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The general anesthetic propofol increases brain *N*-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase

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1 Propofol (2,6-diisopropylphenol) is widely used as a general anesthetic and for the maintenance of long-term sedation. We have tested the hypothesis that propofol alters endocannabinoid brain content and that this effect contributes to its sedative properties.

2 A sedating dose of propofol in mice produced a significant increase in the whole-brain content of the endocannabinoid, *N*-arachidonylethanolamine (anandamide), when administered intraperitoneally in either Intralipid or emulphor-ethanol vehicles.

3 *In vitro*, propofol is a competitive inhibitor (IC_{50} 52 μM; 95% confidence interval 31, 87) of fatty acid amide hydrolase (FAAH), which catalyzes the degradation of anandamide. Within a series of propofol analogs, the critical structural determinants of FAAH inhibition and sedation were found to overlap. Other intravenous general anesthetics, including midazolam, ketamine, etomidate, and thiopental, do not affect FAAH activity at sedative-relevant concentrations. Thiopental, however, is a noncompetitive inhibitor of FAAH at a concentration of 2 mM.

4 Pretreatment of mice with the CB_1 receptor antagonist SR141716 (1 mg kg^{-1}, i.p.) significantly reduced the number of mice that lost their righting reflex in response to propofol. Pretreatment of mice with the CB_1 receptor agonist, Win 55212-2 (1 mg kg^{-1}, i.p.), significantly potentiated the loss of righting reflex produced by propofol. These data indicate that CB_1 receptor activity contributes to the sedative properties of propofol.

5 These data suggest that propofol activation of the endocannabinoid system, possibly *via* inhibition of anandamide catabolism, contributes to the sedative properties of propofol and that FAAH could be a novel target for anesthetic development.

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Abbreviations: 2-AG, 2-arachidonylglycerol; FAAH, fatty acid amide hydrolase; LORR, loss of righting reflex

Introduction

Propofol is a widely used general anesthetic with relatively few adverse side effects and several unique properties compared to other general anesthetics (Trapani *et al*., 2000). For example, propofol has been shown to be antiemetic (Gan *et al*., 1996), induce postrecovery mood alterations (Mortero *et al*., 2001), and is associated with a higher incidence of postoperative dreaming in humans compared to other general anesthetics (Brandner *et al*., 1997). Although propofol has been shown to potentiate endogenous GABAergic neurotransmission and to activate directly $GABA_A$ receptors (Williams and Akabas, 2002), which is a common property of general anesthetics, the unique properties of propofol suggest that interactions with other neurotransmitters or neuromodulators contribute to the clinical profile of this drug. We have tested the hypothesis that sedative doses of propofol affect cannabinoid receptors (CB_1) and brain endocannabinoid systems.

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CB_1 agonists including Δ^9-tetrahydrocannabinol, the principal psychoactive component of the plant *Cannabis sativa*, produce sedation and sleep in mammals (Adams & Barratt, 1975; Adams and Martin, 1996) and have been used historically for their analgesic and sedative properties during surgery. CB_1 cannabinoid receptors are located throughout the central nervous system including the hypothalamus and brainstem (Tsou *et al*., 1998a), regions that have been implicated as sedative sites of action of anesthetics (Nelson *et al*., 2002). In addition, endocannabinoid administration increases the time spent in slow wave and REM sleep, and the CB_1 receptor antagonist SR141716 increases wakefulness in animals, suggesting that endogenous cannabinoid signaling modulates sleep–wake cycles (Santucci *et al*., 1996; Mechoulam *et al*., 1997; Murillo-Rodriguez *et al*., 1998, 2001a). Although clearly divergent in initiation, physiological sleep and drug-induced sedation share some of the same neuronal circuitry (Nelson *et al*., 2002); therefore, an endocannabinoid contribution to anesthesia-induced sedation warrants consideration.

Five endogenous arachidonic acid-derived compounds, including *N*-arachidonylethanolamine (anandamide) and 2-arachidonylglycerol (2-AG), have been identified that bind and activate CB_1 cannabinoid receptors (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995). These endocannabinoids have been hypothesized to serve as retrograde inhibitors of neurotransmitter release in several brain regions including the cerebellum, hippocampus, and nucleus accumbens (Kreitzer & Regehr, 2001; Robbe *et al*., 2001; Wilson & Nicoll, 2001). The effects of anandamide and 2-AG are terminated upon uptake *via* a membrane transporter and/or catabolism by intracellular fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGL), respectively (Di Marzo *et al*., 1994; Hillard & Jarrahian, 2000; Deutsch *et al*., 2001; Dinh *et al*., 2002). FAAH is located primarily within large, principal neurons of the brain, including the hypothalamus and brain stem (Tsou *et al*., 1998b), and is also expressed in high concentrations within the liver (Schmid *et al*., 1985). Initial anatomical characterizations indicate that MGL has a more restricted distribution within the thalamus, cerebellum, hippocampus, and cerebral cortex (Dinh *et al*., 2002). Although the biosynthetic enzymes for anandamide and 2-AG have not been completely characterized, the presence of CB_1 receptors and endocannabinoid-degrading enzymes within brain systems subserving arousal and sleep are consistent with a role for endocannabinoids in drug-induced sedation. The purpose of these studies was to determine whether propofol interacts with the endocannabinoid system, and to determine whether this interaction contributes to its sedative–hypnotic properties.

