

Spinal Carbonic Anhydrase Contributes to Nociceptive Reflex Enhancement by Midazolam, Pentobarbital, and Propofol

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Background: Systemic administration of acetazolamide blocks nociceptive hyperreflexia induced by pentobarbital. The authors assessed the effect of intrathecal carbonic anhydrase inhibitors (CAIs) on nociceptive reflex enhancement by pentobarbital, propofol, and midazolam.

Methods: Twenty-seven rats with chronic indwelling subarachnoid catheters were studied. Nociceptive paw reflex latency (PWL) for paw withdrawal from radiant heat was measured in forelimbs and hind limbs. Measurements were obtained under control conditions, 15 min after lumbar intrathecal injection of 10 μ l artificial cerebrospinal fluid containing the CAIs acetazolamide or ethoxzolamide, and during the 55 min after intraperitoneal injection of three sedative drugs: 30 mg/kg pentobarbital, 50 mg/kg propofol, or 1.9 mg/kg midazolam.

Results: Control values of PWL averaged 10.9 ± 1.5 s in the forelimbs and 11.1 ± 1.6 s in the hind limbs ($P = 0.18$). Intrathecal injection of 50 μ M ethoxzolamide reduced PWL by 8% and 4% in the forelimbs and hind limbs, respectively ($P = 0.01$); all other CAI injections had no effect on PWL. Following anesthetic injection, PWL in the forelimbs was reduced by approximately 35–40% of control values; in the hind limbs, CAI treatment decreased the PWL reduction to 8–16% for pentobarbital ($P < 0.001$), 30–32% for propofol ($P < 0.02$), and 9–16% for midazolam ($P < 0.001$). The hind limb reduction of hyperreflexia by CAI was less for propofol than for midazolam or pentobarbital ($P < 0.002$).

Conclusion: Spinal carbonic anhydrase contributes to nociceptive hyperreflexia induced by pentobarbital and midazolam and to a lesser extent with propofol. These findings are consistent with a role for carbonic anhydrase in nociceptive signal enhancement by these drugs.

LOW concentrations of many general anesthetics decrease the threshold for perception of painful stimulation in humans¹⁻³ and the threshold for nociceptive withdrawal reflexes in rats.⁴⁻⁶ We previously reported that pentobarbital-induced nociceptive hyperreflexia in rats can be blocked by systemic administration of acetazolamide (a carbonic anhydrase inhibitor [CAI]), amiloride (which blocks cellular hydrogen ion transport), and inhibitors of nitric oxide synthase.⁷ On the basis of the latter findings, we have proposed⁷ that nociceptive re-

flex enhancement by pentobarbital may be due to excitatory effects of the drug that are mediated by bicarbonate ion and dependent on carbonic anhydrase (CA). One of the pharmacological actions of pentobarbital is to prolong the open time of the γ -aminobutyric acid type A (GABA_A) receptor ionophore.⁸ Neuronal excitation by activation of GABA_A receptors has been described in central synapses.⁹⁻¹¹ Briefly, the mechanisms for the latter phenomenon proposed by Kaila¹² and Staley *et al.*¹³ involve an anion shift from Cl^- to HCO_3^- ^{13,14} ions. This shift is thought to be caused by a prolongation of the open time of the GABA_A ionophore either by high local concentrations of GABA¹³ or by drugs such as the barbiturates.¹⁵ By catalyzing the conversion of HCO_3^- to CO_2 , CA plays an essential role in the excitatory mechanisms of GABA_A receptor activation.¹³

In the current study, we sought further evidence for a role for CA in anesthetic-induced nociceptive hyperreflexia. The current study focused on two objectives: (1) to examine the role of spinal CA on nociceptive hyperreflexia induced by pentobarbital; and (2) to compare the effects of intrathecal CAI on nociceptive hyperreflexia induced by pentobarbital with that observed with two other drugs, propofol and midazolam.

Materials and Methods

The study protocols, approved by the Faculty of Medicine Animal Care Committee (University of Calgary, Calgary, Alberta, Canada), were designed to comply with the guidelines of the International Association for the Study of Pain and the Canadian Council for Animal Care.

