Oleamide-mediated sleep induction does not depend on perturbation of membrane homeoviscosity

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Abstract To verify whether the sleep-inducing properties of oleamide were related to its ability to perturb membrane homeoviscosity, affecting 5-HT_{2A} receptors, we compared the effects of oleamide and oleic acid, the latter lacking both the sleep-inducing effect and the action on 5-HT_{2A} receptors. In binding studies the two compounds did not directly interact with rat brain cortex 5-HT_{2A} receptors, nor did they increase the affinity of a 5-HT_{2A} agonist, either in vitro or ex vivo. They had similar fluidizing effects, in vitro at high concentrations (≥10 μM), and ex vivo after a dose of 100 mg/kg, and they reduced locomotor activity with similar potency. There thus appears to be no causal relationship between the fluidizing effects of oleamide and its sleep-inducing properties.

Key words: Oleamide; Oleic acid; Sleep; Membrane homeoviscosity; 5-HT_{2A} receptor; Rat

1. Introduction

Oleamide (*cis*-9,10-octadecenoamide) is a sleep-inducing amidated lipid originally discovered in the cerebrospinal fluid of sleep-deprived cats [1]. Among its biochemical effects, oleamide was reported to inactivate gap junction channels [2] and to modulate the activity of some receptors including serotonin (5-HT) 5-HT_2 [3] and 5-HT_7 [4] receptors. Oleamide potentiated the effect of 5-HT on chloride currents of frog oocyte expressing 5-HT_{2A} receptors [3], and also on inositol-phosphate formation in rat P11 cells, which endogenously express the 5-HT_{2A} subtype [4]. It was suggested that these effects were due to an allosteric action of oleamide on 5-HT_{2A} receptors since it did not affect the chloride currents or inositol-phosphate formation. Thus, evidence that 5-HT receptors were involved in sleep regulation or in the effects of sleep deprivation [5–7] suggested that oleamide's sleep-inducing effects may be linked to its actions on 5-HT neurotransmission [3].

Chemically, oleamide is composed of a long alkane chain with a centrally placed *cis* double bond and a primary amide with strong hydrogen bonding potential. It was therefore suggested that this molecule could potentially disrupt membrane homeoviscosity more than its free fatty acid analogs [8]. It was thus opined that oleamide might be a new neuromodulator which, by perturbing membrane homeoviscosity, would selectively affect the conformation and activity of membrane proteins sensitive to the lipid environment, such as 5-HT_{2A} receptors or the gap junction membrane channels [8,9]. This suggestion was reinforced by evidence that oleic acid, which lacks the primary amide group, did not modify the spontaneous sleep-waking cycle [1] and had not effect on either gap junction channels [2] or 5-HT neurotransmission [3,4].

This study was aimed at clarifying how the membrane homeoviscosity perturbation is involved in oleamide's hypnotic effect, with particular regard to the brain 5-HT_{2A} receptor-mediated effects.

2. Materials and methods

2.1. Animals

Male CRL:CD(SD)BR rats (Charles River, Italy), weighing about 250 g, were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Rats were treated intraperitoneally with oleamide, oleic acid or vehicle (ethanol:water, 1:1, 2 ml/kg). Behavioral studies were conducted from 20 to 40 min after the injection, whereas for the ex vivo studies rats were killed by decapitation 30 min after treatment. Untreated rats were used for the in vitro binding studies.

After decapitation, the brain cortices were rapidly dissected out and used to prepare the crude synaptosomal pellet (P_2) [10]. The cortices were homogenized in 20 volumes of 0.32 M sucrose, pH 7.4, and centrifuged for 5 min at $1000 \times g$. The supernatant was centrifuged again at $12\,000 \times g$ for 20 min to yield the P_2 pellet.

2.2. Behavioral measurements

Behavioral studies were conducted from 20 to 40 min after i.p. doses of 12.5, 25, 50 or 100 mg/kg of oleamide or oleic acid. Controls received vehicle alone.

