

## **Endocannabinoids control spasticity in a multiple sclerosis model**

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### **ABSTRACT**

Spasticity is a complicating sign in multiple sclerosis that also develops in a model of chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice. In areas associated with nerve damage, increased levels of the endocannabinoids, anandamide (arachidonylethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG), and of the AEA congener, palmitoylethanolamide (PEA), were detected here, whereas comparable levels of these compounds were found in normal and non-spastic CREAE mice. While exogenously administered endocannabinoids and PEA ameliorate spasticity, selective inhibitors of endocannabinoid re-uptake and hydrolysis—probably through the enhancement of endogenous levels of AEA, and, possibly, 2-arachidonoyl glycerol—significantly ameliorated spasticity to an extent comparable with that observed previously with potent cannabinoid receptor agonists. These studies provide definitive evidence for the tonic control of spasticity by the endocannabinoid system and open new horizons to therapy of multiple sclerosis, and other neuromuscular diseases, based on agents modulating endocannabinoid levels and action, which exhibit little psychotropic activity.

Key words: autoimmune encephalomyelitis • multiple sclerosis • anandamide • 2-arachidonoyl-glycerol • palmitoylethanolamide • cannabinoid

**M**ultiple sclerosis (MS) is a chronic, demyelinating disease of the central nervous system (CNS), where individuals accumulate neurological damage and paresis. In addition, troublesome signs often develop, such as pain, spasticity, and tremor, which are difficult to treat. This condition has prompted some patients to find alternative medicines and to perceive benefit from cannabis use (1). The effects of cannabis and cannabinoids are mediated through cannabinoid receptors (CB) of the type 1 (CB<sub>1</sub>), expressed mainly in the CNS but also in peripheral tissues, and type 2 (CB<sub>2</sub>), expressed almost uniquely in immune cells (2). Recently, we have demonstrated that exogenous agonists of CB receptors, in particular CB<sub>1</sub>, can inhibit spasticity in the Biozzi ABH mouse chronic relapsing experimental allergic encephalomyelitis (CREAE) model of MS (3). This autoimmune demyelinating disease is induced actively by sensitization to CNS myelin (4). Systemic agonists will not discriminate between CB receptors located in centers associated with control of pain, motor, or cognitive functions and therefore may induce unwanted psychoactive effects that will limit medical alleviation of pain/motor deficits. More important to note, we demonstrated that CB antagonism transiently exacerbated spasticity (3). Whilst inverse agonism of antagonists (2) may explain the latter observation, alternatively this finding suggested that endogenous CB ligands are limiting the spasticity that occurs during CREAE. This finding would predict that spasticity would be associated with changes in the equilibrium of the endocannabinoid system (5), which could be manipulated for therapeutic benefit by using inhibitors of endocannabinoid degradation. This study demonstrates that the endocannabinoid system exhibits tonic control of spasticity in an MS-like condition.

## **MATERIALS AND METHODS**

### **CREAE induction**

Biozzi ABH mice were from stock bred at the Institute of Ophthalmology, UCL London, or were purchased from Harlan Olac (Bicester, U.K.). CREAE was induced following subcutaneous injection of 1 mg of syngeneic spinal cord homogenate emulsified in Freund's complete adjuvant (Difco, Poole, U.K.) on day 0 and day 7 as described previously (4). Animals developed a relapsing-remitting disease progression and between 60–80 days post-inoculation developed evidence of spasticity (incidence 50%–60%) (3). Similarly treated CREAE animals that had not yet demonstrated tremor, hind-limb, or tail spasticity were used as non-spastic controls.

### **Assessment of endocannabinoid levels**

The spinal cords were expelled rapidly from the cervical spinal column by using hydrostatic pressure applied through a 19-gauge needle inserted into the lumbar column via a phosphate-buffered saline-filled syringe. Brains were dissected from the cranium. All tissues were frozen in liquid N<sub>2</sub> within 60 s from death (6). AEA, PEA, and 2-AG levels in lipid extracts from mouse brain and spinal cord were assessed by using isotope-dilution gas chromatography/mass spectroscopy essentially as described previously (7, 8). Results were compared by one-way analysis of variance (ANOVA), incorporating a Bonferroni *t*-test. Statistical analysis was performed by using SigmaStat V2 software (SPSS Inc., Chicago, Ill.).

