Brain lipids that induce sleep are novel modulators of 5-hydroxytryptamine receptors

JUAN PABLO HUIDOBO-TORO* AND R. ADRON HARRIS†‡

*Unidad Regulacion Neurohumoral, Departamento de Fisiología, Facultad Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; and
†Denver Veterans Affairs Medical Center and Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262

Communicated by Richard A. Lerner, The Scripps Research Institute, La Jolla, CA, April 8, 1996 (received for review April 2, 1996)

ABSTRACT Amide derivatives of fatty acids were recently isolated from cerebrospinal fluid of sleep-deprived animals and found to induce sleep in rats. To determine which brain receptors might be sensitive to these novel neuromodulators, we tested them on a range of receptors expressed in Xenopus oocytes. cis-9,10-Octadecanamide (ODA) markedly potentiated the action of 5-hydroxytryptamine (5-HT) on 5-HT₁A and 5-HT₂C receptors, but this action was not shared by related compounds such as oleic acid and trans-9,10-octadecanamide. ODA was active at concentrations as low as 1 nM. The saturated analog, octadecanamide, inhibited rather than potentiated 5-HT₂C responses. ODA had either no effect or only weak effects on other receptors, including muscarinic cholinergic, metabotropic glutamate, GABAₐ, N-methyl-D-aspartate, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Modulation of 5-HT₂ receptors by ODA and related lipids may represent a novel mechanism for regulation of receptors that activate G proteins and thereby play a role in alertness, sleep, and mood as well as disturbances of these states.

Recent reports identifying a fatty acid primary amide present in the cerebrospinal fluid of sleep-deprived cats, and the demonstration that synthetic cis-9,10-octadecanamide (ODA) markedly induced sleep when injected into rats (1), raise the possibility of a new family of neuromodulators. ODA appears to be one member of a group of amidated lipids that are normally found in the brain of mammals, including humans (1-3). A key question is the mechanism(s) by which these compounds alter neuronal function. A variety of neurotransmitters have been implicated in sleep (4-6), and we asked whether this compound modified the function of receptors for these neurotransmitters. 5-Hydroxytryptamine (5-HT or serotonin) receptors were of particular interest as they have been suggested to mediate the sleep rebound that follows sleep deprivation (4, 5). To compare all the receptors in the same cell type and to minimize problems of ODA delivery and eliminate the metabolism that occurs in vivo (1), we used the Xenopus oocyte expression system. This well-characterized system can be used to express a multiplicity of brain receptors from cDNAs or mRNAs, and the pharmacological properties of these receptors mimic those of native brain receptors (7-9). The two-electrode voltage clamp technique was used to measure the currents produced by the activation of these receptors by their selective ligands (10, 11).

MATERIALS AND METHODS

Expression of Brain mRNA. Mature Xenopus oocytes were harvested as detailed by Sanna and coworkers (10). Poly(A)+ mRNA was isolated from adult ICR mouse cerebellum or whole brain following the procedure of Dildy-Mayfield and Harris (11). Oocytes were injected with 80 ng of whole brain mRNA per 50 nl or 100 ng of cerebellar mRNA per 50 nl. Oocytes were incubated for 3-5 days in modified Barth's saline media supplemented with antibiotics, pyruvate, and theophylline as described by Sanna and coworkers (10). A 10 mM stock solution of ODA was prepared in ethanol and diluted immediately before use in modified Barth's saline buffer. Oocytes injected with mRNA were tested with 5-HT, carbachol, kainate, quisqualic acid, and (+)-1-amino cyclopentane-trans-1,3-dicarboxylic acid (t-ACPD) as described in Results.

Expression of cRNAs and cDNAs. Xenopus oocytes were injected with 20-30 ng of cRNA coding for the rat 5-HT₂C receptor or with 50 ng of cRNA for the rat 5-HT₂A receptor (12). Preparation of the cRNAs, cytoplasmic injection, and recording was carried out as described (13, 14). Oocytes injected with 20–30 ng of human GABAₐ subunit α₁+β₁ cDNAs (15) were tested with 1-50 μM GABA at 10-min intervals. Nuclear injection of cDNAs and recording were carried out as described by Valenzuela and coworkers (14). To study α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor function, oocytes were injected with 20 ng of rat GluR3 cRNA and tested with kainate as described by Dildy-Mayfield and Harris (13). For N-methyl-D-aspartate (NMDA) receptors, oocytes were injected with 20-30 ng of cDNA for human NMDA R1 and 2A receptor subunits (14) and NMDA concentration-response curves were performed in the presence of 10 μM glycine as described (13).