Chronic Intrathecal Catheter Preparation

Male Sprague-Dawley rats (weight, 300–500 g) were instrumented with chronic indwelling intrathecal catheters, with the distal catheter ports located in the lumbar region as previously described.^{16,17} Briefly, under general anesthesia with halothane (2–3% in oxygen), rats were positioned prone in a stereotactic frame. A length of polyethylene-10 catheter (Becton Dickinson Co., Parsippany, NJ) was inserted through an incision in the atlantooccipital membrane to a position of 8 cm caudal to the cisterna magna at the level of the lumbar subarachnoid space and secured with dental cement. Postoperatively, animals free from motor deficits were allowed to recover from surgery for a week before testing. Animals were housed in groups of two or three, with free access to food and water and a 12-h light–dark cycle. Each

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Received from the Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada. Submitted for publication August 1, 2002. Accepted for publication December 9, 2002. Supported by the Canadian Anesthesiologists Society–Abbott Laboratories, Toronto, Ontario, Canada, and the Canadian Institutes for Health Research, Ottawa, Ontario, Canada. Presented in part at the annual meeting of the American Society of Anesthesiologists, New Orleans, Louisiana, October 15, 2001.

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Table 1. Paw Withdrawal Latencies: Effects of Limb Selection and Intrathecal Carbonic Anhydrase Inhibitor Injection

Condition	n	Forelimb			Hindlimb		P
Control	76	10.9 ± 1.5			11.1 ± 1.6		0.18
Intrathecal CAI		Control	CAI	P	Control	CAI	P
20 μM acetazolamide	24	10.8 ± 1.8	10.7 ± 1.5	0.99	10.4 ± 1.4	10.5 ± 1.5	0.76
50 μM ethoxzolamide	20	11.8 ± 1.4	10.8 ± 0.9	0.01	11.1 ± 1.3	10.6 ± 1.1	0.01

Data are shown as mean (s) ± SD. P values for paired t test.

CAI = carbonic anhydrase inhibitor.

animal was used for up to three experiments with a rest of at least 1 week between each experiment. Correct location of catheters was confirmed by injection of methylene blue dye at postmortem examination.

Study Hypotheses and Design

The studies were designed to test three hypotheses. First, we sought evidence that intrathecal pretreatment with CAI would block the nociceptive reflex enhancement associated with systemic anesthetic administration. Second, by administering the CAI into the lumbar cerebrospinal fluid (CSF), we examined whether the block of nociceptive reflex enhancement would occur in the hind limbs but not the forelimbs, suggesting a drug action localized to the lumbar cord or nerves. Finally, we evaluated whether pretreatment with intrathecal CAI produced a similar degree of blockade of the hind limb hyperreflexia in animals sedated with pentobarbital, midazolam, and propofol. In testing the first hypothesis, the role of CA was evaluated as suggested by Maren¹⁸ using two CAIs, acetazolamide and ethoxzolamide. Dose-response relations were evaluated in concentration ranges that are thought to be specific for CAI with few if any other effects.¹⁸ Acetazolamide was used in concentrations of 0.2, 2, 20, and 200 μM, and ethoxzolamide was used in concentrations of 5 and 50 μM.

Paw Withdrawal Reflex Latency

The observer, blinded to the nature of the intrathecal drug injected, measured paw withdrawal latency (PWL) in the forelimbs and hind limbs using a custom-made Hargreaves box¹⁹ as previously described.⁷ The testing chamber is a box (19 × 28 × 29 cm) with acrylic walls and a glass floor. The floor is heated by a projector lamp bulb (Radius tungsten halogen lamp, model EJY, 19 V, 80 W; General Electric, Glen Allen, VA) that projects through an aperture (5 × 10 mm) in the cover of a housing installed below the glass floor. The circuit of the device consists of a photocell aimed at the aperture from within the housing, the projector bulb, a timer, and a switch. To make a measurement, the observer positions the aperture under the paw and closes the circuit with the switch. When the glass has heated sufficiently to evoke a withdrawal response, the interruption of the light falling on the photocell opens the circuit, turning off the timer and the bulb. With our device, paw with-

drawal latencies can be determined within 0.1 s. Paw withdrawal latencies in untreated animals range from 9–12 s, with a cutoff time of 14 s. Measurements were made sequentially in the four limbs at each measurement time described in the next section.

