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Characterization of the Effects of Adenosine Kinase Inhibitors on Acute Thermal Nociception in Mice

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KOWALUK, E. A., K. L. KOHLHAAS, A. BANNON, K. GUNTHER, J. J. LYNCH, III AND M. F. JARVIS. *Characterization of the effects of adenosine kinase inhibitors on acute thermal nociception in mice.* PHARMACOL BIOCHEM BEHAV **63**(1) 83–91, 1999.—Adenosine (ADO) is an inhibitory neuromodulator that can increase the nociceptive threshold in animals exposed to a variety of noxious stimuli. Inhibition of the ADO-metabolizing enzyme, ADO kinase (AK), provides a means of locally enhancing extracellular ADO concentrations. In the present study, the AK inhibitors 5′-amino,5′-deoxy-ADO (NH_2dADO), 5-iodotubercidin (5-IT), and 5′-deoxy,5-iodotubercidin (5′d-5IT) were examined for their analgesic efficacy in the hot-plate model of acute somatic nociception. Control and drug-treated adult male mice were placed on a 55°C hot plate and the latency to the 10th jump was recorded via a computer driven infrared-beam photosensor. All three AK inhibitors were found to significantly increase jump latencies in a dose-dependent fashion. 5′d-5IT was the most potent AK inhibitor (approx. ED_{50} value = 1 μmol/kg, IP), followed by 5-IT (ED_{50} value =10 μmol/kg, IP), and NH_2dADO (ED_{50} value = 100 μmol/kg, IP). 5′d-5IT was found to be more potent and equally efficacious to morphine (ED_{50} value = 5.2 μmol/kg, IP) in this assay. In a model of persistent chemical pain, the phenyl-*p*-quinone-induced abdominal constriction assay, 5′d-5IT (ED_{50} value = 1.5 μmol/kg, SC) and morphine (ED_{50} value = 3.0 μmol/kg, SC) dose dependently reduced nociception. Pretreatment of mice with either the nonselective ADO receptor antagonist, theophylline (56 μmol/kg, IP), but not the peripherally acting antagonist, 8-(*p*-sulfophenyl)-theophylline (8-PST, 200 μmol/kg, IP) significantly attenuated the antinociceptive effects of 5′d-5IT in the hot-plate assay. Furthermore, the antinociceptive effects of 5′d-5IT were completely blocked by an ADO A_1 receptor selective antagonist, DPCPX, while an ADO A_{2A} receptor selective antagonist, ZM 241385, showed markedly less antagonist activity. The analgesic effects of 5′d-5IT were not blocked by the opioid receptor antagonist naloxone; however, 5′d-5IT could produce additive analgesic effects with morphine when both compounds were administered in combination. The apparent efficacy of 2.5 μmol/kg, IP, of 5′d-5IT was not significantly altered following the repeated administration of this dose twice daily for 4 days. The present data provide evidence for an antinociceptive action of AK inhibitors in the hot-plate test, which, at least for 5′d-5IT, is mediated by an enhancement of ADO's actions at the ADO A_1 receptor subtype, is non-opioid in nature, and which does not exhibit tolerance following repeated administration. © 1999 Elsevier Science Inc.

Adenosine Adenosine kinase Analgesia Hot plate Nociception

THERE is abundant evidence to indicate that the purine nucleoside, ADO, functions as an important modulator of cellular function in mammalian physiology (14). The fact that ADO inhibits neurotransmitter release in both the central and peripheral nervous systems (13) has lead to the hypothesis that ADO provides an inhibitory buffer to excitatory neurotransmission. The inhibitory effects of ADO on cellular excitability are mediated via interactions with different cell surface receptor subtypes (termed P1 receptors: A_1, A_{2A}, A_{2B}, and A_3 receptors), and can result in cellular protection during conditions of physiological stress or trauma including ischemia, seizures, and inflammation.

It has also been demonstrated that the activation of ADO receptors can modulate nociception (35,37). ADO receptor

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agonists administered both systemically (1,17,18,38) and centrally (11,33,38,41) have been shown to produce dose-dependent antinociception in a number of acute pain models such as the tail-flick and hot-plate tests. These effects appear to be ADO receptor mediated because ADO-induced analgesia can be blocked by ADO receptor antagonists such as caffeine and theophylline (1,18). In addition, ADO receptor antagonists when administered alone can produce hyperalgesia (22,29). ADO receptors (5), ADO immunoreactivity (3), ADO deaminase (ADA) (15), and ADO transporters (15) are localized in the spinal cord, which appears to be a major site of action of ADO mediated analgesia (24,37).