Locomotor activity was measured in Makrolon cages ($20.5 \times 28 \times 21$ cm), with two horizontal photocell beams along the long axis, 3 cm above the floor and 18 cm apart. Interruptions of the light beams were recorded by a Paul Fray Ltd computer (Cambridge, U.K.) with Spider software. The room containing the activity cages was closed and ventilated; it had dim indirect lighting, with one 15 W incandescent bulb. A loudspeaker about 1 m above the cages masked extraneous noise. Rats were placed in the cage 20 min after the oleamide, oleic acid or vehicle injection and their locomotor activity was measured for 20 min.

The loss of the righting reflex was used to assess the compounds' sleep-inducing properties, as described previously [11].

2.3. Membrane microviscosity

Membrane microviscosity was determined by the fluorescence polarization (FP) technique [12], using 1,6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes) as a fluorescent probe. The P_2 pellets

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Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; DPH, 1,6-diphenyl-1,3,5-hexatriene

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were resuspended in about 300 volumes of 5 mM phosphate buffer, pH 7.4, and preincubated with 2 μM DPH for 30 min at room temperature. For in vitro studies, the direct effect of oleamide and oleic acid was investigated on control rat brain synaptosomal membrane preparations. The FP value was recorded at 25°C, before and 30 s after the addition of 30 μl of oleamide or oleic acid (30 nM–20 μM), or vehicle into the cuvette. In ex vivo studies the FP value of brain synaptosomal membrane preparations from rats injected i.p. 30 min earlier with oleamide or oleic acid (12.5–100 mg/kg) or vehicle alone, was measured.

The FP value is related to the emission (420 nm), detected through an analyzer oriented parallel (FP_1) and perpendicular (FP_2) to the polarization of the exciting light (365 nm), according to the equation $FP = (FP_2 - FP_1)/(FP_1 + FP_2)$ [12]. Membrane microviscosity (η, poise) is related to the FP value according to the equation $\eta = 2FP/(0.46 - FP)$ [12].

2.4. [^{3}H]Ketanserin binding to 5-HT$_{2A}$ receptors

The P_2 pellets or the crude membrane preparations were resuspended in about 100 volumes of ice-cold 50 mM Tris–HCl buffer, pH 7.4, and 1 ml aliquots of the suspension were incubated with 1 nM [^{3}H]ketanserin (NEN, specific. act. 80.9 Ci/mmol) with or without different concentrations of the compounds to be tested, or vehicle (ethanol), in triplicate. Non-specific binding was determined using 1 μM methysergide.

After 15 min at 37°C [13] the incubation was stopped by rapid filtration under vacuum through GF/B fiber filters, which were washed with ice-cold buffer (3×4 ml), dried and counted in 4 ml of Ultima Gold MV (Packard), in a liquid scintillation spectrometer with about 65% counting efficiency. Inhibition curves were fitted using the equation of the sigmoidal dose-response curve (variable slope) built into GraphPad Prism 2.0a for Power Macintosh (GraphPad Software, San Diego, CA, USA). This analysis gave IC_{50} values (i.e. the drug concentration inhibiting specific binding by 50%) and the slope of the curve, with their relative standard error.

Table 1
In vitro and ex vivo effects of oleamide and oleic acid on the binding properties of 5-HT$_{2A}$ receptors in the rat brain cortex

	[^{3}H]Ketanserin specific binding (% of vehicle)	pIC_{50} of α-methyl-5HT (a 5HT$_{2A}$ agonist)
In vitro		
Vehicle	100	5.96 ± 0.06
Oleamide (30 nM)	104 ± 2	
Oleamide (300 nM)	110 ± 11	6.00 ± 0.03
Oleamide (3000 nM)	103 ± 3	5.82 ± 0.05
Oleic acid (30 nM)	109 ± 2	
Oleic acid (300 nM)	107 ± 2	6.00 ± 0.04
Oleic acid (3000 nM)	104 ± 5	5.84 ± 0.09
Ex vivo		
Vehicle	100 ± 3	5.35 ± 0.08
Oleamide (25 mg/kg)	98 ± 2	5.54 ± 0.05
Oleamide (50 mg/kg)	94 ± 3	5.45 ± 0.10
Oleic acid (100 mg/kg)	92 ± 4	5.38 ± 0.02

