

Adenosine Reduces Glutamate Release in Rat Spinal Synaptosomes

Xinhui Li, Ph.D.,* James C. Eisenach, M.D.†

Background: A₁ adenosine receptor activation reduces hypersensitivity in animal models of chronic pain, but intrathecal adenosine does not produce analgesia to acute noxious stimuli. Here, the authors test whether increased inhibition by adenosine of glutamate release from afferents after injury accounts for this difference.

Methods: Synaptosomes were prepared from the dorsal half of the lumbar spinal cord of normal rats or those with spinal nerve ligation. Glutamate release evoked by the TRPV-1 receptor agonist, capsaicin, was measured. Adenosine with or without adenosine A₁ and A₂ receptor antagonists was applied to determine the efficacy and mechanism of adenosine to reduce capsaicin-evoked glutamate release.

Results: Capsaicin produced a concentration-dependent glutamate release similarly in normal and nerve-injured rats. Capsaicin-evoked glutamate release was inhibited by adenosine or R-PIA (R-N6-(2-phenylisopropyl)-adenosine) in a concentration-dependent manner, with a threshold of 10 nM in both normal and nerve-ligated synaptosomes. Blockade of capsaicin-evoked glutamate release by adenosine was reversed similarly in synaptosomes from normal and spinal nerve-ligated animals by an A₁ adenosine receptor antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine) but not by an A₂ adenosine receptor antagonist DMPX (3',7'-dimethyl-1-propargylxanthine). Capsaicin-evoked glutamate release, as well as its inhibition by adenosine, did not differ between synaptosomes prepared from tissue ipsilateral and contralateral to spinal nerve ligation.

Conclusion: These observations confirm previous neurophysiologic studies that presynaptic adenosine A₁ receptor activation inhibits glutamate release from primary afferents. This effect is unaltered after peripheral nerve injury and thereby is unlikely to account for the enhanced analgesic efficacy of intrathecal adenosine in this setting.

ADENOSINE and synthetic adenosine receptor agonists produce antinociception in a broad range of pain models in animals, including hypersensitivity from nerve injury and inflammation.¹ Interestingly, intrathecal injection of adenosine itself does not produce analgesia to acute noxious stimuli^{2,3} but reduces hypersensitivity induced experimentally or in patients with chronic pain.^{4,5} The primary aim of the current study was to determine whether adenosine-mediated inhibition of spinal glutamate release was increased in an animal model of neuropathic pain, potentially providing an explanation for

this selective effect of intrathecal adenosine in hypersensitive states.

Glutamate is released from primary afferents and is a key neurotransmitter both for nociceptive transmission and sensitization. As such, intrathecal injection of glutamate or other excitatory amino acids produces aversive responses and hyperalgesia in animals.^{6–9} Peripheral noxious stimulation increases the release of glutamate from the rat dorsal spinal cord *in vivo*,¹⁰ whereas pharmacologic and electrophysiologic studies indicate that glutamate receptor antagonists produce antinociceptive effects in rodents and humans.^{9,11–14}

As an excitatory amino acid, glutamate is stored in and released from synaptic vesicles, which are widespread in all spinal cord laminae,¹⁵ including from primary afferent terminals in the superficial and deep dorsal horn.^{16,17} Glutamate could be released from a variety of sources besides primary afferents. The current study focused on glutamate release evoked by capsaicin from spinal cord synaptosomes, which primarily stimulates small primary afferent terminals in the superficial dorsal horn *via* TRPV1 channel activation.^{18–20}

