Pain modulation by release of the endogenous cannabinoid anandamide

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Synthetic cannabinoids produce behavioral analgesia and suppress pain neurotransmission, raising the possibility that endogenous cannabinoids serve naturally to modulate pain. Here, the development of a sensitive method for measuring cannabinoids by atmospheric pressure-chemical ionization mass spectrometry permitted measurement of the release of the endogenous cannabinoid anandamide in the periaqueductal gray (PAG) by *in vivo* microdialysis in the rat. Electrical stimulation of the dorsal and lateral PAG produced CB1 cannabinoid receptor-mediated analgesia accompanied by a marked increase in the release of anandamide in the PAG, suggesting that endogenous anandamide mediates the behavioral analgesia. Furthermore, pain triggered by subcutaneous injections of the chemical irritant formalin substantially increased the release of anandamide in the PAG. These findings indicate that the endogenous cannabinoid anandamide plays an important role in a cannabinergic pain-suppression system existing within the dorsal and lateral PAG. The existence of a cannabinergic pain-modulatory system may have relevance for the treatment of pain, particularly in instances where opiates are ineffective.

analgesia | periaqueductal gray | microdialysis | gas chromatography | mass spectrometry

The components of an elaborate neural system that serves naturally to modulate pain sensitivity have been detailed by Liebeskind and subsequent workers (1, 2). These early studies revealed that electrical stimulation of the periaqueductal gray (PAG) produces analgesia, demonstrating the presence of an analgesia circuit in the brain (1). When elicited from the ventral portion of the PAG, this electrical stimulation-produced analgesia (SPA) is mediated by the release of endogenous opiates (3). However, when elicited from the dorsal or lateral part of the PAG, the analgesic effect of stimulation is mediated by unidentified nonopiate substances (4).

Among the prime candidates for these unknown pain modulatory substances are endogenous cannabinoids- compounds similar to the active ingredient in marijuana. Cannabinoids produce analgesia (5) and dampen the spinal and thalamic neuronal responses to noxious stimuli (6, 7). In attempting to understand the neural basis of cannabinoid analgesia, the PAG was a brain region of interest because of its established role in pain-modulation (8) and the presence of the necessary biological machinery for cannabinoid action (9–12). Like opiates, cannabinoids produce analgesia when microinjected in the PAG (13). However, the anatomical subregions of the PAG that support cannabinoid analgesia were the inverse of those supporting morphine analgesia and opiate-mediated SPA (4, 13, 14): cannabinoids were effective in the dorsal and lateral but not the ventral PAG. These findings led us to speculate that endogenous cannabinoids in the dorsal and lateral PAG may be an important component of the nonopiate analgesia system identified earlier. Herein, we show that electrical stimulation of the dorsal and lateral PAG and the dorsal adjacent area produces analgesia that is blocked by the cannabinoid antagonist SR141716A. Similar stimulation conditions cause the release of anandamide, an endogenous cannabinoid. In addition, intradermal injection of formalin, a chemical irritant that induces prolonged pain, ele-

vates anandamide levels in the dorsal and lateral PAG, indicating that pain itself serves as a trigger for endocannabinoid release.

Materials and Methods

SPA in the PAG. Bipolar stimulating electrodes were stereotaxically placed in the PAG of male Sprague–Dawley rats (Charles River). After recovery from the surgery, pain sensitivity was determined by using the tail flick test before and during electrical stimulation (60-Hz, 50-μs monophasic square waves, 0.01 to 1.8 mA). After establishing stable baseline responses to the noxious stimulus, a range of electrical currents was tested to determine the current that produced a maximal analgesic response, then SR141716A (100 μg/10 μl) or vehicle (60% DMSO or 40/40/20 ethanol/DMSO/H_2O) was injected [intracerebroventricularly (i.c.v.)]. Animals were tested twice: day 1, SR141716A; day 2, vehicle. This allowed the tracking of recovery from the drug effect.

Intradermal Formalin Injections. Dilute formaldehyde (4.0%) was prepared in a solution of mono- and dibasic potassium phosphate (pH 7.4) and injected s.c. (150 μl) into the plantar surface of both hindpaws, modified from the method of Dubuisson and Dennis (15).

