Endocannabinoids at the spinal level regulate, but do not mediate, nonopioid stress-induced analgesia

Richard L. Suplita II a, Tania Gutierrez a, Darren Fegley b, Daniele Piomelli b, Andrea G. Hohmann a,*

a Neuroscience and Behavior Program, Department of Psychology, University of Georgia, Baldwin Street, Athens, GA 30602-3013, USA
b Department of Pharmacology and Center for Drug Discovery, University of California, Irvine, CA 92697-4260, USA

Received 10 August 2005; received in revised form 10 October 2005; accepted 11 October 2005

Abstract

Recent work in our laboratories has demonstrated that an opioid-independent form of stress-induced analgesia (SIA) is mediated by endogenous cannabinoids [Hohmann et al., 2005. Nature 435, 1108]. Non-opioid SIA, induced by a 3-min continuous foot shock, is characterized by the mobilization of two endocannabinoid lipids—2-arachidonoylglycerol (2-AG) and anandamide—in the midbrain periaqueductal gray (PAG). The present studies were conducted to examine the contributions of spinal endocannabinoids to nonopioid SIA. Time-dependent increases in levels of 2-AG, but not anandamide, were observed in lumbar spinal cord extracts derived from shocked relative to non-shocked rats. Notably, 2-AG accumulation was of smaller magnitude than that observed previously in the dorsal midbrain following foot shock. 2-AG is preferentially degraded by monoacylglycerol lipase (MGL), whereas anandamide is hydrolyzed primarily by fatty-acid amide hydrolase (FAAH). This metabolic segregation enabled us to manipulate endocannabinoid tone at the spinal level to further evaluate the roles of 2-AG and anandamide in nonopioid SIA. Intrathecal administration of the competitive CB1 antagonist SR141716A (rimonabant) failed to suppress nonopioid SIA, suggesting that supraspinal rather than spinal CB1 receptor activation plays a pivotal role in endocannabinoid-mediated SIA. By contrast, spinal inhibition of MGL using URB602, which selectively inhibits 2-AG hydrolysis in the PAG, enhanced SIA through a CB1-selective mechanism. Spinal inhibition of FAAH, with either URB597 or arachidonoyl serotonin (AA-5-HT), also enhanced SIA through a CB1-mediated mechanism, presumably by increasing accumulation of tonically released anandamide. Our results suggest that endocannabinoids in the spinal cord regulate, but do not mediate, nonopioid SIA.

Keywords: Anandamide; Endocannabinoid; 2-Arachidonoylglycerol; Periaqueductal gray; Rostral ventromedial medulla; Stress antinociception; Fatty-acid amide hydrolase; Monoacylglycerol lipase; Dorsal horn

1. Introduction

Stress-induced analgesia (SIA) is mediated by the activation of endogenous pain inhibitory systems. Both opioid-dependent and opioid-independent forms of SIA have been identified (see Akil et al., 1986 for review). These mechanisms are differentially activated according to stressor parameters and duration (Lewis et al., 1980a,b). SIA elicited by intermittent foot shock is blocked by opioid antagonists (Lewis et al., 1980a), whereas SIA elicited by continuous foot shock (3 min) is blocked by cannabinoid antagonists (Hohmann et al., 2005). We recently demonstrated that this nonopioid form of SIA is mediated by mobilization of two endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide, in the dorsal midbrain (Hohmann et al., 2005).

Opioid and nonopioid SIA share similar neuroanatomical substrates. For example, opioid and cannabinoid receptors populate brain regions regulating nociceptive responding, such as the periaqueductal gray (PAG) (Martin et al., 1995; Lichtman et al., 1996) and the raphe nuclei of the medulla.
(Herkenham et al., 1991). Like opioids, cannabinoids modulate distinct circuits within the midbrain PAG (Vaughan et al., 2000) and the brainstem rostral ventromedial medulla (RVM) (Meng et al., 1998). The competitive CB1 antagonist/inverse agonist SR141716A (rimonabant) microinjected into either the dorsolateral PAG (dPAG) (Hohmann et al., 2005) or RVM (Suplita et al., in press) also attenuates SIA. By contrast, inhibition of endocannabinoid hydrolysis at these sites enhances SIA (Hohmann et al., 2005; Suplita et al., in press). These data support the existence of supraspinal sites of endocannabinoid analgesic action.