Each rat was allowed to acclimatize in the test chamber until it was resting quietly, which usually took 10–15 min. After control measurements of PWL were recorded, the intrathecal catheters were injected with 10 μl artificial cerebrospinal fluid (aCSF), either plain or containing acetazolamide or ethoxzolamide. Fifteen minutes after intrathecal drug injection, animals received an intraperitoneal injection of one of the three sedative drugs: pentobarbital (30 mg/kg), propofol (50 mg/kg), or midazolam (1.9 mg/kg). The effects of sedative drug injection in each animal were represented by the mean value of the paw withdrawal latency and the mean sedation score calculated from measurements made 5, 15, 25, 35, 45, and 55 min after the intraperitoneal injection. For each animal, one mean value for the PWL following intraperitoneal drug injection was calculated by averaging the measurements obtained during the 55 min after intraperitoneal drug injection. For comparisons between drugs, the individual PWL measurements were expressed as a percentage of the predrug injection value in each animal and averaged over the 55-min postinjection time.

Sedation

After each measurement of PWL, the observer determined the presence or absence of the righting reflex and assigned a sedation score. The righting reflex was considered present if the animal made any effort to reassume the prone position within 1 min after having been placed supine. The sedation score was determined by the response to light touch with a fingertip to the whiskers (0 = fully responsive, 1 = slow response, 2 = unresponsive to whisker stimulation).

Drugs

The dose of pentobarbital (30 mg/kg) used in the current study was the same as that used previously.⁷ Pilot evaluations suggested that the duration of sedation after doses of midazolam of 1.9 mg/kg and propofol of 50 mg/kg would be similar to that induced by 30 mg/kg pentobarbital. Intrathecal CAI concentrations in animals

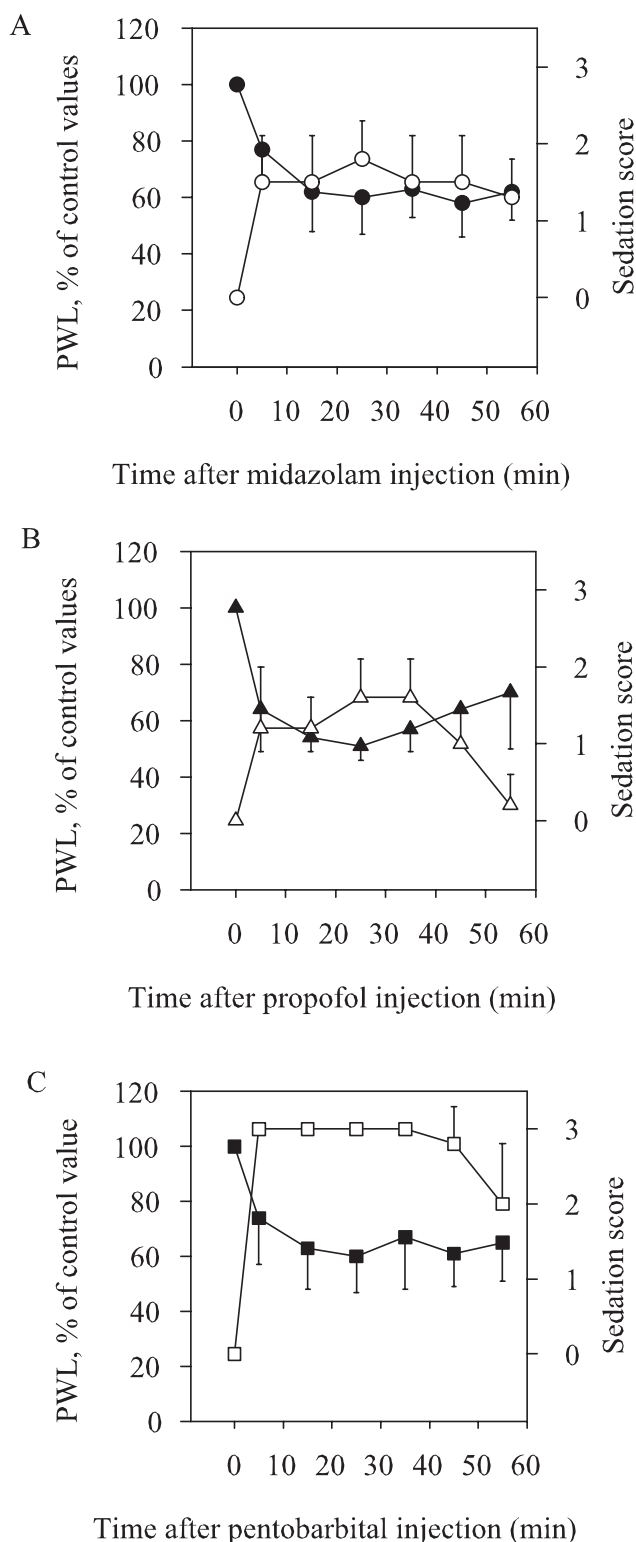


Fig. 1. The time course of the sedation score (empty symbols) and PWL (filled symbols) after intraperitoneal injection of (A) 1.9 mg/kg midazolam ($n = 4$ animals), (B) 30 mg/kg pentobarbital ($n = 11$ animals), or (C) 50 mg/kg propofol ($n = 4$ animals). Mean values \pm SD are shown.

