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Acetic acid conditioning stimulus induces long-lasting antinociception of somatic inflammatory pain

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Abstract

A wide variety of noxious stimuli are known to induce a powerful inhibition of pain sensation evoked at a remote region of the body. Here we show that an intraperitoneal acetic acid (AA) conditioning stimulus produces long-lasting inhibition of formalin-evoked somatic inflammatory pain behavior in mice. This novel long-lasting antinociception was completely blocked by the 5-hydroxytryptamine type 2A/2C (5-$HT_{2A/2C}$) receptor antagonists, ketanserin and ritanserin, but not by the opioid receptor antagonist, naloxone, and α-adrenergic receptor antagonists, phentolamine and yohimbine. In contrast, the 5-$HT_{3/4}$ receptor antagonist, tropisetron, significantly potentiated this long-lasting antinociception. The conditioning stimulus significantly upregulated the levels of both tryptophan hydroxylase immunoreactivity in the medulla oblongata and the 5-$HT_{2A/2C}$ receptor mRNA level in the spinal cord. These results suggested that the visceral noxious stimulus caused a long-lasting augmentation of the serotonergic inhibitory system and downregulated the somatic inflammatory nociceptive transmission.

Keywords: Antinociception; Supraspinal analgesic mechanism; Descending controls; Counterirritation; Acetic acid writhing test; Formalin test; Serotonin; 5-HT receptor; Tryptophan hydroxylase; Nonopioid analgesia

1. Introduction

The phenomenon that pain can be relieved by the experience of pain at another locus has been appreciated for a long time. This counterirritation phenomenon is well documented in many human experiments, and similar behavior can be observed in a large number of animal models (Villanueva and Le Bars, 1995). It has been shown that a wide variety of noxious mechanical, thermal, and chemical stimuli induce a marked inhibition of activities of spinal dorsal horn convergent neurons and it has been suggested that supraspinal neural system mediates the inhibitory control through descending pathways (Le Bars et al., 1979a,b).

The well-known descending pathways for antinociception are the periaqueductal gray (PAG)–rostral ventromedial medulla (RVM)–spinal cord system and the noradrenergic systems derived from pontine A5, A6 (locus coeruleus), and A7 (subcoeruleus) clusters (Fields and Basbaum, 1994). A prominent neurotransmitter involved in the PAG–RVM–spinal cord-mediated antinociception is serotonin (5-hydroxytryptamine, 5-HT), which is released from the terminals of RVM projection neurons in the spinal dorsal horn. The noradrenergic pathways also provide a rich innervation of the spinal cord in a specific pattern. Among the above noradrenergic nuclei, the A7 group innervates the superficial dorsal horn most densely (Kingrey et al., 1997). It is generally considered that endogenous opioids released in various situations inhibit GABAergic inhibitory interneurons in PAG and RVM, which leads to the activation of spinally projecting serotonergic and noradrenergic inhibitory pathways. Systemically applied opioids are believed to activate, at least partially, this descending inhibitory system to induce their analgesia action.

Various noxious conditioning and test stimuli have been employed in the studies of counterirritation phenomena. Most of these stimuli were acute cutaneous in origin, such as mechanical and thermal stimuli applied to the tails or paws evoking acute somatic pain (Le Bars et al., 1979a,b;

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Dickenson and Sullivan, 1987). Several studies have used visceral noxious conditioning stimuli such as intraperitoneal injection of diluted acetic acid (AA) (Calvino et al., 1984) or other algogenic agents (Le Bars et al., 1979a,b; Kraus et al., 1981, 1982), or distension of abdominal viscera (Cadden and Morrison, 1991). Although these visceral conditioning stimuli have been shown to be effective in inducing antinociceptive effects on acute somatic pain, the duration of the evoked antinociception is typically in the range of minutes, making its clinical use unpractical. Furthermore, studies examining the effectiveness of these stimuli on clinically more important pain, like inflammatory and neuropathic pain, have not been reported. Recently, several studies investigating the effects of conditioning nerve injuries on subsequent nociceptive responses/behavior evoked at distant sites have been reported in rats (Danziger et al., 2001; Kalmari et al., 2001; Monhemius et al., 2001; Benoliel et al., 2002). The results obtained seem to be somewhat controversial: inhibitory effects (Monhemius et al., 2001), no effects (Danziger et al., 2001; Kalmari et al., 2001), or even enhanced effects (Benoliel et al., 2002) on the nociceptive responses/behavior were reported.

The aim of this study was to investigate the effects of visceral noxious conditioning stimuli on a behavioral model of somatic inflammatory pain. We employed the AA or magnesium sulfate (MS) injection (intraperitoneally) as the conditioning stimulus and the paw formalin test as the model for somatic inflammatory pain. AA and MS are commonly employed as visceral inflammatory and non-inflammatory pain-inducing agents, respectively (Koster et al., 1959; Gyires and Torma, 1984). The paw formalin test has been widely used as a useful model to characterize acute chemical pain and delayed inflammatory pain (Tjølsen et al., 1992). In this study, we concentrated on examining the formalin-evoked behavior at least more than a week after the conditioning stimuli to explore the possible existence of a long-lasting pain-inhibitory control mechanism. Test stimuli around 1–3 weeks after the conditioning stimuli were used in the studies investigating the impact of nerve injuries, because such intervals were required for full expression of the neuropathic pain-related behaviors (Danziger et al., 2001; Kalmari et al., 2001; Monhemius et al., 2001; Benoliel et al., 2002). Persistent nociceptive input following nerve injuries might also be necessary to fully activate endogenous pain control system (Monhemius et al., 2001).