Cannabinoids produce antinociception through spinal as well as supraspinal mechanisms (for review, see Hohmann and Suplita, 2004). The antinociceptive (Lichtman and Martin, 1991) and electrophysiological (Hohmann et al., 1999c) effects of cannabinoids are attenuated following spinal transection. Nonetheless, a long-lasting residual antinociception remains in spinally transected mice (Smith and Martin, 1992), suggesting the existence of spinal sites of endocannabinoid analgesic action. These data are consistent with the presence of CB1 receptors in the spinal dorsal horn (Tsou et al., 1998a; Hohmann et al., 1999a; Farquhar-Smith et al., 2000). Intrathecally-administered cannabinoids also produce antinociception (Yaksh, 1981; Smith and Martin, 1992; Welch and Stevens, 1992; Welch et al., 1995; Martin et al., 1999) and suppress noxious stimulus-evoked neuronal activity in spinal nociceptive neurons (Hohmann et al., 1998, 1999b; Drew et al., 2000; Kelly and Chapman, 2001), suggesting a functional role for spinal cannabinoid receptors in modulating nociceptive processing. Intrathecal administration of either rimonabant or CB1 antisense oligonucleotides also elicits hyperalgesia (Richardson et al., 1998), suggesting that endocannabinoids may act tonically to suppress nociceptive responding. However, a physiological role for endocannabinoids at the spinal level has not been identified.

Both 2-AG and anandamide fully qualify as endocannabinoids (see Piomelli, 2003 for review). These lipids are synthesized and released on-demand, activate cannabinoid CB1 receptors with high affinity, and are metabolized in vivo by distinct hydrolytic pathways. Within the CNS, 2-AG is present in quantities 170–1000 times greater than those of anandamide (Sugiura et al., 1995; Stella et al., 1997). 2-AG serves as a full agonist at CB1 and CB2 (Sugiura et al., 1999, 2000; Gonsiorek et al., 2000; Savinainen et al., 2001), whereas anandamide is less efficacious (Sugiura et al., 1995; Stella et al., 1997; Savinainen et al., 2001).

2-AG is preferentially degraded by monoacylglycerol lipase (MGL) (Dinh et al., 2002a, b, 2004), whereas anandamide is preferentially hydrolyzed by fatty-acid amide hydrolase (FAAH) (Cravatt et al., 1996). Mutant mice lacking the FAAH gene (FAAH−/−) are impaired in their ability to degrade anandamide and display reduced pain sensation that is reversed by the CB1 antagonist rimonabant (Cravatt et al., 1996; Lichtman et al., 2002). Immunochemical studies have revealed a heterogeneous distribution of FAAH throughout the brain and moderate staining of FAAH-positive cells in the superficial dorsal horn of the spinal cord (Tsou et al., 1998b). In brain, the regional distribution of MGL partially overlaps with that of the CB1 receptor (Dinh et al., 2002a, b). Inhibition of MGL in the midbrain PAG increases 2-AG accumulation and enhances SIA in a CB1-dependent manner (Hohmann et al., 2005), supporting a physiological role for 2-AG in neural signaling. Thus, the anatomical distributions of FAAH and MGL are consistent with a role for these enzymes in terminating the activity of endocannabinoids.

The present studies were conducted to investigate the contribution of endocannabinoids acting at spinal CB1 receptors to nonopioid SIA. We used high-performance liquid chromatography/mass spectrometry (LC/MS) to examine the contribution of 2-AG and anandamide at the spinal level to SIA. We examined the time-course of changes in endocannabinoid levels in the lumbar spinal cord of control rats and rats subjected to foot shock stress. Moreover, we used selective inhibitors of MGL (URB602; Hohmann et al., 2005) and FAAH (URB597; Kathuria et al., 2003) to further elucidate the roles of these endocannabinoids in SIA. We additionally evaluated effects of intrathecal administration of arachidonoylserotonin (AA-5-HT; Bisogno et al., 1998), a FAAH inhibitor that is inactive at phospholipase A2 and CB1 receptors, on SIA. We hypothesized that intrathecal administration of these inhibitors would potentiate nonopioid SIA via a CB1 mechanism. Preliminary results have been reported (Hohmann et al., 2005).

2. Methods

2.1. Subjects

One hundred forty-four adult male Sprague–Dawley rats (320–370 g) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Associations for the Study of Pain (Zimmermann, 1983).

2.2. Drugs

The CB1 antagonist/inverse agonist SR141716A (rimonabant) was a gift from NIDA. The fatty acid amide hydrolase (FAAH) inhibitors URB597 and arachidonoyl serotonin (AA-5-HT) were purchased from Cayman (Ann Arbor, MI). URB602 (biphenyl-3-yl carbamic acid cyclohexyl ester) was synthesized by reacting diimidazole-1-ylmethanone with biphenyl-3-yl amine in acetonitrile in the presence of 4-dimethylaminopyridine and subsequently with cyclohexanol as described previously (Hohmann et al., 2005).