Four different adenosine receptor subtypes have been defined. Adenosine reduces pain transmission mainly through A₁ receptor activation, whereas activation of adenosine A_{2A}, A_{2B}, and A₃ receptor subtypes induces nociception,^{21–23} and mice lacking the A_{2A} and A₃ receptor genes have been shown to be hypoalgesic.^{22,24} However, the mechanisms by which A₁ adenosine receptor agonists cause pain relief are incompletely understood. A₁ adenosine receptor stimulation hyperpolarizes spinal cord dorsal neurons by increasing K⁺ channel conductance.²⁵ Whether A₁ adenosine receptors also act presynaptically to inhibit excitatory neurotransmitter release is unclear. Whole cell patch clamp studies of laminae II neurons demonstrate an inhibition of glutamate release by a presynaptic mechanism by adenosine and A₁ adenosine receptor agonists.^{25,26} In contrast, intrathecal injection of morphine but not an A₁ adenosine receptor agonist reduces glutamate spillover into cerebrospinal fluid in rats after intraplantar injection of formalin, although both drugs reduce pain behavior.²⁷ Similarly, release of substance P and calcitonin gene-related peptide, as markers of C-fiber activity in spinal cord, is reduced by A₁ adenosine receptor agonists in some studies but not in others.^{28–31}

The current study uses a different approach, examining the ability of adenosine to alter capsaicin-evoked glutamate release from spinal cord synaptosomes, which allows for direct study of presynaptic effects in cell

* Assistant Professor of Anesthesiology, † F.M. James, III Professor of Anesthesiology.

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Address reprint requests to Dr. Eisenach: Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina, 27157. Address electronic mail to: jim@eisenach.us. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

populations. A strength of the synaptosomal preparation is the ability to examine *in vitro* a highly enriched subset of synaptic contacts, those that express transient receptor potential V-1 (TRPV-1) receptors and contain glutamate. We chose capsaicin as the stimulant for afferent terminal glutamate release in the current study, because TRPV-1 receptors are maintained or increased in injured³² and adjacent uninjured³³ afferents. In addition, adenosine receptor antagonists were used to study the role of adenosine receptor subtypes in altering glutamate release.

Materials and Methods

Spinal Nerve Ligation

After Animal Care and Use Committee approval from Wake Forest University School of Medicine (Winston-Salem, North Carolina), male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 180–200 g underwent spinal nerve ligation (SNL) during halothane anesthesia as previously described.³⁴ The left L5 and L6 spinal nerves were isolated adjacent to the vertebral column and tightly ligated with 6-0 silk sutures distal to the dorsal root ganglion. After surgery, animals were housed individually with free access to food and water and allowed to recover for at least 2 weeks. Left paw tactile allodynia was confirmed at this time by measuring the hind paw withdrawal threshold in response to application of von Frey filaments, using an up-down method previously described.³⁵ Only animals with a withdrawal threshold less than 4 g after SNL surgery were used. Rats showing neurologic deficits were immediately killed by an overdose of pentobarbital.

Synaptosome Preparation

Spinal nerve-ligated rats or male normal Sprague-Dawley rats (250 g) were used for all experiments. Animals were deeply anesthetized with 1.5–2.1% halothane and then decapitated. The spinal cord was quickly removed and placed in aerated (with 95% O₂–5% CO₂) ice-cold modified Krebs-Ringer's buffer containing 135 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 12.5 mM HEPES, and 10 mM glucose, at pH 7.4. For studies in normal animals, the dorsal half of the lumbar spinal cord was dissected from two rats and homogenized in 14 ml ice-cold sucrose, 0.32 M, and a crude synaptosomal pellet (P₂) was prepared by differential centrifugation with 1,000g for 5 min followed by 15,000g for 20 min as previously described.^{20,36} For glutamate release assay in spinal nerve-injured rats, synaptosomes of lumbar dorsal horn quadrants ipsilateral and contralateral to the nerve injury were collected and prepared similarly from four rats in each experiment.

