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Selective Antiepileptic Effects of *N*-Palmitoylethanolamide, a Putative Endocannabinoid

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Summary: *Purpose:* The purpose of this study was to determine whether *N*-palmitoylethanolamide (PEA), a putative endocannabinoid, would be effective against kindled amygdaloid seizures. For a comparison with earlier work, we also tested the effectiveness of PEA against pentylenetetrazol (PTZ)-induced convulsions.

Methods: Kindling electrodes were implanted bilaterally in the amygdala in 32 Long-Evans rats. After the kindling of generalized (stage 5) seizures, the effects of PEA administration [i.p.; 1, 10, 100 mg/kg in dimethylsulfoxide (DMSO)] were evaluated for anticonvulsant activity. PEA (40 mg/kg, i.p. in DMSO) also was tested for anticonvulsant activity against PTZ-induced convulsions (75 mg/kg, i.p.).

Results: After i.p. administration of PEA, kindled rats displayed an increased latency to clonus at the 1-mg/kg dose. No other dose-dependent effects were noted. When tested against PTZ-induced convulsions, PEA protected against tonic convulsions and prolonged the latency between convulsive episodes.

Conclusions: PEA produces antiepileptic effects, but does not completely suppress seizures. The mechanism of action of PEA remains to be defined. **Key Words:** Kindling—Pentylenetetrazol—Amygdala—Rat.

Considerable evidence in the older literature indicates that the cannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD) can affect convulsions in various preparations (1). For example, McCaughran et al. (2) determined that the main psychoactive compounds found in the cannabis plant, $\triangle 8$ and $\triangle 9$ THC, produce dose-related protection against tonic extension produced by maximal electroshock (MES), as well as suppressing kindled amygdaloid seizures, although only at toxic doses (3). Furthermore, Karler et al. (4) found that CBD also is protective against MES. However, interpretation of these findings was problematic when so little was known about the mechanisms of cannabinoid actions in the brain.

It is now known that endogenous cannabinoids exist in the brain, the action of which are largely neuromodulatory and whose effects are produced at cannabinoid receptors (5). The CB1 receptor is a G protein–coupled receptor found mostly on inhibitory interneurons that are thought to regulate neuronal excitability in the hippocampus and other structures. Thus endogenous cannabinoids such as anandamide and 2-arachidonylglycerol (2-AG), which act on CB1 receptors on hippocampal γ-aminobutyric acid (GABA) ergic neurons (6), therefore may modulate normal and pathologic neural transmission (e.g., 7). Recently, several cannabimimetic compounds have been evaluated for effects on seizures. For example, Wallace et al. (8) determined that THC and the CB1 agonist WIN55,212 are anticonvulsant in the rat pilocarpine model of epilepsy, likely via a CB1 receptor–dependent mechanism that serves to decrease hyperexcitability. Wallace et al. (9) also demonstrated that these compounds suppress tonic hindlimb extension induced by MES. Note, however, that Clement et al. (10) found that anandamide exacerbates convulsions in mice lacking the enzyme fatty acid amide hydrolase, suggesting that cannabinoids can in some circumstances act as proconvulsants.

In contrast to the CB1 receptor, CB2 receptors are found primarily in peripheral tissues and play a role in various analgesic and antiinflammatory responses (11). The compound *N*-palmitoylethanolamide (PEA) was identified by Facci et al. (12) as an endogenous ligand for the CB2 receptor and accumulates in conditions involving degenerative changes to tissues, such as in glutamate-mediated neurotoxicity (13). However, other reports have questioned whether PEA acts at the CB2 receptor (e.g., 14). Skaper et al. (14) reported that, in addition to analgesic and antiinflammatory effects, PEA is effective in protecting cerebellar granule cells from glutamate excitotoxicity. Lambert

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et al. (15) examined the effects of PEA on chemical and electroshock-induced convulsions in mice and found dose-dependent protection against MES at nontoxic doses. They also found that PEA was effective against tonic but not clonic convulsions induced by pentylenetetrazol (PTZ), an antagonist of $GABA_A$ receptors.

