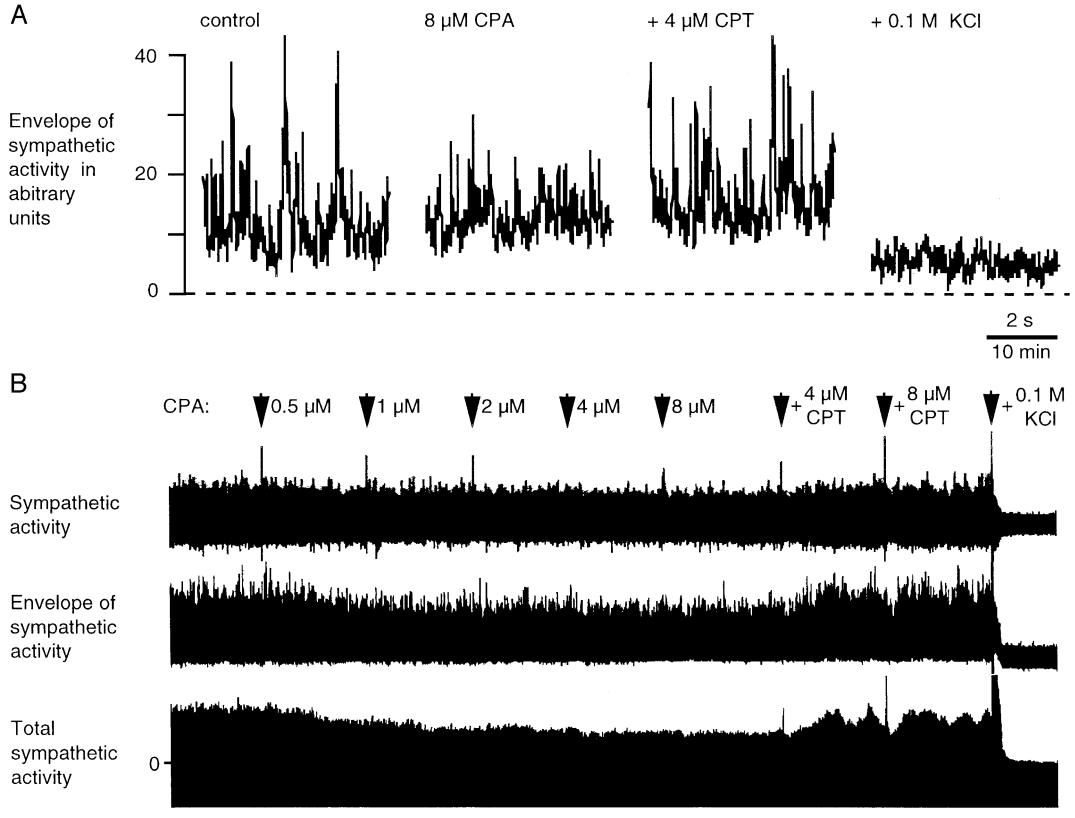


Fig. 1. Original traces show that CPT reverses the CPA-induced reduction of spinal sympathetic activity. (A) Fast traces of the envelope of sympathetic activity show a reduced oscillation of sympathetic activity after CPA (adenosine $A_1$ receptor agonist), which is reversed by adding 4 μM CPT (adenosine $A_1$ receptor antagonist). The background noise of sympathetic activity recording was determined after adding 100 mM KCl to the bath solution. (B) Slow traces show the time course of the reduction of sympathetic activity after cumulatively raising the concentration of CPA in the bath solution. Arrowheads indicate the timing of drug applications. Note that 4 and 8 μM CPT produced comparable effects. The quantitative analysis of changes of sympathetic activity was based on the changes of total sympathetic activity.

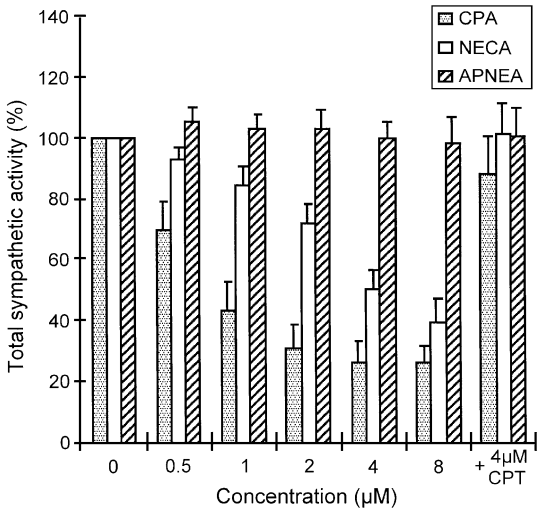


Fig. 2. Effects of different adenosine receptor agonists on the total sympathetic activity. Total sympathetic activity was reduced in a dose-dependent manner by CPA ($n=8$) or NECA (adenosine $A_1/A_2$ receptor agonist, $n=7$), but not by APNEA (adenosine $A_3$ receptor agonist, $n=6$). A further addition of CPT reversed the reduction of sympathetic activity induced by either CPA ($n=4$) or NECA ($n=5$).