2.3. Lipid extractions and LC/MS analyses

For ex vivo experiments, we habituated otherwise naive rats to the guillotine for at least 7 days prior to the experiment and killed them either without exposure to or at various times (2, 7, 15, and 25 min) after foot shock (0.9 mA for 3 min; n = 10 per group). The lumbar spinal cords were quickly dissected and stored frozen (−80 °C) until lipid extraction. Lipids were extracted in methanol/chloroform/water (1:2:0.25), and the organic phase was recovered, evaporated to dryness, reconstituted in chloroform/methanol (1:3, 80 μl) and subjected to LC/MS analysis as described previously (Giufrida et al., 1999; Hohmann et al., 2005).

2.4. Surgical procedures

Rats were anesthetized with a mixture of sodium pentobarbital and ketamine. Intrathecal catheters were constructed from PE10 tubing (Yaksh and
Catheters were implanted through an incision in the atlanto-occipital membrane to a depth of 8.5 cm. The catheter tip extended just rostral to the lumbar enlargement. Catheters were fixed to the skull with a stainless steel screw and dental acrylic. Animals were allowed to recover five to seven days prior to testing.

2.5. Behavioral testing

SIA was quantified behaviorally using the tail-flick test (D’Amour and Smith, 1941). After establishing stable baseline responses to thermal stimulation of the tail (model 33A tail-flick unit, IITC Inc., Woodland Hills, CA), rats received a single intrathecal injection of rimonabant (2 or 20 nmol), URB602 (1 nmol), URB597 (1 nmol), AA-5-HT (1 or 200 nmol) or DMSO vehicle. Drugs were injected in a volume of 10 µl followed by an equivalent volume of saline to flush the catheter. Tail-flick latencies were assessed three times at 2-min intervals immediately following drug administration to assess changes in tail-flick latency induced by the injection itself. Rats were subsequently subjected to continuous foot shock (0.9 mA for 3 min; Hohmann et al., 2005). Tail-flick latencies were reassessed at 2-min intervals for 60 min and averaged for each subject in two trial blocks. A 10-s cut-off latency was employed to prevent tissue damage. The region of the tail subjected to thermal stimulation was slightly varied across trials to eliminate sensitization. In previous studies we showed that tail-flick latencies remain stable throughout the entire observation interval in the absence of the stressor (Hohmann et al., 2005). Following sacrifice, catheter placement was verified by injection of fast green dye followed by dissection.

2.6. Statistical analyses

Time course data were analyzed using repeated measures analysis of variance (ANOVA). Post hoc comparisons were performed using Fisher’s Protected Least-Squares Difference (LSD) test to correct for inflated alpha error, with \( P < 0.05 \) considered significant. Pearson’s correlation coefficients were calculated to assess the relationship between spinal endocannabinoid mobilization and SIA.

3. Results

3.1. Effects of foot shock stress on endocannabinoid levels

To determine whether endocannabinoid release participates in non-opioid SIA, we analyzed 2-AG and anandamide levels in the lumbar spinal cords of rats killed before (non-shock) or at various times after foot shock using LC/MS. Example chromatograms show the coelution of endogenous 2-AG and anandamide with synthetic standards (Fig. 1). ANOVA revealed time-dependent changes in 2-AG levels derived from lumbar spinal cord extracts of shocked rats relative to non-shocked rats \( F(4,44) = 21.106, P < 0.001 \) (Fig. 2a). Post hoc analyses confirmed that 2-AG levels were significantly increased in rats killed at 2 min post shock \( (P < 0.001) \) and decreased at subsequent time points \( (P < 0.05) \). By contrast, anandamide levels in the lumbar spinal cords of rats subjected to foot shock did not differ reliably from controls. Planned comparisons failed to reveal a significant elevation in anandamide levels at either 2 min \( (P = 0.24) \) or 5 min \( (P = 0.21) \) post shock (Fig. 2a). Foot shock also failed to alter either 2-AG or anandamide levels in tissues derived from the occipital cortex (Fig. 2b), an area enriched in cannabinoid CB1 receptors but not implicated in SIA. A significant correlation was observed between SIA and post-shock levels of 2-AG \( (r = 0.96, P = 0.02) \), but not anandamide \( (r = 0.763, P = 0.237) \), in the lumbar spinal cord over the same interval (Fig. 3).