All receptors and drugs were tested in oocytes obtained from at least two different frogs; n refers to the number of oocytes tested. Concentration-response curves were compared by ANOVA, and effects of single concentrations of drugs were compared with use of a t test. Animal care was in accordance with institutional guidelines.

RESULTS

We first asked if ODA would directly alter membrane currents of oocytes injected with brain mRNA. Superfusion of 0.1-64 μM ODA onto oocytes injected with whole brain mouse mRNA did not elicit any currents (Fig. 1A). However, in these same oocytes, nanomolar concentrations of ODA produced a substantial and reversible potentiation of the currents elicited with 5-HT. In contrast, the currents elicited by kainate or carbachol were either unchanged or inhibited by 100 nM ODA (Fig. 1B and C). To test effects of ODA on metabotropic glutamate receptors (mGluRs), mouse cerebellar mRNA was injected into Xenopus oocytes. These cells were tested with either quisqualic acid (a selective agonist of the metabotropic mGluR1 and mGluR5 receptors) or t-ACPD (a preferential agonist at the mGluR2, 3, and 8 receptors) (17, 18). ODA (100

Abbreviations: ODA, cis-9,10-octadecanamide; 5-HT, 5-hydroxytryptamine; t-ACPD, (+)-1-amino cyclopentane-trans-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate; mGluR, metabotropic glutamate receptor.

†To whom reprint requests should be addressed.

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carboxyl group proved critical to the high potency of ODA because oleic acid, the nonamidated fatty acid parent compound, produced only minor potentiation at a concentration of 1 μM, and even 10 μM produced only 75% potentiation of the 5-HT currents (Fig. 2B).

To test whether ODA modified the affinity of the 5-HT2c receptor for its ligand or the efficacy of 5-HT, we compared the 5-HT concentration-response curves in control oocytes and in oocytes pretreated with 100 nM ODA (Fig. 2 C and D). These experiments showed that ODA produced a substantial potentiation of 5-HT action at maximal as well as submaximal concentrations of 5-HT and did not alter the EC50 but, instead, increased receptor efficacy. Results with the 5-HT2α receptor clone also demonstrated that ODA, but not oleic acid, facilitated the 5-HT-induced currents, although the magnitude of the potentiation was not as large as that with the 5-HT2c receptor clone (Fig. 3).

The molecular specificity of fatty acid amides for potentiation of 5-HT responses was determined by testing structural analogs of ODA (Table 1). In addition to the amidation of the carboxy group, it is clear that the olefin bond is also critical for the potentiation. Reduction of the cis-9,10 carbon–carbon double bond yields octadecanamide, a compound that inhibited the 5-HT currents. The trans-9,10 isomer was completely devoid of biological activity in this assay. Likewise, a shift of the cis olefinic bond, from carbons 9,10 to carbons 8,9 resulted in loss of activity. The hydroxylation of carbon 18 resulted in an active compound, although potentiation was reduced in comparison with ODA. Two additional compounds tested exhibited a significant potentiation of the 5-HT responses, although the potentiation occurred with a delay. When tested in the same manner as ODA, 100 nM cis-12,13-octadecanamide produced no potentiation of 5-HT, but subsequent application of 5-HT (20 min later) demonstrated potentiation (169 ± 56%) in all oocytes studied. Likewise, linoleicamide (cis-9,10-cis-12,13-octadecanamide) exhibited a similar behavior in that the 5-HT potentiation was not immediate but delayed; at a concentration of 100 nM, this compound produced 110 ± 18% potentiation.

Because the ligand-gated ion channels are critical for regulation of brain excitability and represent sites of action for sedative-hypnotic drugs (20, 21), it was important to examine effects of ODA on several of these ionotropic receptors. In contrast to traditional sedative drugs (e.g., barbiturates and benzodiazepines), ODA did not enhance inhibitory neurotransmitter activity, but relatively high concentrations (0.3–3 μM) inhibited the maximal GABA(A) response by 26–32% (Fig. 44). With regard to excitatory ionotropic receptors, 0.3–3 μM ODA did not significantly affect the function of AMPA or NMDA receptors (Fig. 4 B and C). The lack of effect of ODA on these excitatory ligand-gated ion channels extended to the cloned 5-HT3 receptor. This receptor was expressed in oocytes and studied as described (11), and ODA was tested with 1 μM 5-HT. ODA produced a nonsignificant potentiation of 33 ± 15%. Furthermore, octadecanamide produced effects identical to ODA on all the ionotropic receptors tested (results not shown). Thus, in contrast to the 5-HT2 receptors, the ligand-gated ion channels showed only weak effects of ODA and did not distinguish between ODA and octadecanamide.