that CPA was more potent than NECA in reducing sympathetic activity ($P<0.01$) at lower doses (1–2 μM). At 8 μM, NECA was comparably as effective as CPA in reducing sympathetic activity ($P=0.623$, by Bonferroni test). In rat striatal membranes, CPA is the most $A_1$-sensitive agonist and gives the most satisfactory results to eliminate the $A_1$ binding of [$^3$H] NECA (Bruns et al., 1986). In agreement with our observations here, the rank order of potency for reduction of sympathetic activity: CPA>NECA also suggests a role of adenosine $A_1$ receptors.

To further clarify whether or not there was an effect elicited by the agonist of adenosine $A_2$ or $A_3$ receptors, the more selective agonist, i.e., CGS21680 or Chloro-IB-MECA, was applied. CGS21680 at a concentration as low as 0.03 μM is effective to activate adenosine $A_2$ receptors (O'Kane and Stone, 1998). At 1 μM, CGS21680 has been demonstrated to change the efficacy of synaptic transmission in the dorsal horn neurons of the adult rat spinal cord (Patel et al., 2001) or inhibit NMDA-induced current in neostriatal neurons (Nörenberg et al., 1997). On the other hand, 0.1–1 μM Chloro-IB-MECA has been used to activate the adenosine $A_3$ receptors in hippocampal CA1 neurons (Dunwiddie et al., 1997). In our tests, bath application of 0.1–4 μM CGS21680 or 0.1–0.8 μM Chloro-IB-MECA to the spinal cord did not cause a significant change in sympathetic activities (Table 1).

### 3.2. CPT reversed the sympathetic activity reduction induced by CPA or NECA

Subsequent to the dose–response tests of adenosine analogs, CPT was further applied to examine if the agonist-induced sympathetic activity reduction could be reversed. An addition of 4 μM CPT consistently reversed the sympathetic activity reduction induced by 8 μM CPA or NECA (Figs. 1 and 2). By antagonizing CPA or NECA effects, CPT could restore sympathetic activity back to a level comparable to that under control conditions (sympathetic activity in the presence of CPT–CPA and CPT–NECA: 88 ± 13% and 102 ± 12%, respectively). In contrast, in the presence of 8 μM APNEA while sympathetic activity remained unaffected, CPT did not exert any discernible effect.

### 3.3. CPT but not DMPX pretreatment significantly attenuated CPA- or NECA-induced reduction of sympathetic activity

Since CPT could reverse the CPA- or NECA-induced reduction of sympathetic activity (Fig. 2), we further clarified whether the effect of these agonists was mainly attributed to an activation of $A_1$-adenosine receptors. In this series of experiments, the water-soluble antagonist, CPT or DMPX, was added into the bath solution prior to CPA or NECA application. DMPX at 10 μM has been used as an effective adenosine $A_2$ receptor antagonist (Cunha et al., 1995), although its cross-reactivity may mildly block the responses elicited by $A_1$-adenosine agonist (Seale et al., 1998). Preliminary trials indicated that neither did 0.5–8 μM CPT nor DMPX pretreatment significantly alter sympathetic activity (Table 1). Under control conditions when CPT was not added into the bath solution, applications of CPA caused a concentration-dependent reduction of sympathetic activity (Fig. 3A). In the presence of only 1 μM CPT, an application of CPA up to 8 μM did not reduce sympathetic activity (Fig. 3A; ANOVA: $P=0.742$). In contrast, a pretreatment of 1 μM DMPX did not affect CPA-induced reduction of sympathetic activity (data not shown). When 8 μM DMPX was added into the bath solution, the reduction of sympathetic activity induced by CPA applications was slightly attenuated but still significant (Fig. 3A; ANOVA: $P<0.001$). Thus, CPT is more potent than DMPX in antagonizing the CPA-induced reduction of sympathetic activity. Similar tests were