In light of the description of the anticonvulsant properties of PEA by Lambert et al., we examined whether antiepileptic effects might be observed against kindled amygdaloid seizures in rats. For comparison, we examined the effects of PEA on PTZ-induced seizures in rats.

METHODS

Animals

Forty-two male Long-Evans rats (Charles River, St. Constant, Quebec, Canada), weighing 275 to 375 g at the time of surgery, were used. Food and water were freely available, and experiments were performed during the light portion of the 12-h light/dark cycle. All procedures were conducted in strict accordance with the guidelines established by the Canadian Council on Animal Care, as approved by the University of Saskatchewan Animal Care Committee.

The effect of PEA on kindled seizures

Surgery

After at least 7 days of daily handling and habituation to housing conditions, the rats were anesthetized with isoflurane (5% initial; 2% maintenance), placed in a stereotaxic instrument, and given a subcutaneous injection of ketoprofen (Anafen; 10 mg/kg) as a preemptive analgesic. All rats received stereotaxic implantation of two kindling electrodes bilaterally into the basolateral nucleus of the amygdala (AM; 2.6 mm posterior to bregma; 4.5 mm lateral to the midline; 9.1 mm ventral from dura). A reference wire and four additional dental screws were anchored to the skull and affixed with dental acrylic.

Initial kindling

One week after surgery, afterdischarge thresholds (ADTs) were determined at one of the two kindling electrodes. Electrical stimulation consisted of a 1-s train of balanced biphasic square-wave pulses (1 ms duration; 60 pps) delivered to the AM at an initial current of 20 μA (base to peak) and increased in increments of 10 μA at 1-min intervals (alternating between left and right AM) until an afterdischarge (AD) was evoked. ADT was arbitrarily defined as the minimum stimulation intensity sufficient to trigger an AD that outlasted the stimulation by $\geq$5 s. Twenty-four hours later, once-daily kindling sessions began, with stimulation applied at 100 μA above the ADT. The duration of AD was measured, and behavioral seizures were classified according to Racine's scale (16). Daily stimulation continued until three consecutive generalized stage 5 seizures were evoked.

PEA administration

On completion of initial kindling, rats received PEA (Tocris, Ellisville, MO, U.S.A.) or dimethylsulfoxide (DMSO) vehicle control (n = 10). PEA was dissolved in pure DMSO (1 ml/kg; Sigma-Aldrich) and injected i.p. at 1 (n = 10), 10 (n = 8), or 100 (n = 4) mg/kg 2 h before the kindling session. Syringes were kept in a 50°C water bath just before injection to prevent precipitate from forming.

Histologic examination and data analysis

At the completion of kindling, rats were killed with CO_2, and brains were removed and fixed in 10% formalin for $\geq$1 week before sectioning. Frozen coronal sections 40 μm thick were taken from the regions of the electrode tracks and stained with cresyl violet. Analysis of variance (ANOVA) was applied to the difference scores between predrug and postdrug trials, and PTZ seizure data (see later) were analyzed with *t* tests or Fisher's Exact test, where appropriate. A level of $p < 0.05$ was considered significant. Values are given as mean $\pm$ SD.

The effect of PEA on PTZ-induced seizures

We attempted to approximate in rats the dose of PEA that is effective in suppressing PTZ-induced convulsions in mice (14). Because the first dose that we tested, 40 mg/kg, was effective in rats, no further doses were tested. Five naive rats received 40 mg/kg of PEA (prepared as described earlier) 2 h before an i.p. injection of PTZ at a dose of 75 mg/kg. Another group of five rats received an i.p. injection of pure DMSO (1 ml/kg). For 45 min after the PTZ injection, the incidence of convulsions and latency to the onset of the first and subsequent clonic or tonic seizure was measured. The latency was defined as the first occurrence of clonus or tonic seizure, respectively, where clonic seizures were characterized by rhythmic jerking or wild running, and tonic seizures, by continuous flexion or extension of muscles. Each rat was used only once and then was killed with CO_2.