2. Materials and methods

2.1. Animals

Adult male C57BL/6J mice (25–30 g, 12–16 weeks; Clea Japan, Japan) were used. Mice were housed in a temperature- and humidity-controlled environment with a 12-h light/dark cycle and free access to food and water. All behavioral experiments were performed in a sound-proof room during the light cycle (7:00 a.m.–7:00 p.m.). Mice were allowed to become acclimatized to the sound-proof room for at least 1 h before starting the experiments. The experiments were conducted in accordance with the ethical guidelines for the study of experimental pain in conscious animals (International Association for the Study of Pain, 1995). The protocols of the pain behavioral studies described in this paper were approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University.

2.2. Visceral conditioning stimuli

Mice were treated with 0.6% AA (0.1 ml/10 g body weight, 250–300 μl ip) or 1.2% MS (0.1 ml/10 g body weight, 250–300 μl ip) solution. The mice were placed individually in transparent observation chambers and the number of writhes per animal within 20 min after the injection was counted. The pain responses were terminated in about 1 h. Then we returned the mice to the home cages. The following observation indicated normal behavior in the mice (i.e., they did not show any signs of pain), although we could not completely exclude the possibility that these conditioning stimuli provoke less evident long-lasting effects. Control experiments were conducted by intraperitoneal injection of physiological saline solution. All mice showed normal body weight gain at least 3 weeks after these visceral and somatic (formalin; see Section 2.3) conditioning stimuli. One to 6 weeks after the AA or saline injection, or 2 weeks after MS injection, we examined the somatic noxious tests described below in the presence or absence of one of the drugs (see Section 2.5). Each mouse was tested at only one of four time points (1, 2, 3, or 6 weeks after one of the conditioning stimuli), and euthanized with excess doses (100–200 mg/kg ip) of sodium pentobarbital (Nembutal; Dainippon Pharmaceutical, Osaka, Japan) after the experiment.

2.3. Somatic noxious conditioning or test stimulus

To study the effect of the visceral noxious conditioning stimuli on the somatic acute pain and prolonged pain, we used the formalin (Tjølsen et al., 1992), von Frey (Chaplan et al., 1994), and paw flick (Hargreaves et al., 1988) tests. Formalin was also employed as a somatic noxious conditioning stimulus in some experiments. The formalin test was conducted as previously described (Saegusa et al., 2000). Briefly, 10 μl of 0.5% paraformaldehyde (PFA) in saline was injected subcutaneously into the dorsal surface of the hindpaw using a 30-gauge needle connected to a microsyringe under light halothane anesthesia. Immediately after the injection, each mouse was placed in the observation chamber. Within 1 min after the injection, the mouse displayed the behavior typical of this model of nociception (i.e., the mouse held the injected paw just off from the floor or displayed licking, biting, and flinching). The time spent

licking and/or biting the injected hindpaw was recorded for 2-min periods at 1–3 and 5–7 min and then at 5-min intervals for 10–45 min after the injection. An initial acute phase [Phase 1 (Ph 1), during the first 7 min after the formalin injection] was followed by a relatively short quiescent period and then by a prolonged tonic response [Phase 2 (Ph 2), beginning 10 min after the formalin injection]. Ph 1 is presumed to be a result of a direct activation of primary afferent nociceptors, and Ph 2 involves peripheral inflammatory events and central sensitization (Tjølsen et al., 1992). The cumulative times spent for the pain-related behaviors during Ph 1 and Ph 2 were analyzed separately. At the formalin concentration used in this study, the pain response appeared to be near maximal, since the magnitude of the pain response evoked by 2% PFA (10 μl) was not significantly different from that evoked by 0.5% PFA in our experimental condition (data not shown).

The assessment of mechanical (von Frey test) and thermal (paw flick test) sensitivities of hindpaws was carried out according to previously described methods (Saegusa et al., 2000). In this study, we assessed the effects of AA conditioning stimulus, which was applied 2 or 3 weeks before. First, mechanical sensitivity was assessed and, 1–2 h after this test, thermal sensitivity was measured. Mechanical sensitivity was evaluated with calibrated von Frey hairs (Stoelting, Wood Dale, IL, USA) by measuring the tactile stimulus producing a 50% likelihood of hindpaw withdrawal response (50% gram threshold), which was determined using the up–down paradigm (Chaplan et al., 1994). Thermal sensitivity was evaluated by measuring withdrawal latency with a Ugo Basile plantar test apparatus (Ugo Basile, Comerio, Italy). Data from right and left hindpaws were combined and averaged in both tests.

2.4. Peripheral inflammatory response

The extent of peripheral inflammation was assessed by measuring the paw volume immediately before (V_{pre}) and 1 h after (V_{post}) formalin injection with plethysmometer (Unicom TK101; Unicom, Chiba, Japan). Percent peripheral inflammation was calculated as follows: % peripheral inflammation=$(V_{post} - V_{pre})/V_{pre} \times 100\%$.