***In Vivo* Microdialysis in the PAG.** Microdialysis probes were constructed as previously described (16). The probes were of a concentric design with 2-mm tips and 40-kDa-cutoff membrane. They were flushed with water, methanol, and the perfusion buffer before use. *In vitro* recovery of [$^2H_8$] anandamide from artificial cerebrospinal fluid (aCSF) was performed with 30% β-cyclodextrins in aCSF [148 mM NaCl/2.7 mM KCl/1.2 mM CaCl$_2$/0.85 mM MgCl$_2$; 1 μl/min, (17)] as the perfusion buffer. The inclusion of β-cyclodextrins in the perfusion buffer produced an approximately 15-fold increase in the recovery of anandamide *in vitro* compared with that in aCSF alone (recovery with aCSF buffer: mean, 0.34%; range, 0.11- 0.65%; recovery with aCSF + 30% β-cyclodextrins: mean, 5.49%; range, 5.09– 5.90%). We also obtained *in vitro* probe blanks with the probes immersed in aCSF.

For electrical stimulation experiments, insulated electrodes with 2-mm exposed tips were secured 1 mm anterior and posterior to the probe (Fig. 3A). For *in vivo* microdialysis, male Sprague–Dawley rats, weighing 320–360 g, were anesthetized with urethane (1.25 g/kg) and placed in the stereotaxic frame. Body temperature was maintained at 37°C with a thermostated rectal probe. The dialysis probe (and the electrodes for electrical stimulation experiments) was lowered into the PAG stereotaxically. The perfusion buffer was maintained at 1 μl/min. Baseline

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samples were collected at least 270 min after probe insertion. Samples were collected on ice. After establishing a stable baseline, electrical stimulation (bipolar, 0.1 msec/1 mA, 60 Hz, 5-sec trains) was delivered for 30 min, or 4% formalin solution was injected in the hindpaws. Approximately 9 hr after probe insertion, the rats were perfused intracardially with saline and 10% formalin. The brains were cryoprotected, cut in 40-μm sections on a cryostat, and stained with cresyl violet for probe and electrode location.

LC/MS Analysis of Microdialysis Samples. The method employed was modified from that of Koga *et al.* (18), who demonstrated that anandamide can be measured by atmospheric pressure chemical ionization–liquid chromatography/mass spectrometry (APCI-LC/MS). Samples from 15- or 30-min collection periods were chromatographed with a pair of 50-mm Zorbax Eclipse XDB C-18, reversed-phase HPLC (4.6 mm i.d., 1 ml/min) columns (Hewlett-Packard) with isocratic 85% methanol/1 mM

ammonium acetate/0.05% acetic acid; the samples were subjected to MS (Hewlett-Packard 1100 series) in the APCI mode with selected ion monitoring (*m/z* of 348.3), fragmentor voltage of 50 V, vaporizer at 325°C, drying gas at 350°C, drying gas flow rate of 7 liters/min, and corona current of 7 μA.