### **Assessment and Modulation of Spasticity**

Quinpirole, rolipram, and *R*(+)WIN-55,212 were purchased from RBI/Sigma (Poole, U.K.). Anandamide, PEA and 2-AG were purchased from Cayman chemical (Ann Harbor Mich.). AM404 (9), AM374 (10), and VDM11 (11) were synthesized as previously described. The CB receptor antagonists SR141617A and SR144465 (2) were supplied by Sanofi Research (Montpellier, France). Ethanolic solutions were evaporated under vacuum and dissolved in PBS:tween 80 (Sigma.) (3) to be administered as a single intraperitoneal or intravenous tail injection. The resistance to flexion of individual hind limbs was measured against a strain gauge (5–8 readings per time point) as described previously (3). The results were expressed as a mean  $\pm$  SEM per group, and the data were analyzed by using repeated measures, ANOVA incorporating a pair-wise Tukey post-hoc test.

## RESULTS AND DISCUSSION

### Endocannabinoid levels in spastic mice

At baseline in normal ABH mice ([Fig. 1](#)), whole brains and spinal cords contained similar levels of the endocannabinoids arachidonylethanolamide (AEA/anandamide, ~29–33 pmol/g) and 2-arachidonoyl glycerol (2-AG, ~5–7 nmol/g) and the non-CB receptor binding, cannabimimetic metabolite (2) palmitoylethanolamide (PEA, ~220–240 pmol/g), as found previously in rat CNS tissue (7, 8). These levels were not changed significantly in non-spastic CREAE remission animals ([Fig. 1](#)), despite the fact that these animals had experienced 2–3 paralytic episodes and would contain demyelinated lesions and axonal loss in the spinal cord (4). In comparison with normal animals, however, endocannabinoids were present in significantly ( $P < 0.05$ ) elevated amounts in the brain of spastic mice ([Fig. 1](#)). Although brain levels were relatively unchanged for AEA compared ( $P > 0.05$ ) with non-spastic mice, there was a modest increase of AEA ( $P < 0.05$ ) in spastic brains compared with levels in normal brains. However, there was a marked increase (~200%) of AEA ( $P < 0.01$ ) and PEA ( $P < 0.05$ ) within the spinal cord of spastic mice ([Fig. 1](#)). This site is one in which major pathological change occurs during CREAE in ABH mice (4, 6). The brain during CREAE is relatively unaffected, but animals do develop lesions within the cerebellum (4), an area implicated in the control of tone. Because of the low levels of AEA (picomoles/gram), subtle changes in the amounts of this metabolite may not be detected as readily in whole brain, as opposed to that in isolated brain regions (8). However, 2-AG is found at 200–800 times higher levels than AEA and here an increase (~70%) in 2-AG levels was found readily in brain and spinal cords ( $P < 0.05$ ) of spastic animals compared with non-spastic controls ([Fig. 1](#)). Although this finding may suggest that 2-AG could serve as a more-easily detectable indicator of endocannabinoid activity during spastic disease, 2-AG has not been detected in micro-dialysates from rat striatum (12) or in human cerebrospinal fluid (CSF) (13), despite its relative abundance in CNS tissues (5, 7, 8). This condition is due possibly to tissue compartmentalization of 2-AG (13), to its very limited release from neuronal cells (14) or to its rapid esterification into phospholipid membranes and hydrolysis (15, 16). This study indicates that the endocannabinoids are up-regulated locally in areas of CREAE-induced damage. Therefore, clinical parameters; that is, spasticity versus non-spasticity and lesion load/topography, need to be considered when samples from MS patients are examined for human confirmation of these animal data. As post-mortem artifactual increases of endocannabinoids may mask changes in human CNS tissue (17), studies in humans will rely on examination of the CSF. Further, studies are also warranted to evaluate whether the levels of functional receptors for

endocannabinoids [to date, AEA has been found to bind to the two CB receptors, as well as to vanilloid receptors] (2, 18, 19) are likewise changed from the non-spastic condition to compensate for the effects of the disease.

