

Anesthetic-like Interaction of the Sleep-inducing Lipid Oleamide with Voltage-gated Sodium Channels in Mammalian Brain

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Background: *cis*-9,10-Octadecenoamide (cOA) accumulates in cerebrospinal fluid during sleep deprivation and induces sleep in animals, but its cellular actions are poorly characterized. In earlier studies, like a variety of anesthetics, cOA modulated γ -aminobutyric acid_A receptors and inhibited transmitter release/burst firing in cultured neurones or synaptoneuroosomes.

Methods: Here, radioligand binding (³H]batrachotoxinin A 20- α -benzoate and mouse central nervous system synaptoneuroosomes) and voltage clamp (whole cell recording from cultured NIE115 murine neuroblastoma) confirmed an interaction with neuronal voltage-gated sodium channels (VGSC).

Results: cOA stereoselectively inhibited specific binding of toxin to VGSC (inhibitor concentration that displaces 50% of specifically bound radioligand, 39.5 μ M). cOA increased (4 \times) the K_d of toxin binding without affecting its binding maximum. Rate of dissociation of radioligand was increased without altering association kinetics, suggesting an allosteric effect (indirect competition at site 2 on VGSC). cOA blocked tetrodotoxin-sensitive sodium currents (maximal effect and affinity were significantly greater at depolarized potentials; $P < 0.01$). Between 3.2 and 64 μ M, the block was concentration-dependent and saturable, but cOA did not alter the V_{50} for activation curves or the measured reversal potential ($P > 0.05$). Inactivation curves were significantly shifted in the hyperpolarizing direction by cOA (maximum, -15.4 ± 0.9 mV at 32 μ M). cOA (10 μ M) slowed recovery from inactivation, with τ increasing from 3.7 ± 0.4 ms to 6.4 ± 0.5 ms ($P < 0.001$). cOA did not produce frequency-dependent facilitation of block (up to 10 Hz).

Conclusions: These effects (and the capacity of oleamide to modulate γ -aminobutyric acid_A receptors in earlier studies) are strikingly similar to those of a variety of anesthetics. Oleamide may represent an endogenous ligand for depressant drug sites in mammalian brain.

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THE fatty acid amide *cis*-9,10-octadecenoamide (cOA) is stimulating much interest as a potential humoral regulator of arousal in mammalian brain. Originally detected in the cerebrospinal fluid of cats subjected to sleep deprivation, this substance causes hypomotility and induces a condition analogous to natural sleep when injected into rats.^{1,2} In contrast, the *trans*-9,10 isomer of octadecenoamide is considerably less active as a hypnotic under these conditions.¹ The biosynthesis of cOA requires ammonia and oleic acid and occurs in rat brain microsomes³ and murine neuroblastoma cells in culture.⁴ A key degradative enzyme, fatty acid amide hydrolase, has also been found in the brain of several mammalian species, including humans.⁵⁻⁷ Although cOA is structurally similar to endocannabinoids, which are cleaved by the same family of amidohydrolases, its *in vivo* effects and pharmacologic profile suggest that it may represent the prototype of a novel family of signaling molecules.

Evidence for involvement of serotonergic pathways in cOA action comes from both cellular and behavioral experiments. In oocytes expressing 5-hydroxytryptamine (HT)_{2a} and 5-HT_{2c} receptors, Ca²⁺-dependent chloride currents induced by 5-HT are selectively augmented by nanomolar concentrations of cOA.⁸ Similar perturbations to 5-HT-stimulated second messenger responses of recombinant 5-HT_{2a} and 5-HT₇ receptors in cultured mammalian cells have been reported.⁹ However, other investigators concluded that, because the binding of guanosine 5'-O-(3-thiotriphosphate) to rat brain membranes is not affected by 1 mM cOA, the hypnotic effects may not be a consequence of G-protein activation.¹⁰ Nevertheless, using a behavioral model of 5-HT receptor activation, it has been demonstrated that cOA acts with low affinity through a cannabinoid receptor to positively modulate 5-HT receptor function in the rat, although effects on phosphoinositide hydrolysis were not detected.¹¹ Recently, cannabinoid receptor antagonists have been shown to block the hypnotic actions of oleamide *in vivo*.¹²

At micromolar concentrations, *cis*-oleamide selectively interferes with communication through rat glial cell gap junctions¹³ and positively modulates inhibitory synaptic currents and both native and recombinant γ -aminobutyric acid (GABA)_A receptors.^{14,15} Only the hypnogenic *cis* isomer exerts this GABA_A modulation in our hands.¹⁶

Recently we reported that micromolar concentrations of cOA (but not *trans*-9,10 isomer of octadecenoamide) markedly inhibit the depolarization of synaptoneuro-

somes, the release of [³H]GABA from synaptosomes, and increases in synaptosomal free calcium in mouse brain preparations when veratridine (but not K⁺) is used as the activator.¹⁶ The electrophysiologic component of this study revealed that cOA (but not the *trans*-9,10 geometric isomer of octadecenoamide) selectively and potently suppresses sustained repetitive firing in cultured pyramidal neurons. Our results are compatible with the idea that an inhibitory effect on sodium channels is also involved in the action of cOA, and we hypothesized that this brain lipid may induce sleep by reciprocal modulation of GABA_A receptors and voltage-gated sodium channels in the brain. Here we have used biochemistry and whole-cell voltage clamp to demonstrate that, in common with a variety of anesthetics, cOA binds directly to neural sodium channels, where it acts as a voltage-state-dependent blocker.