treated with midazolam and propofol (determined in the initial pentobarbital studies) were 20 μ M acetazolamide and 50 μ M ethoxzolamide.

Statistical Analysis

All data are presented as mean \pm SD. The effect of time on PWL during the 55-min observation period was evaluated with the Pearson product-moment correlation coefficient. Effects of intrathecal CAI and limb selection were evaluated by paired *t* test. Using pentobarbital as the sedative drug, the dose-effect relation between intrathecal CAI concentration and PWL were examined with two-factor analysis of variance (ANOVA) using drug concentration as the first factor and upper *versus* lower limbs as the second factor. The effects of intrathecal acetazolamide on nociceptive hyperreflexia in pentobarbital- and propofol-treated animals were compared directly in the same animals using a randomized block design to assign each animal to receive either pentobarbital or propofol. Results from the latter study were analyzed with two-factor ANOVA using drug as the first factor and limb as the second factor influencing the PWL. To compare the effects of intrathecal CAI administration among the three sedative drugs, results from animals that received either 20 μ M acetazolamide or 50 μ M ethoxzolamide intrathecally were pooled for each sedative drug and then compared with two-factor ANOVA using the sedative drug as the first factor and limb as the second factor.

Results

Forty-three rats were enrolled in the study. Nine control animals without catheters were given midazolam or propofol without any CAI. Intrathecal catheters were successfully inserted and maintained in 27 animals; 76 studies were performed on these animals. Results are presented as mean \pm SD.

Control Conditions and Effects of Intrathecal Carbonic Anhydrase Inhibitor Injection

Paw withdrawal latency before any drug administration was approximately 11 s and was similar in the forelimbs and the hind limbs (table 1). Intrathecal injection of CAI had only small effects on PWL. The highest concentration of acetazolamide tested (20 μ M) had no significant effect on PWL. Intrathecal injection of 50 μ M ethoxzolamide was associated with a 4% reduction in PWL in both forelimbs and hind limbs (table 1; $P = 0.01$), without a significant difference between the forelimbs and hind limbs ($P = 0.377$). In two animals, intrathecal injection of 10 μ l aCSF containing 200 μ M acetazolamide caused agitation; this dose was therefore removed from the protocol. None of the remaining