Glutamate Release in Synaptosomes

Glutamate release in synaptosomes was performed as previously described.²⁰ In all synaptosomal experiments, the P₂ pellet was resuspended in 8 ml modified Krebs-Ringer's buffer, aerated with 95% O₂–5% CO₂, and incubated at 37°C for 30 min. The suspension was then centrifuged at 12,000g at 37°C for 4 min, and the resultant pellet was resuspended in 4.5 ml Krebs-Ringer's buffer and aliquoted into a 96-well microplate with 100 μ l in each well. This synaptosome suspension or buffer containing standard concentrations of glutamate (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 μ M) were added to a buffer solution of 150 μ l containing β -nicotinamide adenine dinucleotide (final concentration of 0.5 mM), glutamate dehydrogenase (final concentration of 1.3 units/well), and capsaicin with or without adenosine alone or with antagonists. This assay relies on the generation of β -nicotinamide adenine dinucleotide phosphate hydrate by glutamate dehydrogenase in the presence of glucose, with β -nicotinamide adenine dinucleotide phosphate hydrate being measured fluorometrically.³⁷ Plates were preheated to 37°C, and fluorescence at 460 nm from excitation at 340 nm was recorded at 37°C using a commercial plate reader (FL 600 with KC4 software; Bio-Tek Instruments, Inc., Winooski, VT). An endpoint analysis was performed after 1.5 min of incubation. A standard curve was constructed, and glutamate generation from synaptosome suspensions was determined by linear regression. Values were normalized to concentration of the total protein in synaptosomes as determined by the method of Bradford³⁸ with bovine serum albumin as a standard.

After determination of concentration responses for glutamate release evoked by capsaicin, the inhibition by adenosine to glutamate release evoked by 20 μ M capsaicin was investigated. In addition, antagonism of the effect of adenosine by DPCPX and DMPX was further studied in both normal and spinal nerve-injured animals.

Materials

Adenosine was obtained from Fujisawa USA, Inc. (Deerfield, IL) at 3 mg/ml solution. MgSO₄, KCl, sodium bicarbonate, and glucose were obtained from Fisher Scientific (Fair Lawn, NJ). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) was purchased from Tocris Cookson Inc. (Ellisville, MO). DMPX (3',7'-dimethyl-1-propargylxanthine), R-PIA (R-N6-(2-phenylisopropyl)-adenosine), capsaicin (8-methyl-N-vanillyl-6-nonenamide), glutamate, β -nicotinamide adenine dinucleotide, glutamate dehydrogenase, and remaining chemicals were obtained from Sigma (St. Louis, MO).

Data Analysis

Net glutamate release in synaptosomes exposed to capsaicin with or without other agents was calculated by subtracting glutamate release in wells on the same plate

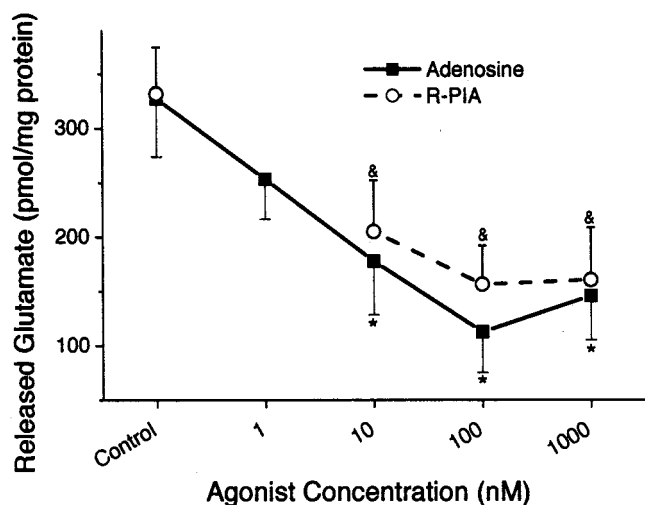


Fig. 1. Glutamate release in spinal synaptosomes from normal rats evoked by 20 μ M capsaicin alone (control) or in the presence of 1–1,000 nM adenosine or 10–1,000 nM R-PIA. Each symbol represents the mean \pm SE of 12 observations. * $P < 0.05$, compared with control by one-way analysis of variance; & = $P < 0.05$ compared to no R-PIA.

incubated in the absence of capsaicin (800–1,000 pmol/mg protein). Data are presented as mean \pm SE. Synaptosome data were analyzed by one- or two-way analysis of variance followed by the Dunnett test. $P < 0.05$ was considered significant.