### **Endocannabinoids inhibit spasticity**

The lack of changes of endocannabinoid levels between normal and non-spastic mice and elevations of endocannabinoids in spastic mice may provide an explanation to previous observations obtained by using CB receptor antagonists (3). In this previous study, it was shown that exogenously administered SR141617A and SR144465, two antagonists selective for CB<sub>1</sub> and CB<sub>2</sub> receptors, failed to affect resistance to flexion of hind limbs (muscle tone) in normal and non-spastic CREAE mice, in contrast to spastic animals, which showed a transient elevation in resistance to flexion of the hind limbs with both antagonists (3). On the basis of the results described above, these observations can now be explained by hypothesizing that the two antagonists block the action of disease-limiting endocannabinoids, whose levels are elevated during spastic disease. Although it is speculated that endocannabinoid levels may increase in an attempt to compensate for the spastic defect, it is possible that endocannabinoids, or other unrelated fatty acid amides, could be elevated transiently as a mere consequence of spasticity, such as increased motor activity (12, 20) or tissue damage (5), rather than exerting a compensatory effect on this sign. While exogenous administration of methanandamide (AM356), an enzymatically stable AEA analog (21), can limit experimental spasticity (3), to suggest a cause-and-effect relationship between endocannabinoids and inhibition of spasticity, the effect of exogenous AEA, 2-AG and PEA was investigated here. All three substances had the capacity to significantly ( $P < 0.01$ ) ameliorate spasticity, although they had differing efficacy profiles (Fig. 2). Whilst AEA and PEA maximally inhibited spasticity within 10–30 min, exogenous 2-AG induced inhibition with a relatively slower onset. (10 mg/kg i.v. Fig. 2, and 1 mg/kg i.v.  $n = 13$  limbs, data not shown). This finding was somewhat surprising because endocannabinoids are susceptible to rapid inactivation through enzymatic hydrolysis (5, 15). Although different cannabinoids have different pharmacokinetics (3), this observation may also suggest that 2-AG is not directly mediating the inhibition, and that this condition could result in part from the actions of other bioactive metabolite(s). For example, 2-AG may also act by slowly inhibiting the degradation of endogenous AEA, thereby increasing its levels (15, see below). Alternatively, 2-AG activates CB<sub>2</sub> receptors more efficaciously than AEA, and this different mechanism of action may also explain the different profile of spasticity inhibition observed here for the two endocannabinoids. As for PEA, this endogenous compound does not exhibit CB<sub>1</sub> or CB<sub>2</sub> agonist activity (2) but may also be capable of enhancing endocannabinoid actions, as shown in suppression of signs of hyperalgesia (22) and inhibition of breast cancer cell proliferation (23), through not fully understood effects (23). Similar to AEA, the levels of this metabolite were found here to be raised in CREAE mice spinal cord (Fig. 1) and to transiently ameliorate spasticity (3) (Fig. 2). Thus, PEA appears to be linked somewhere to the endocannabinoid system, even though it is not an endocannabinoid itself.

### **Inhibition of endocannabinoid degradation in the control of spasticity**

Having established that the endocannabinoids are up-regulated during spastic CREAE, a causal relationship to the anti-spastic effect was sought through manipulation of endocannabinoid levels

and action *in vivo* (Fig. 2). AEA was the first endocannabinoid described, and its biosynthetic/degradation pathways have been best described (5, 24). Because exogenous applied naturally occurring cannabimimetic metabolites—in particular AEA—can limit spasticity (Fig. 2), then augmenting the levels of endogenous AEA might have a therapeutic effect. One route would be to induce its synthesis. AEA has been reported to regulate dopamine-mediated locomotor activity, whereas D<sub>2</sub> receptor agonism with quinpirole induced the release of AEA and augmented the dopamine-inhibiting effect of exogenous AEA in mice (12). However, no amelioration of spasticity was evident following administration of quinpirole alone (1 mg/kg i.v.) (Fig. 3) and 10 mg/kg i.p. (data not shown, n = 8 limbs). This finding suggests that such stimulation either fails to induce sufficient AEA to control spasticity or that AEA production was altered in an anatomical site away from the spastic lesion (20).