Table 1
Effects of the drugs that fail to cause significant changes in total sympathetic activity

| Drug | Concentration (μM) | Total sympathetic activity (%) | *n* |
|---|---|---|---|
| APNEA | 8 | 98 ± 9 | 6 |
| CGS21680 | 4 | 91 ± 7 | 5 |
| Chloro-IB-MECA | 0.8 | 94 ± 11 | 6 |
| CPT | 8 | 116 ± 11 | 9 |
| DMPX | 8 | 116 ± 5 | 5 |
| MRS1191 | 8 | 118 ± 15 | 9 |
| ZM241385 | 4 | 110 ± 7 | 7 |

Total sympathetic activities prior to drug applications were considered as 100%. Drugs were applied directly into the bath solution to achieve a final concentration as indicated above. Data were presented as the mean ± S.E.M.

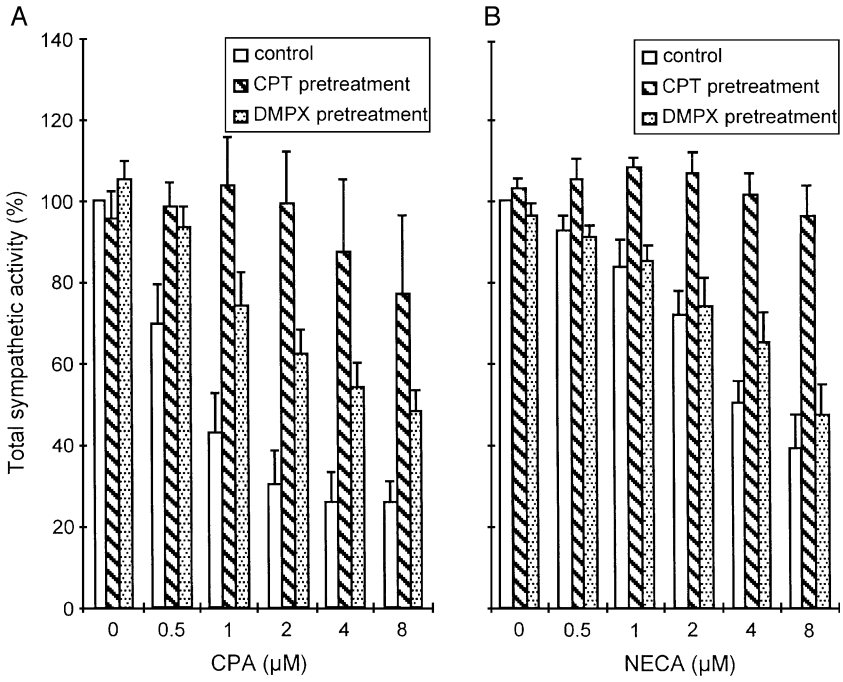


Fig. 3. Effects of CPT or DMPX pretreatment on CPA- or NECA-induced sympathetic activity reduction. The pretreatment was achieved by applying 1 μM CPT or 8 μM DMPX. (A) CPT almost abolishes while DMPX mildly attenuates CPA-induced sympathetic activity reduction ($n = 6–8$). (B) CPT abolishes while DMPX does not affect NECA-induced reduction of sympathetic activity ($n = 7$).

also conducted to evaluate NECA effects. As shown in Fig. 3B, the NECA-induced reduction of sympathetic activity was also attenuated by CPT (ANOVA: $P = 0.708$), but not by DMPX (ANOVA: $P < 0.001$).

### *3.4. ZM241385 or MRS1191 did not affect sympathetic activity*

By application of selective antagonist alone, we further determined whether or not there were endogenous activities of adenosine $A_2$ or $A_3$ receptors in the spinal cord, which might mask their agonistic effects. ZM241385 is a selective adenosine $A_2$ receptor antagonist. At 0.1 μM, ZM241385 is effective to prevent the effects induced by 0.03 μM CGS21680 (Nikbakht and Stone, 2001). MRS1191 at a concentration of 10 μM has been used to antagonize the effect of 1 μM Chloro-IB-MECA (Dunwiddie et al., 1997). In our tests, bath application of 0.1–4 μM ZM241385 or 1–8 μM MRS1191 did not cause significant changes in sympathetic activities (Table 1).