Methods

Drugs and animals

2,6-Diisopropyl phenol (propofol) and analogs were purchased from Aldrich Chemicals (St Louis, MO, U.S.A.) except 4-iodo-propofol, which was synthesized as described previously (Trapani *et al*., 1998). Intralipid vehicle (10%) was purchased from Baxter (Chicago, IL, U.S.A.). The CB_1 receptor agonist Win 55212-2 and antagonist SR141716 were both administered in emulphor vehicle consisting of ethanol, emulphor, and saline in a 1 : 1 : 18 ratio (Cradock *et al*., 1973). In some experiments, propofol was administered in the same vehicle formulation. [^{3}H]CP55940 was purchased from Du Pont NEN (Boston, MA, U.S.A.). [^{3}H]Anandamide and SR141716 were obtained from the Drug Supply Program of the National Institute on Drug Abuse. Deuterated anandamide and 2-AG were obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). All other chemicals were obtained from standard commercial sources. Animals were purchased from Harlan Sprague–Dawley (Madison, WI, U.S.A.). Male rats (250–300 g) were used for brain membrane preparations. Male, ICR mice (25–35 g) were used for righting reflex and brain endocannabinoid measurement experiments. All animals were housed on a 12 : 12 light cycle with lights on at 6 : 00 with *ad lib* access to food and water. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Liquid chromatography/mass spectrometry (LC/MS)

Mice were treated with propofol (100 mg kg^{-1}, i.p.) or vehicle for the length of time indicated followed by rapid decapitation and brain removal. Brains were removed and placed in liquid nitrogen or frozen on a metal surface using dry ice within 2 min of decapitation. Brains were stored at −80°C until extraction. Two methods were used in this study to measure endocannabinoid brain content. Method A was a modification of the method of Gonzalez (Gonzalez *et al*., 1999). Frozen brains were homogenized in three volumes of chloroform : methanol : Tris (50 mM, pH 7.4), 2 : 1 : 1 containing [$^2H_8$] anandamide (84 pmol ml^{-1}) and [$^2H_8$] 2-AG (186 pmol ml^{-1}). The extracted lipids were further purified using an open-bed silica column and the endocannabinoids were eluted with 9 : 1 chloroform : -methanol. The extract was dried and resuspended in 20 μl methanol and the endocannabinoid content was determined using liquid chromatography-atmospheric chemical ionization mass spectrometry ((LC-APCI-MS, Agilent LC-MSD 1100 series, SL model) as described by Walker *et al*., 1999). Samples were separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm, 5 μm diameter) with isocratic mobile phase of 85% methanol/1 mM ammonium acetate/0.05% acetic acid. The flow rate was 0.3 ml min^{-1}, and detection was made in a positive ion mode. Selective ion monitoring was used to detect [$^2H_8$]anandamide (*m*/*z* 356) and anandamide (*m*/*z* 348), [$^2H_8$]2-AG (*m*/*z* 387) and 2-AG (*m*/*z* 379). 2-AG was often seen as a doublet as it isomerizes to 1(3)-AG during extraction; the areas of both peaks were added to obtain total 2-AG.

We have recently developed a new endocannabinoid assay (Method B) that allows for high sensitivity, high recovery of internal standards, and low variability. Brains were weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 84 pmol [$^2H_8$]AEA and 186 pmol [$^2H_8$]2-AG. Tissue was homogenized with a glass rod and sonicated in 4°C water for 1 h followed by incubation overnight at −20°C to precipitate proteins. After centrifugation at 1500 × *g*, the supernatant was removed and evaporated to dryness under N_2 gas, resuspended in methanol, and dried again. The final extract was resuspended in 20 μl methanol. Samples (5 μl) were separated on a reverse-phase C18 column (Kromasil, 250 × 2 mm, 5 μm diameter) using mobile phase A (deionized water, 1 mM ammonium acetate, and 0.005% acetic acid) and mobile phase B (methanol, 1 mM ammonium acetate, and 0.005% acetic acid). Samples were eluted at 300 μl min^{-1} using a linear gradient of 85% solvent B to 100% solvent B over 25 min. Detection was made in a positive ion mode as described above with the following retention times: [$^2H_8$]anandamide (*m*/*z* 356; retention time = 13.70 min), anandamide (*m*/*z* 348; retention time = 13.90 min), [$^2H_8$]2-AG and 1(3)-AG (*m*/*z* 387; retention times = 14.3 and 15.1 min, respectively), and 2-AG and 1(3)-AG (*m*/*z* 379; retention times = 14.5 and 15.3 min, respectively). The quantity of AEA detected in control animals using Method B was three-fold greater than that detected using Method A; however, 2-AG measurements were not changed.

Righting reflex

Anesthetic-induced loss of righting reflex (LORR) was determined in mice as described previously (Irifune *et al*., 1999). Propofol was administered in 10% Intralipid, which is

an aqueous emulsion containing soybean oil (100 mg ml^{-1}), glycerol (22.5 mg ml^{-1}), egg lecithin (12 mg ml^{-1}), and disodium edetate (0.005 mg ml^{-1}). This is the formulation that is used to administer propofol to humans (trade name Diprivan). Control mice received an equal volume of 10% Intralipid. Thiopental was administered in saline and control mice received an equal volume of saline. All solutions were administered by i.p. injection in a volume of 0.01 ml g^{-1} body weight. Mice were pretreated with either SR141716 (1 mg kg^{-1}), Win 55212-2 (1 mg kg^{-1}), or emulphor vehicle, 30 min prior to anesthetic administration. Righting reflexes were determined at 1 min intervals after anesthetic administration; mice were placed on their back onto a plastic platform oriented 45° off the table. If the animal was able to right within 10 s so that both forepaws were on the platform, it was determined to have an intact righting reflex.