Extracellular concentrations of ADO can be enhanced through the inhibition of the primary intracellular ADO metabolizing enzyme, ADO kinase (AK) (7,8). The functional significance of the inhibition of AK in vivo is illustrated by reports that AK inhibitors can increase ADO concentrations (4) and decrease seizure susceptibility when administered directly into the central nervous system (42). Because AK inhibition has been shown to selectively increase ADO levels only in neural tissue undergoing trauma (4), AK inhibition may represent a mechanism to selectively enhance the actions of ADO while minimizing nonspecific side effects associated with ADO receptor agonists (14,40). Consistent with these observations, a recently described AK inhibitor, GP 683 has been shown to reduce the anesthetic requirement in dogs at doses that do not affect cardiovascular function (39).

Endogenous ADO appears to exert a tonic modulation of nociceptive processing because blockade of ADO receptors has been shown to be algogenic (22). Further, the intrathecal administration of an AK inhibitor, NH_2dADO (Fig. 1), has been shown to produce antinociception in tests of acute (24) and persistent (32) pain. The analgesic efficacy of AK inhibition appears to be more robust compared to that produced by inhibition of ADA (24,32). Similarly, inhibition of AK has been shown to be more effective than inhibition of ADA in reducing seizure susceptibility (28). AK inhibitors also appear to be more effective in increasing the release of ADO from spinal cord slices as compared to ADA inhibitors (16).

Demonstrations of the antinociceptive effects of AK inhibition have been primarily based on the pharmacology of intrathecally administered NH_2dADO (24,32), which inhibits AK with nanomolar affinity but has poor cell penetrability and may have limited access to the CNS following systemic administration (25). Other potent AK inhibitors such as 5-iodotubercidin (5-IT) and 5′-deoxy,5-iodotubercidin (5′d-5IT) (Fig. 1) have greater affinity for intracellular AK (7,8) and have in vivo efficacy in animal models of chemically induced seizures (25) and transient ischemia (21,27) following systemic administration. The present studies were conducted to investigate the analgesic effects of these three prototypic AK inhibitors following systemic administration in the hot-plate model of acute somatic nociception.

METHOD

Subjects

Male CF-1 mice (Harlan Farms, Portage, MI), weighing approximately 25–30 g, were used in the present studies. Mice were housed 14 to a cage and maintained in a climate controlled facility with a 12 L:12 D cycle. In all experiments, individual animals were used once. All animal handling and experimental protocols were approved by an institutional animal care and use committee (IACUC), and were conducted in accordance with the ethical principles for pain-related animal research of the American Pain Society.

Test Compounds

NH_2dADO and 5-IT were purchased from Research Biochemicals Inc. (Natick, MA). 5′d-5IT was synthesized at Abbott Laboratories. Morphine sulfate was purchased from Mallinckrodt (St. Louis, MO). ZM 241385 was purchased from Tocris Cookson Inc. (Ballwin, MO). Other test compounds were purchased from Research Biochemicals Inc. (Natick, MA). All test compounds were administered IP at a volume of 10 ml/kg.

Analgesia Testing

Analgesia was measured using a 16-chamber automated hot-plate analgesia monitor (Model No. AHP16AN, Omnitech Elecronics, Columbus, OH) using methodology adapted from Bannon et al. (2) and Decker et al. (9). The temperature of the hot plate was maintained at 55°C. Experimentally naive mice were placed in individual, 9.8 × 7.2 × 15.3 cm (L × W × H) plastic enclosures on the hot plate, and the latency until the tenth jump was recorded. Jumps were recorded by disruption of a photocell beam located 12.5 cm above the surface of the hot plate. Mice were removed from the hot plate after either 10 jumps were made or 180 s (approximately three times control latencies) had elapsed, whichever occurred first. The latency until the tenth jump was used for statistical analysis. These test parameters were employed to accurately assess nociceptive thresholds in both experimentally naive and drug-treated mice (30). Inspection of both vehicle and drug-treated mice revealed no overt tissue damage after hot-plate testing, and these animals were behaviorally indistinguishable from mice that had not undergone hot-plate testing.

