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Acute and late effects on induction of allodynia by acromelic acid, a mushroom poison related structurally to kainic acid

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1 Ingestion of a poisonous mushroom *Clitocybe acromelalga* is known to cause severe tactile pain (allodynia) in the extremities for a month and acromelic acid (ACRO), a kainate analogue isolated from the mushroom, produces selective damage of interneurons of the rat lower spinal cord when injected either systemically or intrathecally. Since ACRO has two isomers, ACRO-A and ACRO-B, here we examined their acute and late effects on induction of allodynia.

2 Intrathecal administration of ACRO-A and ACRO-B provoked marked allodynia by the first stimulus 5 min after injection, which lasted over the 50-min experimental period. Dose-dependency of the acute effect of ACRO-A on induction of allodynia showed a bell-shaped pattern from 50 ag kg^{-1} to 0.5 pg kg^{-1} and the maximum effect was observed at 50 fg kg^{-1}. On the other hand, ACRO-B induced allodynia in a dose-dependent manner from 50 pg kg^{-1} to 50 ng kg^{-1}.

3 *N*-methyl-D-aspartate (NMDA) receptor antagonists and Joro spider toxin, a Ca^{2+}-permeable AMPA receptor antagonist, inhibited the allodynia induced by ACRO-A, but not by ACRO-B. However, other AMPA/kainate antagonists did not affect the allodynia induced by ACRO.

4 Whereas no neuronal damage was observed in the spinal cord in ACRO-A-treated mice, induction of allodynia by ACRO-A (50 fg kg^{-1}) and ACRO-B (50 ng kg^{-1}) was selectively lost 1 week after i.t. injection of a sublethal dose of ACRO-A (50 ng kg^{-1}) or ACRO-B (250 ng kg^{-1}). Higher doses of ACRO-A, however, could evoke allodynia dose-dependently from 50 pg kg^{-1} to 500 ng kg^{-1} in the ACRO-A-treated mice. The allodynia induced by ACRO-A (500 ng kg^{-1}) was not inhibited by Joro spider toxin or NMDA receptor antagonists. These properties of the late allodynia induced by ACRO-A were quite similar to those of the acute allodynia induced by ACRO-B.

5 ACRO-A could increase $[Ca^{2+}]_i$ in the deeper laminae, rather than in the superficial laminae, of the spinal cord. This increase was not blocked by the AMPA-preferring antagonist GYKI52466 and Joro spider toxin.

6 Taken together, these results demonstrate the stereospecificity of ACRO for the induction of allodynia and suggest the presence of a receptor specific to ACRO.
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Abbreviations: ACRO, acromelic acid; aCSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methylisooxazole-4-propionic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CL, confidence limits; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D(−)-2-amino-5-phosphonovaleric acid; GYKI52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; i.t., intrathecal; JSTX, Joro spider toxin-3; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[A,D]cyclohepten-5,10-imine hydrogen maleate; NBQX, 2,3-dihydro-9-nitro-7-sulfamoylbenzen[F]quinoxaline; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NS102, 6,7,8,9-tetrahydro-5-nitro-1*H*-benz[G]indole-2,3-dione-3-oxime; PG, prostaglandin; SNP, sodium nitroprusside; SYM2081, (2*S*, 4*R*)-4-methylglutamic acid

Introduction

Glutamate is the main excitatory neurotransmitter in the central nervous system and mediates fast neurotransmission at the vast majority of excitatory synapses. In addition to neurotransmission, glutamate involves neural plasticity and excitotoxicity *via* *N*-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors (Nakanishi, 1992; Hollmann & Heinemann, 1994). The non-NMDA receptor group is further divided pharmacologically into α-amino-3-hydroxy-5-methylisooxazole-4-propionic acid (AMPA) and kainate subtypes. The ingestion of a Japanese poisonous mushroom *Clitocybe acromelalga* causes marked reddish edema and severe tactile pain (allodynia) (erythromelalgia) in the tips of hands and feet, which continue for a month. By monitoring the lethal effect of the toxins in mice, two isomers of acromelic acid (ACRO), ACRO-A and ACRO-B, were isolated from the poisonous

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mushroom (Konno *et al*., 1983). ACRO contains a partial structure of kainate in the molecule and a single systemic administration of ACRO-A to the rat caused a series of abnormal behavioral symptoms, such as an initial marked tonic extension of the hind limbs, followed often by severe tonic/clonic seizures and a transient flaccid paraplegia, and severe spastic paraplegia remained in surviving rats. Histological examination revealed that ACRO induced selective lesions of interneurons confined to the lower spinal cord without causing neuronal damage in the hippocampus and other regions (Shinozaki *et al*., 1989; Kwak *et al*., 1992a). On the other hand, systemic administration of kainate induces quite different types of behavioral changes from those induced by ACRO-A, and it also induced histological damages in hippocampal CA1 and pyramidal cells, but not in the spinal cord. However, there was no difference in the potency of ACRO-A to increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in primary cultured neurons between the spinal cord and hippocampus (Ogata *et al*., 1994). The regional difference of neuronal damage *in vivo* induced by systemic administration of ACRO-A and kainate could not be explained by the difference in the toxicity between cultured spinal and hippocampal neurons *in vitro* (Tsuji *et al*., 1995). Although a new type of non-NMDA receptors specific to ACRO was suggested to be present in the spinal cord, the selective damage of the lumbar spinal cord by ACRO remains unknown.

Consistent with severe allodynia induced by the ingestion of the poisonous mushroom and selective action of ACRO on the lower spinal cord, the dorsal horn of the spinal cord is an important site for pain transmission and glutamate has been shown to be involved in spinal nociceptive processing. Continuous intrathecal (i.t.) injection of ACRO-A induced long-lasting pure motor, rigid-spastic paraparesis in a dose-dependent manner that was accompanied by selective degeneration of interneurons in the laminae II–IV, which was in marked contrast with nonselective neuronal damage induced by kainate and AMPA (Kwak & Nakamura, 1995a, b). These behavioral and morphological changes were considerably ameliorated by concomitant infusion of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a non-NMDA receptor antagonist, but not by D(−)-2-amino-5-phosphonovaleric acid (D-AP5), an NMDA-receptor antagonist (Kwak & Nakamura, 1995a). Increase in $[Ca^{2+}]_i$ and neurotoxicity to cultured spinal neurons by ACRO-A were completely blocked by the non-NMDA-receptor antagonists 2,3-dihydro-9-nitro-7-sulfamoyl-benzen[F]quinoxaline (NBQX) or/and CNQX (Ogata *et al*., 1994; Tsuji *et al*., 1995). Although ACRO possesses a partial structure of kainate in the molecule, it is a more potent agonist for AMPA-type receptors than kainate-type receptors (Kwak *et al*., 1992b; Smith & McIlhinney, 1992). We previously demonstrated that i.t. injection of NMDA and AMPA induced allodynia through different pathways and that i.t. kainate could not evoke allodynic responses at all (Minami *et al*., 2001). In contrast to extensive studies on mechanisms of induction and maintenance of pain by spinal NMDA receptors (Meller & Gebhart, 1993; Ito *et al*., 2001), studies on those by non-NMDA receptors are limited. Although ACRO can be a cause of prominent allodynic responses that last for a month following ingestion of the poisonous mushroom, the involvement of ACRO in induction of allodynia and its relation to the excitotoxicity remain to be clarified. In the present study, we showed that i.t. injection of ACRO-A induced allodynia at extremely low doses and compared it with the allodynia induced by ACRO-B.

