Effect of 2-(Phosphono-methyl)-pentanedioic Acid on Alldynia and Afferent Ectopic Discharges in a Rat Model of Neuropathic Pain

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Received August 14, 2001; accepted October 31, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Increased glutamate availability in the spinal cord and primary afferent nerves plays an important role in acute and chronic pain. Afferent ectopic discharges from the site of nerve injury constitute a source of abnormal sensory input to the spinal dorsal horn. The ectopic afferent activity is largely responsible for the development of hypersensitivity of dorsal horn neurons and neuropathic pain. Inhibition of glutamate carboxypeptidase II (GCP II) reduces glutamate release generated from N-acetyl-aspartyl-glutamate in nerve tissues and may have an analgesic effect on neuropathic pain. In the present study, we determined the effect of a GCP II inhibitor, 2-(phosphono-methyl)-pentanedioic acid (2-PMPA), on allodynia and ectopic afferent discharges in an animal model of neuropathic pain. Neuropathic pain was induced by partial ligation of the left sciatic nerve in rats. Tactile alldynia was assessed using von Frey filaments applied to the plantar surface of the injured hindpaw. Single unit activity of ectopic discharges was recorded from the sciatic nerve proximal to the site of ligation. Intravenous injection of 50 to 100 mg/kg 2-PMPA significantly reduced alldynia in a dose-dependent manner. Furthermore, 2-PMPA dose-dependently attenuated the ectopic discharge activity of injured sciatic afferent nerves. At a dose of 100 mg/kg, 2-PMPA significantly inhibited the ectopic activity from 14.7 ± 2.1 to 4.4 ± 0.5 impulses/s without altering the conduction velocity of afferent nerves. Therefore, these data suggest that the antiallodynic effect of 2-PMPA may be mediated, at least in part, by inhibition of ectopic afferent discharges at the site of nerve injury.

Peripheral nerve injury elicits a number of electrophysiological and molecular changes in axons proximal to the injury site as well as their neuronal cell bodies of origin. Unlike sensory nerve endings, the mature afferent axons normally are not capable of generating sustained discharges, even when strong natural stimuli are applied. However, a substantial proportion of afferents develop an ectopic repetitive firing capability in the region of injury after nerve injury (Devor, 1991; Devor et al., 1993). Ectopic afferent discharges from the site of nerve injury constitute a source of abnormal sensory input to the spinal dorsal horn (Kajander et al., 1992; Matzner and Devor, 1994). There is a growing appreciation that peripheral and central neuroplasticity are not mutually exclusive, but interact extensively to reinforce the pathological changes that contribute to chronic pain (Campbell et al., 1988; Devor et al., 1993; Sheen and Chung, 1993). The ectopic afferent activity is largely responsible for the development of hypersensitivity of spinal cord dorsal horn neurons and the hyperalgesia and alldynia (abnormal pain sensation in response to innocuous stimuli) associated with neuropathic pain in rats (Kajander et al., 1992; Matzner and Devor, 1994; Yoon et al., 1996). Clinical studies also provide strong evidence that the ectopic discharges from afferent nerves are a source of ongoing spontaneous pain, and this abnormal afferent activity dynamically maintains a state of central hypersensitivity that underlies evoked-pain syndromes such as alldynia and hyperalgesia in patients with painful neuropathies (Graeley et al., 1992; Campero et al., 1998). Thus, drugs capable of suppressing the ectopic afferent activity may provide an effective therapy for the treatment of neuropathic pain. In this regard, the analgesic effect of anticonvulsants may be mediated partly by their action on ectopic afferent discharges (Yaari and Devor, 1985; Pan et al., 1999).