2.5. Pharmacology

The following drugs were used to characterize pharmacologically the long-lasting antinociception: ketanserin tartrate, ritanserin, tropisetron, yohimbine hydrochloride (all from Research Biochemicals International, Natick, MA, USA), naloxone hydrochloride, and phentolamine hydrochloride (both from Sigma, St. Louis, MO, USA). All drugs, except ritanserin, were dissolved in physiological saline and injected intraperitoneally 1 h before formalin tests. Ritanserin was dissolved in dimethyl sulfoxide and diluted to desired concentrations in physiological saline. Intrathecal injection of ketanserin (5 μl) was made 15–20 min before formalin tests according to the procedure of Hylden and Wilcox (1980), using a microsyringe with a 30-gauge needle. The doses of these drugs were determined according to previous studies (Vaccarino et al., 1988; Tasker and Melzack, 1989; Alhaider, 1991; Takeshita and Yamaguchi, 1995; Ringkamp et al., 1999) and our preliminary study.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitative immunoblot analysis

Mice were deeply anesthesized with diethyl ether and decapitated at 2–3 and 6 weeks after AA or saline injection, and medullae oblongatae were dissected. Pooled samples (two medullae per sample) were homogenized in 300 μl of lysis buffer [2% SDS, 1 mM dithiothreitol, 10% glycerol, a protease inhibitor cocktail tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany), and 50 mM Tris–HCl buffer, pH 6.8]. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, CA, USA). Proteins were separated using 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo, Japan). The membrane was blocked with TBS (20 mM Tris–HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween 20 and 5% blocking reagent, and probed with a rabbit anti tryptophan hydroxylase (TPH) antibody (1:200 dilution; Chemicon International, CA, USA) and visualized with the Vistra ECF Western blotting system (an alliance of Amersham International and Molecular Dynamics, CA, USA). The immunoreactive bands were quantified with IMAGE QUANT software (Molecular Dynamics). Eight independent Western blot experiments of four independent medulla samples from AA-conditioned or saline control mice were analyzed. For each set of experiments, the band intensity of the saline control sample was set at 100, and then the relative band intensity of the AA-conditioned sample was calculated as a fraction of this value. For the negative control, the membrane was incubated in the absence of the primary antibody.

2.7. RNA extraction and semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Mice were deeply anesthesized with diethyl ether and decapitated at 2–3 weeks after AA or saline injection, and lumbar spinal cords (L_3–L_6) and medullae oblongatae were dissected. Total cellular RNA was isolated from the pooled (two mice per sample) lumbar spinal cords and medullae by TRIZOL reagent (Gibco BRL/Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized in a 20-μl final volume using 1 μg of total RNA, which had been treated with RNase-free DNase I (Gibco BRL/Life Technologies), by Takara RNA LA PCR kit (Takara Biochemicals, Otsu, Japan). PCR conditions for the simultaneous linear amplification of 5-$HT_{2A/2C}$ receptors and β-actin (289 bp), which was used as an endogenous internal control to com-

pensate for sample-to-sample variation, were determined empirically. Previously described primers (Saucier et al., 1998) were used for amplification of the 5-$HT_{2A/2C}$ receptors. Bands for 5-$HT_{2A/2C}$ receptors consisted of three discrete DNA fragments 542/525 and 430 bp in size. The 542- and 525-bp bands were shown to encode the 5-HT_{2A} and 5-HT_{2C} receptor cDNAs, respectively. The 430-bp amplification product was also shown to correspond to a splice variant of the 5-HT_{2C} receptor. For image analyses, we measured the combined 542/525-bp band intensity using a FluorImager 595 and an IMAGE QUANT software (Molecular Dynamics). Each set of data was calculated as the ratio of 5-HT receptors to β-actin mRNA levels, and the value of AA-conditioned sample was expressed as a percentage of that found in the saline control. At least duplicate amplification was performed from three (for medulla) or four (for spinal cord) independent samples.

2.8. Statistical analysis

Experimental data were expressed as mean ± S.E.M. and tested according to Student's *t* test for the immunoblot and RT-PCR data analyses; Mann–Whitney *U* test for comparisons between two groups; and one-way analysis of variance (ANOVA) or two-way repeated-measures ANOVA followed by Tukey test for multiple comparisons. $P < .05$ was considered statistically significant.

3. Results

3.1. Effects of the conditioning stimuli on the somatic nociceptive responses

AA (0.6%) induced typical writhing responses (19.1 ± 0.42, $n = 129$). Control mice injected with saline never displayed the aversive responses ($n = 131$). One to 6 weeks after the administration, the response to the formalin injection was examined. There was no significant difference in the magnitude of the biphasic nociceptive responses among the naïve and the saline groups (Ph 1: Table 1; Ph 2: naïve: 170.1 ± 8.21 s, $n = 30$; 1 week: 146.7 ± 23.4 s, $n = 12$; 2 weeks: 170.7 ± 6.11 s, $n = 15$; 3 weeks: 159.0 ± 13.5 s, $n = 7$; 6 weeks: 170.9 ± 16.1 s, $n = 6$) (Fig. 1A). One week after the AA conditioning (hereafter referred to as AA 1w), the Ph 2 response of the AA-conditioned group (105.4 ± 8.98 s, $n = 13$) was considerably reduced compared with that of the saline group (referred to as S 1w) (Fig. 1A). Two and 3 weeks after the AA conditioning (AA 2w and AA 3w, respectively), the Ph 2 responses of the conditioned groups (AA 2w: 102.8 ± 9.56 s, $n = 15$; AA 3w: 92.2 ± 14.8 s, $n = 7$) were significantly reduced compared with their respective saline control responses ($P < .01$ for both groups) (Fig. 1A). The magnitudes of the AA-conditioned Ph 2 responses among 1- to 3- week groups were not significantly different. The inhibitory effect, however, was not observed in the mice