## 4. Discussion

Using an in vitro splanchnic nerve-spinal cord preparation, we have applied various adenosine receptor agonists and antagonists to determine which types of adenosine receptors could regulate sympathetic outflow at the spinal levels. Our results demonstrated that an exogenous activation of adenosine $A_1$ receptors in the spinal cord reduced sympathetic activity. Notably, under our in vitro conditions, these adenosine $A_1$ receptors are quiescent, as revealed by the lack of effects of their antagonists. Although we have sought to determine a possible involvement of adenosine $A_2$ or $A_3$ receptors in modulating sympathetic activity, we failed to find significant changes elicited by applications of their selective agonists or antagonists. The effect of a likely $A_2$ receptor agonist, NECA, was attenuated by $A_1$ but not by $A_2$ receptor antagonist. These observations clearly indicate that $A_1$, but not $A_2$ or $A_3$, is the main type of adenosine receptors responsible for regulating spinal sympathetic activity.

In in vivo studies, intrathecal injections of either adenosine $A_1$ or $A_2$ receptor agonist have been reported to decrease blood pressure and heart rate (Koh et al., 1996; Koh et al., 2000). These studies implied the existence of spinal $A_1$ and $A_2$ receptors in down-regulating sympathetic outflow. Using the present in vitro experimental model, we have confirmed the existence of spinal $A_1$ receptors in regulating sympathetic activity. However, we failed to find a significant role of spinal $A_2$ receptors in sympathetic regulation. One of the advantages in conducting experiments under in vitro conditions as we have done here is the ease of controlling the working concentration of drugs and assuring the specificity of agonists by challenging with various antagonists. In contrast, intrathecal regime usually requires a high dose of drugs to elicit discernible responses. This inherent difficulty in methodology may not guarantee the drug specificity and, thus, explains the discrepancy. Alternatively, a possible involvement of $A_2$ receptors in

sympathetic regulation at the spinal levels might depend on the supraspinal inputs, which would not be assessable in our experimental model.

It is intriguing how spinal $A_1$ receptors reduce sympathetic activity. In the CNS, an activation of $A_1$ receptors often leads to the reduction of synaptic transmission (Flagmeyer et al., 1997; Marchi et al., 2002; Oliet and Poulain, 1999). Similar to the observations in the hippocampus (Lambert and Teyler, 1991), a nice work presented by Deuchars et al. (2001) has shown that spinal adenosine $A_1$ receptors reduce excitatory but not inhibitory synaptic inputs onto the lateral horn neurons. Deuchar et al. using electron microscopy also revealed the immunoreactivity of adenosine $A_1$ receptors within both pre- and postsynaptic structures in the intermediolateral cell column (Deuchars et al., 2001). We have previously described that the spinal sympathetic activity is generated, at least partly, by the interplay of γ-amino-butyric acid (GABA), glycine, and glutamate amino acid neurotransmitter activities (Su, 2001). In the caudal part of nucleus tractus solitarius, presynaptic adenosine $A_1$ receptors reduce the glutamate release from the primary afferent terminals (Kato and Shigetomi, 2001). Taken together, it is plausible that, when intraspinal adenosine $A_1$ receptors are activated, glutamatergic synaptic neurotransmission is reduced, leading to a reduced sympathetic activity as we have observed here. However, in our previous attempts to block the activities of ionotropic glutamate receptor by kynurenate, we observed an unstable sympathetic activity, rather than a simple reduction of sympathetic activity as it was incurred by adenosine $A_1$ receptors (Su, 2001). Thus, a presynaptic action of adenosine $A_1$ receptors in reducing excitatory neurotransmission may not be sufficient to reduce spinal sympathetic activity. Another possibility why spinal sympathetic activity was reduced by activating adenosine $A_1$ receptors might be due to a direct postsynaptic effect. In superior cervical ganglion neurons, adenosine acts on their adenosine $A_1$ receptors to produce a voltage-dependent inhibition of $Ca^{2+}$ currents (Zhu and Ikeda, 1993). In CA3 hippocampal neurons, adenosine $A_1$ receptors by reducing adenylate cyclase activities may enhance the $Ca^{2+}$-dependent $K^+$-mediated afterspike hyperpolarizing current (Gerber and Gähwiler, 1994). Such a direct postsynaptic effect of adenosine $A_1$ receptor that reduces neuronal excitability may consequently play a major role in modulating sympathetic outflow at the spinal level.

In summary, we have presented evidence suggesting that an activation of endogenously quiescent $A_1$ receptors in the spinal cord can reduce sympathetic outflow. We have also excluded a direct involvement of $A_2$ and $A_3$ receptors in sympathetic regulation at the spinal levels. It is foreseeable then that a surge of metabolic demands under extreme conditions may trigger the release of endogenous adenosine. By eliciting an overall reduction of sympathetic activity via adenosine $A_1$ receptors, adenosine may down-regulate vasomotor tone and other visceral functions, thereby signifying the sympathetic system to react as part of the protective machinery in case of emergency.

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