Statistics. The area under the peak at approximately 8-min retention time (according to the elution time of anandamide standards) was determined after Gaussian signal averaging set at 0.2 or 0.3 min width (Hewlett-Packard CHEMSTATION software); the area was converted to molar levels by using a regression equation generated from a series of standard concentrations that spanned the range of values found in the microdialysis samples (e.g., Fig. 2*C*). The data from the behavioral experiments were analyzed by the Wilcoxon sign–rank test, and the anandamide levels were analyzed by repeated measures analysis of variance followed by a post hoc analysis of means (the *t* test). $P < 0.05$ was considered statistically significant.
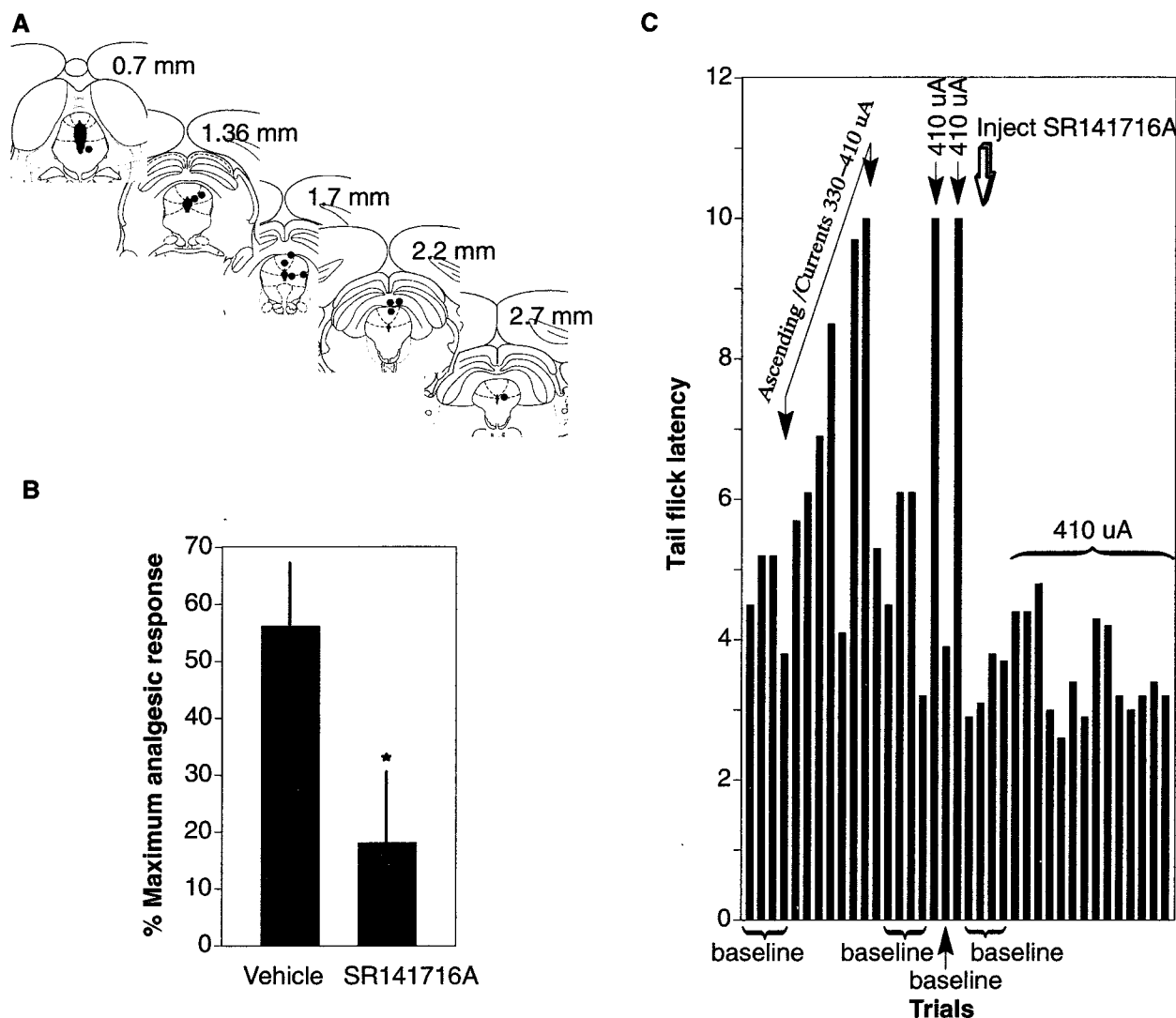
Fig. 1. Cannabinoid receptor-mediated stimulation produced analgesia in the rat. (*A*) Electrode placements (*n* = 11) are shown with anterior–posterior coordinates (21) of the sections (interaural line = 0) to the right of each diagram. Note that the stimulation sites are located in the dorsal aspect of the PAG, and two lie outside the dorsal border of the PAG, an area from which SPA has been elicited in previous studies (22). (*B*) The analgesic effects of this stimulation were antagonized by SR141716A (*P* = 0.02). The long time course (approximately 