CB_1 receptor binding assays

The binding of propofol to the CB_1 receptor was determined *in vitro* by assessing competition with [^{3}H]CP55940 binding in rat forebrain membrane as described previously (Hillard *et al*., 1995). The concentration of [^{3}H]CP55940 used was 2 nM, incubation time was 1 h at ambient temperature, and nonspecific binding was determined in the presence of 10 μM Win 55212-2. Propofol was delivered using DMSO.

[^{3}H]Anandamide uptake into cerebellar granule neurons (CGNs)

Cerebellar granule neurons (CGNs) were prepared from neonatal rats of either sex as described (Hillard *et al*., 1997) and were used on day 7 *in vitro*. Uptake was measured exactly as described (Hillard *et al*., 1997); cells were preincubated with propofol in DMSO or an equivalent amount of DMSO alone for 10 min prior to the addition of [^{3}H]anandamide. Uptake was allowed to continue for 2 min, both media and cellular [^{3}H]anandamide were measured, and uptake was calculated as [^{3}H]anandamide in cells as a fraction of total [^{3}H]anandamide.

FAAH activity assays

FAAH activity was determined in rat forebrain membranes using conversion of [^{14}C]anandamide (labeled in the ethanolamine portion of the molecule) to [^{14}C]ethanolamine using previously published procedures (Edgemond *et al*., 1998). Membranes were preincubated at 30°C with anesthetic for 5 min prior to the addition of [^{14}C]anandamide. The incubation was allowed to continue for 10 min in the concentration–response experiments and for 2 min in the kinetic experiments.

Statistical analyses

For righting reflex experiments, the Kruskal–Wallis test was used to test for differences due to treatment or time. Planned, individual comparisons between drug- and vehicle-treated mice were made using the Mann–Whitney *U*-test. For endocannabinoid measurement, one-way ANOVA, followed by *post hoc* Bonferroni's multiple comparisons test were used to determine statistical differences in mean endocannabinoid content between vehicle- and anesthetic-treated animals. All statistical values as well as IC_{50}, K_m, and V_{max} were calculated using Prism Software (GraphPad, San Diego, CA, U.S.A.).

Results

Propofol increases whole-brain content of endocannabinoids

We utilized LC/MS to quantify anandamide and 2-AG brain content during the peak of propofol-induced LORR. At 8 min after i.p. injection of 100 mg kg^{-1} propofol in Intralipid vehicle, the time point at which propofol-induced LORR was maximal, whole-brain anandamide content was significantly higher in propofol-treated mice compared to vehicle-injected mice (Figure 1a). At 40 min after propofol administration, a time point at which all propofol-treated animals had regained their righting reflex, brain anandamide content did not differ between propofol- and vehicle-treated mice. Propofol administration also tended to increase whole-brain 2-AG content 8 min after administration (Figure 1b); however, this effect did not reach statistical significance. Thiopental (60 mg kg^{-1}) had no effect on anandamide (Figure 1c) or 2-AG (data not shown) brain content in mice killed at 6 min after injection, the peak of the thiopental-induced loss of righting reflex.

Intralipid is an emulsion that is used to administer propofol to humans, which greatly improves its pharmacokinetic and safety profiles (Kanto & Gepts, 1989). However, to determine whether the effects of propofol on brain anandamide content required the coadministration of propofol with Intralipid, we determined the effects of propofol on brain endocannabinoid content using emulphor as a carrier. The time course of propofol's effect on the righting reflex in emulphor vehicle was altered slightly compared to Intralipid vehicle (the onset of action occurred more quickly), but the efficacy of propofol was not different between the two formulations (data not shown). At 8 min following administration of 100 mg kg^{-1} of propofol in emulphor vehicle, brain anandamide content was increased significantly compared to emulphor vehicle-treated mice (Table 1). The effect of propofol on brain anandamide content was not different between the two vehicle formulations. In addition, brain 2-AG content was significantly increased in propofol-/emulphor-treated mice compared to emulphor vehicle-treated mice (Table 1).

Propofol does not affect uptake of anandamide but inhibits FAAH activity in vitro

Several potential mechanisms exist by which propofol administration could increase endocannabinoid brain content including inhibition of membrane transport and inhibition of degradation. Propofol had no effect on the accumulation of anandamide by CGNs, a model system with an operative anandamide carrier (Hillard *et al*., 1997) (Figure 2a). Therefore, we conclude that propofol does not increase extracellular anandamide concentrations *via* transport inhibition.

We determined the effects of propofol on FAAH activity *in vitro*, since FAAH has been shown to be an important regulator of brain anandamide content (Cravatt *et al*., 2001). Propofol delivered in Intralipid produced a concentration-related decrease in the activity of FAAH with an IC_{50} of 14 μM