RESULTS

Kindling

No obvious behavioral changes were associated with i.p. injections of PEA before, during, and after triggering of kindled seizures. PEA produced no protection from stage 5 seizures. In all cases, rats responded to kindling stimulation with a generalized limbic-type seizure after PEA administration. PEA did, however, produce some specific changes in aspects of the kindled seizures. One-way ANOVA indicated that a significant difference existed in the latency to clonus between the final stage 5 seizure before PEA administration (control trial) and the initial seizure under drug administration (drug trial; $F_{3,28} = 5.172$; $p = 0.006$; Table 1). Post hoc analysis indicated that 1 mg/kg PEA significantly delayed the onset of

TABLE 1. *Latency to clonus*

	DMSO	1 mg/kg	10 mg/kg	100 mg/kg
Control trial	5.80 ± 2.36 s	7.90 ± 2.62 s	5.50 ± 1.24 s	3.50 ± 1.50 s
Drug trial	6.70 ± 2.59 s	15.1 ± 3.07 s[a]	6.13 ± 1.65 s	4.25 ± 0.63 s

Data represent time in seconds to reach clonus in the last stage 5 seizure before drug administration (control trial) and the first stage 5 seizure after PEA administration (drug trial). All values are expressed as mean ± SEM.
[a] $p < 0.05$.

clonus (latency, 15.1 ± 9.72 s) when compared with the final stage 5 seizure before PEA administration (7.90 ± 8.31 s; mean difference, 7.20 ± 5.98 s). No significant differences were seen in the 10-mg/kg and 100-mg/kg PEA groups or in DMSO-treated controls (Fig. 1A).

One-way ANOVA indicated that the differences in the duration of clonus were not statistically significant ($F_{3,28} = 0.113$; $p = 0.952$), as measured by the mean difference score of the duration of clonus between the last stage 5 seizure before drug administration and the first seizure after drug administration (Fig. 1B), within each drug group. Data are shown in Table 2. As shown in Fig. 1C, no significant differences in AD duration were found under the various doses of PEA ($F_{3,28} = 0.763$; $p = 0.524$; see Table 3). Thus the mean difference scores of duration of AD of DMSO-treated controls (drug trial, 61.8 ± 25.2 s; control trial, 49.1 ± 24.2 s) did not differ from those of rats treated with PEA at 1 mg/kg (drug trial, 64.2 ± 20.9 s; control trial 48.1 ± 26.9 s), 10 mg/kg (drug trial, 51.4 ± 33.4 s; control trial, 47.4 ± 27.2 s), or 100 mg/kg (drug trial, 30.5 ± 7.04 s; control trial, 25.3 ± 3.30 s).

PTZ-seizures

At the dose tested, PEA suppressed tonic but not clonic convulsions induced by PTZ. Five of five DMSO-treated rats exhibited characteristic tonic convulsions involving body torsion or extension of the extremities, whereas none of the five PEA-treated rats exhibited tonic convulsions ($p < 0.001$, Fisher's Exact test). Instead, the rats treated with PEA progressed directly to clonic seizures without any evidence of tonic episodes. Furthermore, most rats displayed at least two convulsive episodes in 45 min, except for two PEA rats that displayed only one convulsion. PEA- and DMSO-treated rats did not differ in the latency to the first convulsion (DMSO, 118.6 ± 66.8 s; PEA, 155.6 ± 94.9 s; $t(8) = 0.713$; $p = 0.248$) or in the duration of the first convulsion (DMSO, 28.2 ± 11.2 s; PEA, 21.6 ± 11.4 s; $t(8) = -0.923$; $p = 0.167$). However, a significant difference was seen in the latency to the second convulsion. PEA-treated rats displayed a significantly longer latency to the second convulsion (1,456.2 ± 954.4 s) than did DMSO-treated controls (485.8 ± 97.9 s; $t(8) = 2.262$; $p = 0.027$). However, the duration of the second convulsion did not differ between the PEA-treated rats and DMSO-treated controls (DMSO, 45.2 ± 23.4 s; PEA, 30.3 ± 13.6 s; $t(6) = -0.986$; $p = 0.362$).