Blockade of degradation with specific inhibitors may serve as an alternative means of increasing the bio-availability of the endocannabinoids. Furthermore, this strategy would provide some selectivity, as these inhibitors would particularly target areas where elevated levels of the endocannabinoids are being produced and utilized. AEA, and possibly 2-AG, are removed actively from the extracellular space into the cytosol through specific mechanisms (9, 15) and undergo breakdown by fatty acid amide hydrolase (FAAH) (5, 15, 24, 25). We found that spasticity could be ameliorated by injection (10 mg/kg i.v.) of either the competitive re-uptake inhibitor AM404 (9) or the selective FAAH inhibitor, AM374 (10), both of which have been shown to enhance AEA neuromodulatory actions (9, 26) (Fig. 3A). No additive effect was evident by using a combination of both reagents (n = 18, data not shown). These compounds have very low affinity for cannabinoid receptors (9, 10) and have never been shown to behave as CB agonists (27). In fact, there was no evidence for cannabimimetic effects (hypothermia) of AM404 or AM374 at the doses used here *in vivo* (data not shown). Furthermore, significant (P<0.001) anti-spasticity effects were also evident by using doses of AM404 (2.5 mg/kg) and AM374 (1 mg/kg) likely to be sub-threshold for CB<sub>1</sub> agonist control of spasticity (Fig. 3B) (3). These inhibitory effects had a rapid onset before a slow return of CREAE signs over the next few hours (Fig. 2) and were comparable with those obtained following effective CB receptor agonism (3). Both AEA and AM404 may also behave as vanilloid receptor (VR1) agonists (18, 19), and other VR1 agonists have been found to reduce bladder hyper-reactivity in MS (28). Whilst the role of VR1, if any, in control of spasticity has yet to be demonstrated, a similar inhibition (P<0.001) of spasticity (Fig. 3B) by the extremely selective anandamide transporter inhibitor VDM11 (10 mg/kg i.v.), which has essentially no CB or VR-1 agonist activity (11), further supports the hypothesis that endocannabinoids mediate control of spasticity via CB receptors.

Although further studies are required to demonstrate that exogenous inhibitors of endocannabinoid re-uptake (VDM11 and AM404) and hydrolysis (AM374) actually cause amelioration of spasticity by enhancing the tissue levels of endocannabinoids, here we gained indirect evidence that at least AM374 is acting via this mechanism. In fact, the anti-spastic effect of AM374 (1 mg/kg i.v.) was blocked by cannabinoid receptor antagonists (SR141716A and SR144465, both 5 mg/kg i.v.) administered 20 min prior to AM374 (Fig. 3B). Under the same conditions, the two antagonists were shown to counteract the anti-spasticity effects of the CB receptor agonist *R*(+)-WIN-55, 212 [5 mg/kg i.p., change in resistance to flexion at 30 min +7.0 ± 18.1% (P>0.05, n = 11 limbs) compared with baseline in antagonist-pretreated animals, as opposed to -35.5 ± 19.7% (P<0.05, n = 16 limbs) in vehicle-pretreated mice receiving *R*(+)-

WIN-55, 212 alone]. These findings suggest that the inhibitory effect on spasticity by AM374, which does not directly activate CB receptors (10), is due to enhancement of endocannabinoid levels and subsequent stimulation of CB receptors.

