Endocannabinoids control spasticity in a multiple sclerosis model[1]

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SPECIFIC AIMS

Synthetic cannabinoid agonists and antagonists, acting at cannabinoid receptors, inhibit or exacerbate spasticity, respectively, in an animal model of multiple sclerosis (MS) and chronic relapsing experimental allergic encephalomyelitis (CREAE). There are also anecdotal reports on the alleviation of these MS signs in humans by marijuana smoking. Here, we have investigated the existence of a possible tone of endogenous cannabinoids controlling spasticity in CREAE mice by examining whether: 1) endocannabinoid levels in nervous tissues are altered during spasticity; 2) exogenously administered endocannabinoids inhibit spasticity; and 3) pharmacological manipulation of endocannabinoid levels and action alters spasticity.

PRINCIPAL FINDINGS

1. Endocannabinoid levels increase in spastic mice

CREAE was induced in Biozzi ABH mice (Harlan Olac, Bicester, U.K.) after subcutaneous injection of 1 mg of syngeneic spinal cord homogenate emulsifed in Freund's complete adjuvant (Difco, Poole, U.K.) on day 0 and day 7 as described previously. Animals developed a relapsing-remitting disease progression, and between 60 and 80 days post-inoculation they developed spasticity (50%–60% incidence). Similarly treated CREAE animals that had not yet demonstrated tremor, hindquarter, or tail spasticity were used as non-spastic controls. Brains and spinal cords from non-spastic controls, spastic mice, and mice after remission were excised and frozen in liquid N_2 within 60 s from death. Lipids were extracted and purified to be analyzed by gas chromatography-electron impact mass spectrometry (GC-MS), after appropriate derivatization, for the presence of the endocannabinoids anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), and of the AEA congener, palmitoylethanolamide (PEA).

In normal ABH mice (**Fig. 1**), whole brains and spinal cords contained similar levels of AEA (~29–33 pmol/g) and 2-AG (~5–7 nmol/g) and the non-cannabinoid receptor binding, cannabimimetic metabolite, PEA (~220–240 pmol/g). These levels were not significantly changed in nonspastic CREAE remission animals (Fig. 1), despite the fact that these animals had experienced 2–3 paralytic episodes and would contain de-myelinated fibers and axonal loss in the spinal cord. 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 with nonspastic 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 ($P<0.01$) 2-AG and PEA ($P<0.05$) within the spinal cord of spastic mice (Fig. 1). This is the site of major pathological change occurring during CREAE in ABH mice.

These findings may explain the previous observation that exogenously administered SR141617A and SR144465, two antagonists selective for CB_1- and CB_2-type cannabinoid receptors, exacerbate signs of spasticity (i.e., resistance to flexion of hind limbs) in spastic but not in normal and nonspastic remission CREAE mice. It is possible that the antagonists block the action of spasticity-limiting endocannabinoids, whose levels are elevated during the disease. It can be now speculated that endocannabinoid levels increase in an at-

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.00-0399fje To cite this article, use (December 8, 2000) *FASEB J.* 10.1096/fj.00-0399fje

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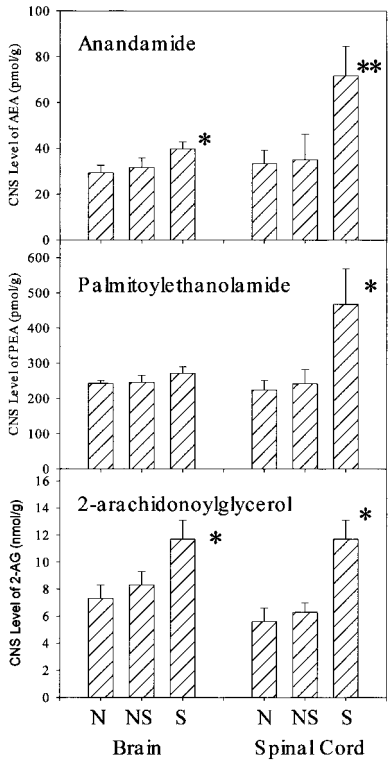

Figure 1. Endocannabinoid levels increase in spastic mice during CREAE. Endocannabinoid levels were measured by isotope-dilution gas chromatography/mass spectroscopy from rapidly frozen brains and spinal cords removed from either normal (N) or postrelapse remission animals showing either spasticity (S)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

tempt to compensate for the spastic defect. However, it is also possible that endocannabinoids or other unrelated fatty acid amides are elevated as a mere consequence of spasticity, such as increased motor activity, or tissue damage, rather than exerting a compensatory effect on this sign. To rule out this possibility, we performed the following experiments.