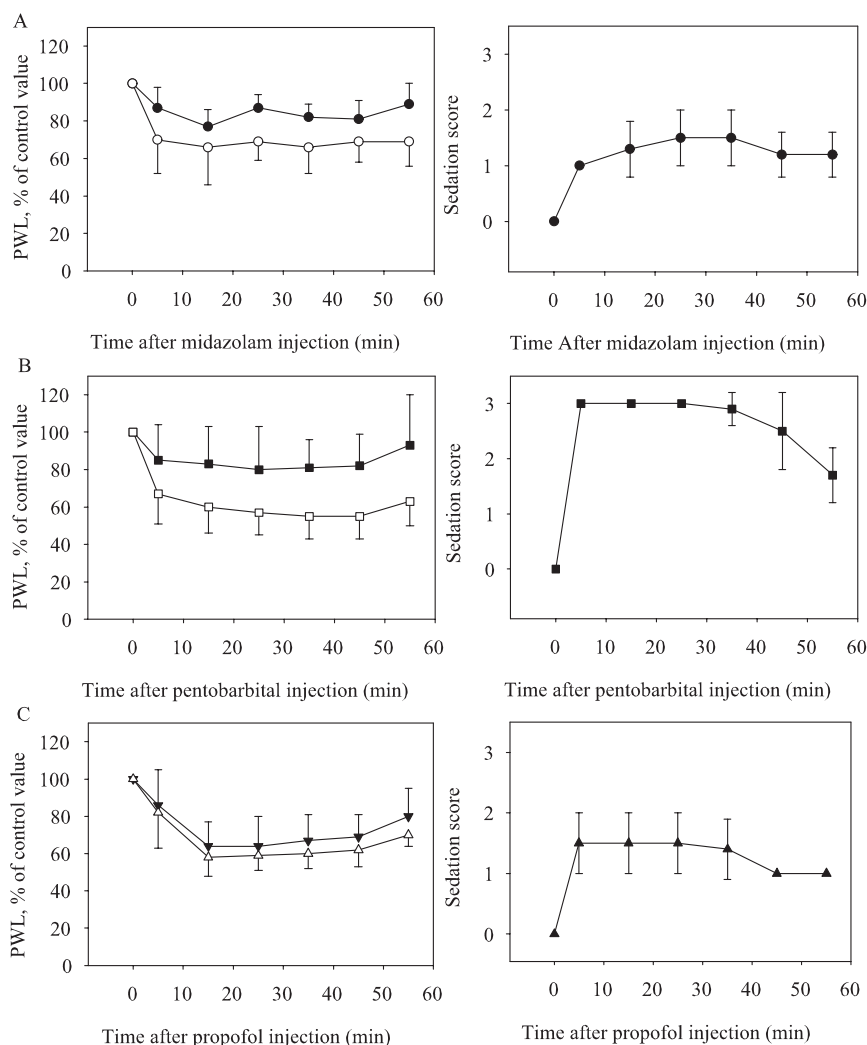


Fig. 2. The effects of lumbar intrathecal pretreatment with 10 μ l acetazolamide, 20 μ M, on sedation scores and PWL after intraperitoneal injection of (A) 1.9 mg/kg midazolam ($n = 5$ animals), (B) 30 mg/kg pentobarbital ($n = 12$ animals), or (C) 50 mg/kg propofol ($n = 7$ animals). The mean PWL during the 55-min observation period was longer in the lower limbs (filled symbols) than in the upper limbs (empty symbols) for all three sedative drugs (see table 3 for details of analysis).

doses of acetazolamide or ethoxzolamide produced any obvious behavioral effects.

Sedation

Midazolam, pentobarbital, and propofol had sedative effects during the 55-min observation period after intraperitoneal injection (figs. 1–3). Animals that received pentobarbital were more deeply sedated than those treated with midazolam or propofol ($P < 0.001$; table 2).

Influence of Intrathecal Carbonic Anhydrase Inhibitor Injection on Paw Withdrawal Latency

In control animals (no intrathecal CAI treatment), PWL in both forelimbs and hind limbs fell rapidly to 60–70% of control values (fig. 1 and table 3) and remained stable during the 55-min observation period. In both the control animals and animals treated with intrathecal CAI, there was no significant correlation between time and PWL during the first 55 min after injection of any of the three sedative drugs ($P > 0.3$).

In animals pretreated with intrathecal injection of 20 μ M acetazolamide or 50 μ M ethoxzolamide, forelimb

PWL values were similar to values observed in control animals (table 2). In contrast, in the hind limbs, intrathecal acetazolamide and ethoxzolamide injection reduced the nociceptive hyperreflexia induced by midazolam and pentobarbital (figs. 2 and 3 and table 3). In propofol-treated animals, intrathecal acetazolamide but not ethoxzolamide decreased hind limb hyperreflexia.

Pretreatment with intrathecal acetazolamide produced a concentration-dependent reduction ($P = 0.002$) in hind limb hyperreflexia (fig. 4). Both 5 and 50 μ M ethoxzolamide reduced hind limb hyperreflexia ($P < 0.01$) to a similar degree.

Comparison of Carbonic Anhydrase Inhibitor Effects on Hyperreflexia Induced by Pentobarbital, Propofol, and Midazolam

When pentobarbital and propofol were compared directly in the same animals, 20 μ M intrathecal acetazolamide blocked hind limb hyperreflexia to a greater degree during sedation with pentobarbital than with propofol (two-factor repeated-measures ANOVA, interaction term, $P = 0.003$; table 3). Since the temporal