**DISCUSSION**

We found that fatty acid amides produce a potent and selective modulation of 5-HT2 receptors expressed in Xenopus oocytes. The selectivity is manifested in the structural requirements of the fatty acid amides (discussed below) and in the receptor specificity.

Of the receptors tested in the present study, only the 5-HT2 receptors were affected by low concentrations of ODA, whereas several other receptors that activate phospholipase C
via G proteins were not sensitive to ODA. This selectivity indicates that the site of action is likely the 5-HT receptor or an associated protein rather than other components of the signaling system (e.g., calcium release) that are common to all these receptors. However, it is important to note that there is a vast family of receptors that couple with G proteins, and it is certainly possible that members of this family, in addition to 5-HT2 receptors, may be sensitive to specific fatty acid amides.

From our structure-activity analysis, it is clear that the site of action of these fatty amides displays strict structural requirements, including amidation of the carboxyl group of the fatty acid and unsaturation at the 9,10 position. The fact that the charged carboxyl group needs to be amidated indicates that the negative charge has to be neutralized, suggesting interaction at a highly hydrophobic site. Likewise, the requirement of a cis conformation, as deduced from the lack of activity of the trans isomer, points to a curved pocket that demands a bent carbon chain for optimal receptor interaction. It is interesting that the analog without an olefinic bond (octadecanamide), which should be able to mimic the conformation of the cis unsaturated analog, is active but produces the opposite action of ODA. The mechanisms by which ODA increases (and octadecanamide decreases) the efficacy of 5-HT receptor activation remain to be defined. These receptors display pronounced desensitization (10, 17, 22), and it is possible that ODA reduces this process, or it could increase the probability of receptor activation of G proteins. The lipid amides are probably allosteric regulators of 5-HT2 receptors, just as many traditional sedative drugs are allosteric regulators of GABA<sub>A</sub> receptors (20). This new class of allosteric regulators may provide a "fine-tuning" of the responsiveness of 5-HT<sub>2</sub> receptors. As noted above, it will be of interest to determine if additional lipid amides serve as allosteric modulators for other members of the vast family of receptors that signal through G proteins.

Little is known about the biogenesis of ODA and related compounds, but the high concentration and large diversity of fatty acids in brain raises the possibility of a family of fatty acid amides (1–3). Although fatty acids are rather weak modulators of neurotransmitter receptors and ion channels (22–24), our results show that amidation of oleic acid markedly enhances its effects on one family of brain receptors. It should be noted that
amidation is also necessary for activity of many neuropeptides (25), suggesting that it may be a common strategy for adapting structural molecules (e.g., proteins and fatty acids) to signaling functions. ODA may be a prototype of a family of biologically active fatty acid amides, with varying lengths of carbon chains, degrees of unsaturation, and position of the carbon–carbon double bonds. In this regard, it is interesting that octadecanamide, a compound derived from the stearate, a prevalent brain fatty acid, had effects on the 5-HT receptor that were opposite to those of ODA. This result raises the possibility of agonist–antagonist or inverse agonist pharmacology among this family of simple, biologically active compounds.

There is ample evidence of the importance of 5-HT in regulation of sleep and, in view of the potent modulation of 5-HT2 receptors by these novel compounds, it is tempting to try to link this effect to their sleep-inducing action. For example, reduction of brain 5-HT by lesions or synthesis inhibitors produces insomnia that is reversed by tryptophan, the precursor of 5-HT (see ref. 19). In addition, pharmacological experiments suggest that effects of sleep deprivation are mediated by 5-HT systems (4–6, 26). However, sleep is a complex process that likely involves multiple neurotransmitter systems.

Table 1. Structure activity relationship for potentiation of 5-HT responses by fatty acid amides in *Xenopus* oocytes injected with cRNA for the 5-HT2C receptor

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of oocytes tested</th>
<th>% Potentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>6</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>ODA</td>
<td>25</td>
<td>365 ± 81*</td>
</tr>
<tr>
<td>Octadecanamide</td>
<td>5</td>
<td>-52 ± 10*</td>
</tr>
<tr>
<td>trans-9,10-Octadecanamide</td>
<td>10</td>
<td>20 ± 10</td>
</tr>
<tr>
<td>cis-8,9-Octadecanamide</td>
<td>7</td>
<td>3 ± 10</td>
</tr>
<tr>
<td>18-Hydroxy, cis-9,10-octadeaconamide</td>
<td>4</td>
<td>142 ± 40*</td>
</tr>
</tbody>
</table>

Values represent the percent potentiation of the response produced by 100 nM 5-HT. For methodological details, see legend to Fig. 2. All compounds were tested at a concentration of 100 nM. Values are mean ± SEM.