Methods

Intrathecal injection and acute effect of ACRO on mechanical allodynia

Male ddY mice were purchased from Shizuoka Laboratory Center (Hamamatsu, Japan). The animals were housed under conditions of a 12-h light–dark cycle, a constant temperature of $22\pm2^{\circ}C$ and $60\pm10\%$ humidity. They were allowed free access to food and water before testing. All animals conformed to the regulations of the Animal Care Committees of Osaka Medical College and Kansai Medical University, and received humane care in accordance with the guidelines of the ethics committee of the International Association for the Study of Pain (Zimmermann, 1983).

Studies on mechanical allodynia were carried out as described previously (Minami *et al*., 2001). A 27-gauge stainless-steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae and drugs in saline (5 μl) were injected slowly into the subarachnoid space of conscious mice weighing 22 ± 2 g. After the i.t. injection, each mouse was placed in an individual $13\times8.5\times13$ cm Plexiglas enclosure with wood chips on the floor and observed. Allodynia was assessed once every 5 min over a 50-min period by light stroking of the flank of the mice with a paintbrush. The allodynic response was ranked as follows: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; 2, vigorous squeaking evoked by the stroking probe, biting at the probe, or strong efforts to escape. The maximum possible scores for allodynia of six mice were $2\times6=12$ in any 5-min period, and $2\times6\times10=120$ for 50 min, and were taken as 100%. To evaluate the effects of agents on allodynia, we assessed the effect on the maximal possible score of allodynia obtained 15 and 10 min after i.t. injection of ACRO-A and ACRO-B, respectively. The animals were used only for a single experiment.

Late effects of ACRO on allodynia and morphology

At 1 week after i.t. injection of a sublethal dose of ACRO-A ($50\,ng\,kg^{-1}$) or ACRO-B ($250\,ng\,kg^{-1}$) into 3-week-old mice, which all survived without behavioral disturbances, induction of allodynia was examined by i.t. injection of various agents. To evaluate the effect of NMDA and non-NMDA receptor antagonists on allodynia in ACRO-A-treated mice, we assessed it on the maximal possible score of allodynia obtained 5 min after i.t. injection of ACRO-A ($500\,ng\,kg^{-1}$). All ACRO-A-treated mice survived without motor disturbances and produced prominent allodynia at $500\,ng\,kg^{-1}$ of ACRO-A. For histochemistry, eight male mice received i.t. injection of ACRO-A ($300\,ng\,kg^{-1}$) to the subarachnoid space and six mice died during convulsions within 15 min after injection. On day 9 after injection, two surviving mice were anesthetized by pentobarbital and perfused transcardially with a fixative (4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4). The spinal cord was excised, immersed in the fixative overnight, and mounted in paraffin. Tissue sections (2 μm in the thickness) were stained by hematoxylin and eosin and

photographed under light field illumination on a Nikon Eclips E1000 microscope (Tokyo, Japan).

Calcium measurement

Slices were prepared from the lumbar spinal cord of 2-week-old mice. After the animal had been anesthetized with ethyl ether, the spinal cord was dissected by quickly cutting the vertebral arcs at the lateral side of thoracic to sacral regions. The spinal cord was immediately placed in artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2/5% CO_2 and maintained in ice-cold aCSF. aCSF contained (in mM): 120 NaCl, 3.1 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 5.0 glucose, 2.0 sodium pyruvate, 0.5 *myo*-inositol, and 0.02 ascorbic acid. Roots and meninges were removed, and lumbosacral segments L3–S2 of the spinal cord were embedded in aCSF containing 3% low-melting agarose. Lumbosacral segments were cut by using a vibrating blade microtome (Leica VT-1000S, Nussloch, Germany), and slices (300-μm thick) obtained from lumbar segments L4–L6 were used for calcium measurement.

After 3-h incubation of the slices in aCSF bubbled with 95% O_2/5% CO_2 at 37°C, slices were loaded with fura-2 by incubating them for 2 h at room temperature in Krebs' solution containing 10 μM fura-2-acetoxymethyl ester (Dojindo Chemical, Kumamoto, Japan) and 0.01% cremophor EL (Sigma-Aldrich, St Louis, MO, U.S.A.). The slices were kept in Krebs' solution for more than 1 h after loading, and then they were placed in the recording chamber that was mounted on an inverted fluorescence microscope (IX-70, Olympus, Tokyo, Japan) and mechanically fixed in place by using an overlaying grid of nylon threads attached to a platinum ring. The slice was superfused in Krebs' solution equilibrated with 95% O_2/5% CO_2 at 3 ml min^{-1}. $[Ca^{2+}]_i$ was measured as a fluorescence ratio obtained with excitation at 340 and 380 nm. Optical signals were recorded by using the Argus-HiSCa imaging system (Hamamatsu Photonics, Shizuoka, Japan) equipped with a cooled charge-coupled device camera.

Chemicals

The following agents were generous gifts: ACRO-A and ACRO-B, from Professor H. Shirahama of Hakkaido University; (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[A,D]cyclohepten-5,10-imine hydrogen maleate (MK-801), from Merck Research Laboratories (Rahway, NJ, U.S.A.); and prostaglandin (PG) E_2 and $PGF_{2\alpha}$, from Ono Central Research Institute (Osaka, Japan). NMDA was obtained from Sigma-Aldrich. AMPA, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI52466), kainate, NBQX, 6,7,8,9-tetrahydro-5-nitro-1*H*-benz[G]indole-2,3-dione-3-oxime (NS102) were purchased from Research Biochemicals Internationals (Natick, MA, U.S.A.). (2*S*, 4*R*)-4-methylglutamic acid (SYM2081) was obtained from Tocris Cookson Ltd (Bristol, U.K.). D-AP5 was supplied by Cambridge Research Biochemicals (Cambridge, U.K.). Joro spider toxin-3 (JSTX) was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

Statistics

Data for mechanical allodynia were analyzed by the Mann–Whitney *U*-test. All results are presented as mean ± s.e.m. $P<0.05$ was considered statistically significant. ID_{50} values with 95% confidence limits (CL) were calculated by use of the computer program of Probit test.