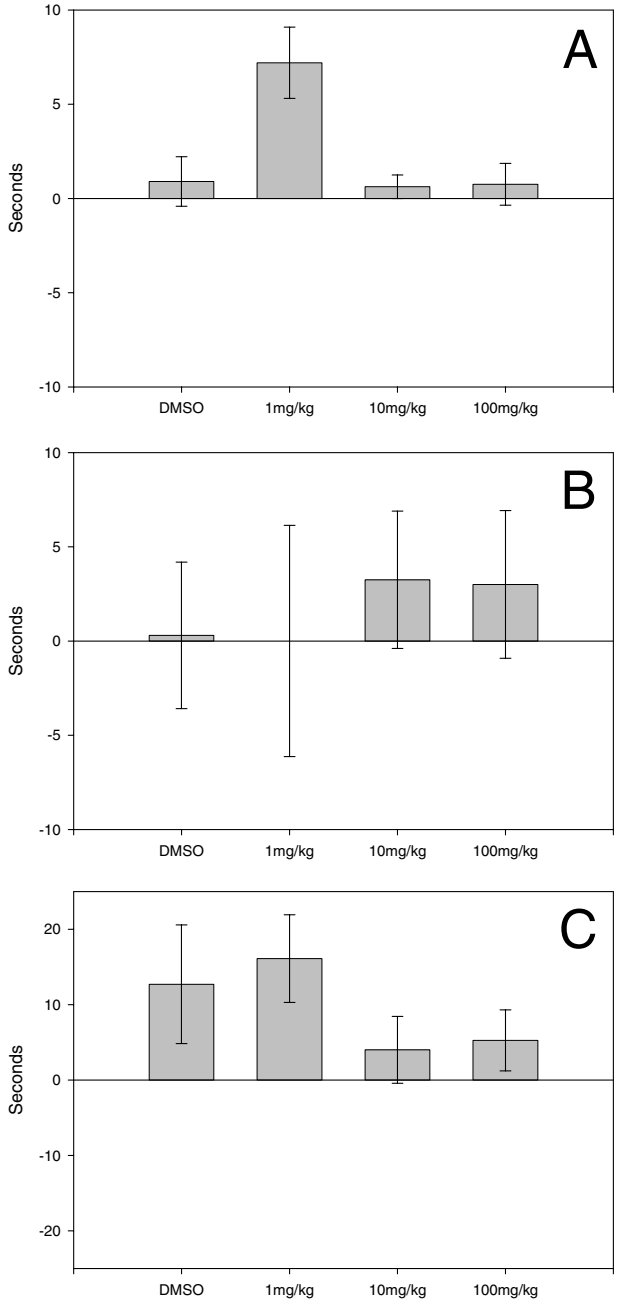

FIG. 1. A: Latency in seconds to clonus. **B:** Duration in seconds of clonus. **C:** Duration of afterdischarge (AD), in seconds. Grey bars represent the mean difference (increase or decrease) between the control and drug trials within each drug group. Error bars represent mean ± SEM.

DISCUSSION

The present experiment confirms in rats the results of a previous report that PEA suppresses the tonic component of PTZ-induced convulsions in mice (15). However, we

TABLE 2. *Duration of clonus*

	DMSO	1 mg/kg	10 mg/kg	100 mg/kg
Control trial	36.0 ± 3.51 s	39.6 ± 8.31 s	30.9 ± 4.38 s	23.3 ± 2.65 s
Drug trial	36.3 ± 2.59 s	39.6 ± 5.56 s	34.1 ± 5.14 s	26.3 ± 4.13 s

Data represent duration of clonus in seconds for the last stage 5 seizure before drug administration (control trial) and the first stage 5 seizure after PEA administration (drug trial). All values are expressed as mean ± SEM.

found that a range of doses of PEA showed no antiepileptic effects against kindled amygdaloid seizures, except for an increased latency to clonus in rats treated with 1 mg/kg of PEA.