*A significant effect of the compound, P < 0.01 (paired Student’s t test).

FIG. 3. ODA potentiation of 5-HT2A responses. Oocytes were injected with 50 ng of cRNA for the rat 5-HT2A receptor and recording was carried out as described for the 5-HT2C receptor (5). (A) Recording from a single oocyte showing the lack of potentiation of 5-HT responses following a 1-min application of 100 nM oleic acid before coapplication with 100 nM 5-HT, and the robust potentiation caused by 100 nM ODA. (B) ODA (100 nM) potentiated the currents induced by either 10 (n = 3), 100 (n = 9), or 1000 (n = 2) nM 5-HT. Values are mean ± SEM. Significant effect of ODA is indicated by * (P < 0.05).

FIG. 4. Effects of ODA on ligand-gated ion channels. (A) GABA<sub>A</sub> receptor. Oocytes injected with 20–30 ng of human GABA<sub>B</sub> subunit α1+β1 cDNAs were tested with 1–50 μM GABA at 10-min intervals as described by Valenzuela and coworkers (5). Concentration-response curves were performed in the same oocytes before (n = 9) and after a 1-min incubation and a 20-sec coapplication of different GABA concentrations with either 0.3 μM (n = 5) or 3 μM (n = 2) ODA. Results are from at least two separate batches of oocytes. Values are mean ± SEM. The E<sub>max</sub> (95% confidence interval) was different for control (103%; 91–115) and 0.3 μM (77%; 69–86) or 3 μM (61%; 50–73). The GABA EC<sub>50</sub> (95% confidence limits) was not different for control (6.1 μM; 4.5–8.3), 0.3 μM (4.9 μM; 3.6–6.6), or 3 μM (4.1; 2.4–7.1). (B) AMPA receptor. Oocytes were injected with 20 ng of rat GluR3 cRNA and tested with kainate as described by Dildy-Mayfield and Harris (10). Experiments were conducted in the absence (n = 4) and in the presence of 0.3 (n = 4) or 3 μM (n = 4) ODA. Values are mean ± SEM. The kainate E<sub>max</sub> and EC<sub>50</sub> values were not significantly different between control and 0.3 or 3 μM ODA. (C) NMDA receptor. Oocytes were injected with 20–30 ng of cDNA for human NMDA R1 and 2A receptor subunits, and NMDA concentration-response curves were performed in the presence of 10 μM glycine as described by Dildy-Mayfield and Harris (11). NMDA responses before (n = 6) and after a 1-min application and 20-sec coapplication of NMDA with 0.3 (n = 5) or 3 μM (n = 4) ODA. Results were derived from at least two separate batches of oocytes. Values are mean ± SEM. The NMDA E<sub>max</sub> and EC<sub>50</sub> values were not significantly different between control and 0.3 or 3 μM ODA.
and brain pathways, and direct extrapolation to the central nervous system from our in vitro system would be speculative. The mechanisms by which ODA and related lipid amides regulate sleep as well as their role in sleep deprivation may be illuminated by studies of acute and chronic effects of these compounds on synaptic transmission mediated by 5-HT2 receptors.

Lastly, it may be important to note that sleep disturbances are often inextricably linked to other behavioral or psychiatric disorders, including anxiety and depression (4, 5). The current therapy for these disorders includes inhibitors of serotonin uptake, suggesting a disturbance of serotonergic function. In addition, pharmacological and biochemical studies suggest that the 5-HT2 receptors may play a role in depression, anxiety, schizophrenia, and alcoholism (27–30). The studies reported here raise the possibility that abnormal sensitivity of 5-HT2 receptors to endogenous fatty acid amides could be responsible for some aspects of psychiatric disorders and sleep disturbances.

We thank Drs. Dale Boger, Richard Lerner, Steve Henritksen, and Benjamin Cravatt for providing the compounds and for many helpful discussions. We thank Dr. David Julius for cDNAs for the 5-HT2A, 5-HT2C, and 5-HT3 receptors; Dr. Paul Whiting for the GABA<sub>A</sub> and NMDA receptor cDNAs; and Dr. Steven Heinemann for the GluR3 cDNA. We are particularly grateful to Dr. C.F. Valenzuela for helpful and expert advice and to V. Bleck for assistance with the illustrations. This work was supported by the Department of Veterans Affairs and National Institutes of Health Grants AA06399, GM47818, and AA03527. J.P.H.-T. is the recipient of a Fundacion Andes Scholar Award for a sabbatical leave of absence to the University of Colorado.