Mechanisms underlying the generation of ectopic discharges from injured afferents are complex and likely involve the interaction between endogenous chemicals and ion channels. After nerve injury, the anterograde transport of proteins and neurotransmitters results in accumulation at the
site of nerve injury, which may be responsible for the generation of ectopic discharges to cause chronic pain (Burchiel and Ochoa, 1991; Devor, 1991). Increased glutamate availability in the peripheral nerve has been shown to play a role in nociception (Davidson et al., 1997; Carlton andCoggeshall, 1999; Du et al., 2001). N-Acetyl-aspartyl-glutamate (NAAG) is hydrolyzed by the neuropeptidase glutamate carboxypeptidase II (GCP II; also termed N-acetylated ω-linked acidic dipeptidase) to liberate N-acetyl-aspartate and glutamate. Both NAAG and GCP II are present in the peripheral nerve and dorsal root ganglia, especially in astrocytic glial cells and nonmyelinating Schwann cells (Berger et al., 1995; Berger and Schwab, 1996). Thus, an important source of glutamate in the peripheral nerve after nerve injury could be derived from NAAG through stimulation of GCP II. 2-(Phosphonomethyl)-pentanedioic acid (2-PMPA) is a potent and selective GCP II inhibitor (Jackson et al., 1996), which reduces glutamate accumulation and increases NAAG in the brain tissue in a rat model of cerebral ischemia (Slusher et al., 1999). NAAG itself could additionally reduce glutamate release via its action on mGlu3 receptors (Slusher et al., 1999). Inhibition of spinal GCP II attenuates nociception caused by inflammation in rats (Yamamoto et al., 2001a,b). We reasoned that inhibition of GCP II may ultimately reduce glutamate levels in the nerve tissue and produce an analgesic effect on neuropathic pain. The major objective of the present study was to examine the effect of 2-PMPA on allodynia and the ectopic discharge activity from the injured afferent nerve in an animal model of neuropathic pain.

Materials and Methods

Male rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 225 to 250 g were used in this study. Under halothane anesthesia, the left sciatic nerve was exposed and isolated at the midthigh, and one-third to one-half of the nerve was ligated tightly with a 5.0 silk suture, according to the method described previously (Seltzer et al., 1990). The animals were allowed to recover for 10 to 14 days after nerve injury. Then, the right jugular vein was cannulated with polyethylene-50 tubing, and the catheter was externalized to the back of the neck under halothane anesthesia. The rats were used for behavioral testing and electrophysiological studies after recovery for at least 3 days after cannulation. We and others have shown that stable tactile allodynia develops within 1 week after nerve ligation and lasts for at least 4 weeks (Seltzer et al., 1990; Pan et al., 1999). Thus, all of the final studies were conducted between 2 and 4 weeks after sciatic nerve ligation. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Penn State University College of Medicine and conformed to National Institutes of Health guidelines on the ethical use of animals. All efforts were made to minimize both the suffering and number of animals used.

Behavioral Assessment of Tactile Allodynia. To evaluate the mechanical sensitivity of the injured hindpaw, rats were placed in individual plastic boxes on a mesh floor and allowed to acclimate for 30 min. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the left hindpaw with sufficient force to bend the filaments for 6 s. Brisk withdrawal or paw flinching was considered as a positive response. In the absence of a response, the filament of next greater force was applied. In the presence of a response, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal response was calculated by using the “up-down” method, as described in detail previously (Chaplan et al., 1994; Pan et al., 1999). Each trial was repeated two to three times at approximately 2-min intervals. Three separate groups of animals, each consisting of eight rats, were used for behavioral studies. After obtaining a consistent baseline, a single dose (25, 50, or 100 mg/kg) of 2-PMPA was injected intravenously. The paw-withdrawal threshold was determined every 15 to 30 min up to 3 h after injection of 2-PMPA. Motor dysfunction was evaluated by testing the ability of the animals to stand and ambulate in a normal posture and to place and step with the hindpaw. We assessed the motor function in a simple manner by grading the ambulation behavior of rats as follows: 2 = normal; 1 = limping; 0 = paralyzed.