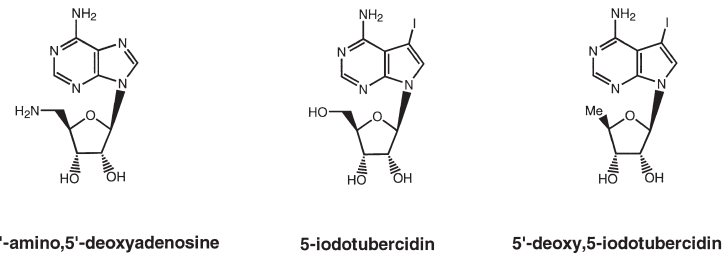

FIG. 1. Structures of 5′amino,5′deoxyadenosine (NH_2dADO, MW = 439), 5-iodotubercidin (5IT, MW = 392), and 5′-deoxy,5-iodotubercidin (5′d-5IT, MW = 376).

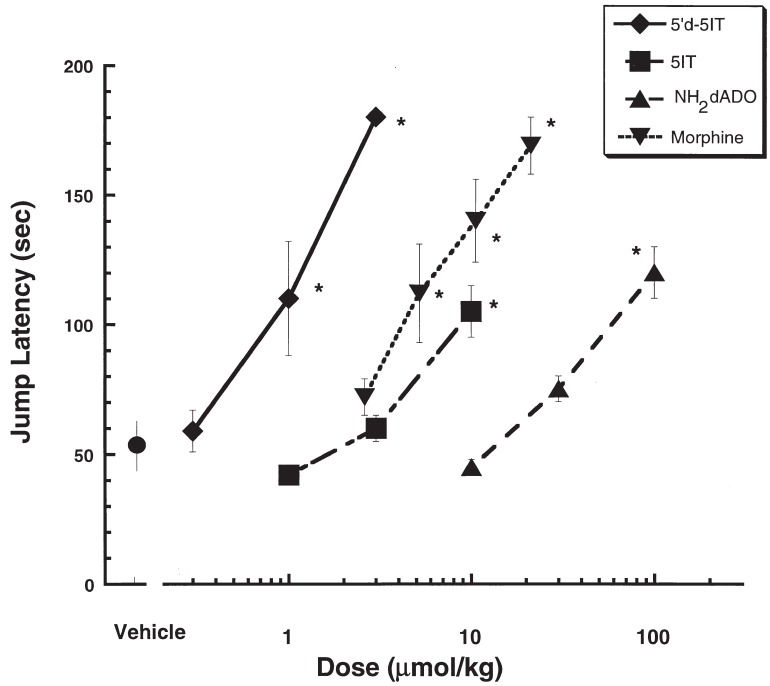

FIG. 2. Dose–response curves for the antinociceptive effects of AK inhibitors and morphine in the hotplate test. Values represent mean (±SEM) values from individual experiments for each dose–response determination. Jump latencies following vehicle administration were averaged across each experiment. 5′d-5IT, $F(3, 28) = 16.23$, $p < 0.01$; 5IT, $F(3, 29) = 10.46$, $p < 0.01$; NH_2dADO, $F(3, 29) = 17.31$, $p < 0.01$; and morphine, $F(4, 30) = 11.34$, $p < 0.01$, produced dose-dependent increases in jump latencies. AK inhibitors were administered IP 30 min before testing. *Indicates $p < 0.05$ compared to vehicle-treated mice.

The antinociceptive effects of 5′d-5IT and morphine were also assessed in a model of persistent chemical pain, the phenyl-*p*-quinone–induced abdominal constriction assay (6). Mice were administered phenyl-*p*-quinone (68 μmol/kg, IP dissolved in 5% ethanol). Test compounds were administered subcutaneously, 30 min before phenyl-*p*-quinone. The presence of characteristic stretching or writhing responses was noted during a 10-min period beginning 5 min after the injection of phenyl-*p*-quinone. Mice displaying one or more of these nociceptive responses were categorized as responders, and mice who did not display these behaviors were regarded as nonresponders (9).

AK inhibitors and morphine were routinely administered IP 30 min prior to hot-plate testing. In the antagonist studies, ADO receptor antagonists or naloxone were administered IP 30 min prior to the AK inhibitors or morphine, respectively. To assess the effects of repeated administration, 5′d-5IT (2.5 μmol/kg) or vehicle was administered IP twice daily at 12-h intervals for 4 days, and the animals were tested on the fifth day.

The vehicle for the AK inhibitors and ADO receptor antagonists was 10% DMSO/34% hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO) in distilled water, and saline was used as the vehicle for morphine. Experimental and control groups contained six to eight mice each.