### **Inhibition of endocannabinoid signaling exacerbates spasticity**

As CB receptor antagonism very transiently exacerbates spasticity (3), we studied the effect on spasticity of inhibiting CB receptor signaling (Fig. 3A). CB receptors are negatively coupled through  $G_{i/o}$  proteins to adenylate cyclase, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine inhibits receptor agonism (29). Rolipram, a selective inhibitor of cAMP-selective phosphodiesterase IV, has anti-inflammatory effects and inhibits the immunological processes that drive the development of EAE (30). However, in agreement with its possible counteraction of endocannabinoid-induced inhibition of cAMP levels, rolipram (10 mg/kg i.v.) induced a transient increase ( $P < 0.001$ ) in limb (Fig. 3A) and tail spasticity ( $n = 15/15$ ,  $P < 0.001$  compared with normal animals  $n = 0/5$  (Fig. 3C–D). Furthermore, limb tremor became evident in some mice ( $n = 6/15$ ). The exacerbation was not evident in normal animals, where rolipram appeared to have a sedative effect and was very transient (Fig. 3A), consistent with that observed previously with CB antagonists (3). This finding again suggests that compensatory mechanisms are activated rapidly after exacerbation of spasticity in CREAE mice and substantiates further the involvement of the endocannabinoid system in the tonic down-regulation of this sign.

### **CONCLUSIONS**

Whilst this study appears to provide strong evidence for a role of endocannabinoids in the control of spasticity, the specific function of each endocannabinoid requires further elucidation and may be assessed through the use of specific inhibitors once these are generated. The equilibrium of the endocannabinoid system appears to be altered significantly during spastic events in CREAE, possibly in response to abnormal neuronal signaling and/or neurodegenerative effects in damaged nerves. However, this phenomenon does not control spasticity as adequately as it may be possible by administering exogenous CB agonists (3)—including drugs based on endocannabinoids that have been reported to have very low potential for physical dependence (31)—or by manipulating endocannabinoid endogenous levels. This manipulation may minimize some of the undesirable psychoactive effects associated with  $CB_1$  agonism and may have implications for symptom control in MS and other neuromuscular disease conditions. Parallels can already be seen in the treatment of depression where serotonin re-uptake inhibitors are clinically preferable to receptor agonists. The finding of increased amounts of endocannabinoids in these damaged tissues may open even wider horizons for therapeutic intervention in MS with little psychotropic side effects.

### **ACKNOWLEDGMENTS**

The authors acknowledge the support of the Multiple Sclerosis Society of Great Britain and Northern Ireland, the Medical Research Council, the Wellcome Trust, the National Institute on Drug Abuse (grant DA09789), the Ministero per l'Università e la Ricerca e Scientifica e Tecnologica (grant 3933), and the Associazione Italiana per la Sclerosi Multipla.