Recording of Single-Unit Activity of Afferent Nerves. Alldynic conditions were first verified in all rats before afferent nerve recording experiments. Rats were anesthetized with an intraperitoneal injection of sodium phenobarbital (45 mg/kg). The right jugular vein and left carotid artery were cannulated for administering drugs and monitoring the blood pressure, respectively. The trachea was cannulated and the rat was ventilated artificially with a respirator (SAR-830; IITC Inc./Life Science, Woodland Hills, CA). Arterial blood gases were analyzed with a blood gas analyzer and maintained within physiological limits. Throughout the experiment, body temperature was maintained in the range of 37–38°C with a circulating water heating pad and heat lamps. The fascia and sheath overlying the left sciatic nerve were removed carefully. The nerve was draped on a platform and covered with warm mineral oil. Small nerve filaments were teased gently from the nerve segment proximal to the ligated site under an operating microscope (model M900; D. F. Vasconcellos S.A., Sao Paulo, Brazil). Single-unit afferent nerve activity was recorded with a bipolar stainless electrode. The filaments in the distal-cut end of the sciatic nerve were dissected gradually until the single-unit activity of afferents was isolated. The action potential of the nerve was amplified, filtered with a bandpass filter of 100 to 1000 Hz, and monitored through an audiomplifier (model AM8; Grass Instruments, West Warwick, RI) and a storage oscilloscope (TDS 210; Tektronix, Wilsonville, OR). The neurogram was recorded on a thermal-sensitive recorder (model K2G; Astro-Med, West Warwick, RI). The single-unit afferent was identified initially by examining the wave form and the spike amplitude on the oscilloscope at a rapid sweep speed, as well as by checking the recorded sound frequency related to each spike activity. Furthermore, the signals were digitized at a sampling rate of 20 kHz and recorded into a Pentium computer through an analog-to-digital interface card for subsequent off-line analysis. An amplitude threshold was set for the recorded action potential of nerve fibers. When an event was detected, the associated wave form (6 ms) was extracted and displayed continuously in a separate software oscilloscope window. Single-unit recording was ensured by checking the constancy of the shape and polarity of the displayed spike wave form. Discharge frequency was quantified using a software program (Experimental Workbench; DataWave Technology Inc., Longmont, CO).

After the spontaneous discharge activity of a single-unit afferent was identified, the baseline discharge was recorded for 10 to 15 min. Then, 2-PMPA was injected intravenously at the dose of 25, 50, or 100 mg/kg. We have shown previously that intravenous injection of saline has no effect on the baseline spontaneous ectopic discharges (Pan et al., 1999). We used the following two criteria to ensure that the recorded activity was ectopic discharges originating from the neuromas (Pan et al., 1999): 1) recorded nerve fibers had no receptive field in the peripheral tissue; and 2) at the end of recordings, the ectopic discharge activity was increased by mechanical stimulation of the neuroma but was not altered by transecting the nerve distal to the neuroma site. In separate normal rats, we determined whether intravenous injection of 2-PMPA had any effect on the responses of normal afferent fibers to mechanical stimulation, because normal afferent fibers usually have no spontaneous discharges. After the receptive fields of afferents were precisely located on the skin, the conduction velocity and afferent responses to stimulation of the receptive field with von Frey filaments were examined before and 30 min after intravenous injection of 100 mg/kg 2-PMPA.
The conduction velocity of normal and injured afferent fibers was measured by electrical stimulation of the sural and the sciatic nerve proximal to the ligated site, respectively. A bipolar stimulating electrode was placed distal to the recording site to electrically evoke the action potential of the afferent (Pan et al., 1999). Conduction time was determined by measuring the time interval from the signal of electrical stimulation to recording of the evoked afferent’s action potential displayed on the oscilloscope. C- and Aδ-fiber afferents were classified as those with a conduction velocity < 2.0 and 2.0 to 15 m/s, respectively. Those with a conduction velocity > 15 m/s were considered to be Aβ-fiber afferents.

The ectopic discharge activity of afferents was averaged during control and for 30 to 45 min after 2-PMPA treatment. All the behavioral data collected were normally distributed, as determined by the Komogorov-Smirnov test. Thus, these data are presented as mean ± S.E.M., and parametric tests were chosen for statistical analysis. Differences in the paw-withdrawal threshold in response to mechanical stimulation before and after nerve ligation, the conduction velocity, and the evoked response of normal afferents by mechanical stimulation before and after 2-PMPA treatment were compared using Student’s paired t test. The effect of 2-PMPA on allodynia and afferent ectopic activity was determined by repeated measures analysis of variance followed by Dunnett’s post hoc test. P < 0.05 was considered to be statistically significant.