Statistics

Jump latency data were analyzed using one-way ANOVA as described by Decker et al. (9). Where appropriate, Fisher's Protected Least-Significant Difference (FLSD) was used for post hoc analysis. The level of significance was set at $p < 0.05$. ED_{50} values were estimated using least-squares linear regression. The chi-square statistic was used to evaluate statistical significance in the abdominal constriction assay ($p < 0.05$).

RESULTS

The three AK inhibitors examined in the present study (NH_2dADO, 5IT, and 5′d-5IT) were found to significantly increase the jump latency of mice placed on a 55°C hot plate in a dose-dependent fashion (Fig. 2). 5′d-5IT was the most potent AK inhibitor (ED_{50} value = 1 μmol/kg, IP) compared to 5IT (ED_{50} value = 10 μmol/kg, IP), and 5′NH_2dADO (ED_{50} value = 100 μmol/kg, IP). 5′d-5IT exhibited full efficacy in this test at a dose of 3 μmol/kg, IP, whereas similar efficacy was not observed at 3- or 33-fold higher doses of 5IT or 5′NH_2dADO, respectively. For comparison, morphine was also found to dose-dependently increase jump latency (ED_{50} value = 5.2 μmol/kg, IP) with maximal efficacy observed at a dose of 21 μmol/kg, IP (Fig. 2). At their respective ED_{50} values, the mean number of jumps for mice treated with 5′d-5IT (7.4 ± 1.2; mean ± SEM) or morphine (8.2 ± 1.1) were similar. Both 5′d-5IT and morphine also produced dose-dependent antinociceptive effects ($p < 0.05$) in a model of persistent chemical pain, the abdominal constriction assay (Fig. 3).

Additional experiments were conducted to further characterize the antinociceptive effects of the most potent AK inhibitor, 5′d-5IT. The antinociceptive effects of 5′d-5IT in the hotplate test could be significantly attenuated by pretreatment

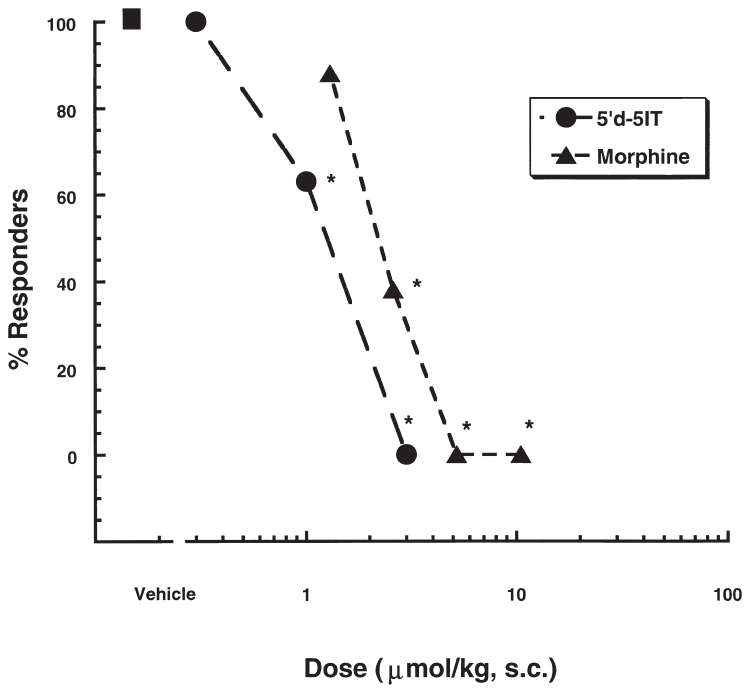

FIG. 3. Dose–response curves for 5′d-5IT and morphine to reduce nociception in the abdominal constriction assay. % Responders represents the percentage of mice displaying the prototypic abdominal constriction response relative to the number tested. Test compounds were administered SC to individual groups of mice ($n = 8$) 30 min before phenyl-*p*-quinone. *Indicates $p < 0.05$ compared to vehicle-treated mice.