Materials and Methods

Materials

[benzoyl-2,5-³H]Batrachotoxinin A 20- α -benzoate ([³H]BTX; specific activity 34 Ci/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). The sodium channel activators veratridine and scorpion (*Leiurus quinquestriatus*) venom were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). The *cis*-9,10 and *trans*-9,10 isomers of oleamide were synthesized according to published procedures.^{14,17} All other chemicals and saline constituents for electrophysiologic studies were from Sigma (Poole, Dorset, United Kingdom) or Merck Ltd. (Poole).

Neuroblastoma Cell Culture

Murine NIE115 neuroblastoma cells were supplied by Dr. D. E. Ray (MRC Toxicology Unit, University of Leicester, United Kingdom). Confluent cells were subcultured twice weekly and grown on small glass coverslips in Dulbecco's modified Eagle medium (containing Glutamax-1; Gibco, Paisley, Renfrewshire, United Kingdom) supplemented with 10% fetal calf serum and 50 μ g/ml gentamicin. Cells were incubated at 37°C in 5% CO₂ in triple-vented 35-mm culture dishes. In some experiments, 2% dimethyl sulfoxide was added to the growth medium, which increased Na⁺ current density in the clamped somata but did not qualitatively alter oleamide sensitivity. Cells were selected for electrophysiologic experiments 24–36 h after plating.

[³H]BTX Binding Assay

Synaptoneurosomal fractions were isolated from the brains of male CD1 mice (20–25 g; Charles River Laboratories, St. Constance, Quebec, Canada) in sodium-free isolation saline (130 mM choline chloride, 5.4 mM KCl, 5.5 mM glucose, 0.8 mM MgSO₄, and 50 mM HEPES,

adjusted to pH 7.4 with Tris base) as previously described.¹⁸ Batrachotoxinin is a full agonist at the alkaloid neurotoxin recognition site on voltage-gated Na⁺ channels, which functionally facilitates hyperpolarizing shifts in channel activation and markedly impairs channel inactivation. These properties are exploited by neurochemists to chemically elicit ion flux through the normally voltage-responsive ion channel. [³H]BTX binding was determined according to published methods^{18,19} with minor modifications. For equilibrium assays, binding medium (60 μ l) consisting of isolation saline containing [³H]BTX (final concentration, 2.2–175 nM as required), scorpion venom (30 μ g), together with bovine serum albumin and tetrodotoxin (final concentration, 1 mg/ml and 1 μ M, respectively), was thoroughly mixed with the appropriate oleamide isomer (in 0.8 μ l dimethyl sulfoxide) or dimethyl sulfoxide (solvent control). The scorpion venom promotes levels of specific binding to site II on the Na⁺ channel macromolecule. The binding reaction was then initiated by adding synaptoneurosomes (100 μ l; approximately 150 μ g protein), and incubations were continued at 37°C for 50 min. To terminate the reaction, 3 ml of ice-cold wash saline (163 mM choline chloride, 1.8 mM CaCl₂, 0.8 mM MgSO₄, and 5 mM HEPES, adjusted to pH 7.4 with Tris base) was added, and after brief vortexing, bound [³H]BTX was separated from free radioligand by rapid vacuum filtration through Whatman GF/C filters (Mandel Scientific, Guelph, Ontario, Canada). After two 5-ml washes with ice-cold saline, the radioactivity present on filters was quantitated using liquid scintillation counting. Nonspecific binding, determined in the presence of 0.25 mM veratridine, amounted to 17.9 \pm 1.4% of total binding with [³H]BTX at 10 nM.

Potential effects of the sleep modulator on the time course of association of [³H]BTX with site 2 on the sodium channel complex were investigated by preincubating synaptoneurosomes with cOA (100 μ M) or the solvent control in binding medium devoid of [³H]BTX for 10 min at 37°C. [³H]BTX (10 nM) was then added, and the increase in specific binding was monitored up to 30 min.

The effect of cOA on the kinetics of [³H]BTX:sodium channel complex dissociation was examined by equilibrating synaptoneurosomes with radioligand (10 nM) in binding medium for 50 min at 37°C. At this point, either veratridine (plus solvent control) or veratridine plus cOA (at 100 μ M or 200 μ M) was added and the incubations continued from 1–30 min before determination of specifically bound [³H]BTX-A.

Electrophysiology

Cells adhering to glass coverslips were transferred to a plexiglas recording chamber rigidly mounted on the stage of a Nikon diaphot inverted microscope (Nikon, Kingston, Surrey, UK). Cells were continually superfused

with saline containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM HEPES, pH 7.3, and supplemented with 0.1% dimethyl sulfoxide and 0.1% bovine serum albumin. Tetrodotoxin and cOA were applied quantitatively in this superfusing solution (flow rate, ~5 ml/min; bath volume, < 0.5 ml). The whole-cell patch-clamp technique was used to voltage clamp the cells with a single microelectrode. A Burleigh (Harpenden, Herts, United Kingdom) PCS-5000 micromanipulator was used. Pipettes (3–4 MΩ) were fabricated from thin-walled borosilicate glass (1.6-mm OD; Hilgenberg-GMBH, Malsfeld, Germany) on a Mecnex electrode puller (Mecnex Ltd., Geneva, Switzerland). The pipette solution consisted of 10 mM NaCl, 20 mM TEA-Cl, 110 mM CsCl, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, pH 7.4. To reduce stray capacitance, the tips of the electrodes were silanized by immersion in Sigmacote (Sigma, Poole) after back-filling. Only spherical and unclumped cells were used in the study, and 75–90% series resistance compensation was applied directly from the Axopatch 200 preamplifier (Axon Instruments Ltd., San Diego, CA). Because I/V curves for the sodium channel shift to more negative potentials in the first few minutes after beginning cell dialysis, at least 6 min was allowed after membrane rupture before taking data. Analog data were filtered at 5 KHz and were digitized at 20–40 KHz using a CED1401 plus laboratory interface for analysis using patch and voltage-clamp software (CED, Cambridge, United Kingdom). Leak currents and residual capacitive artefacts were deducted offline in software. Details of, and rationale for, pulse protocols are given in Results or associated figure legends. All electrophysiologic experiments were conducted at room temperature (20–24°C). Conductance (g) through Na⁺ channels was calculated by dividing the peak Na⁺ current by the driving force (V-E_r), where E_r is the observed reversal potential in the clamped cell. Relative conductance was fitted to a Boltzmann function of the following form:

$$(g/g_{\max}) = 1/(1 + \exp[(V_{1/2} - V)/k])$$

where g_{\max} is the peak conductance, $V_{1/2}$ is the voltage at which half-maximal conductance occurs, k is the slope factor, and V is the command voltage.

Statistical Analysis

In binding studies, the inhibitor concentration which displaces 50% of specifically bound radioligand (IC_{50}) of cOA was calculated using the probit method.²⁰ Effects of cOA on equilibrium binding parameters (K_d and binding maximum) of [³H]BTX were determined according to Bennett.²¹ Least squares regression analysis was used to fit lines to data as appropriate. Quantitative electrophysiologic data were analyzed using Microsoft Excel 97 (Microsoft Corp., Seattle, WA) and GraphPad Prism 3 software (San Diego, CA), and are cited as mean ± SEM (n). P values were generated using the Student paired t test.

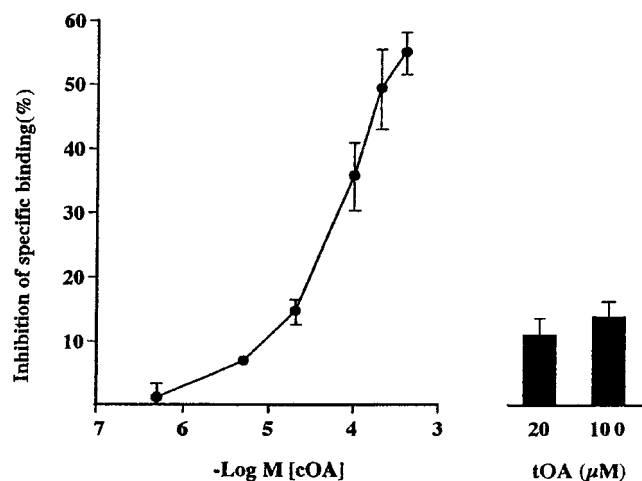


Fig. 1. Concentration-dependent inhibition of the specific binding of [³H]BTX by *cis*- and *trans*-oleamide to mouse brain synaptoneuroosomes. Data points represent mean ± SEM of three independent experiments.

Results

Binding Studies

cis-9,10-Octadecenoamide inhibited the specific binding of 10 nM [³H]BTX to mouse brain synaptoneuroosomes in a concentration-dependent fashion and gave an IC_{50} of 39.5 μM (fig. 1). The *trans* isomer of oleamide was considerably less effective, achieving 22% and 27% inhibition of [³H]BTX binding at 20 μM and 100 μM, respectively. The mechanism by which cOA interacts with site 2 of voltage-sensitive sodium channels was investigated using Scatchard analysis of [³H]BTX binding and by examining the effect of this sleep inducer on [³H]BTX binding kinetics. Figure 2 shows the Scatchard analysis of the specific binding of [³H]BTX to synaptoneuroosomes in the absence and presence of cOA. Regression analysis of these data demonstrated that the

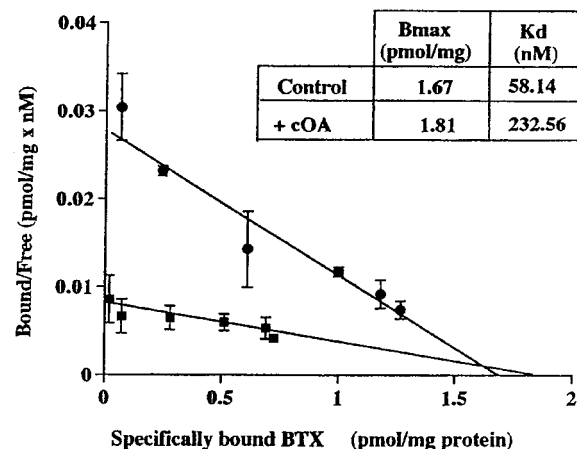


Fig. 2. Scatchard analysis of the effect of cOA on [³H]BTX binding to mouse brain synaptoneuroosomes. Synaptoneuroosomes were equilibrated with increasing concentrations of [³H]BTX in the presence (■) or absence (●) of 100 μM cOA. Values show means ± SEM of three independent experiments.