Results

Acute induction of mechanical allodynia by ACRO

Mechanical allodynia (tactile pain) is a prominent symptom suffering from food regio-isomers, ACRO-A and ACRO-B, which possess a constitutional moiety of kainic acid (Figure 1). We previously reported that i.t. injection of NMDA and AMPA, but not kainate, resulted in prominent agitation responses to tactile stimuli applied to the flank, such as licking, biting, and escape from the stimuli (Minami *et al*., 2001). Figure 2a presents time courses of allodynia evoked by ACRO-A (50 fg kg^{-1}), ACRO-B (50 ng kg^{-1}), and kainate (5 μg kg^{-1}). Consistent with our previous study, kainate did not induce allodynic responses to innocuous stimuli applied to the flank. On the other hand, ACRO-A and ACRO-B induced allodynia 5 min after i.t. injection. These responses were long-lasting and did not disappear by 50 min. The time courses of allodynia induced by ACRO-A and ACRO-B were similar to those by NMDA (500 ng kg^{-1}) and AMPA (500 ng kg^{-1}) reported previously. When the scores of allodynia obtained for the overall 50 min were cumulated and expressed as a per cent of the maximum possible score, ACRO-A produced allodynia over a wide range of dosage from 50 ag kg^{-1} to 0.5 pg kg^{-1} and the dose–response curve showed a bell-shaped pattern (Figure 2b). The significant ($P<0.01$) effect was observed at 5–500 fg kg^{-1} and the allodynic effect was lost at 50 pg kg^{-1} of ACRO-A. In contrast, ACRO-B induced allodynia in a dose-dependent manner from 50 pg kg^{-1} to 50 ng kg^{-1}. Most mice exhibited strong spontaneous agitation immediately after i.t. injection at 250 ng kg^{-1}. Spontaneous agitation, scratching behaviors, and tonic convulsions continued at 500 ng kg^{-1} of ACRO-B, which led to death within 15 min. Similarly, i.t. injection of 500 ng kg^{-1} of ACRO-A induced strong spontaneous agitation for the first 3–5 min, intermittent behavioral activation such as scratching, jumping and tonic convulsion and caused death within 15 min. Although a lethal dose of ACRO-A was similar to that of ACRO-B, the potency of ACRO-A to induce allodynia was unexpectedly low as compared with that of ACRO-B as well as NMDA and AMPA.

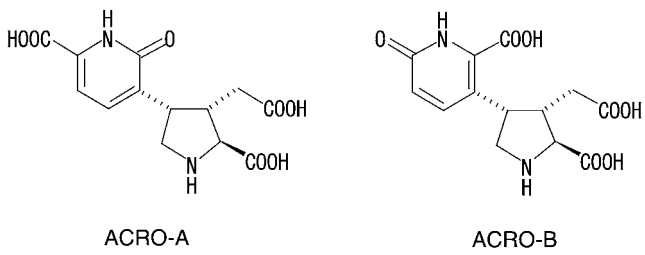

Figure 1 Chemical structures of ACRO-A and ACRO-B.

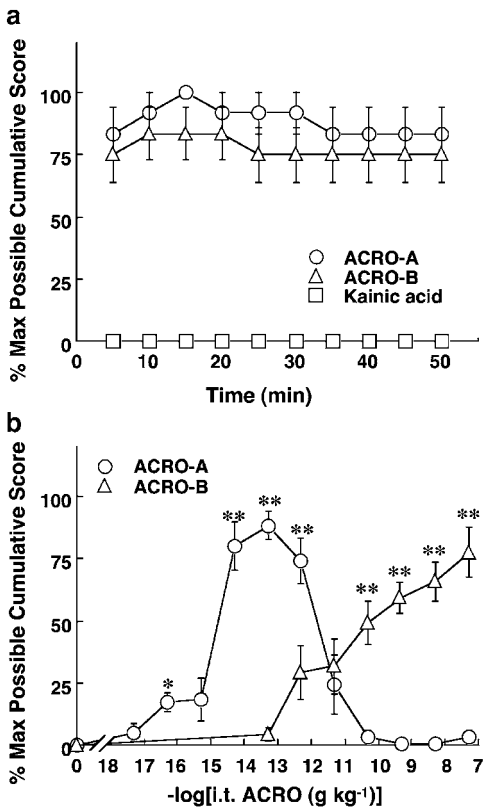

Figure 2 Acute effect of ACRO on induction of allodynia. (a) Time courses of allodynia. ACRO-A (50 fg kg^{-1}), ACRO-B (50 ng kg^{-1}), or kainate (5 μg kg^{-1}) was injected into the subarachnoid space of conscious mice. Studies on allodynia were carried out as described in 'Methods'. Each column (mean $\pm$ s.e.m.) represents the per cent of the maximum possible cumulative score of six mice evaluated every 5 min. Thus, the maximum possible cumulative score for allodynia in any 5-min period was 12 (2×6) and was taken 100%. (b) Dose dependency of allodynia. Various doses of ACRO-A or ACRO-B were injected into the subarachnoid space of conscious mice. Data (mean $\pm$ s.e.m., $n = 6$) are expressed as a per cent of the maximum possible cumulative score over the 50-min experimental period. Thus the maximum possible cumulative score of allodynia for 50 min was 120 ($2 \times 6 \times 10$) and was taken 100%. $^*P < 0.05$; $^{**}P < 0.01$, as compared with the saline-injected group (Mann–Whitney U-test).

Characterization of ACRO-induced acute allodynia

Since ACRO-A was less potent in the depolarizing activity of rat dorsal root fiber than ACRO-B (Ishida & Shinozaki, 1991) and the effective dose of ACRO-A for induction of allodynia was extremely low (Figure 2b), we examined whether AMPA/kainate receptor antagonists could attenuate the ACRO-induced allodynia. The nonselective AMPA/kainate receptor antagonist NBQX did not inhibit allodynia induced by either ACRO-A or ACRO-B (Figure 3a and b). We further examined the effects of AMPA-preferring (GYKI52466) and kainate-preferring (NS102 and SYM2081) receptor antagonists. None of them blocked the allodynia induced by ACRO-A and ACRO-B, either. However, the Ca^{2+}-permeable AMPA/kainate receptor antagonist JSTX, blocked the induction of allodynia by ACRO-A in a dose-dependent manner with an ID_{50} value (95% CL) of 101 ng kg^{-1} (24.8–239 ng kg^{-1}). But JSTX did not affect the allodynia by ACRO-B at doses up to 5 μg kg^{-1}.