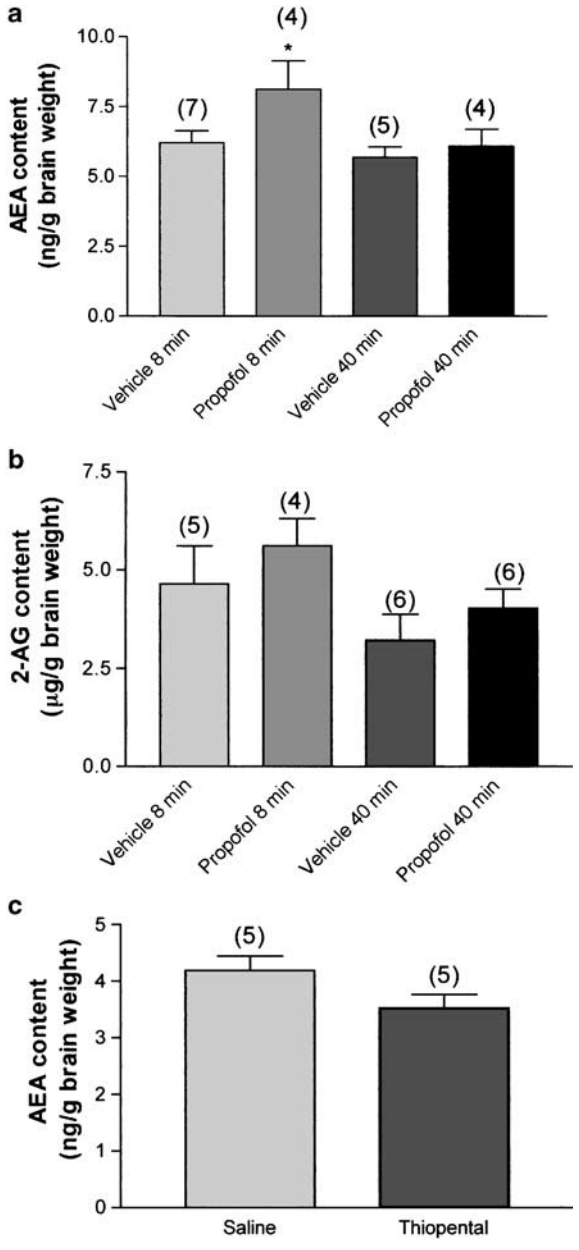

Figure 1 Effects of propofol (100 mg kg^{-1}, i.p. in Intralipid vehicle) and thiopental (60 mg kg^{-1}, i.p. in saline) on brain endocannabinoid content as determined using Method A. (a) Propofol significantly increased anandamide content at 8 min, but not at 40 min after administration (*$P<0.05$, Bonferroni's multiple comparisons test). (b) The effect of propofol on 2-AG content at 8 and 40 min after drug administration; no significant changes were observed. (c) Thiopental administration did not significantly affect brain anandamide content at 6 min after administration. Data are presented as mean (±s.e.m.) endocannabinoid content. Number of mice in each group is indicated in parentheses.

(Figure 2b). Propofol delivered in DMSO also inhibited FAAH activity *in vitro*, although with a higher IC_{50} (52 μM; Table 3). For purposes of comparison, unlabeled anandamide exhibited an IC_{50} value of 3 μM (Figure 2b). Kinetic analyses (Figure 2c) revealed that propofol (at a concentration of 17 μM in Intralipid) is a competitive inhibitor of FAAH, increasing the K_m for anandamide (control: 1.5 ± 0.5 μM; propofol: 4.6 ± 1.6 μM) without affecting the V_{max} (control: 1.9 ± 0.2 nmol min^{-1} mg protein^{-1}; propofol: 1.9 ± 0.3 nmol min^{-1} mg protein^{-1}). In support of this conclusion, Lineweaver–Burk analysis of the data resulted in a significant difference in the slopes of the double reciprocal plots (95% confidence intervals (CIs) $0.36-0.45\times10^{-3}$ for control and $2.1-3.7\times10^{-3}$ for propofol) and no significant difference in *Y*-intercepts (95% CIs $5-10\times10^{-4}$ for control and $-61-44\times10^{-4}$ for propofol).

We have determined the effects of several structural analogs of propofol on FAAH activity and find that the presence and size of the substituents at the 2 and 6 positions are critical determinants of potency (Table 2). Among the analogs examined, 2,6-di-sec-butyl phenol was the most potent inhibitor of FAAH, while the bulkier 2,6-di-tert-butyl phenol and the smaller 2,6-dimethyl phenol were both considerably less potent. Removing one of the isopropyl groups (i.e. 2,6-diisopropyl compared to 2-isopropyl) also resulted in loss of potency. It is noteworthy that 4-iodo propofol, which potentiates GABAergic transmission but does not induce LORR after i.p. injection (Lingamaneni *et al*., 2001), did not inhibit FAAH activity.

Effects of other general anesthetics on FAAH activity

To determine whether inhibition of FAAH is a common property of general anesthetics, we tested the ability of etomidate, midazolam, ketamine, and thiopental to inhibit FAAH activity. Of these anesthetics, only thiopental inhibited FAAH *in vitro* (Table 3 and Figure 2b). However, the IC_{50} value for thiopental (610 μg ml^{-1} or 2 mM) is considerably greater than the minimally effective plasma concentration for anesthesia in humans of 19 μg ml^{-1} (Barash *et al*., 2001) and the concentrations required to potentiate GABA neurotransmission (26 μM; Cordato *et al*., 1999). Thiopental, at a concentration of 2 mM, is a noncompetitive inhibitor of FAAH, reducing the V_{max} (control 1.8 ± 0.1 nmol min^{-1} mg protein^{-1}; thiopental 1.1 ± 0.1 nmol min^{-1} mg protein^{-1}) without affecting the K_m for anandamide (control 1.6 ± 0.3 μM; thiopental 1.4 ± 0.4 μM).