Table 1
Summary of the Ph 1 duration of the formalin-induced licking/biting behavior

Conditioning stimulation[a]		Antagonist (dosage, route)	Ph 1 (s)[b]	*n*
Naïve			13.2 ± 1.15	30
1 week	Acetic acid		13.5 ± 1.41	13
1 week	Saline		13.3 ± 1.17	12
2 weeks	Acetic acid		14.3 ± 2.25	15
2 weeks	Saline		13.6 ± 1.42	15
3 weeks	Acetic acid		11.7 ± 1.10	7
3 weeks	Saline		13.4 ± 2.41	7
6 weeks	Acetic acid		15.3 ± 1.31	6
6 weeks	Saline		12.6 ± 2.18	6
2 weeks	$MgSO_4$		14.9 ± 3.40	7
2 weeks	Saline		18.1 ± 4.62	7
First[c]	Formalin		15.3 ± 1.85	12
2 weeks later[c]	Formalin		17.9 ± 1.40	12
2 weeks	Acetic acid	Ketanserin (1 mg/kg ip)	17.3 ± 2.08	8
2 weeks	Saline	Ketanserin (1 mg/kg ip)	13.7 ± 3.16	9
2 weeks	Acetic acid	Ketanserin (0.3 nmol it)	13.1 ± 2.38	6
2 weeks	Saline	Ketanserin (0.3 nmol it)	15.7 ± 2.49	8
2 weeks	Acetic acid	Ketanserin (1 nmol it)	15.5 ± 3.11	8
2 weeks	Saline	Ketanserin (1 nmol it)	16.6 ± 3.24	7
2 weeks	Acetic acid	Saline (5 μl it)	18.4 ± 4.71	7
2 weeks	Saline	Saline (5 μl it)	20.5 ± 5.24	8
3 weeks	Acetic acid	Ketanserin (1 mg/kg ip)	11.4 ± 2.42	5
3 weeks	Saline	Ketanserin (1 mg/kg ip)	14.4 ± 2.76	6
2 weeks	Acetic acid	Ritanserin (1 mg/kg ip)	15.0 ± 1.64	6
2 weeks	Saline	Ritanserin (1 mg/kg ip)	16.3 ± 1.64	5
2 weeks	Acetic acid	Tropisetron (3 mg/kg ip)	11.1 ± 2.15	9
2 weeks	Saline	Tropisetron (3 mg/kg ip)	13.0 ± 1.99	10
2 weeks	Acetic acid	Phentolamine (3 mg/kg ip)	15.7 ± 2.33	8
2 weeks	Saline	Phentolamine (3 mg/kg ip)	12.5 ± 1.28	8
2 weeks	Acetic acid	Yohimbine (3 mg/kg ip)	11.6 ± 1.39	7
2 weeks	Saline	Yohimbine (3 mg/kg ip)	15.9 ± 2.21	7
2 weeks	Acetic acid	Naloxone (3 mg/kg ip)	12.4 ± 3.19	8
2 weeks	Saline	Naloxone (3 mg/kg ip)	15.4 ± 2.82	7
2 weeks	Acetic acid	Naloxone (30 mg/kg ip)	11.5 ± 1.94	6
2 weeks	Saline	Naloxone (30 mg/kg ip)	11.7 ± 1.98	6

[a] Represents a type of conditioning stimuli and a conditioning stimulus–test stimulus (formalin) interval.

[b] Mean licking/biting behavior (s) ± S.E.M. during Ph 1 period.

[c] In the case of using formalin as a conditioning stimulus, we first administered the formalin into the right hindpaw (first) and, 2 weeks later, the test formalin stimulus was applied into the contralateral left hindpaw (2 weeks later).