Depolarization of postsynaptic neurons activates NMDA receptors, which in turn triggers a cascade of biochemical events such as the production of PGs in the cells. As shown in Figure 3c, the NMDA receptor antagonists D-AP5 and MK-801 inhibited the induction of allodynia by ACRO-A, but not by ACRO-B, with ID_{50} values (95% CL) of 10.8 μg kg^{-1} (5.0–23.9 μg kg^{-1}) and 1.3 μg kg^{-1} (0.56–3.0 μg kg^{-1}), respectively. On the other hand, the 60-min pretreatment of mice with oral administration of indomethacin (5 mg kg^{-1}), a nonsteroidal anti-inflammatory drug that inhibits PG production, did not attenuate ACRO-induced allodynia at all (Figure 4a).

We further examined the effect of morphine on ACRO-induced allodynia. As shown in Figure 4b, simultaneous i.t. injection of morphine did not inhibit the allodynia induced by ACRO-A (50 fg kg^{-1}) and ACRO-B (50 ng kg^{-1}) up to 5 μg kg^{-1}.

Late effect of ACRO on induction of allodynia

To clarify the relation between induction of allodynia and neurotoxicity by ACRO, we injected a high dose of ACRO-A (50 ng kg^{-1}) or ACRO-B (250 ng kg^{-1}) into the subarachnoid space of conscious mice. Mice treated by ACRO-A or ACRO-B returned in healthy and apparently unaffected conditions within 30 min and moved freely without paralysis and paraplegia. When induction of allodynia was examined 1 week after i.t. injection, both ACRO-A (50 fg kg^{-1}) and ACRO-B (50 ng kg^{-1}) could not induce allodynia in mice pretreated by either ACRO-A or ACRO-B (Figure 5). Interestingly, $PGF_{2\alpha}$ induced allodynia in mice pretreated by ACRO-B, but not by ACRO-A. Other substances, that is, PGE_2, NMDA, AMPA, arginine, a substrate of nitric oxide synthase, and sodium nitroprusside (SNP), a nitric oxide (NO) donor, that were previously shown to induce allodynia by i.t. injection (Eguchi *et al*., 1999), could produce allodynic responses in ACRO-pretreated mice.

Although it could not evoke allodynia at 50 fg kg^{-1} in ACRO-A-pretreated mice (Figure 5a), ACRO-A induced allodynia at 500 ng kg^{-1} that caused death within 15 min in naive mice (Figure 6a). Interestingly, the dose–response curve of ACRO-A for late effect on induction of allodynia in the pretreated mice (Figure 6b) was quite similar to that of ACRO-B for acute effect on induction of allodynia in naive mice (Figure 2b). Furthermore, the late effect of ACRO-A (500 ng kg^{-1}) on induction of allodynia was not affected by GYKI52466, JSTX, D-AP5, and MK-801 (Figure 7), similar to the acute effect of ACRO-B in naive mice (Figure 3b and d). Since JSTX induced continuous seizures at a dose of 500 ng kg^{-1} or higher in ACRO-A-treated mice, the effect of JSTX on the late effect by ACRO-A was not examined at higher doses.

Action site of ACRO-A in the spinal cord

To study the action site of ACRO-A, we examined $[Ca^{2+}]_i$ response to ACRO-A *in situ* using 24 spinal slices from lumbar segments L4–L6 of 16 2-week-old mice. Figure 8a illustrates a fura-2-loaded lumbar slice preparation under low magnification and the representative of time courses of $[Ca^{2+}]_i$ increase induced by 3.3 μM ACRO-A. Although the $[Ca^{2+}]_i$ increase considerably varied from slice to slice, ACRO-A could produce a rapid and transient increase in $[Ca^{2+}]_i$ in a dose-

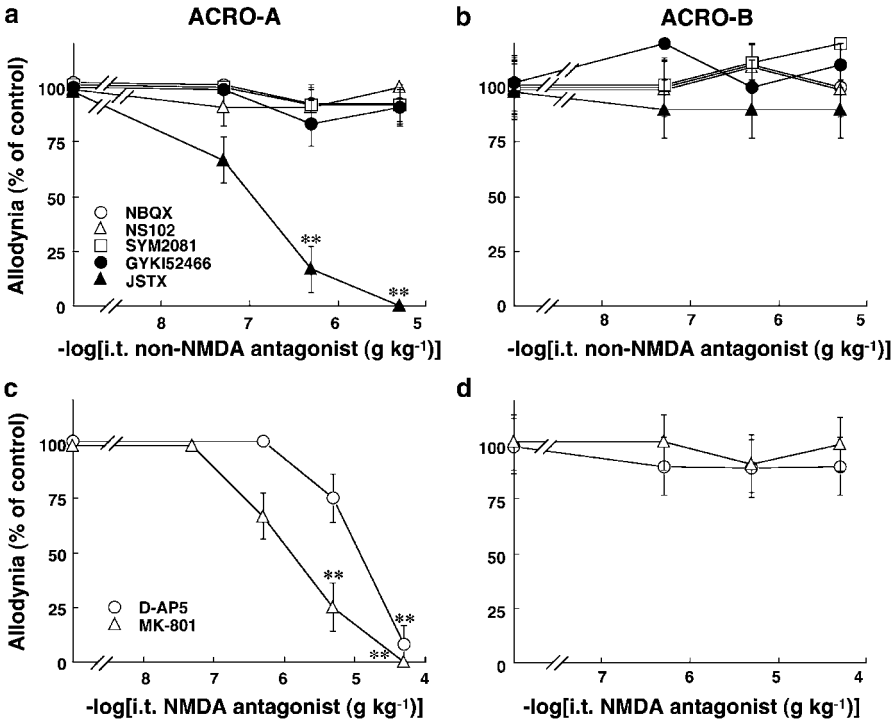

Figure 3 Effect of non-NMDA and NMDA receptor antagonists on ACRO-induced allodynia. (a, b) Effect of non-NMDA receptor antagonists on allodynia induced by ACRO-A and ACRO-B. Indicated doses of the nonselective non-NMDA antagonist NBQX, the kainate antagonists NS102 and SYM2081, the AMPA antagonist GYKI52466 or the Ca^{2+}-permeable AMPA receptor antagonist JSTX were i.t. injected simultaneously with ACRO-A (50 fg kg^{-1}, a) or ACRO-B (50 ng kg^{-1}, b) into the subarachnoid space of conscious mice. (c, d) Indicated doses of the NMDA antagonsits D-AP5 and MK-801 were i.t. injected simultaneously with ACRO-A (50 fg kg^{-1}, c) or ACRO-B (50 ng kg^{-1}, d) into the subarachnoid space of conscious mice. Effects of various agents on the allodynia were evaluated 15 and 10 min after i.t. injection of ACRO-A and ACRO-B, respectively. The scores of allodynia induced by ACRO-A and ACRO-B alone were 100 and 83.3%, respectively, and were taken as 100%. $^{**}P<0.01$, as compared with the ACRO-A alone-injected group (Mann–Whitney *U*-test).

dependent manner from 1 to 33 μM, which was more obvious in the deeper laminae than in the superficial laminae (Figure 8a and b). Weak but significant $[Ca^{2+}]_i$ increase was observed in the medial part of the deeper laminae by low doses of ACRO-A from 10 nM to 1 μM. AMPA-preferring receptor antagonist GYKI52466 itself gradually increased $[Ca^{2+}]_i$ in both superficial and deeper laminae of the spinal slice, but it did not affect a rapid increase in $[Ca^{2+}]_i$ by 100 nM ACRO-A (Figure 8c). Similarly, JSTX (10 μM) did not inhibit the ACRO-A-induced $[Ca^{2+}]_i$ increase, but rather enhanced it (Figure 8d).