Results

Adenosine Effect in Normal Rats

The effects of adenosine on evoked glutamate release were determined using a fixed capsaicin concentration of 20 μ M, which we previously demonstrated to release approximately 300 pmol/mg protein.²⁰ Adenosine reduced capsaicin-evoked glutamate release in spinal synaptosomes from normal rats in a concentration-dependent manner, with a threshold of 10 nM and maximum inhibition of approximately 50% (fig. 1). In addition, this evoked glutamate release was also inhibited by R-PIA, a specific adenosine A₁ receptor agonist, in a similar manner. To examine adenosine receptor subtype involved in inhibition of glutamate release, a fixed concentration of 1 μ M adenosine was used, which reduced capsaicin-evoked glutamate release approximately 50%. DPCPX, a highly selective A₁ adenosine receptor antagonist, prevented inhibition by adenosine in a concentration-dependent manner, with threshold of 100 nM and with complete blockade of adenosine inhibition at 1 μ M (fig. 2A). In contrast, the selective A₂ adenosine receptor antagonist had no effect on adenosine inhibition of capsaicin-evoked glutamate release, even at a 10-fold higher concentration of 10 μ M (fig. 2B).

Adenosine Effect in SNL Rats

Capsaicin evoked glutamate release in a concentration-dependent manner in spinal synaptosomes ipsilateral

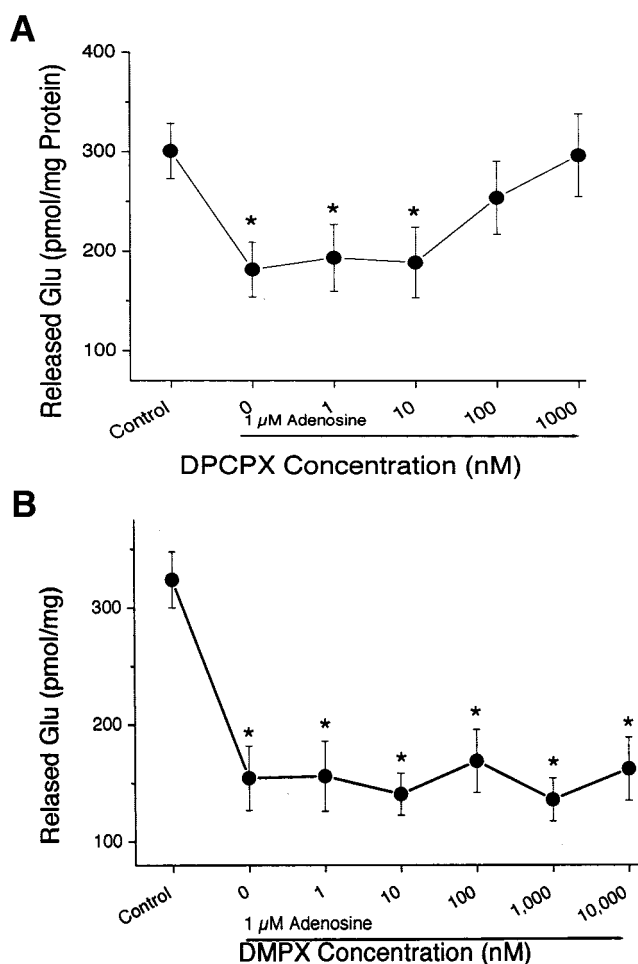


Fig. 2. Glutamate (Glu) release in spinal synaptosomes from normal rats evoked by 20 μ M capsaicin alone (control) or in the presence of 1 μ M adenosine or (A) with the A₁ adenosine receptor antagonist DPCPX, 1–1,000 nM (each symbol represents the mean \pm SE of 18 observations), or (B) with the nonspecific A₂ adenosine receptor antagonist DMPX, 1–10 μ M (each symbol represents the mean \pm SE of 8 observations). * $P < 0.05$, compared with control by one-way analysis of variance.