3.2. Assessment of SIA

In all studies, baseline tail-flick latencies did not differ between groups prior to administration of drug or vehicle. Moreover, latencies recorded just prior to foot shock, following injection of either drug or vehicle, did not differ between groups. Post-shock latencies were higher than baseline measures in all studies \( (P < 0.0001) \), demonstrating that continuous foot shock for 3 min elicited robust antinociception. Fig. 3 shows the temporal correspondence between SIA and foot shock-induced endocannabinoid accumulation in the lumbar spinal cord.
3.3. Pharmacological inactivation of CB1 receptors

Intrathecal injection of rimonabant (2 nmol or 20 nmol) failed to suppress post-shock tail-flick latencies relative to vehicle-treated controls \[ F(2,18) = 0.104, P = 0.9017 \] (Fig. 4).

3.4. Spinal inhibition of monoacylglycerol lipase (MGL)

Intrathecal administration of the MGL inhibitor URB602 (1 nmol) increased post-shock tail-flick latencies \[ F(2,17) = 8.954, P < 0.003 \] relative to either vehicle controls \( P < 0.002 \) or animals receiving URB602 co-administered with rimonabant \( P < 0.004 \) (Fig. 5). Rimonabant (2 nmol) blocked the URB602-induced potentiation of stress antinociception. Tail-flick latencies observed in animals receiving URB602 co-administered with rimonabant did not differ from vehicle \( P = 0.7529 \).

3.5. Spinal inhibition of fatty-acid amide hydrolase (FAAH)

Intrathecal administration of either URB597 (1 nmol) or AA-5-HT (1 nmol or 200 nmol) increased post-shock tail-flick latencies relative to controls \[ F(3,32) = 20.765, P < 0.0001 \] and \[ F(2,14) = 4.571, P < 0.03, \] respectively] (Fig. 6). Co-administration of rimonabant (2 nmol) with either AA-5-HT or URB597 blocked the potentiation of SIA induced by each compound \( P < 0.0001 \) and \( P < 0.04 \), respectively). Furthermore, both the high (200 nmol) and low (1 nmol) dose of AA-5-HT increased post-stress tail-flick latencies relative to controls \( P < 0.0001 \) for either dose). Post hoc analyses revealed that the 1 nmol dose of AA-5-HT was as effective as the 200 nmol dose in potentiating stress antinociception. Administration of either dose of AA-5-HT enhanced stress antinociception from 9–60 min post foot shock.

4. Discussion

We have previously demonstrated that nonopioid SIA is mediated by mobilization of two endocannabinoids, 2-AG and anandamide, in the midbrain PAG (Hohmann et al., 2005). The present results extend these findings and suggest that endocannabinoid actions at spinal CB1 receptors modulate SIA. Foot shock stress induced time-dependent changes in the
levels of 2-AG, but not anandamide, in the lumbar spinal cord. However, intrathecal administration of rimonabant failed to attenuate nonopioid SIA. Nonetheless, selective inhibitors of MGL and FAAH, administered via the same route, increased both the magnitude and duration of SIA through a CB1-dependent mechanism. A parsimonious interpretation of these findings is that inhibition of MGL or FAAH prevented the deactivation of spinal 2-AG and anandamide, respectively, magnifying nonopioid SIA. Thus, our findings suggest that spinal endocannabinoids regulate, but do not mediate, nonopioid SIA.

It is possible that the placement of chronic indwelling intrathecal catheters elevated anandamide levels in the behavioral studies, and that this change in endocannabinoid tone was augmented by the FAAH inhibitors. However, changes in endocannabinoid tone due to catheter placement appear unlikely because intrathecal rimonabant administration did not alter tail-flick latencies in the presence or absence of foot shock. Moreover, Fos immunocytochemical studies suggest that spinal compression induced by catheter placement does not induce appreciable neuronal activation (Hohmann et al., 1999b).
Electrophysiological studies have provided evidence both for (Chapman, 1999) and against (Harris et al., 2000; Morisset and Urban, 2001) the existence of a tonic endocannabinoid tone at the spinal level. However, in our study, intrathecal rimonabant administration failed to suppress either basal nociceptive thresholds (determined immediately prior to the foot shock) or nonopioid SIA. Hyperalgesic effects of spinal rimonabant (Richardson et al., 1997) may reflect sensitivity of supraspinally-organized (i.e. hot plate) relative to spinally organized (e.g. tail-flick) pain behaviors in measuring lowered nociceptive thresholds. It is also possible that exposure to uncontrolled stress (e.g. due to handling) mobilizes endocannabinoids in behavioral studies (Patel et al., 2005), thereby resulting in a CB1-dependent apparent hyperalgesia.