No neuronal damage in the spinal cord by a high dose of ACRO-A

After systemic or i.t. administration of a high dose of ACRO-A into rats, there was extensive degeneration of interneurons in the gray matter with a glial reaction selectively at the lumbosacral spinal cord (Shinozaki *et al*., 1989; Kwak & Nakamura, 1995a). To examine whether selective loss of ACRO-induced allodynia was ascribed to similar histological changes, we administered ACRO-A to mice. When ACRO-A was i.t. injected at 5–50 ng kg^{-1}, no mice died and they showed no behavioral disturbances 1 week after injection. Histological examination did not show any significant change in the spinal cord (data not shown). When a higher dose (300 ng kg^{-1}) of ACRO-A was injected, six out of eight mice died within 15 min during convulsions, whereas two mice survived and did not show any abnormal behavioral signs on day 9 after injection. Then, the spinal cords of these mice were histologically examined, and compared with those of saline-injected mice. The number of microglia with dark nuclei was increased particularly in the dorsal horn and around the central canal of ACRO-A-injected mice (Figure 9a), as compared with saline-injected mice (Figure 9b), suggesting that ACRO-A may produce gliosis in the gray mater of mouse spinal cord. Other changes, including neuronal degeneration, were not observed by Bodian's silver stain, Nissl stain, and Luxol fast blue stain (data not shown). Apoptosis was not detected by the TUNEL method in the spinal cord of ACRO-A- and saline-treated mice (data not shown).

Discussion

ACRO and kainic acid are natural products related structurally to glutamate and share neuroexcitatory and neurotoxic properties with it. We previously showed that i.t. injection of NMDA and AMPA, but not kainate, induced allodynia in mice (Minami *et al*., 2001). In the present study, we demonstrated that i.t. ACRO-A induced allodynia at doses from 50 ag kg^{-1} to 500 fg kg^{-1}, which was much lower than that of a lethal dose of 500 ng kg^{-1}. Difference in the capability to induce allodynia between ACRO and kainate may explain severe allodynia in the tips of extremities accompanied by ingestion of the poisonous mushroom containing ACRO. In

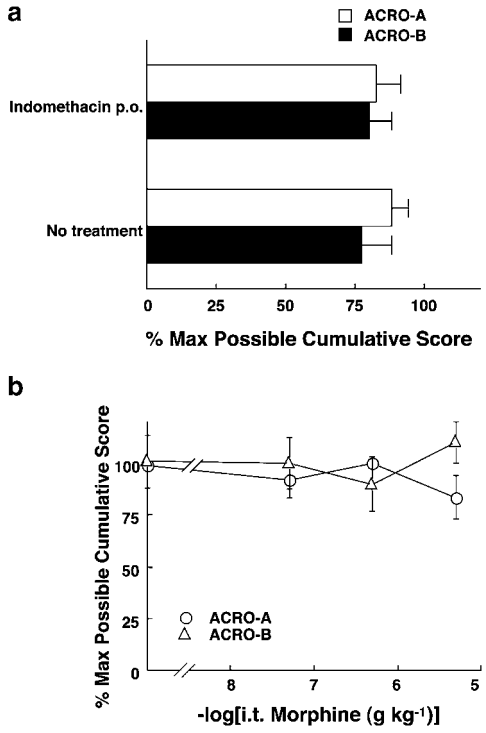

Figure 4 Effect of indomethacin and morphine on ACRO-induced allodynia. (a) Effect of oral indomethacin on ACRO-induced allodynia. Indomethacin (5 mg kg^{-1}) was orally administered 60 min before i.t. injection of ACRO-A (50 fg kg^{-1}, open column) or ACRO-B (50 ng kg^{-1}, closed column). Data (mean ± s.e.m., $n = 6$) are expressed as a per cent of the maximum possible cumulative score over the 50-min experimental period. (b) Effect of i.t. morphine on ACRO-induced allodynia. Indicated doses of morphine were i.t. injected simultaneously with ACRO-A (50 fg kg^{-1}) or ACRO-B (50 ng kg^{-1}). The effect of morphine on the allodynia was evaluated 15 and 10 min after i.t. injection of ACRO-A and ACRO-B and data (mean ± s.e.m., $n = 6$) are expressed as described in the legend for Figure 3.

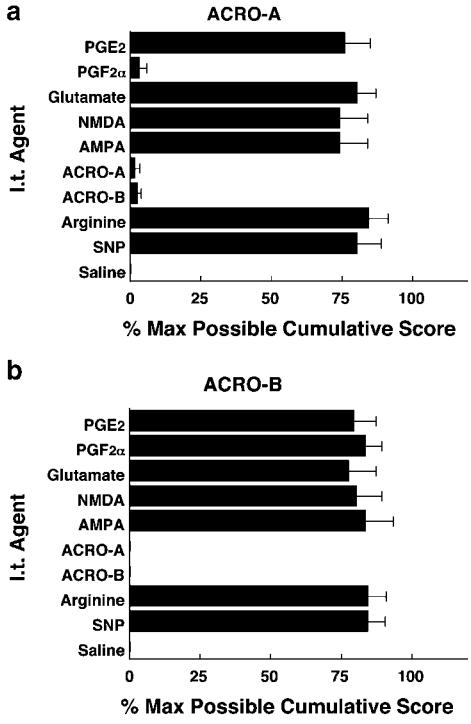

Figure 5 Late effect of pretreatment of ACRO on induction of allodynia. A high dose of ACRO-A (50 ng kg^{-1}) or ACRO-B (250 ng kg^{-1}) was i.t. injected into mice. At 1 week after i.t. injection, PGE_2 (500 ng kg^{-1}), $PGF_{2\alpha}$ (50 μg kg^{-1}), glutamate (50 ng kg^{-1}), NMDA (500 ng kg^{-1}), AMPA (500 ng kg^{-1}), ACRO-A (50 fg kg^{-1}), ACRO-B (50 ng kg^{-1}), arginine (250 μg kg^{-1}) or SNP (500 ng kg^{-1}) was i.t. injected and allodynia was assessed for 50 min as described in the legend for Figure 2b.