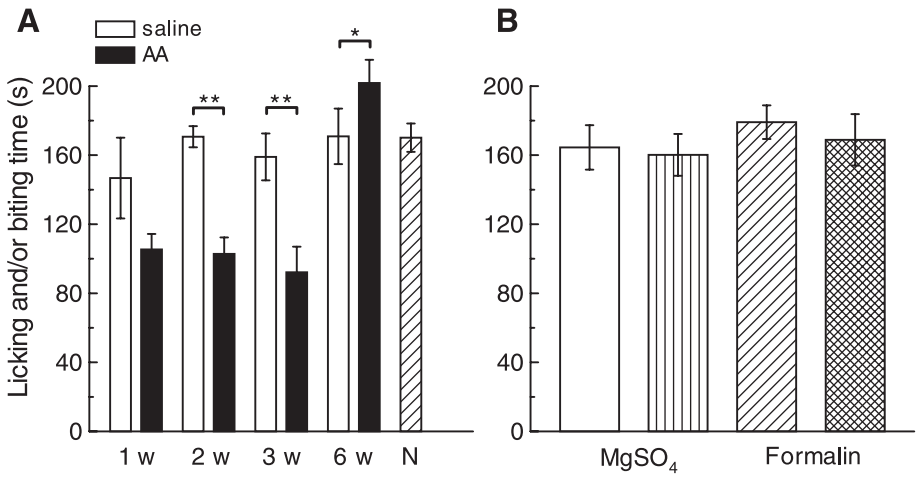

Fig. 1. Effects of the noxious conditioning stimuli on the formalin-induced Ph 2 response. (A) Time course of AA-evoked inhibition of the Ph 2 response. AA-induced significant (**$P<.01$ after Mann–Whitney U test) inhibition at the two time points (2 and 3 weeks) compared with their corresponding saline control. In contrast, the Ph 2 response was significantly (*$P<.05$, after Mann–Whitney U test) potentiated at 6 weeks. For comparison, the Ph 2 response of naïve animals (N) is shown (diagonal up-hatched bar). (B) Effect of magnesium sulfate ($MgSO_4$; MS) or formalin conditioning stimuli on the Ph 2 response. In contrast to AA, MS did not induce significant inhibition of the Ph 2 response (white bar, saline control; vertical hatched bar, mice conditioned with MS 2 weeks before). In the case of formalin stimulation, the conditioning formalin stimulus (diagonal up-hatched bar) was applied to the right hindpaw, and test formalin stimulus (diagonal cross-hatched bar) was applied to the left hindpaw 2 weeks later. This conditioning stimulus also had no effect on the Ph 2 response.

conditioned 6 weeks before, and a significant facilitatory effect was revealed (201.8 ± 13.4 s, $n=6$; $P<.05$) (Fig. 1A). The Ph 1 responses were not affected by the AA conditioning stimuli (Table 1). The acute mechanical and thermal thresholds were also not affected by the AA stimuli conditioned 2 and 3 weeks before (50% gram threshold: AA 2w: 0.52 ± 0.10 g, $n=5$; S 2w: 0.61 ± 0.11 g, $n=5$; AA 3w: 0.57 ± 0.14 g, $n=5$; S 3w: 0.58 ± 0.08 g, $n=5$; withdrawal latency: AA 2w: 15.5 ± 1.16 s, $n=5$; S 2w: 16.0 ± 1.14 s, $n=5$; AA 3w: 16.2 ± 0.98 s, $n=5$; S 3w: 16.6 ± 1.27 s, $n=5$). In addition, the conditioned stimuli had no effect on the formalin-induced peripheral inflammation (AA 2w: 28.5 ± 3.06%, $n=6$; S 2w: 32.3 ± 5.15%, $n=6$). Thus, the AA conditioning stimulus was found to induce a long-lasting inhibition (more than 3 weeks) of the formalin-induced somatic inflammatory pain without affecting the sensitivities of acute chemical, mechanical, and thermal pain, and this inhibition did not seem to be attributable to the reduced peripheral inflammation.

We next investigated whether this long-lasting inhibition could be induced by other conditioning stimuli like MS and formalin. All the mice injected with MS displayed typical writhing responses (9.1 ± 0.91, $n=9$). However, 2 weeks after this visceral stimulus, formalin-induced Ph 1 and Ph 2 responses were not inhibited (MS: Ph 1: Table 1; Ph 2: 160.2 ± 12.1 s, $n=7$; S: Ph 1: Table 1; Ph 2: 164.5 ± 12.9 s, $n=7$) (Fig. 1B). In the case of formalin conditioning, we first administered the formalin into the right hindpaw (Ph 1: Table 1; Ph 2: 179.1 ± 9.74 s, $n=12$), and 2 weeks later, the test formalin was injected into the left hindpaw to avoid possible reduction of Ph 2 response due to the damage to sensory fibers and the surrounding tissues at the first injection site. This somatic pain conditioning stimulus did not inhibit both Ph 1 and Ph 2 responses induced by the second formalin injection (Ph 1: Table 1; Ph 2: 168.9 ± 14.9 s, $n=12$) (Fig. 1B). These results suggest that the long-lasting inhibition of the Ph 2 response appears to be preferentially induced by the visceral inflammatory stimulus.

3.2. 5-$HT_{2A/2C}$ receptor antagonists reverse the long-lasting antinociception

Several studies have indicated the involvement of supraspinal neural network in the counterirritation phenomena (see Introduction). To examine the pharmacological characteristics of the present long-lasting antinociception, we assessed the effects of several 5-HT and α-adrenergic receptor antagonists.

Ketanserin (1 mg/kg ip), a 5-$HT_{2A/2C}$ receptor antagonist, completely blocked the AA-evoked inhibition of the formalin-induced Ph 2 response (AA 2w: 192.5 ± 4.90 s, $n=8$; S 2w: 177.0 ± 9.65 s, $n=9$; AA 3w: 189.0 ± 30.6 s, $n=5$; S 3w: 151.6 ± 19.8 s, $n=6$) (Fig. 2A). Ritanserin (1 mg/kg ip), another 5-$HT_{2A/2C}$ receptor antagonist, also antagonized the inhibition (AA 2w: 175.5 ± 21.8 s, $n=6$; S 2w: 197.5 ± 12.3 s, $n=5$) (Fig. 2A). In contrast, a 5-$HT_{3/4}$ receptor antagonist, tropisetron (3 mg/kg ip), did not reverse, but further reduced, the Ph 2 response (AA 2w: 68.9 ± 14.3 s, $n=9$; S 2w: 184.2 ± 7.37 s, $n=10$; $P<.05$) (Fig. 2A).