20 hr) of SR141716A precluded following recovery on the test day. (*C*) Sample data from a single rat. After the establishment of a stable baseline of response, the threshold for SPA was determined. The current was raised in ascending steps until a level of stimulation (410 mA) that produced complete (10-sec tail-flick latency) analgesia was reached. After injection of SR141716A (100 μg/10 μl, i.c.v.), the stimulation no longer produced analgesia.
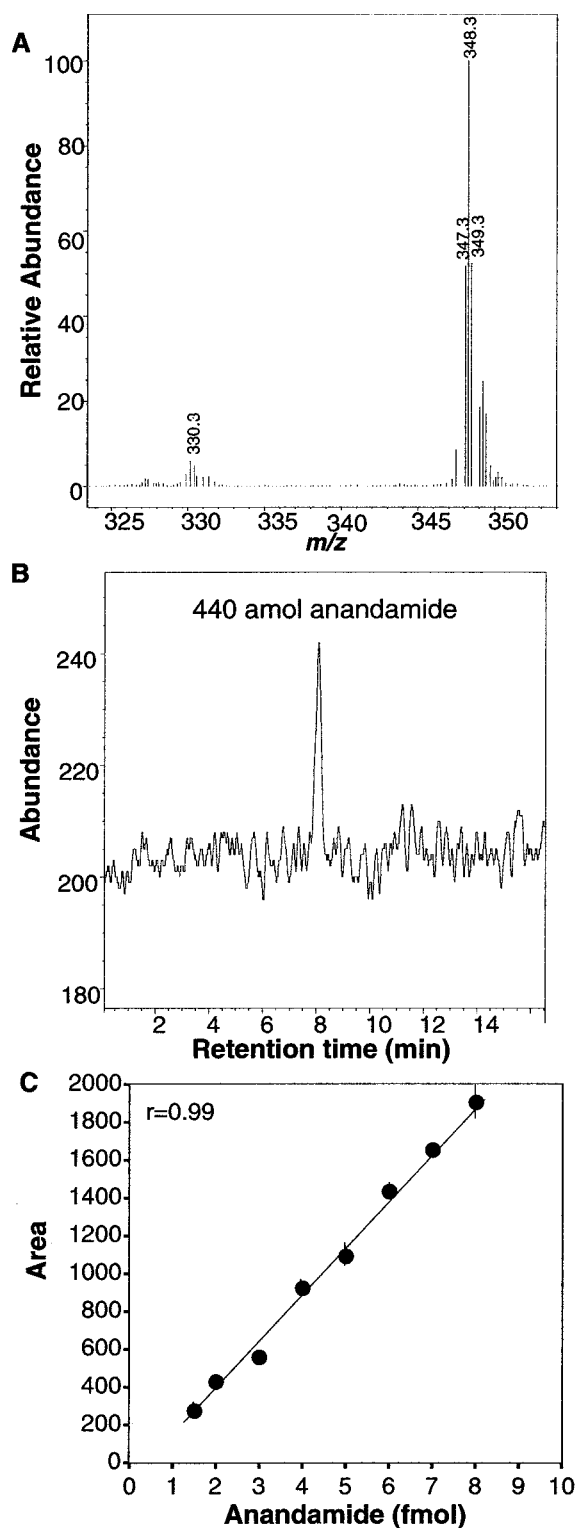
Fig. 2. Sensitive, precise measurement of anandamide was performed with LC/APCI MS. (*A*) The mass spectrum reflects the minimal fragmentation as desired for maximal sensitivity; the spectrum shown mainly represents the distribution of naturally occurring anandamide M^+ isotopes around the major peak at 348.3. The minor peak at 330.3 is identical to the mass of anandamide minus water. The data are in excellent agreement with spectra obtained by Koga *et al.* (18). (*B*) Detection of 440 amol of anandamide. This method was developed by using a Hewlett Packard 1100 series mass selective detector in selected ion monitoring mode at molecular weight 348.3, that of the principal anandamide M^+ isotope (23). (*C*) Synthetic anandamide analyzed by APCI-LC/MS with the parameters described. The response, assessed by peak area,