2. Exogenously administered endocannabinoids inhibit spasticity

To assess whether endocannabinoids do limit spasticity in CREAE mice, the effect of exogenous AEA, 2-AG and, for a comparison, PEA on hind-limb resistance to flexion in these mice was investigated. Ethanolic solutions of the test compounds were evaporated under vacuum and dissolved in PBS:tween 80 (Sigma, U.K.) to be administered as a single 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. All three substances significantly ($P<0.01$) ameliorated spasticity (not shown). Although 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. and 1 mg/kg i.v. $n=13$ limbs). Although different cannabinoids have different pharmacokinetics, this observation may also suggest that 2-AG is not mediating the inhibition directly. For example, 2-AG may act by slowly inhibiting the degradation of endogenous AEA and thereby increasing its levels (see below). Alternatively, it is known that 2-AG activates CB_2 receptors more efficaciously than AEA, and this may also explain the different profile of spasticity inhibition observed here for the two endocannabinoids. As for PEA, this endogenous compound does not exhibit cannabinoid receptor agonist activity but is capable of enhancing some AEA actions, through not fully understood effects. 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. Pharmacological manipulation of endocannabinoid levels/action modulates spasticity

To suggest a cause-and-effect relationship between endocannabinoid levels and actions and inhibition of spasticity, we studied the effect on spasticity of substances counteracting endocannabinoid inactivation or cannabinoid receptor-induced signal transduction. We found that, at doses of 10 mg/kg (i.v.), AM404 and VDM11, two inhibitors of the AEA membrane transporter, which facilitates AEA re-uptake by cells, significantly ameliorated spasticity; and so did a selective inhibitor of AEA enzymatic hydrolysis, AM374 (**Fig. 2*a*,*b***). These compounds have very low affinity for and efficacy at cannabinoid receptors. 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 ($P<0.001$) antispasticity effects were also evident by using doses of AM404 (2.5 mg/kg) and AM374 (1 mg/kg) very likely to be subthreshold for CB_1 agonist control of spasticity (Fig. 2*b*). 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 observed previously following effective cannabinoid receptor agonism. The antispastic effect of AM374 (1 mg/kg i.v.) was blocked by the CB_1 and CB_2 cannabinoid receptor antagonists (SR141716A and SR144465, both 5 mg/kg i.v.) administered 20 min before AM374 (Fig 2*b*). These findings suggest that the inhibitory effect on spasticity—at least of AM374, which does not directly activate CB_1 and CB_2 receptors—is indeed due to enhancement of endocannabinoid levels and subsequent stimulation of cannabinoid receptors.

We studied the effect on spasticity of counteracting cannabinoid receptor signaling (Fig. 2*a*). CB_1 and CB_2 receptors are coupled through $G_{i/o}$ proteins to inhibition of adenylate cyclase, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine inhibits receptor agonism. A selective inhibitor of cAMP-selective phos-