and contralateral to nerve injury (fig. 3). There was no difference in capsaicin-evoked glutamate release in synaptosomes ipsilateral or contralateral to nerve injury. Capsaicin-evoked glutamate release at a capsaicin concentration of 20 μ M was similar in synaptosome from normal and nerve-injured animals, and the capsaicin concentration–effect relation in tissue from nerve-injured animals in the current study did not differ from that in normal animals, which we reported previously.²⁰ Using a fixed capsaicin concentration of 20 μ M, adenosine reduced glutamate release similarly in synaptosomes made from spinal cord ipsilateral and contralateral to nerve injury, with a threshold of 10 nM and a peak inhibition of approximately 50% (fig. 4). The concentration–effect relation of adenosine did not differ between synaptosomes from normal and nerve-injured animals. We did not repeat the entire antagonist concentration–response study in synaptosomes from nerve-injured ani-

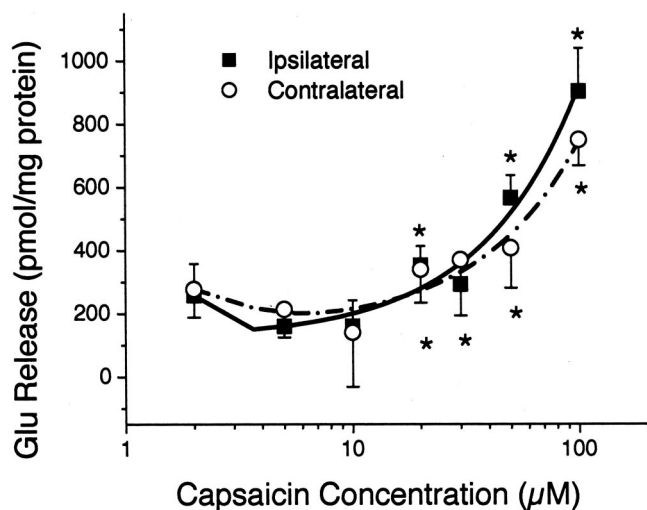


Fig. 3. Glutamate (Glu) release in spinal synaptosomes from lumbar spinal cord tissue ipsilateral (filled squares) or contralateral (open circles) to spinal nerve ligation by 2–100 μM capsaicin. Each symbol represents the mean \pm SE of nine observations. * $P < 0.05$, compared with no-capsaicin control by two-way analysis of variance. $P > 0.05$, F value 0.0176, no difference between ipsilateral and contralateral.

mals, but a concentration of 1 μM DPCPX completely blocked adenosine inhibition (1 μM) of capsaicin-evoked release in synaptosomes ipsilateral and contralateral to nerve injury, whereas 1 μM DMPX had no effect (fig. 5).

Discussion

In addition to neural plasticity after nerve injury that results in chronic pain, other changes occur which alter