The binding of 1 nM [^{3}H]ketanserin was measured on crude synaptosomal preparations from rat brain cortex. For the in vitro experiments, the binding was measured in the presence of oleamide and oleic acid or vehicle (1% ethanol, final concentration). Each value is the mean ± S.E.M. of four replications. For the ex vivo experiments, rats were injected intraperitoneally with oleamide and oleic acid, or vehicle (ethanol:water, 1:1, 2 ml/kg). The brain cortex of two rats per group were pooled and used for the binding experiments. Each value is the mean ± S.E.M. of three replications. The inhibition curve of α-methyl-5HT (four concentrations from 10 to 3000 nM) was also plotted in the different conditions and the relative pIC_{50} were calculated (with their S.E.M.).
No significant differences were found by Student's *t*-test on these parameters.

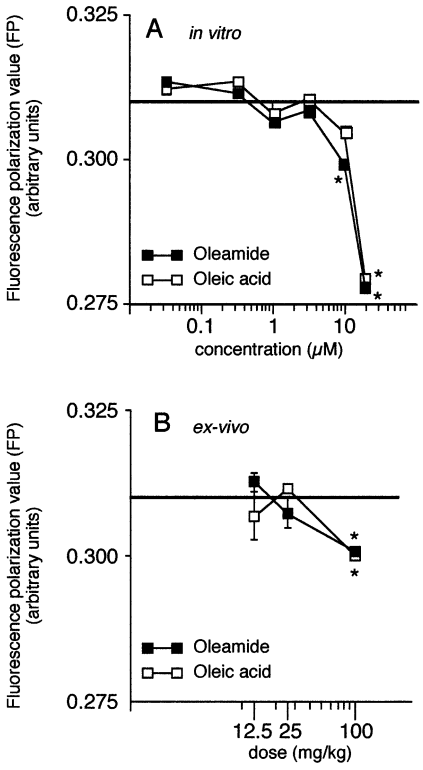

Fig. 1. Effects of oleamide and oleic acid on the membrane fluidity of rat brain cortical synaptosomes. A: For in vitro studies, the fluorescence polarization (FP) value of synaptosomal membranes of control rats was determined 30 s after the addition of the increasing concentrations of oleamide or oleic acid or vehicle (1% ethanol, final concentration) into the cuvette. B: For ex vivo studies, rats were treated intraperitoneally with different doses (12.5–100 mg/kg) of oleamide or oleic acid. Controls received vehicle alone (ethanol: water, 1:1, 2 ml/kg). Thirty minutes after treatment, rats were killed, synaptosomal membranes prepared and the FP measured. Each value is the mean ± S.E.M. of 6–9 measures from three different experiments. The horizontal lines in (A) and (B) indicate the mean FP, expressed as arbitrary units, of the brain synaptosomal membranes of vehicle-treated rats. *: $P < 0.05$ different from vehicle or vehicle-treated rats (Student's *t*-test).

3. Results

3.1. In vitro studies

Fig. 1A shows the in vitro effects of different concentrations of oleamide or oleic acid on the membrane fluidity of a crude synaptosomal preparation (P_2) from rat brain cortex. Concentration-response curves were very similar for the two compounds, which significantly lowered the FP value at 30 μM. Oleamide at 10 μM, but not oleic acid, slightly but significantly reduced the FP value of the crude synaptosomal preparation (Fig. 1A). This last finding was not reproduced when crude membrane preparations from rat brain cortex were used (data not shown).