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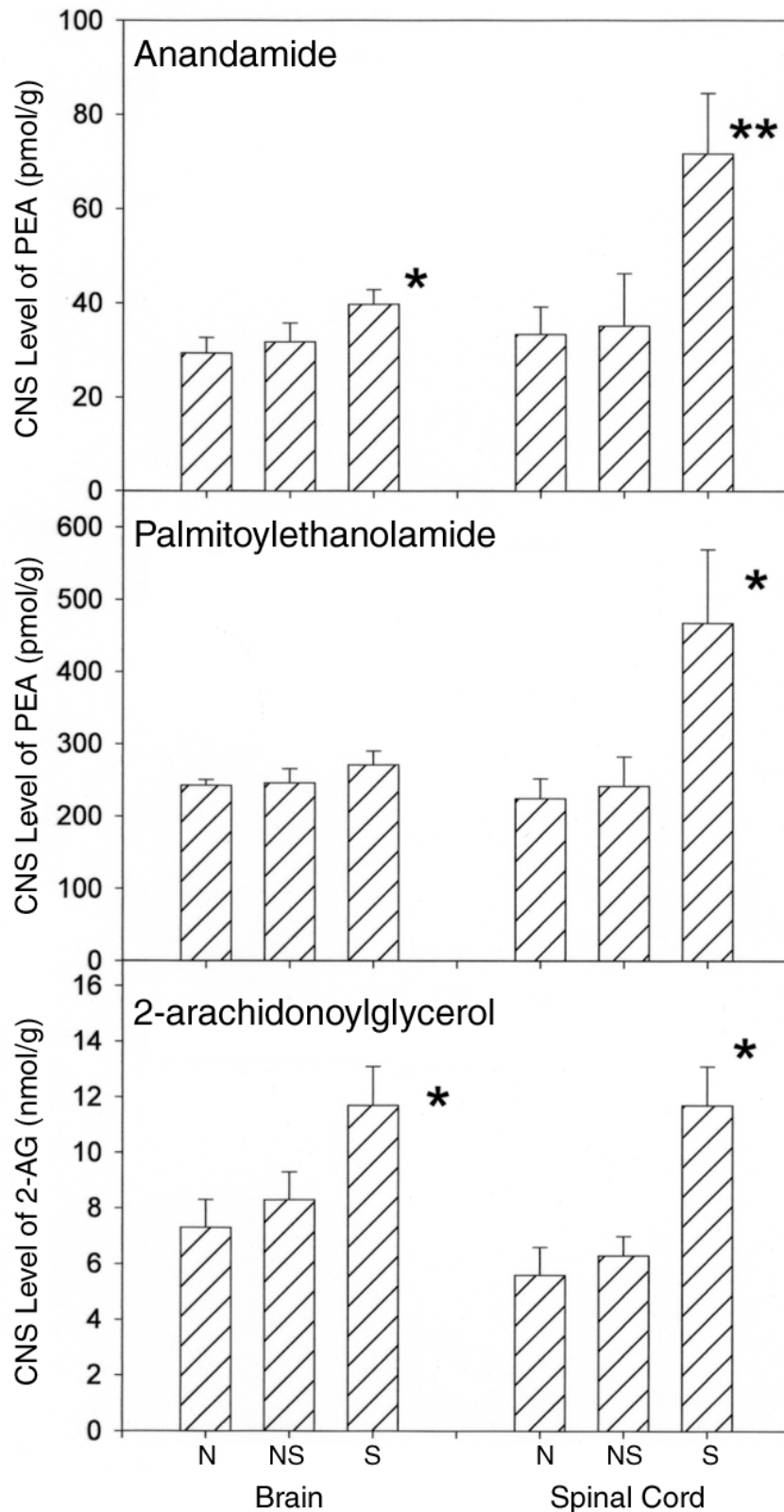