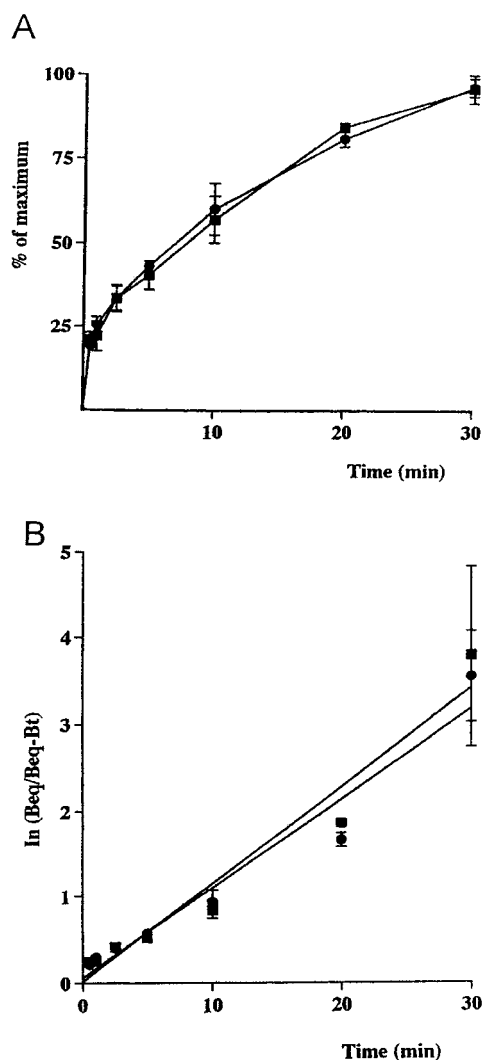


Fig. 3. Inability of cOA to affect the rate of association of [³H]BTX with sodium channels in mouse brain synaptoneurosomes. Both linear (A) and logarithmic (B) transformations of association data are displayed in the presence (■) or absence (●) of 100 μM cOA. Values are mean ± SEM of three independent experiments.

sleep modulator produces a fourfold increase in the K_d of [³H]BTX binding, without affecting the number of binding sites (binding maximum) available to the radioligand. Substances that reduce the association of [³H]BTX likely decrease binding by interacting with closed channels; however, no discernible affect of cOA on the time course of association of [³H]BTX with its binding site was detected (figs. 3A and B). The ability of cOA to interact with activated sodium channels in synaptoneurosomes was investigated by determining its effect on the dissociation of radioligand from the [³H]BTX: sodium channel complex. Application of either 100 or 200 μM cOA (combined with a saturating concentration of veratridine) to synaptoneurosomes previously equilibrated with [³H]BTX caused a concentration-related increase in the rate of dissociation of radioligand over and above that of veratridine alone (fig. 4).

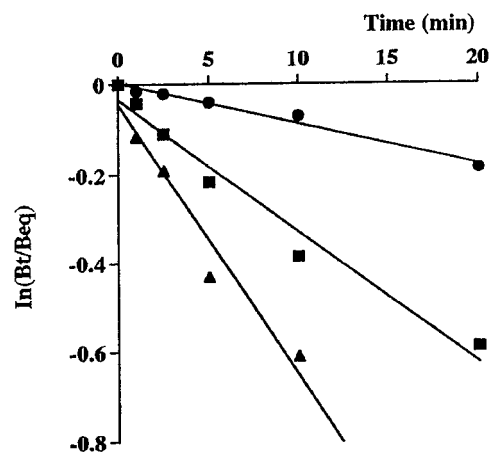


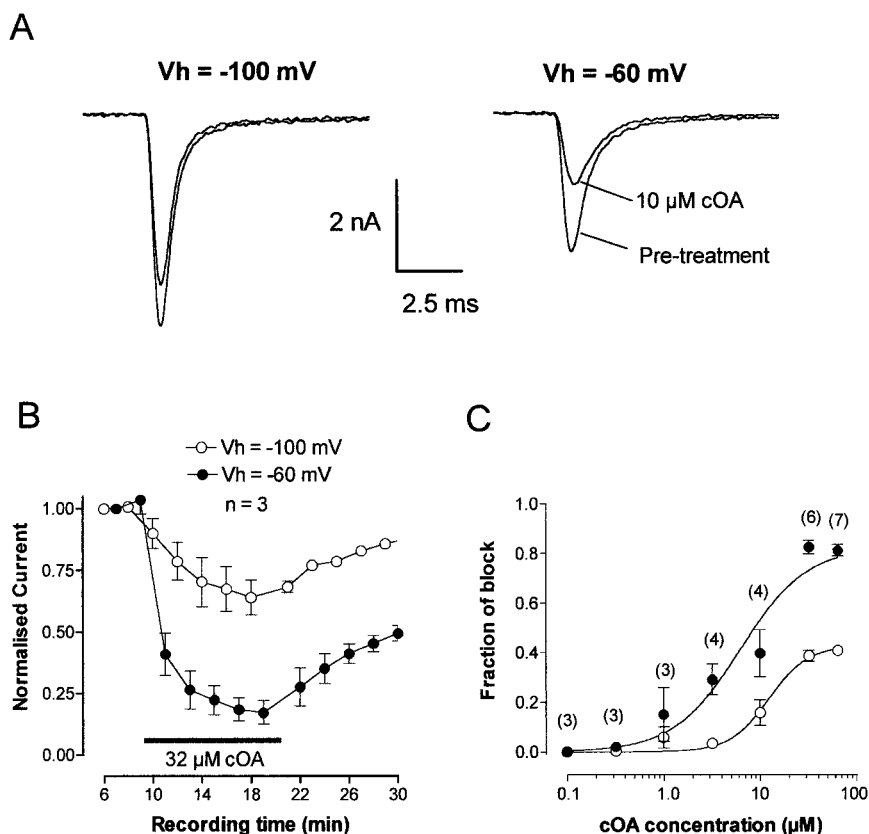
Fig. 4. Dissociation of [³H]BTX from mouse brain synaptoneurosomes. After incubation of [³H]BTX with synaptoneurosomes for 50 min to achieve equilibrium, the dissociation was initiated by adding either 0.25 mM veratridine in the absence (●) or presence of cOA (■, 100 μM; or ▲, 200 μM). Binding of [³H]BTX was then measured at the times indicated. Data points represent mean values of four independent experiments.