Pretreatment with the CB_1 receptor antagonist attenuates the LORR induced by propofol but not thiopental

As has been shown previously (Irifune *et al*., 1999), we found that propofol (50–200 mg kg^{-1}, i.p. in Intralipid) produces a dose-related LORR in mice (data not shown). A dose of 100 mg kg^{-1} produced peak LORR 7–10 min after injection (Figure 3a) with recovery in most mice by 30 min (data not shown). Pretreatment with the CB_1 receptor antagonist, SR141716 (1 mg kg^{-1}, i.p.), reduced the number of mice that lost their righting reflex in response to propofol administration and reduced recovery time in those mice that did lose the reflex (Figure 3a). The majority of the mice pretreated with SR141716 exhibited no behavioral signs of sedation in response to propofol. In addition, mice treated with 1 mg kg^{-1} SR141716 did not exhibit signs of psychomotor excitation. Conversely, pretreatment of mice with the CB_1 receptor agonist Win 55212-2 (1 mg kg^{-1}, i.p.) resulted in a potentiation of the LORR produced by propofol at times greater than 10 min after propofol administration (Figure 3a). This dose of Win 55212-2 alone did not produce a LORR in mice.

Table 1 Propofol increases whole-brain anandamide content regardless of extraction method or vehicle used in drug administration

	Anandamide content		*2-AG content*	
Treatment	*ng g^{-1} wet weight*	*% Vehicle*	*μg g^{-1} wet weight*	*% Vehicle*
Intralipid	19.2±0.7 (10)		2.3±0.2 (10)	
Propofol/Intralipid	23.5±0.9 (10)**	122±5	2.6±0.2 (10)	113±8
Emulphor	19.9±0.8 (8)		3.4±0.5 (7)	
Propofol/emulphor	23.0±0.9 (8)*	116±5	4.7±0.4 (7)*	138±11

Male mice were injected with either vehicle alone or vehicle containing 10 mg ml^{-1} propofol (100 mg kg^{-1}). At 8 min after the injection, the mice were killed and brains were extracted and assayed using method B. Anandamide and 2-AG content were determined in each brain extract using isotope dilution and LC/MS as described. The numbers in parentheses represent the number of mice in each treatment group. % Vehicle refers to the percent increase in the presence of propofol compared to vehicle-treated mice. Values are given as mean±s.e.m. *$P<0.05$ compared to vehicle treatment alone using unpaired *t*-test; **$P<0.01$ compared to vehicle treatment alone using unpaired *t*-test.

In agreement with previous studies (Lowson *et al*., 1990), i.p. injection of thiopental also produced a rapid LORR in mice and was approximately two-fold more potent than propofol (Figure 3b). Pretreatment with SR141716 had no effect on the number of animals that lost their righting reflex after administration of 60 mg kg^{-1} thiopental (Figure 3b). Although thiopental inhibited FAAH activity, the lack of an increase in anandamide concentrations and lack of effect of SR141716 on thiopental-induced LORR suggest that activation of endocannabinoid systems does not contribute to the sedative effects of thiopental *in vivo*. The dose–response relation for thiopental was very steep; so it was not possible to determine the effects of SR141716 pretreatment on higher thiopental doses at which FAAH inhibition could theoretically result in an increase in anandamide.

Propofol does not bind to the CB_1 receptor

To exclude the possibility that direct activation of CB_1 receptors by propofol contributes to the antagonistic effects of SR141716 on propofol-induced LORR, we determined whether propofol binds to the CB_1 receptor. Propofol did not compete for the binding of the high-affinity ligand [^{3}H]CP55940 to the CB_1 receptor of rat forebrain at concentrations as high as 100 μM (data not shown). These data further suggest that the antagonistic effects of SR141716 on propofol-induced LORR are due to inhibition of endocannabinoid activation of CB_1 receptors.

Discussion

The main findings of this study are that i. p. administration of the general anesthetic propofol increases whole-brain content of the endocannabinoid anandamide, and blockade of CB_1 receptors by SR141716 attenuates propofol-induced LORR. While these data support the hypothesis that activation of CB_1 receptors by endogenously synthesized anandamide contributes to the sedative–hypnotic effects of propofol, the causal relation between these observations awaits further study.

We have also demonstrated that propofol is a competitive inhibitor of the anandamide-degrading enzyme, FAAH, which is consistent with the effect of propofol to increase brain anandamide content. Propofol administration also produces a more variable increase in brain content of 2-AG, which is consistent with data that 2-AG is a substrate for FAAH *in vitro* (Goparaju *et al*., 1998), but that alternative processes are present in the brain, including the recently characterized monoglycerol lipase (Dinh *et al*., 2002), to inactivate 2-AG. In fact, the metabolism of 2-AG is not altered in FAAH$^{-/-}$ mice (Lichtman *et al*., 2002), suggesting that inhibition of MGL or another metabolic pathway by propofol could underlie the increase in 2-AG observed in this study.

Our data are in agreement with the conclusions of Cravatt *et al*. (2001), based upon the finding of highly elevated brain content of anandamide in FAAH$^{-/-}$ mice, that FAAH-mediated catabolism maintains low brain anandamide content. When FAAH is inhibited or is absent, anandamide content is elevated. Propofol did not affect anandamide accumulation in CGNs, suggesting that propofol does not increase synaptically available anandamide *via* transport inhibition. *In vitro* data also indicate that propofol does not itself bind to the CB_1 cannabinoid receptor. Therefore, we hypothesize that propofol inhibits FAAH *in vivo*, resulting in an increase in anandamide concentrations in brain and activation of CB_1 receptors. The partial reversal of propofol-induced LORR by the CB_1 receptor antagonist SR141716 indicates that activation of CB_1 receptors is required for full sedative efficacy of propofol following i.p. administration. One caveat of this interpretation is that FAAH inhibition will result in the accumulation of other fatty acid amides in addition to anandamide (Lichtman *et al*., 2002), which could act through non-CB_1 receptor mechanisms that are sensitive to SR141716. This is however unlikely, since non-CB_1 effects of SR141716 have been seen only at doses four to 10 times greater than that used in the present study (Darmani & Pandya, 2000; Fride *et al*., 2003).