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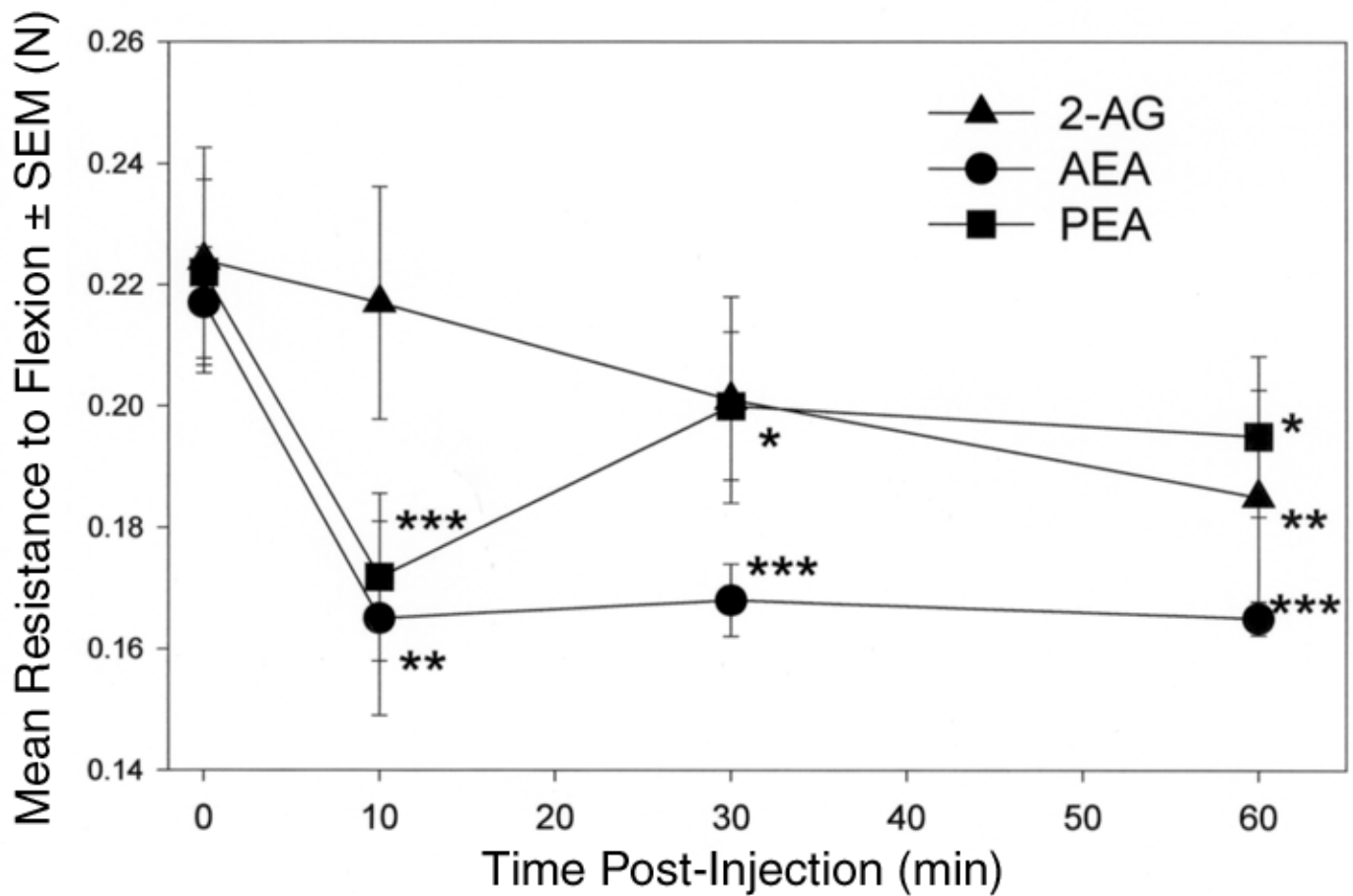
*Received July 19, 2000; revised October 19, 2000.*