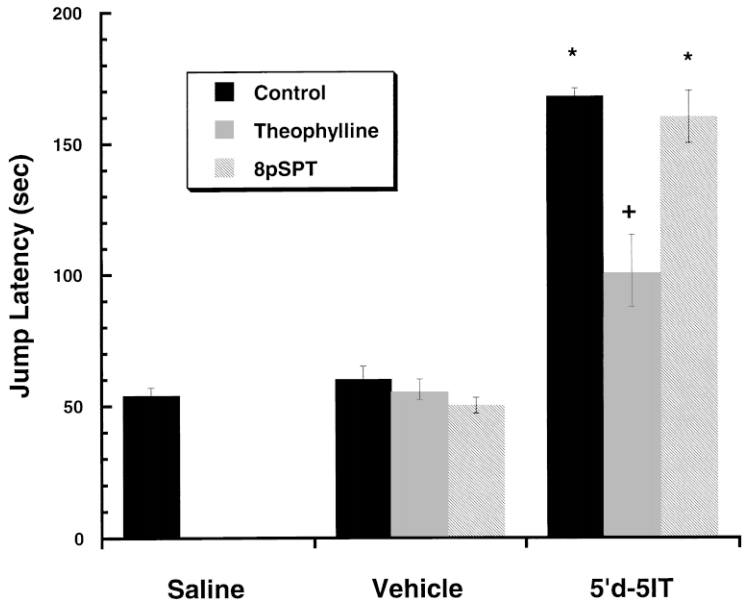

FIG. 4. Antagonism of the antinociceptive effects of 2 μmol/kg 5′d-5IT by theophylline (56 μmol/kg) and 8-PST (200 μmol/kg), $F(6, 48) = 20.5$, $p < 0.01$. 5′d-5IT was administered IP 30 min before testing, and the respective ADO receptor antagonists were administered IP 30 min before 5′d-5IT. An additional group of mice were injected with saline as a control for the injection procedure. Values represent mean (±SEM). *Indicates $p < 0.05$ compared to vehicle-treated mice. +Indicates $p < 0.05$ compared to 5′d-5IT–treated mice.

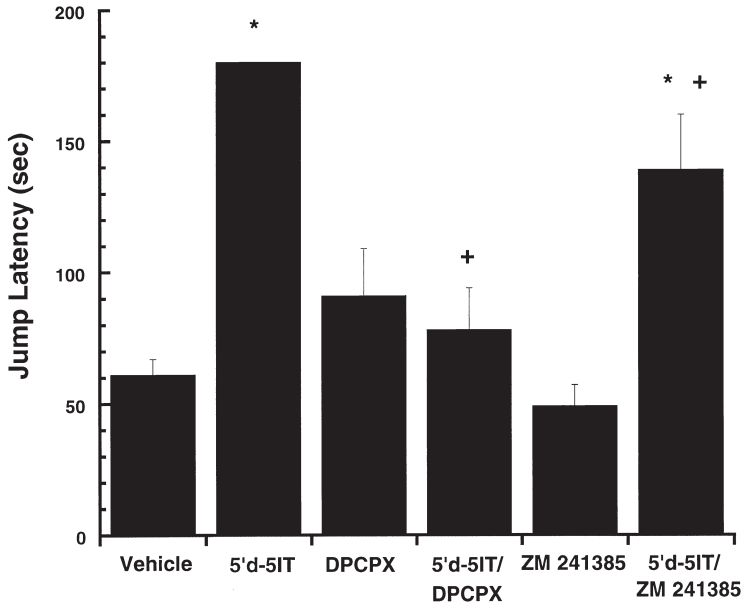

FIG. 5. Antagonism of 5′d-5IT (2 μmol/kg, IP)-mediated analgesia by DPCPX (33 μmol/kg, IP) and ZM 241385 (30 μmol/kg, IP), $F(5, 43) = 14.83$, $p < 0.01$. ADO receptor antagonists were administered IP 30 min before 5′d-5IT. Values represent mean (±SEM). *Indicates $p < 0.05$ compared to vehicle-treated mice. +Indicates $p < 0.05$ compared to 5′d-5IT–treated mice.

with the nonselective ADO receptor antagonist theophylline (56 μmol/kg, IP), but not by the peripherally acting ADO receptor antagonist 8-PST (200 μmol/kg, IP) (Fig. 4). Time course experiments revealed that the analgesic effects of 5′d-5IT persisted for at least 60 min, with the jump latencies of 5′d-5IT (1 μmol/kg, IP)-treated mice returning to control latencies by 2 h (data not shown). Pretreatment of mice with the ADO A_1 selective antagonist, DPCPX (33 μmol/kg, IP) com-