The effects of propofol on $GABA_A$ receptor function have been extensively studied and there exists a causative relation between propofol's ability to potentiate GABA neurotransmission (Trapani *et al*., 1998; Bai *et al*., 1999; Mohammadi *et al*., 2001) and its sedative effects. For example, administration of the $GABA_A$ receptor antagonists, bicuculline and gabazine, attenuate the LORR induced by propofol (Irifune *et al*., 1999; Nelson *et al*., 2002). In addition, a particularly extensive structure–activity study has demonstrated that the efficacies and potencies of a series of propofol analogs significantly correlate with potentiation of $GABA_A$ receptors (Krasowski *et al*., 2001). In spite of this evidence, our data suggest that propofol-induced sedation also requires activation of the CB_1 cannabinoid receptor, since the LORR was significantly reduced in mice pretreated with a modest (1 mg kg^{-1}) dose of the CB_1-selective antagonist, SR141716. We conclude from these data that activation of both $GABA_A$ and CB_1 cannabinoid receptors are required for full sedative

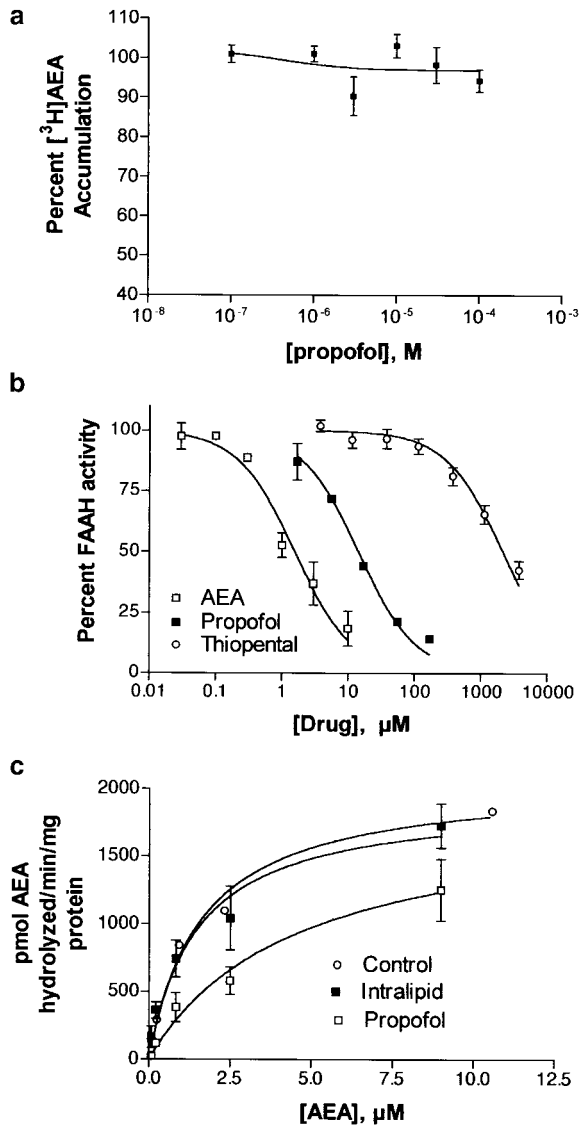

Figure 2 (a) Effect of propofol on [^{3}H]anandamide uptake in cultured CGNs. Propofol was delivered in DMSO. In the same experiments, unlabeled anandamide reduced the uptake of [^{3}H]anandamide to $48\pm2\%$ of control. Data shown are the results of two experiments carried out in triplicate. (b) Effects of anandamide, propofol, and thiopental on rat brain membrane FAAH activity. Drugs were administered in DMSO (anandamide), Intralipid (propofol), or buffer alone (thiopental). Membranes were preincubated with drugs for 10 min prior to the addition of [^{14}C]anandamide. FAAH activity was determined using conversion of [^{14}C] anandamide to [^{14}C] ethanolamine during a 5 min incubation. Each experiment was carried out in triplicate and repeated three times. (c) Effects of Intralipid or propofol (final concentration, 17 μM) in Intralipid on the equilibrium constants for anandamide hydrolysis by FAAH. Control curve was carried out in the presence of buffer alone. Membranes were preincubated with drugs for 5 min prior to the addition of [^{14}C]anandamide. FAAH activity was determined using conversion of [^{14}C]anandamide to [^{14}C]ethanolamine during a 2 min incubation. The control data are that of a single experiment carried out in triplicate, the other data points represent the mean$\pm$s.e.m. for three experiments.

efficacy of propofol following i.p. administration. Whether these two processes occur in series or parallel is among the questions that remain to be answered.