contrast to similar lethal doses of ACRO-A and ACRO-B, the dose–response curve of ACRO-A was quite different from that of ACRO-B, a regio-isomer of ACRO-A (Figure 2b), suggesting the stereospecificity for the induction of allodynia. Consistent with these previous binding studies demonstrating that ACRO-A is a more potent agonist for AMPA-type receptors rather than kainate-type receptors (Kwak *et al*., 1992b; Smith & McIlhinney, 1992), ACRO-A could induce allodynia at extremely low doses (Figure 2a and b). However, the allodynia induced by ACRO-A was not blocked the AMPA/kainate receptor antagonists except for the Ca^{2+}-permeable non-NMDA receptor antagonist JSTX. AMPA receptors are multimeric assemblies of GluR1-GluR4 subunits and a subset of receptors lacking the GluR2 subunit are Ca^{2+}-permeable. Although some kainate receptors have significant Ca^{2+} permeability, the majority of Ca^{2+}-permeable non-NMDA receptors are thought to be AMPA receptors that are located in lamina I and outer lamina II, a region strongly innervated by nociceptors (Engelman *et al*., 1999). They are expressed in subsets of superficial dorsal horn neurons such as inhibitory interneurons and putative projection neurons (Spike *et al*., 1998; Albuquerque *et al*., 1999). JTSX administered spinally produced a significant facilitation of C-fiber response, but not of the A-fiber response, of dorsal horn neurons (Stanfa *et al*., 2000). In behavioral studies, however, JSTX administered i.t. blocked mechanical allodynia evoked by thermal injury, and thermal hyperalgesia and mechanical allodynia induced by intraplantar injection of carrageenan in rats, but had no effect in the formalin test and on allodynia after spinal nerve ligation in rats at 3–5 μg (Sorkin *et al*., 1999; 2001). The authors suggested a role of Ca^{2+}-permeable AMPA receptors in mediating spinal sensitization in selected pain models. We also demonstrated here that the allodynia evoked by ACRO-A was attenuated by simultaneous i.t. injection of JSTX in a dose-dependent manner (Figure 3a), which seemed to be approximately one order lower than that reported in rats.

Acute and late neurotoxicity of ACRO-A has been extensively studied. A single systemic administration of ACRO-A caused long-lasting rigid-spastic paraparesis in the rat, which was accompanied by selective degeneration of spinal cord interneurons (Shinozaki *et al*., 1989; Kwak *et al*., 1992a). Continuous i.t. infusion of ACRO-A was also reported to cause selective damage to the spinal interneurons and a marked decline of choline acetyltransferase activity, a marker of α-motoneurons, accompanied by long-lasting rigid-spastic paraparesis (Kwak & Nakamura, 1995a, b). Morphological changes by ACRO-A resembled those by kainate rather than by AMPA. Irrespective of the route of administration, the selective damage to the spinal interneurons seemed to be common to neurotoxicity by ACRO-A in rats and it was partially ameliorated by concomitant administration of the AMPA/kainate antagonist CNQX. In contrast, surviving mice

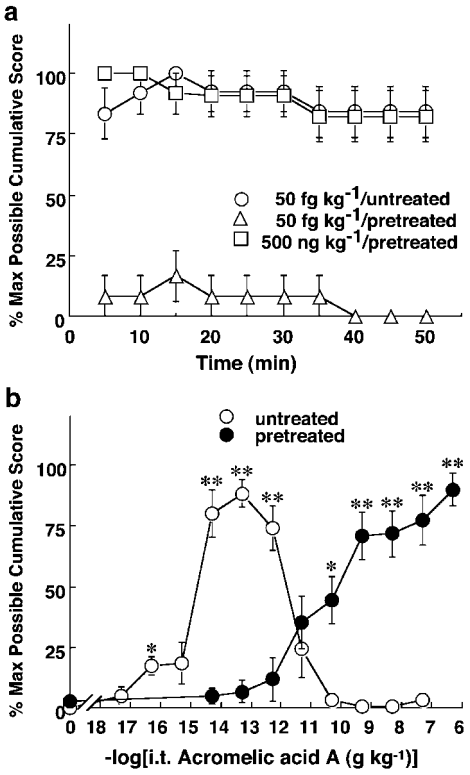

Figure 6 Characterization of late effect of ACRO-A on induction of allodynia. (a) Time courses of allodynia. A high dose of ACRO-A ($50\,ng\,kg^{-1}$) was i.t. injected into mice. At 1 week after i.t. injection, $50\,fg\,kg^{-1}$ or $500\,ng\,kg^{-1}$ of ACRO-A was injected into the subarachnoid space of conscious mice. Allodynia was assessed for 50 min as described in the legend for Figure 2a and a time course of allodynia induced by ACRO-A ($50\,fg\,kg^{-1}$) shown in Figure 2a is presented for comparison. (b) Dose dependency of allodynia. Various doses of ACRO-A were injected into the subarachnoid space of mice 1-week after i.t. injection of ACRO-A ($50\,ng\,kg^{-1}$). Allodynia was assessed for 50 min as described in the legend for Figure 2b and a time course of allodynia induced by ACRO-A shown in Figure 2b is presented for comparison. $^{*}P<0.05$; $^{**}P<0.01$, as compared with the saline-injected group (Mann–Whitney *U*-test).

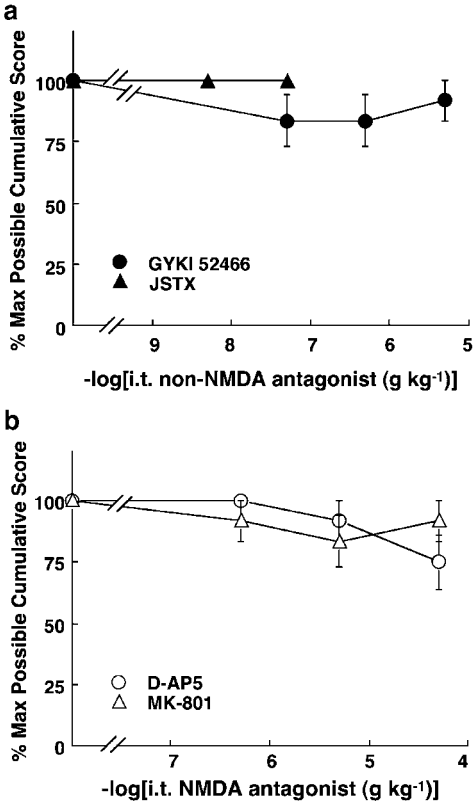

Figure 7 Effect of non-NMDA (a) and NMDA (b) receptor antagonists on ACRO-A-induced allodynia in pretreated mice. A high dose of ACRO-A ($50\,ng\,kg^{-1}$) was i.t. injected into mice. At 1 week after i.t. injection, $500\,ng\,kg^{-1}$ of ACRO-A was injected into the subarachnoid space of conscious mice. Effect of non-NMDA receptor antagonists on allodynia induced by ACRO-A. Indicated doses of the AMPA antagonist GYKI52466, the Ca^{2+}-permeable AMPA receptor antagonist JSTX or the NMDA antagonsits D-AP5 and MK-801 were i.t. injected simultaneously with ACRO-A ($500\,ng\,kg^{-1}$) into the subarachnoid space of conscious mice. Effects of various agents on the late allodynia were evaluated 5 min after i.t. injection of ACRO-A as described in the legend for Figure 3. The score of allodynia induced by ACRO-A was 100% and taken as 100%.