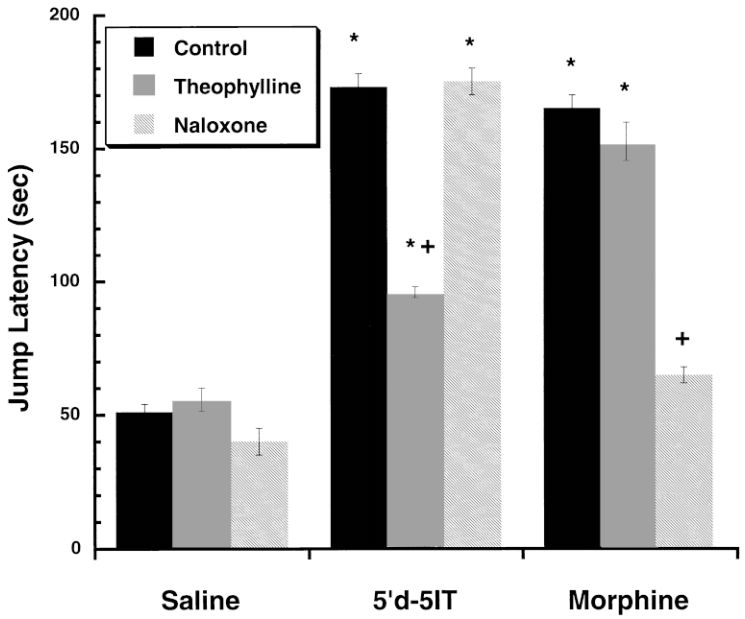

FIG. 6. Antagonism of 5′d-5IT (2 μmol/kg, IP) and morphine (16 μmol/kg, IP)-mediated analgesia by theophylline (56 μmol/kg) and naloxone (14 μmol/kg, IP), $F(8, 62) = 44.78$, $p < 0.01$. Antagonists were administered 30 min before 5′d-5IT or morphine. Values represent mean (±SEM). *Indicates $p < 0.05$ compared to vehicle-treated mice. +indicates $p < 0.05$ compared to mice treated with 5′d-5IT or morphine.

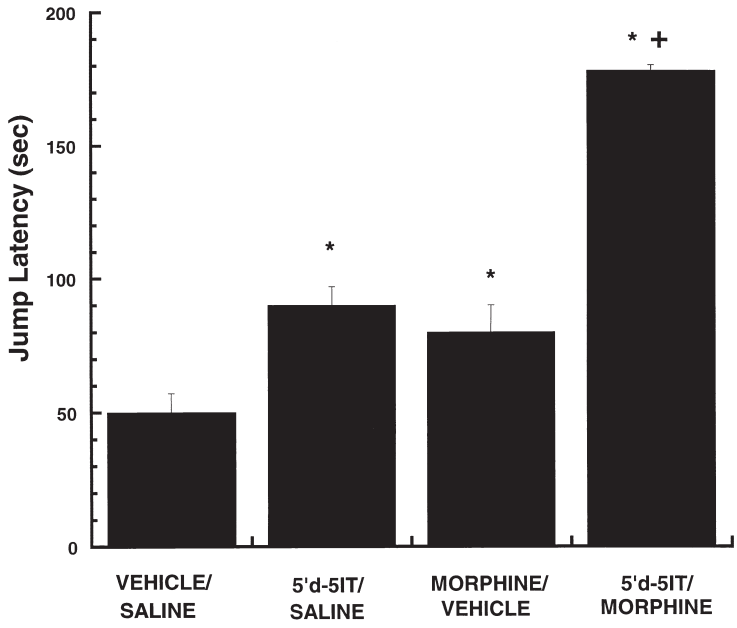

FIG. 7. Additive antinociceptive effects of 5′d-5IT and morphine. When administered alone, 5′d-5IT (1 μmol/kg, IP) and morphine (5.2 μmol/kg, IP) produced mimimal antinociception, whereas the simultaneous administration of these doses of 5′d-5IT and morphine, respectively, produced maximal analgesia in the hotplate test, $F(3, 29) = 39.08$, $p < 0.01$. Values represent mean (±SEM). *Indicates $p < 0.05$ compared to vehicle-treated mice. +Indicates $p < 0.05$ compared to either agonist administered alone.

pletely blocked the antinociceptive effects of 2 μmol/kg, IP 5′d-5IT, whereas the A_{2A} selective antagonist, ZM 241385 (30 μmol/kg, IP) only slightly attenuated 5′d-5IT induced analgesia (Fig. 5).