The sedative effects of thiopental were not inhibited by SR141716, which indicates that the sedative effects of the general anesthetics do not universally require CB_1 receptor involvement. These data also rule out a nonspecific effect of SR141716 to increase wakefulness (Santucci *et al*., 1996), regardless of the mechanism for sedation. However, CB_1 receptor agonists have been shown to prolong the duration of sleep following isoflurane exposure in mice (Schuster *et al*., 2002). In addition, we demonstrate in this study that the CB_1 receptor agonist, Win 55212-2, significantly potentiates and prolongs the LORR induced by propofol. Therefore, it is our current hypothesis that coactivation of the CB_1 receptor can potentiate the sedative response to any general anesthetic. What makes propofol unique is that it combines enhancement of $GABA_A$ function and increased endocannabinoid content and that both of these pharmacologic effects contribute to its sedative efficacy.

It is our conclusion that the activation of the CB_1 receptor by propofol occurs subsequent to an increase in brain anandamide content. This conclusion is supported by the findings that whole-brain content of anandamide was increased during the LORR induced by propofol and returned to

Table 2 Effects of structural analogs of propofol on FAAH activity *in vitro*

Compound	*IC_{50} value for FAAH inhibition (95% confidence interval)*
2,6-di-tert-butyl phenol	No effect at 300 μM
2,6-di-sec-butyl phenol	9 μM (5,14)
2,6-diisopropyl phenol (propofol)	52 μM (31,87)
2,6-dimethyl phenol	No effect at 300 μM
2-isopropyl phenol	159 μM (57,445)
3-isopropyl phenol	128 μM (67,244)
4-isopropyl phenol	155 μM (49,487)
2-sec-butyl phenol	35 μM (22,53)
4-iodo-2,6-diisopropyl phenol	No effect at 300 μM

The IC_{50} values were determined from concentration–response curves carried out using at least six concentrations of the analog. Each experiment was repeated three times using different brain membrane preparations. FAAH activity was determined in rat brain membranes using conversion of [^{14}C]anandamide to [^{14}C]ethanolamine during a 10 min incubation. The concentration of [^{14}C]anandamide was 0.2 nM. All analogs were delivered in DMSO.

Table 3 Effects of various intravenous general anesthetics on FAAH activity *in vitro*

Anesthetic	*IC_{50} value for FAAH inhibition (95% confidence interval)*
Propofol in Intralipid vehicle	14 μM (11, 18.5)
Thiopental	2520 μM (2049, 3098)
Midazolam	No effect at 8 μM
Methohexital	No effect at 350 μM
Ketamine	No effect at 42 μM
Etomidate	No effect at 12 μM

The IC_{50} values were determined from concentration–response curves carried out using at least six concentrations of propofol and thiopental; the others were only tested at the concentrations indicated. Each experiment was repeated at least three times. FAAH activity was determined using conversion of [^{14}C]anandamide to [^{14}C]ethanolamine during a 10 min incubation. The concentration of [^{14}C]anandamide was 0.2 nM.

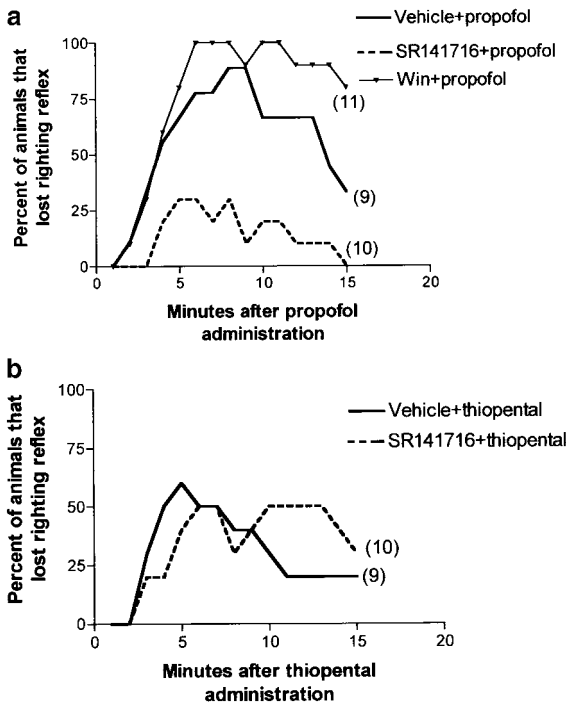

Figure 3 (a) Effects of SR141716 or Win 55212-2 on propofol ($100\,mg\,kg^{-1}$, i.p. in Intralipid vehicle) induced loss of righting reflex. Mice were injected i.p. with SR141716 ($1\,mg\,kg^{-1}$, i.p.), Win 55212-2 ($1\,mg\,kg^{-1}$), or an equivalent volume of emulphor vehicle 30 min before propofol administration. After propofol administration, the mice were scored for the presence or absence of a righting reflex every minute. Kruskal–Wallis nonparametric analysis of the data demonstrated significant effects ($P<0.0001$) of both time and treatment on the LORR. *Post hoc* analysis of the data using planned comparison, Mann–Whitney *U*-tests reveal a significant effect of both SR141716 ($P<0.001$) and Win 55212-2 ($P<0.05$) on the response to propofol. (b) Effects of SR141716 on thiopental ($60\,mg\,kg^{-1}$, i.p. in saline)-induced loss of righting reflex. Mice were injected i.p. with SR141716 ($1\,mg\,kg^{-1}$, i.p.) or an equivalent volume of emulphor vehicle 30 min before thiopental administration. After thiopental administration, the mice were scored for the presence or absence of a righting reflex every minute. Pretreatment with SR141716 had no effect on the response of the mice to thiopental. The number of animals in each group are indicated in parentheses.

baseline at 40 min when the righting reflex was again intact. Our finding that the general anesthetic thiopental, which was insensitive to inhibition by SR141716, had no effect on anandamide brain content argues that the elevation of endocannabinoids was not a result of the anesthesia-induced sedation. This finding also suggests that the effect of propofol in increasing anandamide does not result from enhancement of $GABA_A$ receptor signaling, since thiopental and propofol share this mechanism of action. Finally, we find that propofol has no direct effect on the CB_1 receptor in radioligand-binding studies, which is consistent with an indirect effect of propofol on endogenous CB_1 receptor ligand availability.