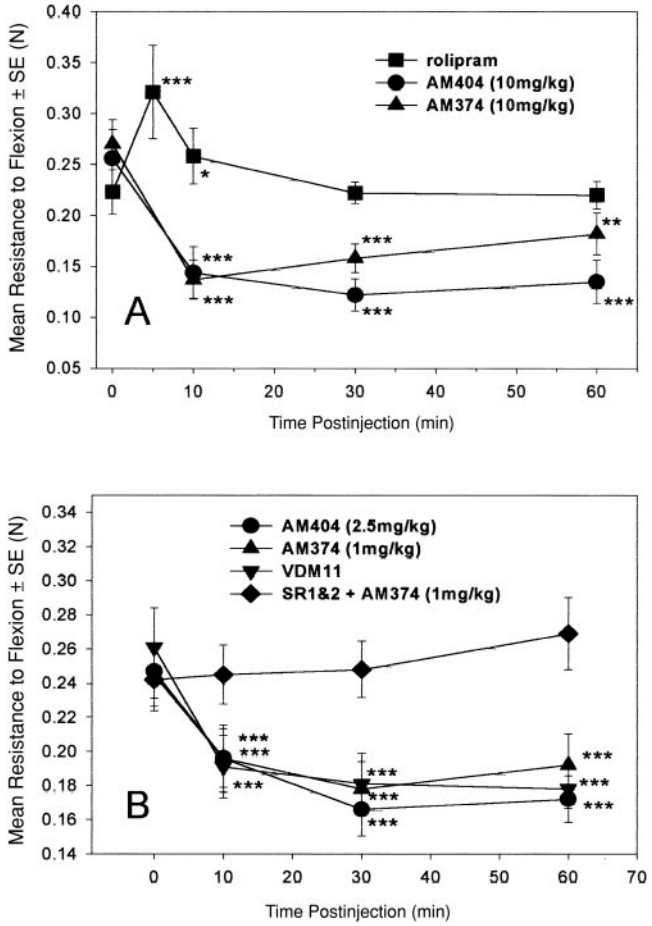

Figure 2. Spasticity is under tonic control of the endocannabinoid system. The forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>5 mice/group) before and following injection i.v. with: ***a***) rolipram (10 mg/kg. n=12 limbs), AM404 (10 mg/kg. n=8 limbs), 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 injection i.v. 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 followed by pair-wise Tukey post-hoc test.)

phodiesterase IV, rolipram (10 mg/kg i.v.) induced a transient increase (P<0.001) in limb (Fig. 2*a*) and tail spasticity (n=15/15, P<0.001 compared with normal animals (n=0/5). 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 (not shown), and was very transient (Fig. 2*a*) consistent with that observed previously with cannabinoid receptor antagonists. This finding again suggests that compensatory mechanisms are rapidly activated after exacerbation of spasticity in CREAE mice and substantiates further the involvement of the endocannabinoid system in the tonic downregulation of this sign.

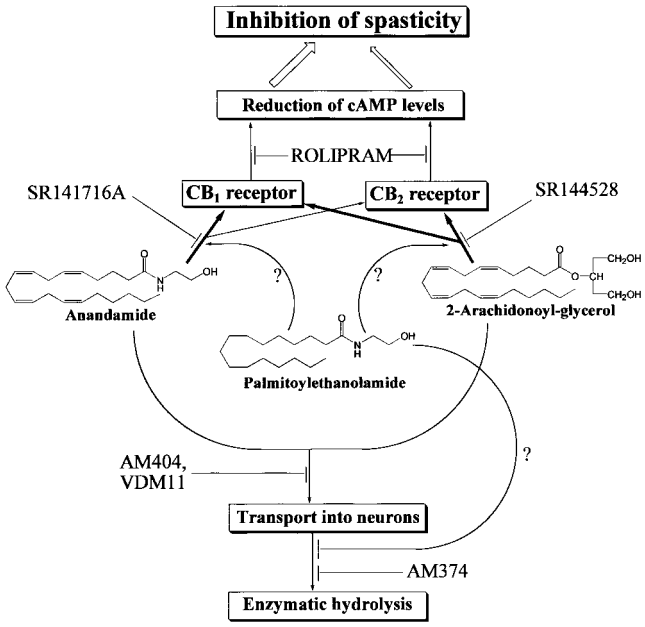

Figure 3. Endocannabinoids tonically inhibit spasticity in CREAE mice. Substances enhancing the tone of endocannabinoids; for example, the inactivation inhibitors AM404, VDM11, and AM374, ameliorate spasticity. Whereas substances counteracting this tone; for example, cannabinoid receptor antagonists SR141716A and, to a lesser extent, SR144528, or the phosphodiesterase IV inhibitor, rolipram, exacerbate spasticity. Palmitoylethanolamide does not activate CB_1 or CB_2 receptors and has beneficial effects on spasticity through unkown mechanisms, possibly by enhancing anandamide action or retarding endocannabinoid degradation. Thin arrows denote activation, thick arrows denote strong activation, blunt arrows denote inhibition, and open arrows denote "leads to."

CONCLUSIONS

We have shown the existence of a possible antispastic tone of endocannabinoids in the brain and, particularly, spinal cord of CREAE mice. 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. This phenomenon, however, does not appear to control spasticity as adequately as what may be possible by administering exogenous cannabinoid receptor agonists or by manipulating endocannabinoid endogenous levels (**Fig. 3**). 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. It remains to be established: 1) if the expression of cannabinoid receptors in nervous tissues is also altered; 2) the role and precise mechanism of action of AEA, 2-AG, and PEA; and 3) the relative role of CB_1 and CB_2 receptors, in CREAE mice. FJ