On the other hand, phentolamine (an α_1/α_2-adrenergic receptor antagonist, 3 mg/kg ip; AA 2w: 87.2 ± 7.64 s, $n=8$; S 2w: 172.4 ± 16.5 s, $n=8$) and yohimbine (an α_2-adrenergic receptor antagonist, 3 mg/kg ip; AA 2w: 89.9 ± 9.40 s, $n=7$; S 2w: 183.0 ± 21.0 s, $n=7$) had no effect on the AA-evoked inhibition of the Ph 2 response (Fig. 2B). Furthermore, neither low dose (3 mg/kg ip; AA 2w: 106.4 ± 8.47 s,

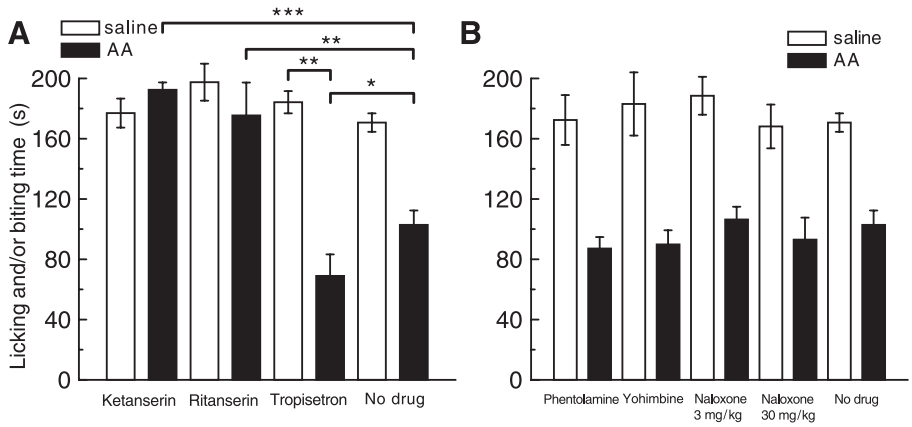

Fig. 2. Pharmacological analysis of the AA-evoked long-lasting inhibition of the Ph 2 response. (A) Effect of 5-HT receptor antagonists on the AA-evoked inhibition of the Ph 2 response. Pretreatment of the 5-$HT_{2A/2C}$ receptor antagonist, ketanserin (1 mg/kg ip) or ritanserin (1 mg/kg ip), completely blocked the inhibition evoked by the AA conditioning stimuli applied 2 weeks before. $^{**}P<.01$, $^{***}P<.001$, when compared with No drug group by two-way ANOVA followed by Tukey test. Tropisetron (3 mg/kg ip), a 5-$HT_{3/4}$ receptor antagonist, did not reverse but further significantly potentiated the inhibition ($^{*}P<.05$, by two-way ANOVA followed by Tukey test). (B) Effect of α-adrenergic receptor antagonists and an opioid receptor antagonist on the AA-evoked inhibition. An α_1/α_2-adrenergic receptor antagonist, phentolamine (3 mg/kg ip), an α_2-adrenergic receptor antagonist, yohimbine (3 mg/kg ip), and low (3 mg/kg ip) and high (30 mg/kg ip) doses of an opioid receptor antagonist, naloxone, did not reverse the inhibition. For comparison, the Ph 2 responses of the AA-conditioned and saline control groups are shown in (A) and (B) (No drug).

$n=8$; S 2w: 188.5 ± 12.6 s, $n=7$) nor high dose (30 mg/kg ip; AA 2w: 93.1 ± 14.6 s, $n=6$; S 2w: 168.1 ± 14.6 s, $n=6$) of naloxone had any effects on the AA-evoked inhibition (Fig. 2B). None of the intraperitoneally administered antagonists significantly affected the Ph 2 response of the saline control group (Fig. 2) and the Ph 1 response (Table 1) in either AA or saline group.

We further examined the effect of the intrathecal administration of ketanserin to investigate the site of action. Intrathecal saline itself tended to depress the Ph 2 responses of AA 2w-conditioned (87.5 ± 15.8 s, $n=7$) and S 2w-conditioned (137.6 ± 23.3 s, $n=8$) groups, compared with their respective control group [i.e., drug-naïve AA 2w-conditioned (102.8 ± 9.56 s, $n=15$) and S 2w-conditioned (170.7 ± 6.11 s, $n=15$) groups mentioned above] (see Section 3.1), although these depressant effects were not significant. However, intrathecal ketanserin (0.3 and 1 nmol) dose-dependently antagonized the AA 2w-evoked inhibition of the Ph 2 responses (0.3 nmol: 114 ± 14.2 s, $n=6$; 1 nmol: 154.9 ± 21.0 s, $n=8$) without affecting saline control groups (0.3 nmol: 143.3 ± 9.14 s, $n=8$; 1 nmol: 150.5 ± 6.42 s, $n=7$). The slight depressant effects on the Ph 2 responses by the intrathecal saline injection might be due to the stress response derived from the intrathecal injection and/or skin incision for the injection (Hylden and Wilcox, 1980). The Ph 1 responses were not affected by the intrathecal administration of ketanserin or saline (Table 1).

3.3. AA conditioning stimulus upregulated TPH immunoreactivity in the medulla oblongata and 5-$HT_{2A/2C}$ receptor mRNA in the spinal cord

To ascertain the involvement of 5-HT in this long-lasting antinociception, we investigated the expression level of TPH, the rate-limiting enzyme for the biosynthesis of 5-HT. Within the central nervous system (CNS) of the vertebrate, TPH is shown to be selectively expressed in the serotonergic neurons of the brainstem raphe nuclei and adjacent reticular formations (for a review, see Joh, 1997).