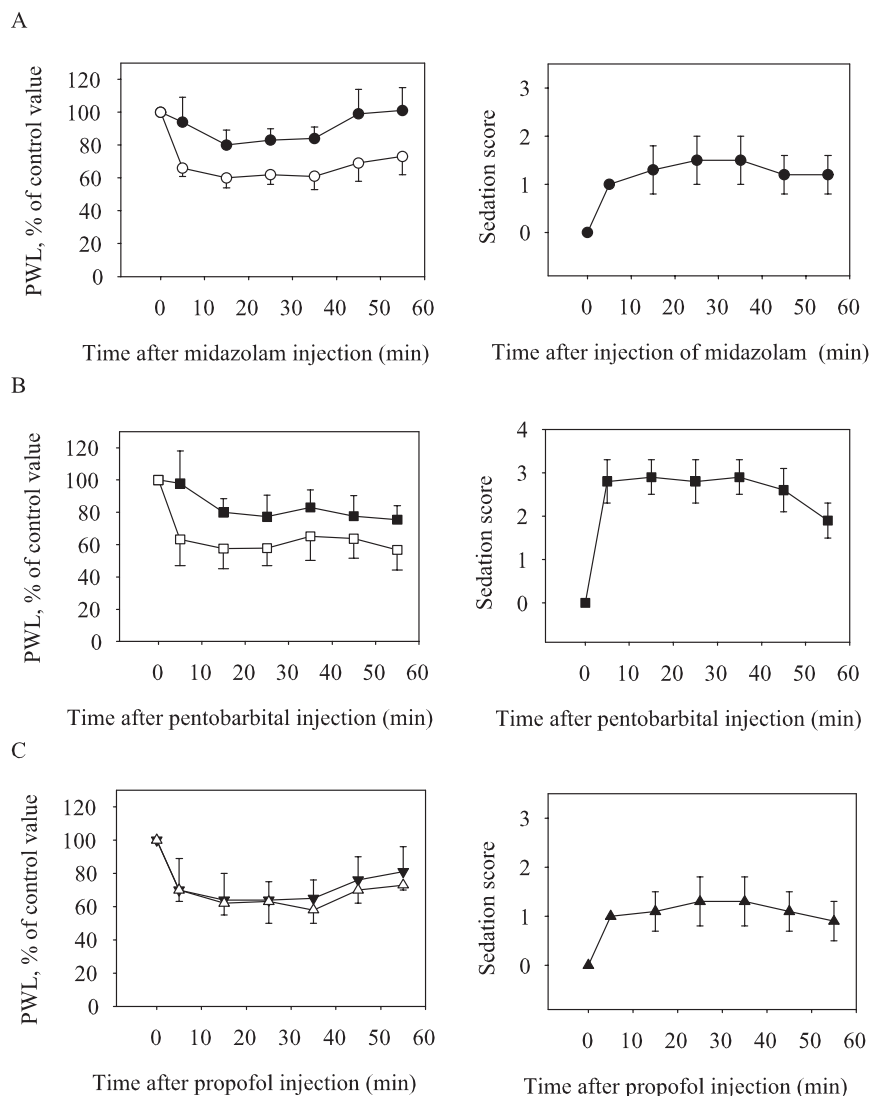


Fig. 3. The effects of lumbar intrathecal pretreatment with 10 μ M ethoxzolamide, 50 μ M, on the time course of PWL and sedation scores after intraperitoneal injection of (A) 1.9 mg/kg midazolam ($n = 6$ animals), (B) 30 mg/kg pentobarbital ($n = 8$ animals), or (C) 50 mg/kg propofol ($n = 8$ animals). The mean PWL during the 55-min observation period was longer in the lower limbs (filled symbols) than in the upper limbs (empty symbols) for midazolam and pentobarbital but not for propofol (see table 3 for details of analysis).

profiles of results for 20 μ M acetazolamide and 50 μ M ethoxzolamide were similar (figs. 2 and 3), the data were pooled for comparison of the results between midazolam, pentobarbital, and propofol. The effect was significantly less ($P = 0.02$) with propofol ($65 \pm 10\%$ in hind limbs *vs.* $71 \pm 10\%$ in forelimbs) than with either midazolam (67 *vs.* $87 \pm 10\%$) or pentobarbital (60 ± 9 *vs.* $83 \pm 9\%$).

Discussion

Previously, we have reported⁷ that systemic administration of acetazolamide blunts the nociceptive hyperreflexia induced by pentobarbital without altering the pre-pentobarbital reflex latency. In the current study, the results show (table 1) that intrathecal administration of 20 μ M acetazolamide did not alter forelimb or hind limb

Table 2. Sedation Scores

	Intrathecal Drug						<i>P</i>
	<i>n</i>	aCSF*	<i>n</i>	20 μ M acetazolamide	<i>n</i>	50 μ M ethoxzolamide	
Midazolam	4	1.3 ± 0.5	5	1.1 ± 0.3	6	1.3 ± 0.3	0.69
Pentobarbital	8	$2.8 \pm 0.3^\dagger$	12	$2.6 \pm 0.3^\dagger$	8	$2.7 \pm 0.3^\dagger$	0.28
Propofol	5	1.1 ± 0.5	8	1.3 ± 0.3	7	1.1 ± 0.3	0.40

Mean sedation scores \pm SD during the 55-minute observation period after sedative drug injection.