Electrophysiology

All patch-clamped cells were found to express fast inward currents in response to step depolarization. From a holding potential of -80 or -100 mV, 0.1-1 μM tetrodotoxin produced complete block of evoked whole-cell inward currents (n = 6) as previously reported for this cell line in the ionic environment used.^{22,23} Preliminary experiments showed that oleamide exerted a reversible block of peak Na⁺ currents (evoked at 0.1 Hz) but that the onset and recovery kinetics were relatively slow, confirming earlier observations in neural membranes (fig. 5).¹⁶ Subsequently, 12-15-min incubations were used after collection of control data to ensure equilibration of the lipid amide with cell membranes and its presumptive receptor site. Under these conditions, cOA caused tonic inhibition of Na⁺ currents in response to a voltage step to 0 mV (0.5 Hz) in a voltage-dependent manner. cOA 10 μM resulted in a 40 ± 9% reduction in peak current at a holding potential of -60 mV, compared with 16 ± 5% at a holding potential of -100 mV (n = 4; $P < 0.05$; fig. 5A). The fractional block was concentration-dependent at the two holding potentials used (fig. 5C), and curves appeared to saturate at 64 μM. At negative holding potentials, the curve was displaced to the right (log of concentration required to block 20% of the peak current was increased significantly; $P < 0.01$; n = 4), increasing the EC₂₀ from approximately 2 μM at -60 mV to approximately 10.5 μM at -100 mV. The maximal cOA fractional block was significantly enhanced at depolarized holding potentials (maximal block at -100 mV, 40.8 ± 0.02%; at -60 mV, 81.1 ± 0.02%; n = 7; $P < 0.0001$).

cis-9,10-Octadecenoamide 3.2-64 μM inhibited Na⁺ currents in a manner that was largely independent of activation voltage (fig. 6). No significant shifts in reversal potential for the peak evoked currents were detected even

Fig. 5. (A) Currents evoked by brief voltage steps to 0 mV from the indicated holding potentials (Vh) were differentially sensitive to the blocking action of 10 μM cOA (upper trace on superimposed sweep) under equilibrium conditions. Each trace is the average of five sweeps applied at 0.5 Hz. (B) The onset and reversal of the cOA block exhibited slow kinetics. cOA application is indicated by the horizontal bar. Data depict the mean responses (\pm SEM) derived from three replicated experiments. (C) The effects of cOA were concentration-dependent and appeared to approach saturation at 64 μM . Tonic block was clearly influenced by holding potential: the substantial shift in EC₂₀ for the two curves and the difference in maximal fractional block for the two holding potentials were both highly significant, as indicated in text. Figures in brackets indicate number of cells examined.



at high cOA concentrations ($P > 0.5$; $n = 4$ at 10 μM and $n = 6$ at 32 μM). Conductance-voltage curves were virtually superimposable before and after application of cOA (order of administration was randomized in these experiments), as shown in figure 6D. Compounding data from replicated experiments confirmed that V_{50} values were not significantly altered by the lipid amide even at the relatively high concentration of 32 μM ($P > 0.05$; $n = 11$).

Steady state inactivation curves (prepulse of 90 ms between -120 mV and -20 mV before a test pulse to $+10$ mV) were fit to a single Boltzmann and showed a concentration-dependent hyperpolarizing shift in the presence of cOA. The maximum value of this shift in the V_{50} for inactivation was -15.4 ± 0.9 mV at 32 μM cOA ($n = 6$; fig. 7A). At concentrations greater than 3.2 μM , the negative shifts were significant ($P < 0.05$) and concentration-dependent. The magnitude of this shift was fitted to the Hill equation (fig. 7B), yielding an apparent EC₅₀ of 11.6 μM (\pm log SEM of 0.057), a slope of 2.8 ± 1.0 (17 *df*), and a tendency to saturate at 64 μM , which was the highest concentration we could formulate at room temperature (fig. 7B). Recovery from inactivation was studied by eliciting current with a 10-ms test pulse to 0 mV from a holding potential of -100 mV with increasing time intervals after a 100-ms prepulse between -100 and 0 mV (fig. 8A). Normalized current data were plotted against recovery interval and fit to a single exponential for each cell examined, confirming that 10 μM cOA

slowed recovery from inactivation, with mean τ increasing from 3.7 ± 0.4 ms to 6.4 ± 0.5 ms ($n = 6$; $P < 0.001$). Compounded data for recovery from inactivation are shown in figure 8B. In control physiologic salines, the peak Na^+ current in these cells did not decrement significantly during a train of 50-ms stimulus pulses to evoke maximal currents at 0.5–10 Hz (not shown). Under these conditions, 10–32 μM cOA did not show detectable frequency-dependent facilitation of block (fig. 9) at frequencies up to 10 Hz ($P > 0.1$; $n = 5$ at 10 μM). Visual inspection of data at other concentrations revealed no frequency dependent fade, but these data were not subjected to statistical analysis.