All of the experimental procedures were approved by the Brown University Animal Care and Use Committee.

Results

Electrical stimulation of the dorsal and lateral PAG produced profound analgesia, which was measured by the loss of a nocifensor reflex, the tail-flick response to thermal pain. Animals that were rendered analgesic by the stimulation were treated with SR141716A, a potent and selective cannabinoid antagonist (19, 20) and the vehicle. The cannabinoid antagonist markedly decreased the analgesic efficacy of electrical stimulation, whereas the vehicle failed to produce an effect (Fig. 1, $P < 0.05$). These findings led us to the hypothesis that anandamide, an endogenous cannabinoid, was released by electrical stimulation of the dorsal and lateral PAG.

In contrast to the commonly used method of GC/MS, which offers a picomole detection limit (10^{-12} mole), the improved method with LC/MS permitted the detection of the endogenous cannabinoid anandamide (Fig. 2*A*) at amol levels (Fig. 2*B*). Amounts of synthetic anandamide between 1.5 and 8.0 fmol, the levels spanning the range found in our microdialysis samples, produced a linear response (Fig. 2*C*). This response is linear over at least four orders of magnitude.

The establishment of an improved method for analyzing anandamide allowed the measurement *in vivo* of its release in the PAG. The basal level of anandamide found in 15-min dialysis samples was 2.79 ± 0.27 fmol. The endogenous material exhibited an elution pattern identical to that of synthetic anandamide (Fig. 3*B*). Histological examination of Nissl-stained sections revealed that all probes were placed within the caudal 2/3 of the PAG (Fig. 3*C*). Electrical stimulation delivered by using pulse parameters similar to those used in the behavioral experiment produced a marked increase in the extracellular levels of anandamide (Fig. 4*A*; $P < 0.05$).

Pain-processing neurons in the spinal cord send profuse projections to the PAG (24, 25). Therefore, it seemed plausible that noxious stimulation would induce the release of anandamide. Subcutaneous injections of dilute formalin in the hindpaws, which cause prolonged pain behavior in rats (15), were employed in combination with microdialysis in the PAG in urethane-anesthetized rats to investigate this possibility. This prolonged nociceptive stimulation stimulated the release of anandamide (Fig. 4*B*, $P < 0.001$).

Discussion

The levels of anandamide found in the dialysates in the present study were similar to those of other neurotransmitters or neuromodulators that are important in pain and found in the PAG. For example, dialysates from the PAG contained low femtomole levels of the endogenous opioid, [Met]enkephalin (26, 27), whereas substance P and neurotensin were found in attomole ranges (27–29), when the values were corrected for our particular collection period. The elevated anandamide levels observed after either electrical or painful stimulation are indicative of heightened levels of anandamide in the extracellular space; they probably reflect even greater increases in concentration at the site of release (for review, see ref. 30). The levels in the extracellular space are attenuated because of transmitter removal mechanisms (31–33).

Experiments conducted approximately 20 years ago revealed the existence of nonopiate factors released in the PAG that serve as chemical messengers in the selective and effective system the