by a single i.t. injection of $300\,ng\,kg^{-1}$ of ACRO-A did not produce marked histological changes in the lumbar spinal cord except for gliosis in the gray matter (Figure 9) and motor disturbances. Although the discrepancy of histological changes in the spinal cord, possibly associated with long-lasting rigid-spastic paraparesis, between rats and mice, could be ascribed to the difference in duration of exposure to ACRO or species, a precise reason remains unknown. A single i.t. injection of ACRO-A or ACRO-B ($500\,ng\,kg^{-1}$) produced spontaneous agitation, scratching behaviors, and tonic convulsions, which led to death within 15 min. In binding studies, it was shown that ACRO-A was 3-fold less potent than ACRO-B in inhibiting the binding of [^{3}H]-kainate to the rat spinal cord (Kwak *et al*., 1992b). Interestingly, while most naive mice died during convulsions within 15 min after a single i.t. injection of $300\,ng\,kg^{-1}$ of ACRO-A, ACRO-A-treated mice all survived without motor disturbances and produced prominent allodynia at $500\,ng\,kg^{-1}$ of ACRO-A (Figure 6a and b). These results demonstrated that an action site(s) of neurotoxicity by ACRO may be different from that of induction of allodynia by ACRO-A.

Activation of AMPA receptors produces the depolarization of postsynaptic neurons followed by activation of NMDA receptors and the production of NO and PGs in spinal neurons. We have demonstrated that many substances involved in spinal synaptic transmission, such as PGE_2, $PGF_{2\alpha}$, NMDA, AMPA, and NO, could induce allodynia when injected intrathecally (Eguchi *et al*., 1999; Ito *et al*., 2001). We previously showed that the allodynia induced by i.t. AMPA was blocked by NMDA receptor antagonist D-AP5 (Minami *et al*., 2001). Here we demonstrated that induction of allodynia by ACRO-A, but not by ACRO-B, was inhibited by the NMDA receptor antagonists MK-801 and D-AP5 (Figure 3c and d), suggesting that glutamate released by ACRO-A may activate NMDA receptors to induce allodynia. In fact, ACRO-A stimulated the release of glutamate from cultured neurons (Tsuji *et al*., 1995). This notion is supported by the observation that the allodynia induced by i.t. glutamate, NMDA, arginine and SNP was not affected by 1-week pretreatment of ACRO-A or ACRO-B (Figure 5). Although no effects of ACRO pretreatment on induction of allodynia by these substances suggest that the activation of NMDA and NO production was

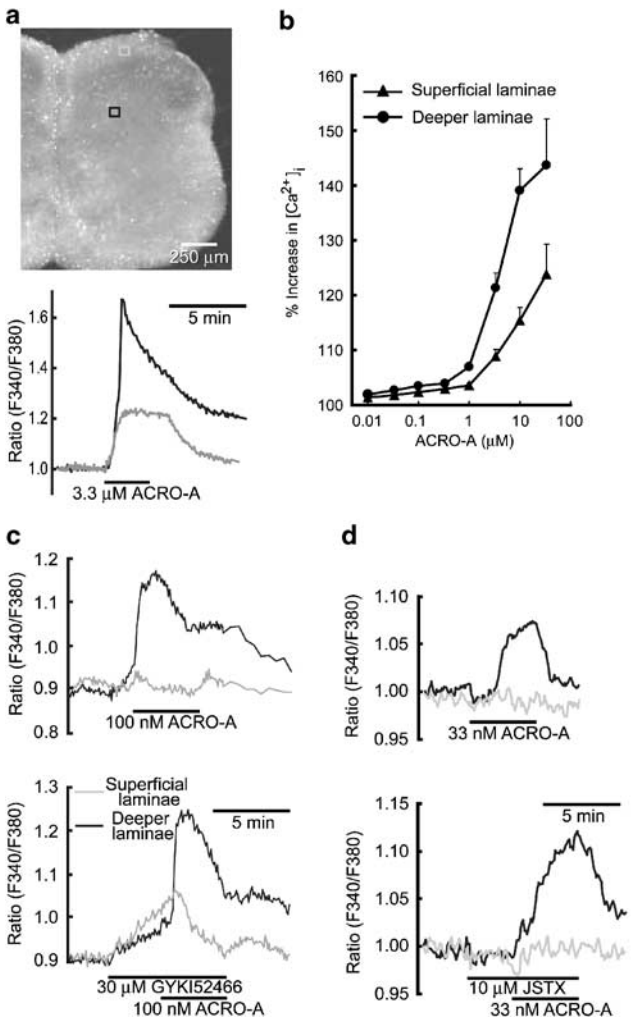

Figure 8 $[Ca^{2+}]_i$ changes by ACRO-A in the spinal cord. (a) Fluorescence image of fura-2-loaded lumbar spinal cord excited at 380 nm and time courses of $[Ca^{2+}]_i$ changes by 3.3 μM ACRO-A. (b) Dose-dependency of $[Ca^{2+}]_i$ changes by ACRO-A. Data (mean $\pm$ s.e.m., $n = 20$–51) are expressed as per cent increase in $[Ca^{2+}]_i$ calculated by dividing the peak $[Ca^{2+}]_i$ after stimulation of ACRO-A by the basal $[Ca^{2+}]_i$ before stimulation. (c) Effect of GYKI52466 on $[Ca^{2+}]_i$ change by 100 nM ACRO-A. (d) Effect of JSTX on $[Ca^{2+}]_i$ change by 33 nM ACRO-A. $[Ca^{2+}]_i$ was measured as a fluorescence ratio obtained with excitation at 340 and 380 nm as described under 'Methods'.