Discussion

Our results demonstrate that cOA is capable of inhibiting the binding of [^3H]BTX to synaptoneurosome, providing the first evidence for an interaction of this natural sleep-inducing substance with site 2 of voltage-gated sodium channels in mammalian brain.²⁴ Modulation of voltage-sensitive sodium channels was proposed in our previous report,¹⁶ which described antagonism of a number of veratridine-dependent responses in functional synaptic preparations and suppression of sustained repetitive firing in pyramidal cells by cOA at low micromolar concentrations. The potency of cOA as an inhibitor in the [^3H]BTX binding assay ($\text{IC}_{50} = 39.5$ μM)

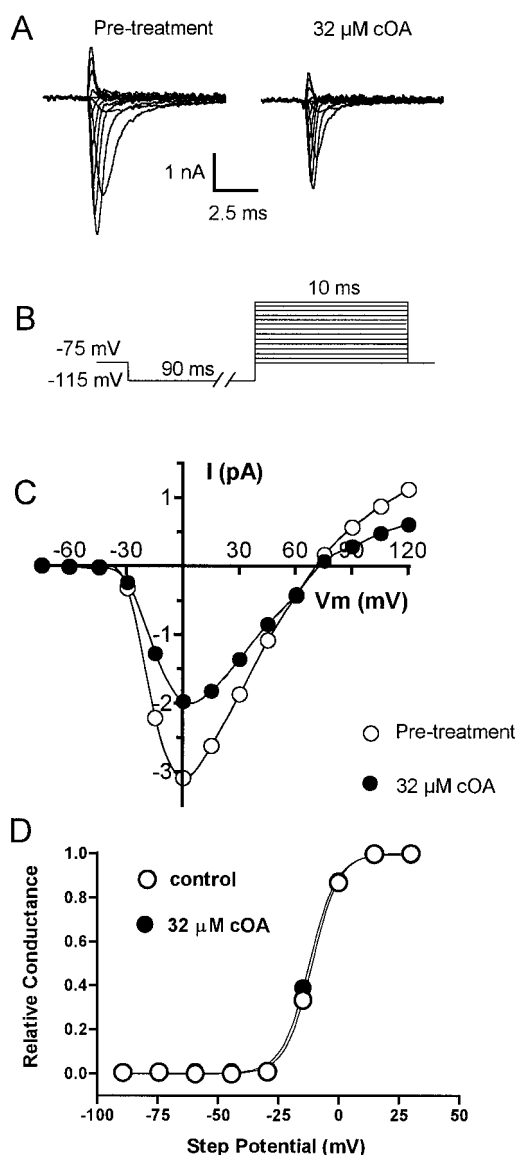


Fig. 6. (A) Families of peak current responses to a 10-ms depolarizing step of increasing magnitude, from the same cell, before and after equilibration with 32 μM cOA. Details of voltage protocols are given below. (B) Cells were clamped at -75 mV and then hyperpolarized to -115 mV to completely remove inactivation before the 10-ms test pulse. Test pulses were applied between -75 and $+120$ mV using 15-mV increments. Twin pulses were repeated at 0.33 Hz. (C) Current-voltage plot for the data depicted demonstrates that the currents reverse close to the Nernstian Na⁺ equilibrium potential under these conditions ($+68$ mV in our salines), that both inward and outward currents were antagonized, and that cOA does not alter the measured reversal potential. No linear trend was observed when comparing extent of block to test potential. (D) Normalized conductance-voltage plots (derived from a different cell) were virtually superimposable even at these relatively high concentrations, suggesting the block did not reflect a shift in voltage dependence of activation gating (the midpoint of the curves, V_{50} , was not significantly altered in replicated experiments).

compares favorably with these results, given the differences in the level of functional complexity and the different requirements of these assays. In addition, marked inhibitory effects on [³H]BTX binding were only observed

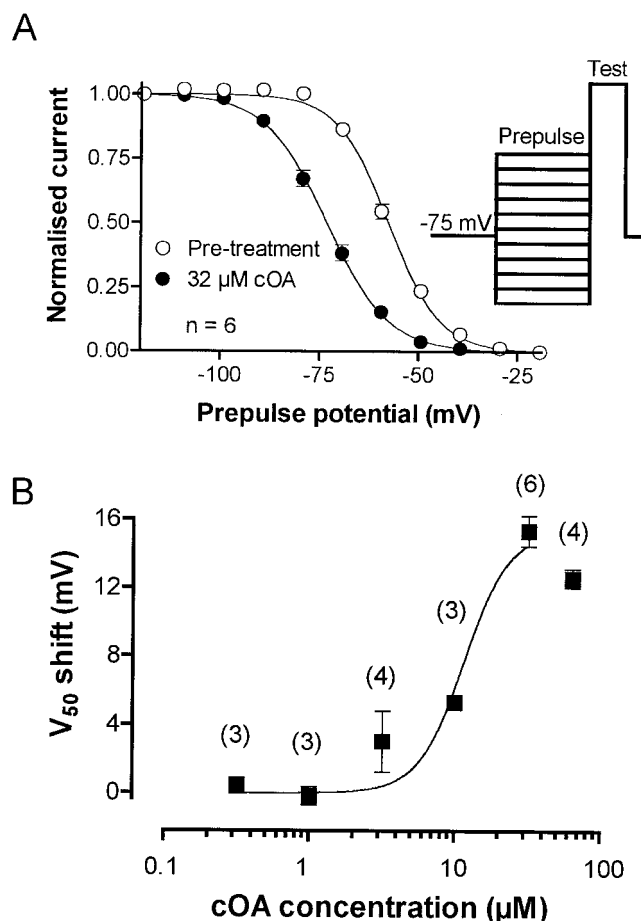


Fig. 7. (A) Steady state inactivation curves before and after equilibration with 32 μM cOA from cells exposed to the indicated voltage protocol (right). Prepulse duration was 90 ms (15-mV increments), and the 10-ms test pulse was to $+10$ mV. The incrementing protocol was repeated every 2 s. After cOA treatment for 15 min, the midpoint for the curve was shifted by approximately 15 mV in the hyperpolarizing direction for this group of cells. (B) The size of the shift in V_{50} was concentration-dependent and saturated at 32 μM . Data were fitted to the Hill equation with two variable parameters (zero and observed maximum response were designated as constant minimal and maximal values, respectively). Derived parameters for EC_{50} , Hill slope, and their associated errors are given in text.