was linear over at least four orders of magnitude. Shown are anandamide levels from 1.5 to 8.0 fmol (in 10-μl volumes), covering the range measured in microdialysis samples. Although the range was restricted, the response was highly linear as determined by regression analysis. The points shown represent means of three samples, and the vertical bars represent SEMs.
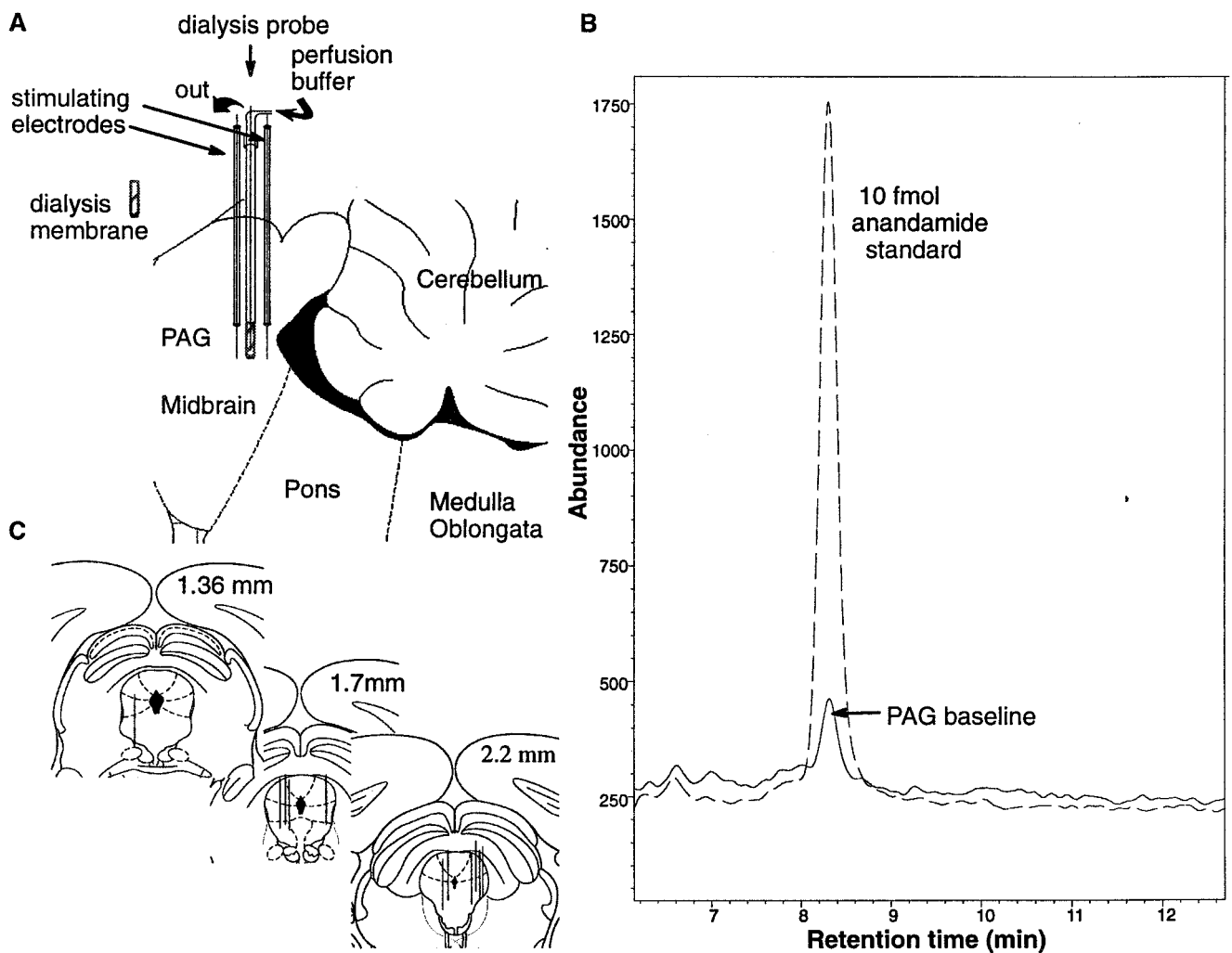
Fig. 3. Collection and analysis of anandamide by microdialysis in rat PAG. (*A*) The preparation used to examine the effects of electrical stimulation of the PAG on extracellular levels of anandamide. Bipolar stimulation of the PAG was accomplished by stainless steel electrodes that, except for 2 mm at the tips, were insulated; electrodes were implanted 1 mm rostral and caudal to the dialysis probe, as shown. Stimulation consisted of 60-Hz constant current pulses, 0.1 ms duration, 1 mA, parameters similar to those used previously for studies of SPA. The dialysis probe had a concentric design described by this laboratory (16) with 2-mm exposed membrane. The buffer was composed of aCSF in 30% β-cyclodextrins, as required to prevent the anandamide from adhering to surfaces within the probe. Probe recovery ranged from 3% to 6%. (*B*) Co-elution of a synthetic anandamide standard and the endogenous anandamide recovered from a microdialysis probe inserted in the PAG of a urethane-anesthetized rat. Samples were chromatographed and analyzed as described. (*C*) Placements of dialysis probes in the PAG. Numbers refer to distance (in mm) from the anterior to the interaural line (21). Placements for electrical stimulation experiments are on the right, and placements for formalin experiments are on the left.