* For midazolam and propofol, control animals did not have catheters implanted and did not receive intrathecal artificial CSF (aCSF). *n* represents the number of animals. $^\dagger P < 0.001$ for one-way analysis of variance comparing midazolam, pentobarbital, and propofol within each intrathecal drug category.

aCSF = artificial cerebrospinal fluid.

Table 3. Mean Paw Withdrawal Latency Values (% of Control) during Sedation with Pentobarbital, Propofol, and Midazolam

Condition	n	Forelimb	Hindlimb	P
20 μ M pentobarbital + acetazolamide	8	60 \pm 8	92 \pm 18	0.001
50 μ M pentobarbital + ethoxzolamide	8	61 \pm 13	84 \pm 8	0.007
Pentobarbital + aCSF	9	59 \pm 11	63 \pm 9	0.220
20 μ M propofol + acetazolamide	7	63 \pm 7	68 \pm 6	0.015
50 μ M propofol + ethoxzolamide	7	66 \pm 5	70 \pm 12	0.170
Propofol	5	61 \pm 6	60 \pm 8	0.420
20 μ M midazolam + acetazolamide	5	66 \pm 13	84 \pm 6	0.006
50 μ M midazolam + ethoxzolamide	6	65 \pm 6	91 \pm 6	0.001
Midazolam	4	66 \pm 8	63 \pm 11	0.400

Mean paw withdrawal latency during the 55 min after anesthetic injection, expressed as percent of preanesthetic control, \pm SD. Acetazolamide and ethoxzolamide were administered intrathecally into the lumbar region. *P* is the significance level for the paired *t* test between forelimbs and hindlimbs.

reflex latency. Following treatment with pentobarbital, forelimb withdrawal latencies in animals treated with 20 μ M acetazolamide (60 \pm 8%) were similar to those of control animals pretreated intrathecally with aCSF (59 \pm 11%; table 3). In contrast, hind limb reflex latencies in the acetazolamide-treated animals remained at 92 \pm 18% of control values (table 3). The observation that lumbar intrathecal injection of acetazolamide blocked pentobarbital-induced hyperreflexia in the hind limbs but not the forelimbs is consistent with a spinal site of action. The results suggest that spinal CA makes a major contribution to the hyperreflexia induced by systemic administration of pentobarbital, midazolam, and propofol. These findings also support a specific physiologic role for spinal CA and bicarbonate ion since the effective concentrations of acetazolamide were within the values conventionally considered to inhibit CA without producing ancillary side effects.¹⁸

The results following intrathecal injection of ethoxzolamide show some important differences with those

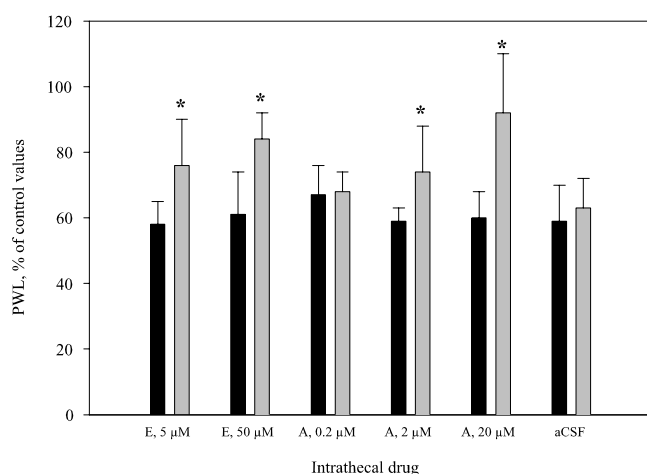


Fig. 4. Paw withdrawal latency (percent of preanesthetic values, mean values \pm SD) in forelimbs (black bars) and hind limbs (gray bars). Nociceptive withdrawal hyperreflexia was induced with intraperitoneal pentobarbital (30 mg/kg). There was a concentration-dependent reduction of hind limb hyperreflexia with acetazolamide (A; *P* = 0.002, interaction term 2-way analysis of variance). **P* < 0.01 relative to upper limb values. E = ethoxzolamide.

with acetazolamide. Intrathecal injection of 50 μ M ethoxzolamide produced a 10% reduction in reflex latency in both the forelimbs and hind limbs. Since this was not seen with either systemic or intrathecal treatment with acetazolamide, these findings suggest that ethoxzolamide may have additional effects on reflex latency. The latter may be due to either systemic effects following reabsorption from CSF or to redistribution to CSF that bathes the forelimb neural structures.