with the strongly hypnotic *cis* isomer. Thus, it appears that perturbation of the [³H]BTX binding domain on voltage-gated sodium channels is mechanistically relevant to the sodium channel blocking action of this sleep inducer.

cis-9,10-Octadecenoamide caused a decrease in the affinity of sites labeled by [³H]BTX without influencing the total number of sites accessed by this radioligand. Our Scatchard data therefore support the idea of competitive inhibition of [³H]BTX binding by this sleep modulator. In addition, because the kinetic analyses revealed that 100 μM cOA was unable to affect the association rate of radioligand but accelerated its dissociation, we infer that an indirect allosteric inhibitory mechanism is responsible for the reduction in affinity. In addition, the dissociation experiments suggest that cOA may inhibit [³H]BTX binding by interacting preferentially with so-

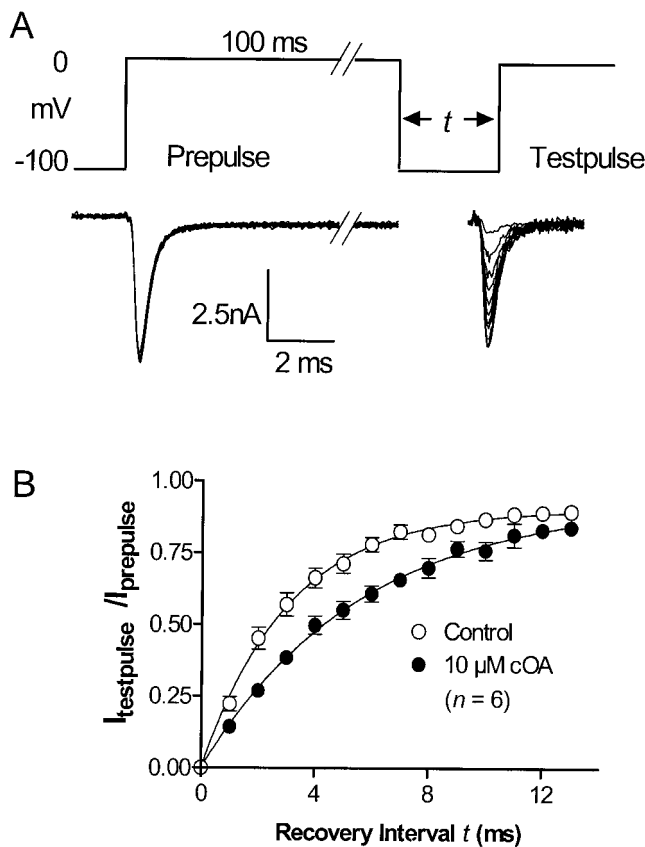


Fig. 8. (A) Twin pulse protocol (applied every 4 s) used to study recovery from inactivation. As the interpulse interval, t , was increased (1-ms increments), evoked current amplitudes were proportionately increased (superimposed current sweeps from a control cell are depicted, lower right). (B) The recovery from inactivation was well fitted by a single exponential and cOA prolonged the time constant for this process (values given in text).

dium channels to which [^3H]BTX and scorpion venom are already bound (*i.e.*, an allosterically modified conformation of the channel), whereas our failure to slow the association of this radioligand with cOA likely excludes its binding to BTX-free (resting or able to be activated) channels.²⁵ Overall, electrophysiologic and binding data suggest a preferential association with inactivated or toxin-modified channel conformations.

The inhibitory profile of cOA on alkaloid neurotoxin binding to sodium channels shows an interesting parallel with those reported for the class I antiarrhythmics,²⁶ anticonvulsants,²⁷ and both local and general anesthetics.^{19,28} The mechanism of competitive inhibition of [^3H]BTX binding by cOA we describe is evidently distinct from the unsaturated insecticidal *N*-alkylamides, which bear some structural similarity to fatty acid amides but reduce the affinity of voltage-gated sodium channels for [^3H]BTX by decreasing its rate of association.²⁹

The negative shifts in inactivation curves and slowing of recovery from inactivation are entirely consistent with those exerted by depressant drugs, including anticonvulsants or local and general anesthetic molecules.^{30,31}