brain uses to suppress pain. These results as well as prior findings indicate that anandamide fulfills the requirements of such a nonopiate mediator of endogenous pain suppression. Cannabinoid receptors (8, 9), cannabinoid receptor mRNA (10), and the anandamide-degrading enzyme fatty acid amide hydrolase (11, 12) are located in the PAG. Cannabinoid agonists applied to the dorsal and lateral PAG produce analgesia (13). Conversely, the systemic or spinal administration of the cannabinoid antagonist SR141716A and spinal CB1 receptor knockdown each produce hyperalgesia (34–36). Here we showed that electrical stimulation of this area simultaneously produces the local release of anandamide and analgesia that is reversed by a cannabinoid antagonist. It appears that pain triggers this cannabinergic pain-suppression system, because a noxious chemical stimulus applied to the hindpaws also causes the release of anandamide in the PAG. These data support the existence of endogenous cannabinergic circuitry in the dorsal and lateral PAG that is triggered by pain and promotes analgesia through the release of anandamide.

The PAG has a recognized role in pain modulation (7, 40). It is both informed of incoming noxious stimuli (24) and capable of mediating analgesia of both opiate and nonopiate types (4, 7, 14). In particular, the dorsal and lateral PAG is active in subcutaneous pain paradigms, such as thermal tail-flick and subcutaneous formalin (38, 39). This probably results from activation of the profuse projections from lamina I of the spinal dorsal horn (25), which comprises mainly the nociceptive-specific neurons, i.e., neurons that respond only to painful stimuli (40–42). The PAG suppresses pain by descending modulation of the spinal cord, which appears to be the dominant mechanism of cannabinoid analgesia for acute pain (43). Support for this notion stems from the findings that spinal transection virtually eliminates the ability of systemically administered cannabinoids to suppress pain-evoked responses in the spinal cord (43), and intrathecal administration of the α_2-adrenergic antagonist yohimbine attenuates the analgesic effects of a systemic cannabinoid (44). The PAG is interconnected with amygdaloid and medullary circuits that mediate descending modulation of spinal
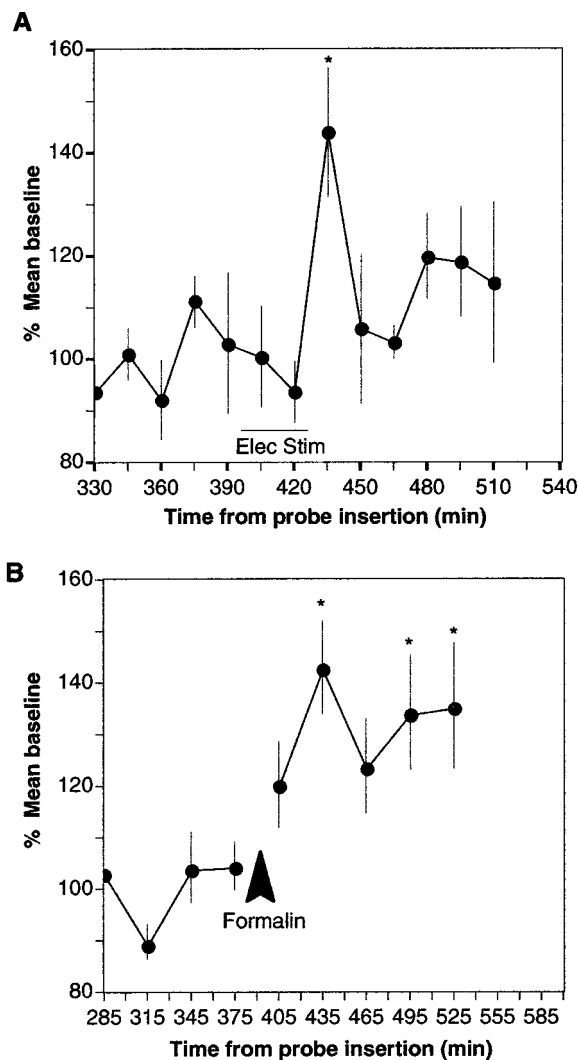
Fig. 4. The release of anandamide in the PAG of the rat stimulated by electrical depolarization or pain. (*A*) Increased extracellular levels of anandamide after electrical stimulation of the PAG in urethane-anesthetized rats. After the establishment of stable baseline values, electrical stimulation (bipolar 0.1 msec/1 mA, 60-Hz, 5-sec trains with 5-sec rest intervals) was delivered for 30 min. Microdialysis samples were collected in 15-min intervals and analyzed by HPLC with detection by APCI–MS, with selected ion monitoring mode at molecular weight 348.3 ($n = 5$, $P < 0.05$, in repeated measures analysis of variance). The asterisks mark points that are significantly different from the baseline average by posthoc test ($P < 0.05$). The delay in measurement presumably reflects the time needed to produce anandamide in the extracellular space with sufficient overflow to achieve recovery by microdialysis. (*B*) Increased extracellular levels of anandamide in the PAG after induction of prolonged pain in urethane-anesthetized rats. After the establishment of a stable baseline, a 4% 150-μl formalin solution was injected subcutaneously in both hindpaws. The samples shown span 30-min intervals ($n = 6$; $P < 0.001$, repeated measures analysis of variance).