We find that propofol is a competitive inhibitor of FAAH *in vitro*. In the absence of an emulsion-type vehicle, the IC_{50} concentration of propofol was $52\,\mu M$ or $9.3\,\mu g\,ml^{-1}$. Brain concentrations of propofol have been demonstrated to be $20\,\mu g\,ml^{-1}$ 10 min after injection of mice with $200\,mg\,kg^{-1}$ i.p. (Lingamaneni *et al*., 2001); extrapolation of these data suggest that brain propofol concentrations following sedative doses are sufficient to inhibit FAAH. In addition, the FAAH-inhibitory concentrations of propofol are very close to concentrations of propofol that affect GABAergic transmission ($2–30\,\mu M$; Trapani *et al*., 1998; Bai *et al*., 1999; Sanna *et al*., 1999). It is noteworthy that propofol was more potent at FAAH inhibition *in vitro* when delivered in Intralipid than in DMSO. Since Intralipid had no effect on FAAH when added alone (data not shown), it is possible that the presence of Intralipid reduced the amount of propofol adhering to the glass tubes or alternatively, potentiated the access of propofol to its binding site on FAAH.

We have investigated the ability of a series of structural analogs of propofol to inhibit FAAH *in vitro*. The structure–activity relation for propofol inhibition of FAAH suggests that the presence and size of substitutions at the 2 and 6 positions are critical determinants of potency. These same molecular characteristics have been shown to be important determinants of anesthetic potency and efficacy (James & Glen, 1980), and for potentiation of $GABA_A$ receptor function (Krasowski *et al*., 2001; Mohammadi *et al*., 2001). Interestingly, one analog of propofol, 4-iodo-2,6-diisopropyl phenol, does not induce LORR after i.p. administration in rodents (Sanna *et al*., 1999), but does potentiate $GABA_A$ receptor function (Trapani *et al*., 1998). It has been argued that the lack of efficacy is due to pharmacokinetic factors (Lingamaneni *et al*., 2001), since 4-iodo-2,6-diisopropyl phenol does produce sedation in rats after i.v. administration (Lingamaneni *et al*., 2001) and has other behavioral effects after i.p. administration (Sanna *et al*., 1999). We found that 4-iodo-2,6-diisopropyl phenol does not inhibit FAAH, which is consistent with the hypothesis that inhibition of FAAH contributes to the sedative efficacy of propofol and its derivatives after i.p. administration. Taken together, these data support a correlation between the ability of propofol and its analogs to inhibit FAAH activity and induce an LORR after i.p. administration, and suggest that propofol inhibition of FAAH activity contributes to its sedative–hypnotic efficacy.

Other investigators have suggested that the endocannabinoid system may contribute to sleep processes. For example, exogenous anandamide administration increases time spent in REM and non-REM sleep, and administration of SR141716 increases time spent in wakefulness at the expense of sleep (see Introduction). In addition, circadian alterations in CB_1 receptor mRNA and protein have been demonstrated within the pons (Martinez-Vargas *et al*., 2003). In addition to sleep and sedation, there are several other effects of propofol that mirror the effects produced by anandamide. For example, propofol accelerates the extinction of learning in single-trial, passive avoidance tasks (Pang *et al*., 1993), an effect that also occurs with the FAAH substrate/inhibitor oleamide (Murillo-Rodriguez *et al*., 2001b) and the endocannabinoid anandamide (Murillo-Rodriguez *et al*., 1998). In addition, propofol has been demonstrated to decrease intraocular pressure (Sator-Katzenschlager *et al*., 2002), induce cellular apoptosis (Tsuchiya *et al*., 2002), and have antiemetic properties (Tsuchiya *et al*., 2002), as have CB_1 cannabinoid receptor agonists (Sanchez *et al*., 1998; Darmani, 2001; Porcella *et al*., 2001; Stamer *et al*., 2001; Tramer *et al*., 2001). It remains to be seen whether these and other similarities between propofol and endocannabinoids are due to a common mechanism, that is, CB_1 receptor activation.

In conclusion, our data demonstrate that propofol sedation after i.p. administration requires CB_1 receptor activation for

full efficacy. Since propofol does not affect the CB_1 receptor directly and elevates whole-brain anandamide content, we hypothesize that propofol activates CB_1 receptor indirectly *via* an increase in anandamide concentrations in brain region(s) yet to be determined. At this stage, however, we cannot rule out other explanations, including the possibility that the two observations of increased anandamide content and inhibition of propofol effect by SR141716 are coincidental and not causative. Propofol, but not other anesthetics, inhibits FAAH activity *in vitro*, and we hypothesize that this property of propofol results in increased activation of CB_1 receptors during propofol-induced sedation. These data cast new light on the mechanisms of action of propofol, the role of endocannabinoids in sleep regulation, and support the multimechanism hypothesis of general anesthetic action. In addition, these data suggest that pharmacological manipulations of the endogenous cannabinergic system could provide viable targets for future drug discovery and anesthesia research.

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