Results

Effect of 2-PMPA on Mechanical Allodynia. Paw-withdrawal threshold in response to application of von Frey filaments before sciatic nerve ligation was 22.7 ± 2.1 g (n = 24). The mechanical threshold decreased significantly (2.5 ± 0.7 g, P < 0.05) within 7 days after nerve ligation and remained stable for at least 3 weeks in the animals studied. Two rats were excluded from the study because the paw-withdrawal threshold was > 8 g, 2 weeks after nerve ligation.

Intravenous injection of 25 mg/kg 2-PMPA did not significantly change the paw-withdrawal threshold in response to application of von Frey filaments in eight rats. Intravenous injection of 50 (n = 8) and 100 (n = 8) mg/kg 2-PMPA both significantly increased the paw-withdrawal threshold in a dose-dependent manner (Fig. 1). 2-PMPA administration was not associated with any overt behavioral changes, such as sedation or agitation, in rats receiving the above doses of 2-PMPA. The motor function, assessed by testing the animals’ ability to stand and ambulate in a normal posture and to place and step with the hindpaw, was not altered by the 2-PMPA treatment. All rats received a score of 2 after intravenous injection of 100 mg/kg 2-PMPA.

Electrophysiological Recording Studies. A total of 27 afferents were recorded from the injured left sciatic nerve in 27 rats. These afferents exhibited typical spontaneous bursting discharge activity (Fig. 2). The conduction velocity was measured in 19 of 27 afferents studied. There were 10 Aδ-fibers with a conduction velocity ranging from 3.4 to 13.8 m/s. The 3 C-fibers had a conduction velocity of between 0.6 and 1.5 m/s. The remaining 6 afferents were Aβ-fibers with a conduction velocity between 18.5 and 44.8 m/s. In 7 afferent fibers, intravenous injection of 25 mg/kg 2-PMPA did not significantly affect the spontaneous ectopic activity (Fig. 3).

Intravenous injection of 50 (n = 10) and 100 (n = 10) mg/kg of 2-PMPA both significantly decreased the spontaneous ectopic discharges recorded from injured sciatic afferent nerves (Figs. 2 and 3). 2-PMPA had a similar inhibitory effect on ectopic discharges recorded from all three types of afferent nerves. The inhibitory effect of 2-PMPA on the ectopic discharge activity lasted 60 to 90 min in all animals tested.

In 10 normal rats, intravenous injection of 100 mg/kg 2-PMPA did not alter the response of 10 normal afferent fibers to mechanical stimulation, evoked by application of calibrated von Frey filaments with bending forces of 2, 5, and 25 g applied to the receptive fields of the afferents (Fig. 4).

In 10 normal afferents, there were 5 Aδ-fibers (conduction velocity = 8.4 ± 1.7 m/s), 2 C-fibers (conduction velocity = 1.1 ± 0.4 m/s), and 3 Aβ-fibers (conduction velocity = 26.8 ± 3.6 m/s). We observed that 2-PMPA had no effect on the spontaneous activity of 3 Aδ-fibers and 2 Aβ-fibers in normal rats (data not shown). Intravenous injection of 100 mg/kg 2-PMPA had no effect on the conduction velocity of these 10 afferent nerves.

![Fig. 1. Time course of the effect of intravenous injection of 25, 50, and 100 mg/kg 2-PMPA on allodynia induced by partial sciatic nerve ligation in rats. The mechanical thresholds were determined by the paw-withdrawal response to von Frey filaments. Data are presented as means ± S.E.M. (n = 8 in each group). * P < 0.05 versus pretreatment control.](image-url)
Discussion

The major finding of the current study is that systemic administration of 2-PMPA attenuated allodynia and inhibited the ectopic discharge activity from the injured peripheral afferent nerve in rats. We observed that intravenous injection of 2-PMPA dose-dependently attenuated allodynia caused by partial sciatic nerve ligation. Furthermore, we found that the similar dose of 2-PMPA significantly inhibited the discharge activity recorded from injured afferent fibers but had no effect on the conduction velocity. Therefore, these data suggest that the inhibitory action of 2-PMPA on the generation of ectopic afferent discharges likely constitutes a mechanism by which 2-PMPA produces an antiallodynic effect in this animal model of neuropathic pain.