Effect of PEA on PTZ-induced convulsions

Contrary to earlier characterization of CB2-receptor expression in the brain (e.g., 13), various reports demonstrated that CB2 receptors are found only outside the central nervous system (e.g., 5). If PEA were acting at the CB2 receptor, the effect of PEA on PTZ-induced convulsions might be mediated by interactions in the periphery, and in particular with immune tissue. Immune mast cells implicated in various inflammatory reactions express the CB2 receptor type that recognizes PEA (12) and may act to decrease glutamatergic activity after excessive excitatory stimulation (13). The mechanism by which this may occur has yet to be resolved, but it is proposed to involve G protein modulation of cellular events. However, it remains unclear to us exactly how the putative peripheral effects of PEA would suppress tonic convulsions. One possibility that has not been considered is that PEA affects distribution of PTZ to the brain; this perhaps warrants investigation.

An alternative explanation is based on the suggestion that PEA may not act as a CB2 agonist, as shown at least in human recombinant (17) and rat spleen preparations (18). One possible site of action involves vanilloid receptors (VRs), on which anandamide acts as a full agonist (19), and PEA, as a "modest partial agonist" (20). Recent studies suggested that PEA may have entourage-like effects at VR1 receptors (21), inhibiting the hydrolysis of anandamide and resulting in enhanced intracellular stimulation of VR1 receptors by anandamide (22). VR1 has been located in the brain (23), and the functional consequences of VR1 activation involve a nonselective cation influx, Ca^{2+} influx, depolarization of membrane, and glutamate release (24), none of which is consistent with antiepileptic properties. However, VR1 agonists readily desensitize this complex (25), resulting in decreased influx of Ca^{2+} and reduction of glutamate release; this could underlie VR1-mediated antiepileptic effects. Although our data do not directly address these ideas, the possibility that PEA is acting on VR1 receptors is attractive, and comprehensive examination of VR1 involvement in seizures is needed.

TABLE 3. *Duration of afterdischarge*

	DMSO	1 mg/kg	10 mg/kg	100 mg/kg
Control trial	49.1 ± 7.65 s	48.1 ± 8.51 s	47.4 ± 9.63 s	25.3 ± 1.65 s
Drug trial	61.8 ± 7.97 s	64.2 ± 6.60 s	51.4 ± 11.8 s	30.5 ± 3.52 s

Data represent duration of afterdischarge (AD) in seconds in the last stage 5 seizure before drug administration (control trial) and the first stage 5 seizure after PEA administration (drug trial). All values are expressed as mean ± SEM.

DMSO, dimethylsulfoxide.

Effect of PEA on kindled amygdaloid seizures

We observed increased latency to clonus in rats treated with 1 mg/kg PEA. This effect could be produced by several candidate mechanisms, including an action of PEA on the VR. As an alternative to the involvement of the VRs, PEA might modulate the increase in endocannabinoids, such as anadamide or 2-arachydonylglycerol, induced by depolarization or activity (26, 27), including seizure activity (28). Our data do not offer a basis for discriminating between these potential mechanisms.

Specificity in the antiepileptic effects of PEA might be indicated by the differential effects of PEA we observed in the two experimental paradigms: suppression of tonic episodes of PTZ-induced convulsions but no effect on clonic convulsions, and delay to clonus in kindled amygdaloid seizures. At least two potential explanations may elucidate these different effects: First, the molecular substrates of tonic and clonic seizures and their sensitivity to the effects of endocannabinoids may differ. Second, separate anatomic substrates of tonic and clonic seizures may be involved. PTZ-induced tonic convulsions are thought to mimic primary generalized seizures by activation of the reticular core (e.g., 29), whereas limbic kindling involves primary triggering of AD in the forebrain followed by secondary generalization to structures responsible for clonus, some of which also are located in the forebrain (e.g., 30). PEA may suppress primary generalized tonic seizures induced by PTZ, but not secondarily generalized clonic seizures triggered in the forebrain, by differentially influencing receptor expression or the availability of endogenous cannabinoids that specifically suppress ictal activity in the reticular formation or other brainstem structures.

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