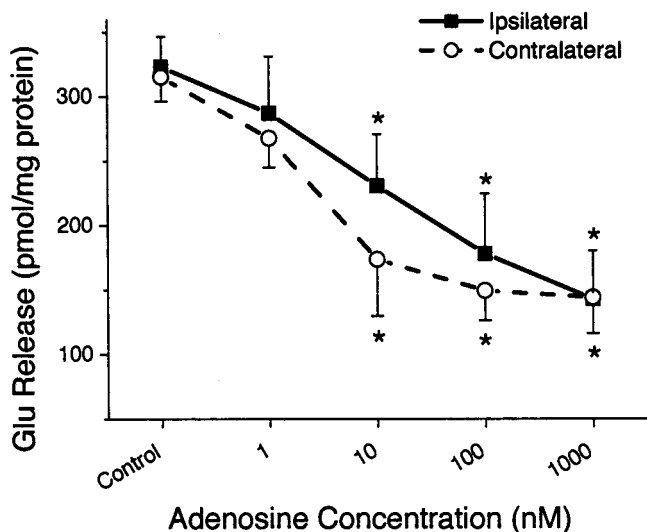


Fig. 4. Glutamate (Glu) release in spinal synaptosomes from lumbar spinal cord tissue ipsilateral (filled squares) or contralateral (open circles) to spinal nerve ligation by 20 μM capsaicin alone (control) or in the presence of 1–1,000 nM adenosine. Each symbol represents the mean \pm SE of eight observations. * $P < 0.05$, compared with control by two-way analysis of variance. $P > 0.05$, F value 0.314, no statistical difference in lumbar spinal tissue between contralateral and ipsilateral to spinal nerve ligation.

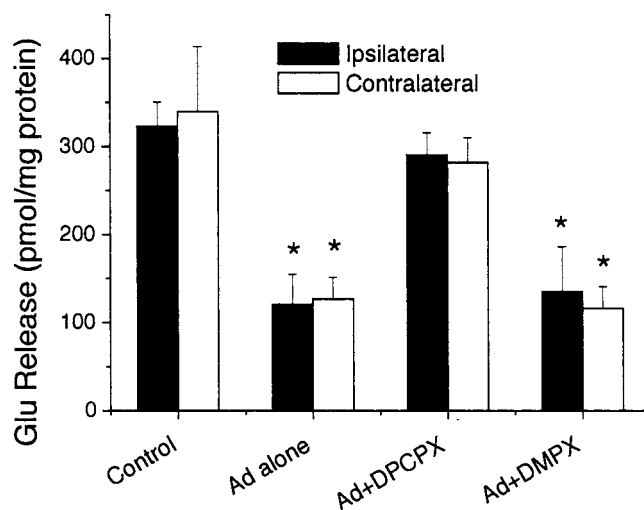


Fig. 5. Glutamate (Glu) release in spinal synaptosomes from lumbar spinal cord tissue ipsilateral (filled bars) or contralateral (open bars) to spinal nerve ligation by 20 μM capsaicin alone (control) or with 1 μM adenosine (Ad alone), or this concentration of adenosine plus the A_1 adenosine receptor antagonist DPCPX, 1 μM (Ad+DPCPX), or the A_2 adenosine receptor antagonist DMPX, 1 μM (Ad+DMPX). Each bar represents the mean \pm SE of 11 observations. * $P < 0.05$, compared with control by two-way analysis of variance. $P > 0.05$, F value 0.0015, no difference between ipsilateral and contralateral tissue in any experimental condition.

potency and efficacy of analgesics. An example of the latter is the efficacy of intrathecally administered adenosine, which acts on A_1 adenosine receptors in the spinal cord for analgesia.^{3,4}

Analgesic Effect of Adenosine

In normal animals and humans, intrathecal adenosine produces no analgesia to acute noxious stimuli^{2,3} but reduces hypersensitivity in neuropathic pain.^{4,5} In contrast, intrathecal injection of small-molecule A_1 adenosine receptor agonists does increase nociceptive thresholds in normal animals,³⁹ perhaps because it penetrates the cord to a greater extent before being removed or metabolized. The current study, using tissue homogenates to prepare synaptosomes, bypasses most of these barriers. The major finding of the current study is that this shift in efficacy of stimulation of spinal A_1 adenosine receptors in settings of nerve injury and hypersensitivity is not due to increased efficacy of adenosine to inhibit glutamate release from primary afferent terminals.