Although high doses of anandamide activate transient receptor potential vanilloid 1 (TRPV1) (for review, see van der Stelt and Di Marzo, 2005), several observations suggest that this mechanism is not responsible for the enhancements of SIA observed here. First, systemic administration of the TRPV1 antagonist capsazepine, at a dose that completely blocks capsaicin-induced antinociception, does not alter nonopioid SIA in our paradigm (Suplita et al., in press). Second, CB1 and TRPV1 show minimal colocalization at the axonal level in the spinal cord, with CB1 localized predominantly to laminae I and II interneurons (Farquhar-Smith et al., 2000) and TRPV1 localized primarily to nociceptive primary afferent terminals (Caterina et al., 1997; Caterina and Julius, 2001). Third, anandamide activation of CB1 and TRPV1 typically induces opposing effects with distinct time courses (see De Petrocellis and Di Marzo, 2005). However, we observed only enhancements—not attenuation—of SIA following intrathecal administration of FAAH inhibitors and no change in SIA following antagonist treatment. Fourth, rimonabant completely blocked the potentiation of SIA induced by intrathecal administration of inhibitors of MGL or FAAH, at doses that were insufficient to reverse SIA when administered alone. Together, these observations suggest that anandamide acts through CB1 rather than TRPV1 at the spinal level to modulate SIA.

The foot shock-induced increases in 2-AG levels were smaller than those observed previously in the dorsal midbrain of the same subjects (Hohmann et al., 2005). This observation is consistent with the inability of spinally administered rimonabant to block nonopioid SIA. By contrast, a tenfold lower dose of rimonabant produced a robust suppression of SIA when microinjected into the dorsolateral PAG. Our results collectively suggest that supraspinal sites of action play a pivotal role in endocannabinoid-mediated SIA.

Our data suggest that spinal inhibition of MGL prolongs the duration of action of 2-AG, thereby enhancing endocannabinoid tone at spinal CB1 receptors to magnify SIA. This enhancement occurred in the absence of reliable changes in spinal anandamide levels. Thus, the antinociceptive effects of MGL inhibitors are not dependent upon concurrent elevations in anandamide that were induced in the midbrain PAG following exposure to the same stressor (Hohmann et al., 2005).

Spinal inhibition of FAAH also potentiated SIA via a CB1-dependent mechanism. However, foot shock did not reliably increase spinal anandamide levels. Our failure to observe enhancements in anandamide accumulation may reflect lower absolute levels of anandamide relative to 2-AG and consequently higher variability in these measurements. Our data are consistent with the ability of FAAH inhibitors to selectively enhance accumulation of anandamide, but not 2-AG (Cravatt et al., 2001; Lichtman et al., 2002; Kathuria et al., 2003; Fegley et al., 2005). FAAH may regulate both the distance endocannabinoids diffuse to engage CB1 receptors and the duration of endocannabinoid actions (Egertová et al., 2003) to increase accumulation of tonically released anandamide. It is also possible that the extent of on-demand anandamide synthesis may be underestimated in the present work due to the rapid metabolism of this lipid by FAAH. Mapping the distribution of MGL, FAAH and CB1 in the spinal cord could further elucidate the anatomical and functional relationship between cells that degrade 2-AG and those expressing CB1.

Cannabinoid receptor agonists such as Δ9-tetrahydrocannabinol (THC) have limited therapeutic applications at present, mainly because of their undesirable psychoactive effects. However, pharmacological agents that protect endocannabinoids such as 2-AG and anandamide from inactivation may lead to a more circumscribed spectrum of physiological responses than those produced by direct cannabinoid agonists. Ideally, this strategy would enhance endocannabinoid activity only at sites with on-going biosynthesis and release, thereby averting undesirable side effects. The possible use of drugs that inhibit endocannabinoid hydrolysis to treat pain in humans has thus propagated both hope and concern (Cravatt and Lichtman, 2003). FAAH is widely distributed throughout the body (Deutsch and Chin, 1993; Cravatt et al., 1996; Matsuda et al., 1997; Watanabe et al., 1998; Maccarrone et al., 2000) and implicated in the metabolism of a variety of anandamide analogues (Desarnaud et al., 1995; Cravatt et al., 1996; Bisogno et al., 1997; Lang et al., 1999a,b; Tiger et al., 2000). Our data demonstrate that local enhancements of endocannabinoid actions at the spinal level are sufficient to potentiate SIA. Additional experiments will be necessary to determine whether inhibitors of endocannabinoid degradation may find therapeutic applications in the treatment of pain and stress-related disorders.

Acknowledgments

The authors would like to thank Nathan Bolton for technical assistance. This work was Supported by DA14022, DA14265 (to A.G.H.) and DA12447, DA3412 (to D.P.).

References