The current findings provide support for a role of CA-dependent, GABA_A receptor-mediated neuromodulation in nociceptive signal transfer in the spinal cord. Although we are not aware of previous reports of this phenomenon in the spinal cord, many laboratories^{10,12,13,15,20,21-23} have investigated a similar process referred to as the anion-shift hypothesis^{10,13} in mammalian hippocampal circuits. Briefly, the anion-shift hypothesis states that under conditions that prolong the open time of the GABA_A receptor channel, there is a shift of ion flux from Cl⁻ to HCO₃⁻ through the channel. This shift results in an HCO₃⁻-mediated depolarizing excitatory current that converts the GABA-mediated postsynaptic membrane response from inhibitory to excitatory. The consequence is facilitation of synaptic transmission and synaptic plasticity.¹³ Extracellular alkaline pH transients lasting approximately 30 s have been observed following GABA_A receptor stimulation with GABA or muscimol²¹ in comparison to the excitatory effect that persists for at least an hour in the current study. The GABA_A receptor-mediated ion shift can be initiated by an activity-related increase in GABA release, by prolonging the open time of the channel pharmacologically or by activation of CA.^{9,10,13} Voipio and Kaila¹⁴ have referred to these modulatory processes as activity-dependent, bicarbonate-mediated excitatory "volume" transmission. Carbonic anhydrase activators enhance, while CAIs impair spatial learning and memory in conscious animals.^{24,25} Sun and Alkon¹⁰ have reviewed recent studies that indicate that in the hippocampus, carbonic anhydrase plays a key role in signal transmission in neuromodulation of signal transmission in hippocampal neural circuits and "functions as an effective attentional gate."

We are unaware of any previous reported evidence supporting CA gating of nociceptive transmission in the spinal cord.

It is of interest that intrathecal CAI had a smaller effect on hind limb nociceptive reflex latency after propofol injection. Although propofol induced a similar magnitude of hyperreflexia as with pentobarbital and midazolam, intrathecal administration of CAI did not reduce the hyperreflexia induced in the lower limbs as much as it did with pentobarbital and midazolam. Since midazolam and propofol produced similar levels of sedation (table 2), the difference in CAI effect on hind limb hyperreflexia in the propofol group cannot be simply explained by the differences in levels of sedation. Midazolam, pentobarbital, and propofol all alter the decay kinetics of GABA_A receptor-mediated currents by slowing deactivation and/or desensitization of GABA_A receptors.^{26–28} Perhaps a detailed understanding of the differences in functional and pharmacological properties among these drugs can help to explain the difference in CAI sensitivity that we have found in the current study.

The findings of the current study may have clinical relevance to the neural mechanism(s) of some aspects of the excitement phase of anesthesia. Excitement during emergence from anesthesia presents a number of management problems, such as arterial hypertension, agitation, delirium, and increased responsiveness to pain. Our findings suggest the latter component may be due to increased transmission of nociceptive impulses achieved by interaction with a CA-mediated gating system similar to that seen in hippocampal pathways.⁹ We have previously presented evidence²⁹ to support a role for bicarbonate-mediated, CA-dependent mechanisms in the enhancement of synaptic transmission by pentobarbital in rat hippocampus *in vitro*. The high degree of correlation between the pharmacodynamics of nociceptive reflex enhancement and activation of the hippocampal electroencephalogram during barbiturate sedation is consistent with a common pharmacological mechanism for these two “excitatory” phenomena.³⁰

In summary, spinal CA contributes to nociceptive hyperreflexia induced by the systemic administration of pentobarbital, midazolam, and propofol. The smaller effect of spinal CAI in propofol-treated animals may indicate that there are additional important factors mediating hyperreflexia induced by propofol. The current findings are consistent with the concept that pentobarbital, midazolam, and, to a lesser extent, propofol interact with mechanisms of carbonic anhydrase gating of nociceptive impulse transmission.

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