Adenosine Reduces Capsaicin-evoked Glutamate Release

The primary assumption underlying the current study is that capsaicin-evoked glutamate release from dorsal spinal cord synaptosomes reflects neurotransmitter release from C fiber primary afferents. Observations regarding A_1 adenosine receptors and TRPV-1 receptors support this assumption. There is anatomical and functional evidence for the presence of A_1 adenosine recep-

tors on primary afferents. Although A₁ adenosine receptor immunostaining in the spinal cord does not overlap with some markers of C fibers, the same study observed A₁ adenosine receptor immunoreactivity on dorsal root ganglion cell bodies, indicating a presence on primary sensory afferents.⁴⁰ In addition, adenosine or A₁ adenosine agonists reduce K⁺ or electrical field-evoked calcitonin gene-related peptide release from spinal cord *in vitro*, and this effect is blocked by previous exposure to capsaicin, consistent with a primary afferent source.^{29,31,41} Therefore, although there is clear evidence for expression of A₁ adenosine receptors on spinal cord neurons,⁴² they are also expressed on afferent terminals. With regard to TRPV-1 receptors, although some spinal cord cell bodies express patchy immunostaining for this receptor, all TRPV-1 immunostaining in the superficial dorsal horn is abolished by dorsal rhizotomy, and fewer than 4% of neurokinin 1-expressing neurons in lamina I of the spinal cord exhibit TRPV-1 immunostaining.^{43,44} Indeed, a high proportion of neurokinin 1-expressing projection neurons in the superficial dorsal horn receive synaptic contact from TRPV-1-expressing afferents,⁴⁵ consistent with the assumption that capsaicin evoked glutamate release from spinal cord synaptosomes in the current study primarily reflects neurotransmitter release from C-fiber terminals.

Adenosine Reduces Glutamate Release in SNL Rats

Adenosine inhibited capsaicin-evoked glutamate release in a concentration-dependent manner in spinal synaptosomes from normal rats and from those with SNL-induced hypersensitivity (current study).²⁰ Although we did not duplicate this full concentration response in normal rats in the current study, comparison of the current results from the raw data from the previous study and comparison of the effects of 20 μ M capsaicin in normal and nerve-injured animal synaptosomes in the current study consistently showed no difference in either threshold or maximum efficacy of adenosine between these groups. These results, coupled with previous observations that SNL affects neither spinal cord A₁ adenosine receptor number nor A₁ adenosine receptor-induced G-protein coupling, suggest that A₁ adenosine receptors are not up-regulated,^{46,47} either in number or function, on central terminals of primary afferents after SNL. We recognize that the current study did not address the role of A₁ adenosine receptors on capsaicin-insensitive primary afferents and that these afferents, especially A β fibers,⁴⁸⁻⁵⁰ are those involved in transducing mechanical allodynia, a hallmark of this model.

Physiologic Activity of Adenosine A₁ Receptor Activation

The current study confirms a wealth of electrophysiologic,^{51,52} neurotransmitter release,^{29,31,41} and behavioral evidence supporting the primary role of the A₁

adenosine receptor subtype in producing antinociception.^{53,54} Intrathecal adenosine reduces mechanical hypersensitivity in rats with SNL by a mechanism involving A₁ adenosine receptors.⁵⁵ That these observations are relevant to the human condition is supported by the observation that intrathecal adenosine also reduces areas of allodynia in patients with chronic neuropathic pain.⁵

In summary, capsaicin-evoked glutamate release from dorsal spinal cord synaptosomes is inhibited in a concentration-dependent manner by adenosine or R-PIA, blocked by A₁ but not A₂ adenosine receptor-selective antagonists, consistent with a direct inhibitory effect of adenosine on presynaptic terminals of primary afferents *via* actions on A₁ adenosine receptors. There is no change in capsaicin-evoked glutamate release from spinal cord synaptosomes after SNL, nor is there a change in adenosine-mediated inhibition or the receptor subtype on which adenosine acts. These results suggest that the increased efficacy of intrathecal adenosine in settings of hypersensitivity does not reflect increased potency or efficacy to inhibit neurotransmitter release from TRPV-1 expressing primary afferents.

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