nociceptive responses (for review, see ref. 45). Each of these areas is involved in cannabinoid analgesia (13). The results presented here indicate that the endogenous cannabinoid anandamide produces analgesia after its release in the PAG from neurons that are depolarized either electrically or by neuronal inputs activated by painful stimuli. In light of the marked

analgesic effects of cannabinoids applied to the periphery and the spinal cord (46, 47), and to at least six brain areas in addition to the PAG (13), endogenous cannabinoids may act in other areas as well (48).

When the cannabinergic system is compared with the endogenous opiate system, similarities and differences emerge. As discussed above, it is clear that the opiate and cannabinoid mechanisms partially overlap anatomically; both are present in the PAG and other pain-processing areas, such as the rostral medulla, the amygdala, and the spinal dorsal horn (refs. 49 and 50; for review, see ref. 45). However, there are clear distinctions between the sites within the PAG that mediate the actions of cannabinoids and those that mediate the actions of opiates, and recent findings have demonstrated that cannabinoid and opiate receptors occur on different peripheral fiber types (51).

An apparent difference between the cannabinoid system and the opioid system is the degree to which they are tonically active. The endogenous opiate system is activated by intense stimuli such as stress (52), high threshold electrical stimulation (2), or intense prolonged pain (53). This phenomenon is consistent with the neurobiology of peptides, which typically occur as cotransmitters (54, 55), and are preferentially released when the neurons achieve high firing rates (56, 57).

By contrast, the administration of a cannabinoid antagonist substantially enhanced pain sensitivity, and hyperalgesia was observed after CB1 receptor knockdown. Both occurred in pain tests that do not produce significant stress or fear (34, 35, 47). The basal levels of anandamide that we measured are probably indicative of tonic pain suppression by endogenous cannabinoids. The presence of a tonically active pain-suppression mechanism became apparent from the hyperalgesia produced by lesions of the spinal dorsolateral funiculus, a major pathway of brainstem suppression of pain at the spinal level (58, 59). It is conceivable that endogenous cannabinoids are the factors that drive this tonic pain-suppression mechanism. Thus, the endogenous cannabinoid and the opioid pain-modulation systems differ not only in their anatomical locations, but also functionally, in the persistent maintenance of decreased pain perception in the cannabinergic system. Both systems become more active in response to painful stimulation. The tonic pain-modulatory actions of the endogenous cannabinergic system raise the possibility that system dysfunction leads to spontaneous pain, hyperalgesia, or allodynia.

Cannabinoids have been used to treat pain for centuries. In ancient China, hemp extract was used as a surgical anesthetic (60), and archeological finds in Israel have revealed its use against the pain of childbirth (61). Cannabis is still used to treat pain, despite its illegal status in most parts of the world (62). The spontaneous and stimulated release of anandamide in a pain-suppression circuit suggests that drugs that inhibit the reuptake of anandamide or block its degradation may form the basis of a modern pharmacotherapy for pain, particularly in instances where opiates are ineffective.

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