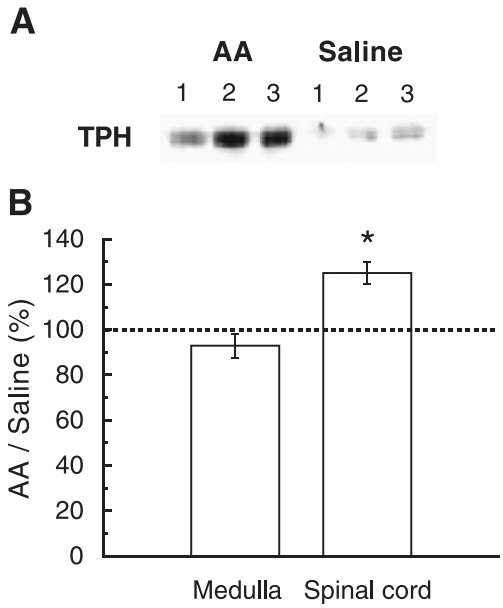

Fig. 3. AA-conditioned stimulus increases TPH protein and 5-$HT_{2A/2C}$ receptor mRNA expression. (A) Representative Western blots for TPH ($M_r=53$ kDa) in the medullae oblongatae from mice treated with AA or saline 2 weeks before. Lanes 1–3 were loaded with 50, 100, and 150 μg of proteins from AA- or saline-treated mice, respectively. (B) Semiquantitative RT-PCR analyses of 5-$HT_{2A/2C}$ receptors from mice conditioned with AA 2 weeks before. Within the linearity of the PCR reaction, the amount of 5-$HT_{2A/2C}$ receptor mRNA relative to β-actin mRNA has been calculated and expressed as percentage of the saline controls. Control level (100%) is shown by dotted line. $^{*}P<.05$, after Student's paired comparison t test.

By quantitative Western blotting analysis, we found that the level of TPH immunoreactivity of AA 2–3w-conditioned mice medullae oblongatae was markedly upregulated compared with that of saline control mice (506 ± 195%, 393 ± 93.3%, and 245 ± 43.1% of saline control at 50-, 100-, and 150-μg lanes, respectively, calculated by Student's *t* paired comparison test, $n = 8$, $P < .05$; Fig. 3A). In contrast, the immunoreactivity returned to saline control level 6 weeks after the AA conditioning (89.4 ± 21.6%, 122 ± 30.1%, and 127.2 ± 31.1% of saline control at 50-, 100-, and 150-μg lanes, respectively, $n = 8$).

Finally, the expression level of mRNA-encoding 5-$HT_{2A/2C}$ receptors was measured by semiquantitative RT-PCR method in both medulla and spinal cord of AA 2–3w-conditioned and saline control mice. Fig. 3B indicated that amplification of AA-conditioned spinal cord (125 ± 4.73% of saline control, calculated by Student's *t* paired comparison test, $n = 8$, $P < .05$), but not medulla (92.9 ± 5.4% of saline control, $n = 6$) samples resulted in significantly higher levels of transcripts compared with those of saline-treated controls.

4. Discussion

In this study, we have shown that an intraperitoneal administration of AA evokes extremely long-lasting inhibition of somatic inflammatory pain in mice. The conditioning stimulus, however, had no effect on acute somatic (mechanical, thermal, and chemical) pain, suggesting the presence of pain type specificity of the conditioning effect. Our results also indicated the plastic nature of descending pain modulation system in mice. In a previous electrophysiological experiment, the Ph 2 neuronal response induced by formalin stimulation was shown to be inhibited by acute pinch conditioning stimulation in rats, but the evoked inhibition lasted for only about 10 min (Dickenson and Sullivan, 1987). Recently, formalin test responses (both Ph 1 and Ph 2) were shown to be reduced by the conditioning partial sciatic nerve injury, which was conducted 7–10 days before (Monhemius et al., 2001). This study suggested that long-term changes in nociceptive processing, which occur following nerve injury, might be necessary to fully activate the endogenous antinociceptive mechanism mediated at least in part via PAG. Details of the activation and extinction of this mechanism are unknown at present. Furthermore, it is possible that the effects of various conditioning nerve injuries vary considerably with respect to the modality of the test noxious stimulus (Danziger et al., 2001; Kalmari et al., 2001; Monhemius et al., 2001; Benoliel et al., 2002). To the best of our knowledge, our report is the first work showing that the activation of long-lasting inhibitory control of somatic inflammatory pain can be evoked by visceral noxious conditioning stimuli. Our model system would contribute to the exploration of the plastic mechanisms of endogenous pain control systems and to the development of novel analgesic drugs that do not share with the side-effect liabilities of narcotic analgesics.

The AA- and MS-induced writhing tests are commonly employed as visceral inflammatory and noninflammatory pain tests, respectively (Koster et al., 1959; Gyires and Torma, 1984). Therefore, we now postulate that visceral inflammation might be an important factor to induce this long-lasting antinociception. The average writhing response to MS was much weaker than that to AA treatment; thus, the dose of MS used in this study might not be sufficient to induce antinociception. However, this does not seem to be the case. Indeed, increases up to five times that of MS concentration did not further increase the writhing number (unpublished results). Thus, the difference of writhing number between AA- and MS-induced writhing tests may represent the different character of pain, although it is still possible that MS (and formalin) conditioning stimulus may evoke antinociceptive effects on formalin-induced pain behavior at different time points, and this possibility should be tested in the future study. Since intraperitoneal injection of AA directly activates visceral and somatic nociceptors innervating the peritoneum, and induces inflammation not only in subdiaphragmatic visceral organs but also in subcutaneous muscle walls, the antinociceptive effect observed in AA conditioning may not be purely visceral in origin. Although further studies are necessary to understand the precise mechanism of this antinociception, this is not likely to be mediated by nonspecific stress response involving humoral and/or hormonal mechanism.