GCP II is found in the brain tissue, spinal cord, dorsal root ganglia, and nonmyelinating Schwann cells (Berger et al., 1995; Berger and Schwab, 1996). GCP II is a membrane-bound metallopeptidase that catabolizes NAAG to N-acetyl-aspartate and glutamate (Robinson et al., 1987; Stauch et al., 1989). 2-PMPA is a potent and selective GCP II inhibitor (Jackson et al., 1996) that can reduce brain ischemic damage by reducing local glutamate accumulation and increasing NAAG (Slusher et al., 1999). It has been shown that 2-PMPA inhibits spinal dorsal horn neurons in the rat inflammation.
Glutamate and nerve injury models (Carpenter et al., 2000). Intrathecal injection of 2-PMPA also depresses both the phase 1 and phase 2 flinching behaviors in rats caused by formalin injection (Yamamoto et al., 2001b). In the present study, systemic injection of 2-PMPA significantly attenuated allodynia induced by sciatic nerve injury in rats. These data provide additional evidence that GCP II inhibitors have a therapeutic effect on neuropathic pain. Although the precise analgesic mechanisms of 2-PMPA on neuropathic pain could not be determined by the data from the present study, our data suggest that the antiallodynic effect of 2-PMPA in this model of neuropathic pain may be due to its inhibitory effect on ectopic afferent discharges. Consistent with this notion, we observed that similar doses of 2-PMPA attenuated significantly the ectopic discharges from injured afferent nerves. 

Generation of ectopic discharges from injured afferents is considered to be one of the important mechanisms underlying chronic neuropathic pain. Na\(^+\) and Ca\(^{2+}\) channels are known to contribute importantly to the generation of ectopic discharge activity following nerve injury (Matzner and Devor, 1994; Xiao and Bennett, 1995). Glutamate has been implicated in the generation and maintenance of neuropathic pain because systemic administration of glutamate receptor antagonists relieves neuropathic pain (Hao and Xu, 1996; Sutton et al., 1999; Ta et al., 2000). One important source of glutamate accumulation is likely through the GCP II pathway. Both NAAG and GCP II are present in the peripheral nerve (Berger et al., 1995; Berger and Schwab, 1996). There are several lines of evidence indicating that glutamate in the peripheral nerve plays a role in nociception. For instance, glutamate has been shown to be released from the sciatic nerve during stimulation (DeFeudis, 1971). NMDA, kainate, and AMPA receptors are located on axons of peripheral nerves (Carlton et al., 1995). Local peripheral injection of glutamate and its receptor agonists induces nociceptive reflexes (Ault and Hildebrand, 1993a,b), excitation and sensitization of nociceptive afferents (Du et al., 2001), and mechanical allodynia and hyperalgesia, which are blocked by co-injection of selective glutamate antagonists (Carlton et al., 1995; Zhou et al., 1996). Furthermore, tissue inflammation also increases production of glutamate in the peripheral tissue and afferent endings, and local administration of NMDA and non-NMDA receptor antagonists is effective in alleviating painful conditions (Davidson et al., 1997; Carlton and Coggeshall, 1999). The role of peripheral glutamate in nerve injury-induced plasticity and neuropathic pain has not been documented previously. In the neuroma site, the glutamate accumulation at the site of nerve injury may also play an important role in the generation of abnormal discharge activity. It is known that activation of ionotropic glutamate receptors causes Ca\(^{2+}\) as well as Na\(^+\) influx (Hollmann and Heinemann, 1994). Thus, local glutamate buildup may play a role in the generation of abnormal afferent barrage. We recently have found that both NMDA and kainate receptors are up-regulated at the site of sciatic nerve ligation (unpublished data). Thus, local accumulation of glutamate may contribute to the development of ectopic discharges from injured afferent nerves. The inhibitory action of 2-PMPA on the ectopic impulse activity from injured peripheral afferents has not been studied previously. As demonstrated in the present study, intravenous injection of 50 to 100 mg/kg 2-PMPA significantly attenuated the ectopic afferent activity generated from the site of nerve ligation. Thus, in addition to the inhibitory effect of 2-PMPA on sensitized spinal neurons caused by nerve injury (Carpenter et al., 2000), the effect of 2-PMPA on the ectopic afferent activity may contribute to its antiallodynic activity by directly reducing nociceptive afferent inputs to the dorsal horn neurons. Our data indicate that 2-PMPA has a rapid effect on ectopic discharges, which is consistent with its effect on tactile allodynia. Thus the antiallodynia produced in this model may be highly dependent on the ectopic afferent barrage. Alternatively, the effect of intravenous injection of 2-PMPA on ectopic discharges may not account entirely for its antiallodynic effect. The antiallodynic effect of 2-PMPA may be a result of its combined central and peripheral actions. Data from this study provide a new rationale for the use of GCP II inhibitors as potential analgesic agents for neuropathic pain treatment.