The antinociceptive actions of 5′d-5IT do not appear to be mediated via an interaction with opioid receptors because pretreatment with naloxone (14 μmol/kg, IP) significantly attenuated the analgesic actions of morphine (16 μmol/kg, IP) but not 5′d-5IT (2 μmol/kg, IP) (Fig. 6). Conversely, pretreatment with theophylline (56 μmol/kg, IP) significantly attenuated the analgesic actions of 5′d-5IT, but was ineffective against morphine (Fig. 6). The analgesic effects of 5′d-5IT and morphine appear to be additive because the combined administration of minimally effective doses of 5′d-5IT (1 μmol/kg, IP) and morphine (5.2 μmol/kg, IP) was found to be fully efficacious in the hot-plate test (Fig. 7).

Finally, no significant attenuation of the analgesic effects of 5′d-5IT were observed following the repeated administration of 2.5 μmol/kg, IP, 5′d-5IT twice daily for 4 days (Fig. 8).

DISCUSSION

The present data demonstrate that, following systemic administration, AK inhibitors can alleviate acute thermal nociception as measured by the hot-plate test in mice. The antinociceptive potency of 5′d-5IT was significantly greater than that observed for 5IT and NH_2dADO. 5′d-5IT was also found to be more potent than, and equally efficacious to, morphine in this test of acute nociception. 5′d-5IT, like morphine, also produced dose-dependent antinociceptive effects in the abdominal constriction assay, a model of persistent chemical pain (6). The greater relative potency of 5′d-5IT as compared to the other AK inhibitors following systemic administration in the hot-plate test is consistent with its subnanomolar potency for inhibition of AK and its apparent increased ability to penetrate into the central nervous system (CNS) (25,26). These data are also in agreement with previous data demonstrating that 5′d-5IT has greater in vivo efficacy as an anticonvulsant as compared to 5IT and NH_2dADO in the pentylenetetrazol-induced seizure model in mice (25). Although NH_2dADO has been shown to produce analgesia following intrathecal administration (24,32), its relatively weak antinociceptive effect following systemic administration may reflect a poor ability to penetrate into the CNS (25) or unfavorable pharmacokinetics.

5′d-5IT–induced antinociception appears to be mediated via an activation of ADO receptors in the CNS because the nonselective ADO receptor antagonist, theophylline, was found to significantly attenuate the antinociceptive effects of this AK inhibitor, while the peripherally acting ADO receptor antagonist, 8-PST was ineffective. It should be noted that 8-PST is approximately 10-fold more potent than theophylline at ADO A_1 receptors (20), and 8-PST was used at an approximately fourfold higher dose relative to theophylline, a dosage that has been shown to selectively block the peripheral effects of ADO agonists (19). Because both theophylline and the highly selective ADO A_1 receptor antagonist, DPCPX, were found to be equally effective in blocking 5′d-5IT-induced analgesia, the antinociceptive effects of 5′d-5IT appear to be mediated via a selective interaction with the A_1 receptor. This notion is further supported by the relatively weak ability of the highly A_{2A} selective antagonist, ZM 241385 (34) to attenuate 5′d-5IT analgesia, and is consistent with other reports that activation of spinal A_1 receptors mediates the antinociceptive