This long-lasting antinociception was insensitive to the α-adrenergic and opioid receptor antagonists. The dosages we employed in this study were within the range of commonly used ones (Vaccarino et al., 1988; Tasker and Melzack, 1989; Alhaider, 1991; Takeshita and Yamaguchi, 1995; Ringkamp et al., 1999). Although C57BL/6J mice are known to be relatively less sensitive to α_2 and opioid analgesics among several mouse strains (for a review, see Lariviere et al., 2001), intraperitoneal administration of an α_2-agonist, dexmedetomidine (50 μg/kg), or morphine (3–10 mg/kg) produced quite robust analgesia (assayed by thermal nociceptive test such as tail flick test and hot plate test) and these analgesia were almost completely antagonized by yohimbine (3 mg/kg ip) and naloxone (1 mg/kg ip) (unpublished observations). Furthermore, warm water (34 °C) swim stress-induced analgesia, which is known to be induced by an opioid-dependent mechanism (Amit and Galina, 1986), was clearly observed in C57BL/6J mice and this analgesia was also completely blocked by naloxone (10 mg/kg ip) (unpublished observations). These observations indicated that we selected quite appropriate dosages, and more importantly, it is possible to induce endogenous opioid-dependent analgesia in C57BL/6J mice.

Contrary to the α-adrenergic and opioid antagonists, the 5-$HT_{2A/2C}$ receptor antagonists blocked—whereas the 5-$HT_{3/4}$ receptor antagonist potentiated—the antinociception. In addition, the AA conditioning stimulus markedly upre-

gulated TPH protein in the medulla oblongata, showing a good temporal correlation with the antinociceptive activity. These results suggest that this long-lasting antinociception is induced by prolonged activation of the serotonergic system in RVM–spinal cord axis directly and/or indirectly via higher brain structures such as PAG neurons in response to the conditioning stimulus. The results of paw volume measurement and intrathecal administration of ketanserin also supported the CNS—probably spinal cord—origin of this antinociception. AA-induced upregulation of 5-$HT_{2A/2C}$ receptor mRNA expression in the spinal cord might also contribute to the prolonged antinociception. Future experiments at the protein level, such as a Western blot or an immunohistochemical study, could be undertaken to examine the upregulation of 5-$HT_{2A/2C}$ receptor. Consistent with our results, a recent in situ hybridization study showed a marked upregulation of 5-HT_{2A} receptor mRNA in rat spinal dorsal horn following peripheral inflammation (Zhang et al., 2001). These observations might indicate an interesting possibility that expression of 5-HT_{2A} receptor in the spinal dorsal horn is regulated by peripheral inflammation.

Accumulated evidence indicates that the spinal 5-HT_2 receptor type contributes to antinociception (Takeshita and Yamaguchi, 1995; Sasaki et al., 2001), although pronociceptive roles of this receptor are also reported (Eide and Hole, 1991; Kjøsvik et al., 2001). The nature and the level of contribution of each 5-HT receptor subtype in the spinal serotonergic pain modulation system are currently under debate (for a review, see Millan, 1997). In contrast with our results, a significant reduction of both Ph 1 and Ph 2 responses in formalin test has been reported in pharmacological studies using 5-HT agonists (Takeshita and Yamaguchi, 1995; Sasaki et al., 2001) and in a study using the partial sciatic nerve ligation as a conditioning stimulus (Monhemius et al., 2001). The reason for this difference is currently under investigation.

There are at least two possible sites for 5-$HT_{2A/2C}$ receptors contributing to the antinociception. One site is on the somata of the inhibitory interneurons such as GABAergic and/or glycinergic neurons in the spinal dorsal horn. There are some data suggesting that the activation of inhibitory interneurons by 5-HT plays a role in mediating a spinal antinociception (Sugiyama and Huang, 1995; see also Millan, 1997). The other site is on the central terminals of primary afferent neurons. Serotonin is known to depolarize the central terminals of primary afferent neurons and inhibit synaptic transmission between nociceptive primary afferents and dorsal horn second-order neurons by suppressing neurotransmitter release (Khasabov et al., 1999). The mechanism involving 5-HT_2 receptor type activation has been proposed to play a role in the primary afferent depolarization (Thompson and Wall, 1996).

This antinociceptive effect presented here, however, does not continue permanently. Six weeks after the conditioning, the sensitized animal showed hyperalgesic behavior against the formalin test. At present, we do not know the exact mechanism of this apparent rebound phenomenon. An interesting possibility is that the 5-$HT_{3/4}$ receptor-meditated facilitatory mechanism, which appears to be concurrently activated by the AA conditioning stimulus but masked by the prominent 5-$HT_{2A/2C}$ receptor-mediated antinociceptive mechanism, might be longer-lived than the antinociceptive mechanism. Further studies are necessary to characterize this pain facilitatory mechanism.

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