We found that 2-PMPA selectively attenuated the ectopic discharge activity from neuromas but did not affect the conduction velocity of afferent nerves. Thus, the inhibitory effect of 2-PMPA on ectopic discharges is due to inhibition of generation, but not conduction, of afferent nerves. Inhibition of GCP II by 2-PMPA can decrease glutamate accumulation and increase NAAG in neurons and brain tissues (Slusher et al., 1999; Thomas et al., 2000). NAAG is a partial antagonist at the NMDA receptor and is also a type II metabotropic receptor agonist with a high degree of specificity for mGluR3 receptors (Neale et al., 2000). Both of these effects may limit glutamate receptor activation. However, 2-PMPA itself has no significant affinity for all known glutamate receptors tested including NMDA, kainate, AMPA, and glutamate transporters (Slusher et al., 1999). More importantly, decreased glutamate availability from NAAG by inhibition of GCP II could decrease the excitability of the neuroma at the injured nerve. GCP II is located extensively in Schwann cells of the peripheral nerve and may be involved in the signaling between axons and Schwann cells during nerve degeneration or regeneration following nerve injury (Berger et al., 1995; Urazaev et al., 2001). At this time, little is known about GCP II, NAAG, and glutamate metabolism at the site of nerve injury. We speculate that nerve injury may be associated with an increased activity of GCP II, which converts NAAG to glutamate in the Schwann cells. Increased glutamate...

**Fig. 4.** Lack of inhibitory effect of intravenous injection of 100 mg/kg 2-PMPA on the responses of normal afferent nerves (n = 10) elicited by von Frey filaments (VFH) applied to the receptive field of sciatic afferent nerves in normal rats. Data are presented as means \(\pm\) S.E.M. Receptive fields of all normal afferent fibers were located on the left hindpaw.
buildup in the Schwann cells and periaxial space could contribute to generation of ectopic discharge activity at the site of injury. Data from the present study suggest that GCP II inhibitors may offer a new strategy for the treatment of neuropathic pain. It should be acknowledged that this is the first study showing the therapeutic potential and the likely sites of action of GCP II inhibitors for neuropathic pain. It is far from clear how decreased glutamate and increased NAAG contribute to the data obtained in this study. Additional studies are needed to define the role of local glutamate and NAAG in the pharmacological actions of 2-PMPA on ectopic afferent discharges and alldynia associated with neuropathic pain.

In summary, intravenous injection of 2-PMPA attenuated significantly the alldynia induced by partial sciatic nerve ligation in rats in a dose-dependent manner. By directly recording single-unit activity of afferent fibers, we found that similar doses of 2-PMPA also inhibited the ectopic discharge activity from the injured nerve site. Therefore, our study suggests that the analgesic effect of 2-PMPA may be mediated, at least in part, by inhibition of ectopic afferent discharges through decreased glutamate accumulation and increased NAAG at the site of nerve injury.

Acknowledgment

The authors gratefully acknowledge the secretarial assistance of Pamela Myers.

References


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