Table 1 shows the in vitro effects of different concentrations of oleamide and oleic acid on the binding properties of 5-HT$_{2A}$ receptors in a crude synaptosomal preparation (P_2) from rat brain cortex. Up to 3000 nM, neither compound affected [^{3}H]ketanserin binding to 5-HT$_{2A}$ receptors or the IC_{50} (i.e. the affinity) of α-methyl-5-HT, a 5-HT$_{2A}$ agonist.

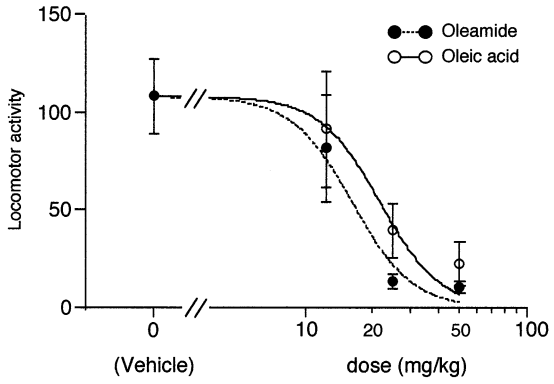

Fig. 2. Effects of oleamide and oleic acid on spontaneous locomotor activity. Oleamide or oleic acid was injected intraperitoneally to rats at the doses indicated, and motility was monitored in observation cages with infrared beams, between 20 and 40 min after treatment. Controls received vehicle alone (ethanol:water, 1:1, 2 ml/kg). Each value, expressed as number of interruptions of the light beams, is the mean ± S.E.M. of 4–8 rats. Locomotor activity was significantly lower ($P < 0.05$) after doses ≥25 mg/kg of both compounds than after vehicle, according to Student's *t*-test.

3.2. Behavioral effects

Rats treated with 25 and 100 mg/kg of oleamide, but not with 100 mg/kg of oleic acid, showed very rapid loss of the righting reflex, more pronounced at the higher oleamide dose. The loss of righting reflex lasted for 90–150 min (data not shown). Both oleamide and oleic acid suppressed locomotor activity between 20 and 40 min after treatment, with very similar effects, the ED_{50} being respectively 16.4 mg/kg (95% confidence limit: 15.4–30.8) and 21.8 mg/kg (95% confidence limit: 11.3–23.6) (Fig. 2).

3.3. Ex vivo studies

As shown in Fig. 1B, synaptosomal membranes (P_2) from the brain cortices of rats treated with 100 mg/kg of oleamide or oleic acid were slightly but significantly more fluid than those of vehicle treated rats. The fluidizing effect was not apparent at lower doses (12.5 and 25 mg/kg) (Fig. 1B).

Table 1 shows that oleamide or oleic acid did not modify the specific binding of the 5-HT_{2A} antagonist [^{3}H]ketanserin and the affinity of the 5-HT_{2A} agonist, measured ex vivo on a P_2 preparation from rat brain cortex.

4. Discussion

The present study indicates that oleamide, but not oleic acid, induces loss of righting reflex in rats, thus confirming its sleep-inducing effect [1,14]. However, our data do not show that this hypnotic effect is related to any alteration of brain membrane homeoviscosity. The main finding was that, at variance with forecasts [8], both oleamide and oleic acid have fluidizing effects, with similar potency. In vitro studies showed superimposable dose-effect curves, high concentrations (≥10 μM) being required to significantly reduce membrane fluidity. Ex vivo studies on brain membranes from rats treated with oleamide or oleic acid showed a slight fluidizing effect only after 100 mg/kg of both compounds, and no such effect at lower doses. However, the loss of righting reflex was observed starting from 25 mg/kg of oleamide, whereas oleic acid was ineffective even at 100 mg/kg, clearly indicating a lack of a causal relationship between the fluidizing effects of oleamide and its sleep-inducing effect. This relationship was previously suggested only on the basis of the predicted effects on membrane homeoviscosity [8]; however, this is the first study evaluating these properties of oleamide and oleic acid.