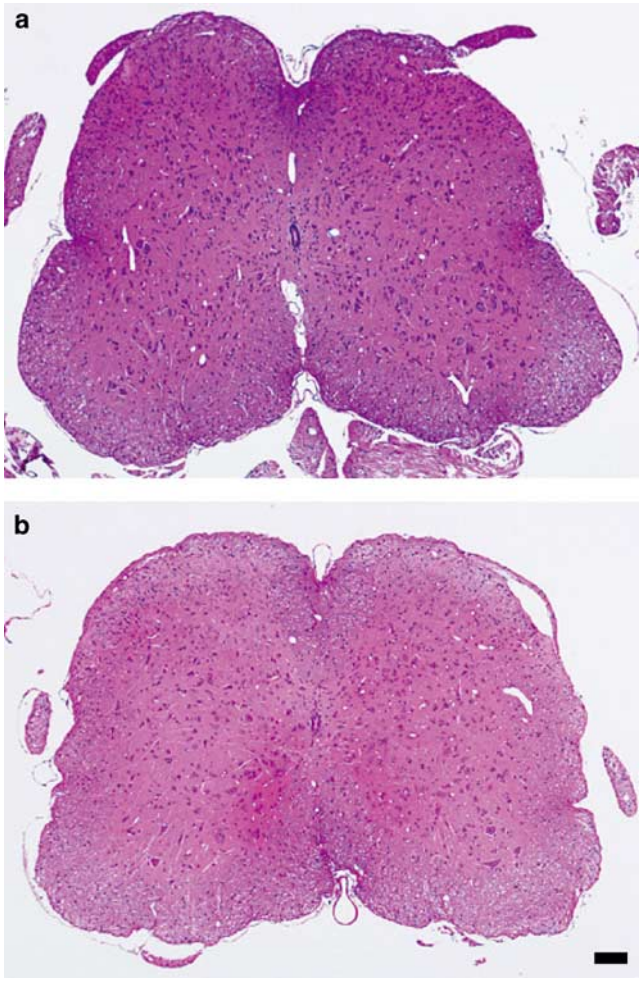

Figure 9 Transverse spinal sections after i.t. injection of ACRO-A or saline. The spinal cord of the mouse that survived for 9 days after i.t. injection of 300 ng kg^{-1} of ACRO-A (a) or saline (b) was embedded in paraffin, and transverse sections at L3–L4 spinal level were stained by hematoxylin and eosin. Scale bar = 100 μm.

downstream of ACRO, the possibility that a subset of neurons susceptible to ACRO-A and ACRO-B was damaged and other neurons responsive to NMDA as well as AMPA were intact in the spinal cord cannot be neglected. Since the allodynia by ACRO-A was not affected by oral administration of indomethacin (Figure 4a), it may not be mediated by the production of PGs in the spinal cord. Interestingly, the 1-week pretreatment of mice by a sublethal dose of ACRO-A abolished the induction of allodynia by $PGF_{2\alpha}$, but not PGE_2 (Figure 5a). We previously showed that neonatal capsaicin treatment abolished the allodynia by PGE_2, but not by $PGF_{2\alpha}$ (Minami *et al*., 1999). Treatment of neonatal animals with capsaicin is known to cause necrotic changes in small-sized neurons in dorsal root ganglia, a substantial loss of unmyelinated C-fibers in peripheral nerves and degeneration of axonal terminals in the superficial layer of the dorsal horn. Opioid analgesia is assumed to be produced by the presynaptic inhibition of stimulation-evoked release of neurotransmitters from primary afferent C fibers and by hyperpolarization of neurons in the dorsal horn. We also previously showed that simultaneous i.t. injection of morphine abolished the allodynia induced by PGE_2, but not by $PGF_{2\alpha}$ (Minami *et al*., 1999). Here morphine did not inhibit the allodynia induced by ACRO-A and ACRO-B (Figure 4b). In agreement with our recent study demonstrating that functional FP-expressing cells that are involved in $PGF_{2\alpha}$-induced allodynia exist in the deeper layer of the dorsal horn (Muratani *et al*., 2003), the increase in $[Ca^{2+}]_i$ by ACRO-A was more prominent in the deeper layer than in the superficial layer in the dorsal horn (Figure 8a and b). It has been known that micromolar concentrations of ACRO-A are generally required to elicit electrophysiological responses and $[Ca^{2+}]_i$ increase in hippocampal and spinal neurons, in marked contrast to the nM dissociation constant in brain and spinal membranes. These increase in $[Ca^{2+}]_i$ induced by ACRO-A was abolished by the AMPA/kainate antagonist NBQX (Ogata *et al*., 1994) and the activation of non-NMDA receptor induced Ca^{2+} influx from outside the cells, mainly *via* voltage-dependent Ca^{2+} channels,

which are insensitive to JSTX, and, in part *via* Ca^{2+}-permeable non-NMDA receptor. Consistent with no effect of GYKI52466 on induction of acute and late allodynia by ACRO-A (Figures 3a and 7a), GYKI52466 did not affect the $[Ca^{2+}]_i$ increase by ACRO-A (Figure 8c). To verify the inhibitory effect of JSTX on ACRO-A-induced allodynia and differentiate it from neurotoxicity by ACRO, we extensively pursued spinal cells responsive to a low dose of ACRO-A and found them in the medial part of the deeper layer of the dorsal horn. The dose–response curve of ACRO-A for $[Ca^{2+}]_i$ increase in the deeper laminae seemed to be biphasic, but the $[Ca^{2+}]_i$ increase by a low dose of ACRO-A was not attenuated by JSTX (Figure 8d). Since Ca^{2+}-permeable AMPA receptors are expressed in subsets of superficial dorsal horn neurons such as inhibitory interneurons and putative projection neurons, JSTX may not directly antagonize the $[Ca^{2+}]_i$ increase by ACRO-A in deeper dorsal horn neurons but modulate neural circuits that are involved in induction of allodynia by ACRO-A. As a research tool, neonatal capsaicin treatment has been employed widely to study mechanisms of pain transmission mediated by unmyelinated C fibers such as thermal hyperalgesia, in animals. ACRO-A treatment can be a novel tool to study mechanisms of pain transmission such as the induction of allodynia by ACRO and $PGF_{2\alpha}$, possibly mediated by myelinated Aβ fibers in the spinal cord.

In summary, the present study demonstrates that i.t. injection of ACRO showed the stereospecificity for induction of allodynia, the mechanism of which may be different from that of the neurotoxicity of ACRO. In addition to the stereospecificity for the induction of allodynia, selective loss of induction of allodynia by ACRO with the pretreatment of ACRO (Figure 5) supports the previous notion that there exists a different type of non-NMDA receptor that is selective to ACRO-A in the spinal cord (Tsuji *et al*., 1995). Although the properties of late allodynia induced by ACRO-A (Figures 6 and 7) resembled those of acute allodynia induced by ACRO-B (Figures 2b, 3c and d), an exact reason remains unknown. The approach to identify a putative ACRO receptor is in progress in our laboratory.

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