Fig. 1



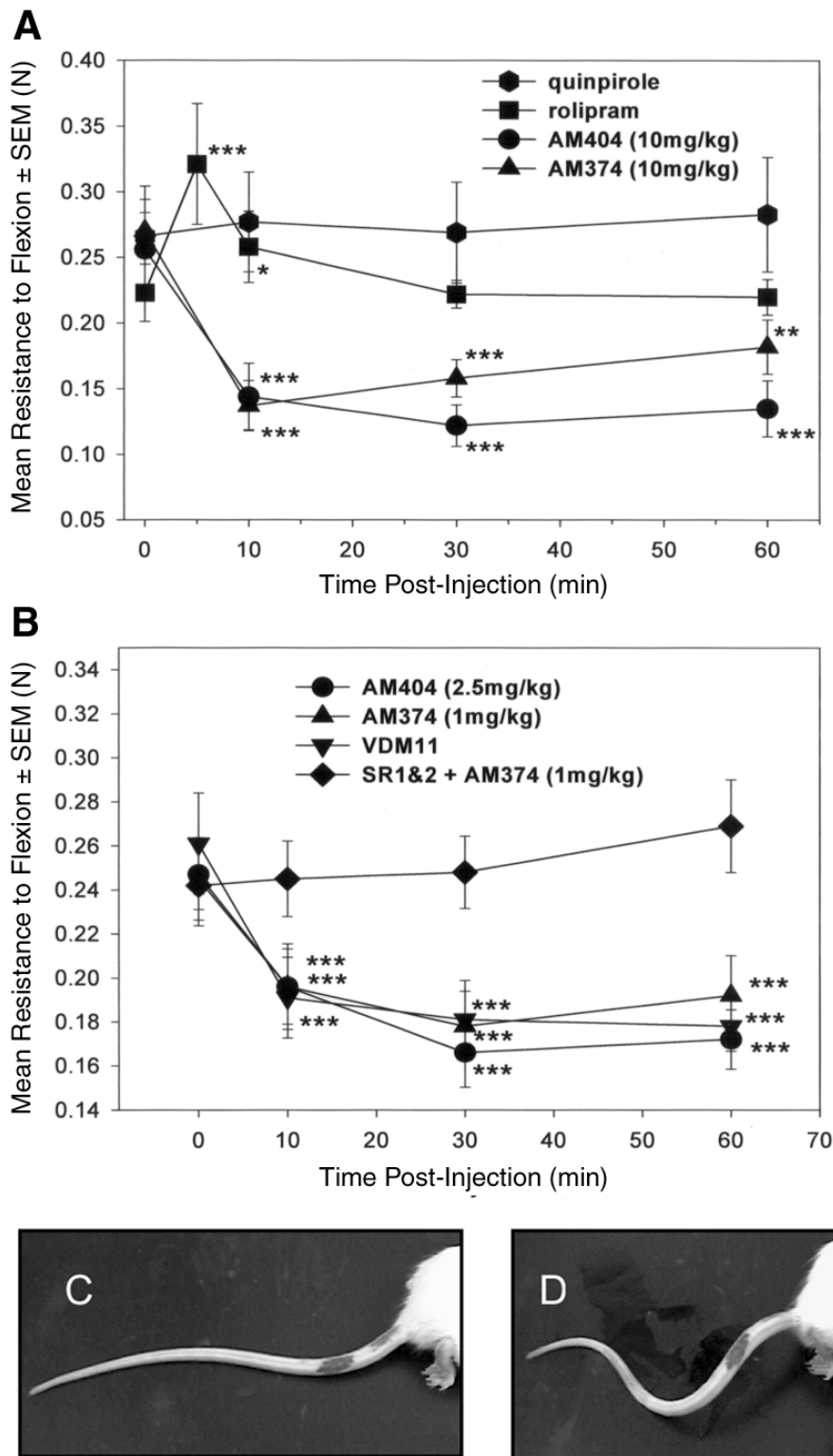
**Figure 1. Endocannabinoid levels increase in spastic mice during CREAE.** Endocannabinoid levels were measured by isotope-dilution gas chromatography/mass spectroscopy from rapidly frozen brains and spinal cords removed from either normal (N) or post-relapse remission animals showing either spasticity (S) or no clinical evidence of spasticity (NS), following the induction of CREAE (1). Results represent mean  $\pm$  SD from 10–16 spinal cords/group (2 spinal cords pooled per sample) and 6–9 brains/group (\* $P < 0.05$  \*\* $P < 0.01$  compared with normal values by ANOVA).

Fig. 2



**Figure 2. Spasticity is limited by endogenous cannabimimetic substances when exogenously administered.** The forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and after i.v. injection with 10 mg/kg anandamide (n = 11 limbs), 2-archidonoyl-glycerol (n = 10 limbs) and as previously reported (3) palmitoylethanolamide (n = 14 limbs) (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$  compared with baseline values by ANOVA.)

**Fig. 3**



**Figure 3. Spasticity is under tonic control of the endocannabinoid system.** The forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>5 mice/group) before and following i.v. injection with: **A**) rolipram (10 mg/kg, n = 12 limbs); quinpirole (1 mg/kg, n = 8 limbs); AM404 (10 mg/kg, n = 8 limbs); or AM374 (10 mg/kg, n=7 limbs); **B**) AM404 (2.5 mg/kg, n = 10 limbs); VDM11 (10 mg/kg, n = 11 limbs); AM374 (1 mg/kg, n = 15 limbs); or AM374 (1 mg/kg, n = 12 limbs) 20 min after i.v. injection with 5 mg/kg of both SR141617A and SR144465 (SR1&2). (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$  compared with baseline values by ANOVA.) **C–D**) Tail spasticity developed following **D**) rolipram.