We also confirmed that oleamide suppresses locomotor behavior, with an ED_{50} very similar to those described [14,15]. This effect was found using ethanol as vehicle, unlike Cheer et al. [14], who found that oleamide was only active if dissolved in oily solvent. Our finding that oleic acid induced hypomotility in rats with a potency similar to oleamide suggests that the sleep-inducing and sedative effects of oleamide might be dissociated and mediated by different mechanisms.

The possibility that the decrease of membrane homeoviscosity, shared by oleamide and oleic acid, plays a role in the sedative effects of the two compounds appears unlikely because of the different doses required to produce the two effects (about 20 mg/kg for the sedative effect and ≥100 mg/kg for the fluidizing effects). However, it cannot be excluded that an in vivo effect on membrane fluidity might be underestimated ex vivo, i.e. after preparation of the synaptosomal membranes.

Our results also exclude that an alteration of membrane homeoviscosity underlies the potentiation of 5-HT_{2A}-mediated responses, previously described in vitro with much lower concentrations of oleamide (below 1 μM), but not oleic acid [3,4]. However, these effects are controversial, since oleamide's potentiating action was not found on different cell lines [16] nor in rat brain cortical slices [15]. Controversial results have been also reported regarding a direct effect of oleamide on 5-HT_{2A} receptors, since no effect was found on [3H]ketanserin binding [14] whereas 500 nM oleamide modestly, but significantly, enhanced the affinity of 5-HT for 5-HT_{2A} receptors [15]. In our studies, similar concentrations of oleamide did not directly interact with rat brain cortex 5-HT_{2A} receptors, nor did they increase the affinity of a 5-HT_{2A} agonist, either in vitro or ex vivo. Thus, although endogenous oleamide levels rise in the cerebrospinal fluid of sleep-deprived rats from 100 to about 500 nM [14], thus being compatible with the reported potentiating effects, the interaction of oleamide with 5-HT_2 receptors has yet to be clarified.

Mechanisms other than direct 5-HT_2 receptor potentiation are likely to be involved in some of the effects. Cheer et al. [15] reported that oleamide, from 2 to 10 mg/kg, modified 5-HT_2 receptor-mediated behavior in rats, in a manner that appeared to involve the activation of cannabinoid CB_1 receptors (i.e. the oleamide effects were mimicked by CB_1 agonists and antagonized by CB_1 antagonists). Furthermore, rats treated intraperitoneally with oleamide show the characteristic 'tetrad' of behaviors induced by cannabinoids [17]. CB_1 receptors could be activated by oleamide itself with affinity of 10 μM, as suggested by Basile et al. [15], or oleamide could amplify the effects of the endogenous CB_1 ligand, anandamide [17]. Oleamide is in fact a competitive substrate of fatty-acid amide hydrolase (FAAH), the enzyme responsible for anandamide inactivation [18,19], and concentrations ≥5 μM are required to inhibit FAAH-mediated hydrolysis of anandamide [17]. Our membrane homeoviscosity data indirectly imply that these high concentrations of oleamide are likely to be reached in the brain 30 min after sleep-inducing doses.

On the other hand, the effects of oleamide on locomotor activity are unlikely to be mediated by anandamide, as pre-

viously suggested [17], for two reasons: (1) this effect was not [14] or very slightly [15] antagonized by CB_1 antagonists and (2) the effect is shared by oleic acid (this study) which is not a substrate of FAAH.

In conclusion, we report for the first time that oleamide and oleic acid both increase membrane microviscosity in vitro and ex vivo. These data clearly indicate a dissociation between the fluidizing and the sleep-inducing effects, the latter being seen only with oleamide, but not oleic acid. The interaction of oleamide with rat brain 5-HT_{2A} receptors was ruled out as well. Both compounds reduce locomotor activity, although further studies are needed to demonstrate a causal relationship with their fluidizing effect. Our data also indicate that the sedative and the sleep-inducing effects of oleamide are mediated by separate mechanisms.

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