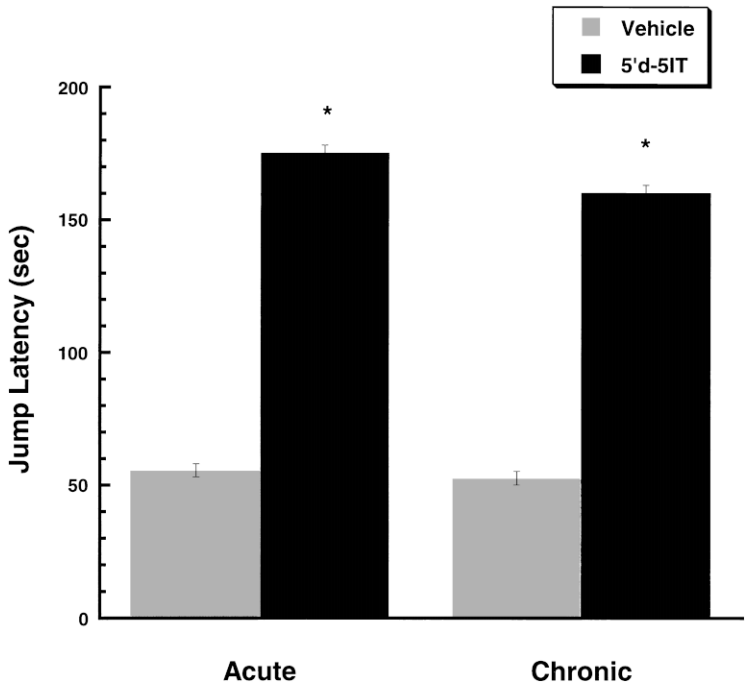

FIG. 8. Lack of tolerance of the antinociceptive effects of 5′d-5IT following the repeated administration of 5′d-5IT (2.5 μmol/kg, IP) twice daily for 4 days, $F(3, 37) = 205.15$, $p < 0.01$. The antinociceptive effects of 5′d-5IT were assessed in individual groups of mice following a single administration of 2.5 mg/kg, IP. 5′d-5IT (acute) or 12 h after the last of eight repeated administrations of 5′d-5IT. Values represent mean (±SEM). *Indicates $p < 0.05$ compared to vehicle-treated mice.

effects of ADO (18,24,32). The dose of ZM 241385 (30 μmol/kg, IP) used in the present study has been shown to fully block the in vivo activation of A_{2A} receptors by endogenous ADO in the rat (23,34).

Although the analgesic efficacy of 5′d-5IT is similar to that produced by morphine, the antinociceptive effects of the AK inhibitor do not appear to be mediated through an interaction with opioid receptors, because naloxone, at a dose that fully blocked morphine analgesia, did not block the antinociceptive effects of 5′d-5IT. Similarly, the ADO receptor antagonist, theophylline, can readily attenuate 5′d-5IT induced analgesia at a dose that has no effect on the analgesic effects of morphine. These data suggest a lack of interaction between opioid and ADO-mediated antinociception following systemic administration. In contrast, activation of spinal opioid receptors has been reported to stimulate ADO release from spinal cord in vitro (35), which may contribute to spinal, but not supraspinal, antinociception in vivo (24,35). Thus, the putative interaction between ADO and opioid sytems may be mediated by pharmacological actions at different physiological sites (i.e., supraspinal vs. spinal) or may reflect the relative contributions of opioid and ADO mechanisms in attenuating acute thermal nociception as assayed by the hot plate compared to other tests of acute nociception (10,12,36).

The antinociceptive effects of 5′d-5IT, however, do appear to be additive with the analgesic effects of morphine. The systemic administration of a combination of minimally effective doses of 5′d-5IT and morphine produced significantly greater analgesia than was observed by either agent administered alone. While an isobolographic analysis of the interaction between 5′d-5IT and morphine was not conducted in the present studies, these findings are consistent with similar observations of additive interactions between NH_2dADO and the μ-opioid agonist DAMGO in increasing rat tail-flick latencies (24). However, unlike the propensity of morphine to produce analgesic tolerance (1), no significant differences in 5′d-5IT analgesia were observable following the repeated administration of 5′d-5IT for 4 days.

AK inhibitors have previously been shown to reduce body temperature and motor activity (8,25), and the possibility exists that these effects could contribute to the antinociceptive effects of 5′d-5IT. However, the hot-plate test has been shown to be insensitive to agents that lack analgesic activity but impair motor behavior (31). The present data demonstrate that 5′d-5IT is approximately 10- and 100-fold more potent than 5IT or NH_2dADO, respectively, in the hot-plate assay. However, there is much less separation in the potency of these AK inhibitors to reduce motor activity (5′d-5IT ED_{50} = 1 μmol/kg; 5IT ED_{50} = 2 μmol/kg; NH_2dADO ED_{50} = 6 μmol/kg) (25). The differential potency and efficacy of 5′d-5IT compared to the other AK inhibitors in the hot-plate test coupled with the similar efficacy and potency of 5′d-5IT to reduce nociception in the abdominal constriction assay suggests the analgesic effects of this AK inhibitor can be separated from its other effects. Further, the anticonvulsant (25), antiischemic (21), and anesthetic enhancing effects (39) of AK inhibitors appear to be separable from their peripheral side effects.

The results of these studies indicate that systemically administered AK inhibitors can produce antinociceptive effects in the mouse hot-plate test. The demonstrated potent activity

of 5′d-5IT to alleviate acute thermal nociception suggests that the localized enhancement of extracellular ADO concentrations via inhibition of AK provides a novel analgesic mechanism having full efficacy like that provided by opioids. The present data also indicate that 5′d-5IT may represent a more useful ligand to investigate the behavioral pharmacology of AK inhibitors following systemic administration as compared to NH_2dADO and 5IT.

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