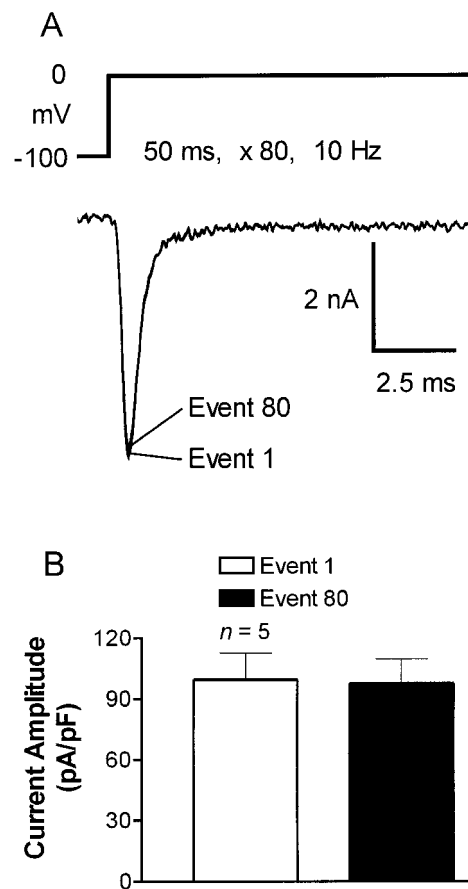


Fig. 9. (A) Step depolarizations (100 mV) were applied as a train with the characteristics described in the top component of the figure. In the lower traces, the first and last events in the train are virtually superimposable, even in the presence of 10 μM cOA. (B) Compounded data from five replicated experiments confirm that no frequency-dependent cumulative block is apparent in the presence of cOA.

These effects are likely to underpin the highly isomer-selective effects of the lipid on burst firing¹⁶ we previously reported in current-clamped rat neurones. The suppression of sustained repetitive firing by *cis*- (but not *trans*-) oleamide was strikingly similar to the effects of the anticonvulsant lamotrigine in similar experiments on the cultured cortical cells.³²

Experiments to confirm the isomer selectivity of oleamide interactions with voltage-clamped cloned Na^+ channels are now underway. We could see no distinctive effect of cOA on channel activation kinetics or voltage dependence of activation gating, nor did the compound influence transmembrane gradients for Na^+ secondary to indirect effects on membrane transport or ionic buffering. The neuroprotective agent riluzole has a similar mode of action, and, *in vivo*, such drugs are strongly depressant at high concentration and have been described as novel anesthetics.³³ Furthermore, sedation has long been an undesirable side effect of anticonvulsant drugs in a clinical setting.³⁴

The tonic blocking effects noted were not noticeably

augmented by high-frequency stimulation of the channel complex. Frequency-dependent fade or open-channel block is a feature of many clinically useful drugs, which may reflect, in part, access of charged molecules at physiologic pH to an intracellular blocking site *via* the polar lumen of the open channel. Molecules such as quinidine are notable for their use dependence, which reflects cumulative enhanced binding of drugs to open channels and failure to unbind completely between pulses.³⁵ The latter form of frequency dependence has been correlated with aromaticity in uncharged drug molecules.³⁵ Oleamide has a calculated log P of approximately 6.5 and, despite its amphiphilic character, bears no charge or aromatic residue. These physicochemical properties may result in relatively rapid off kinetics (fast unbinding, compared with rates of oleamide association with channel proteins) and explain the lack of use dependence in our experiments. The slow onset and recovery times for functional effects are presumably a reflection of partitioning of the extremely hydrophobic molecule into the membrane plane adjacent to its site of action at the channel. In the neuroblastoma cells, the molecule appears to promote inactivation or stabilize the inactivated conformation of the channel. The binding data suggest an interaction of cOA with site 2 of the sodium channel complex. However, the kinetics of modulation of oleamide with the channels in both biochemical and electrophysiologic experiments is notably slow, and the binding interaction with site 2 is indirect. We cannot exclude the possibility that this involves liberation of cytoplasmic messengers and reflects an allosteric effect secondary to changes in phosphorylation of the voltage-gated channel complex. In independent studies, cOA is unable to modulate the binding of guanosine 5'-O-(3-thiotriphosphate) to neural membranes at the concentrations used here, suggesting that G-protein-linked receptors *per se* are not targets for the hypnogenic lipid.¹⁰

Our experiments demonstrate that oleamide has the capacity to concurrently exert inhibitory effects on presynaptic Na⁺ channels and postsynaptic GABA_A receptors. Both channels are widespread in the central nervous system (CNS), and both are crucial for arousal. For these reasons, the effects reported here are consistent with CNS depression and may contribute to the hypnogenic effects of the molecule. However, both of the ion channel targets appear to respond to levels of oleamide above those measured in the cerebrospinal fluid of sleep-deprived or "sleepy" laboratory animals (0.4 μM).² At first glance, this questions the physiologic relevance of the reciprocal modulation proposed here. However, this line of argument does not take into account the scope for physiologic synergy between Na⁺ channels involved in fast axonic conduction in virtually all CNS neurones and the most prevalent inhibitory channel in the brain.^{36,37} More importantly, it ignores the physical

properties of oleamide and the widespread distribution of fatty acid amide hydrolase enzymes in the brain.³⁸ Oleamide has a log P of 6.5; because it is made in biologic membranes and avidly degraded there, it may not be accurate to assume that concentrations in bulk cerebrospinal fluid reflect those seen at the membrane plane at localized sites of action or in key sleep nuclei. Yost *et al.*¹⁵ disclosed that exogenous oleamide is not anesthetic, nor does it synergize anesthetics in laboratory animals. A more recent study demonstrated that, like anesthetics, intraperitoneal oleamide potentiated the sedative action of diazepam and sodium pentobarbital.³⁹ However, the *in vivo* actions of the synthetic molecule are likely to be limited by hepatic and serum hydrolases and uptake into non-neural lipid compartments, hence reducing effective concentrations in the CNS. The *in vivo* effects of oleamide in rodents were very controversial until it was appreciated that its formulation was crucial (lipid-containing vehicles are essential for hypnogenic and psychomotor effects), suggesting that oleagenous excipients may quench or retard the substantial metabolism of oleamide in the periphery and CNS.²

It is an intriguing possibility that oleamide may represent an endogenous ligand for depressant drug binding sites in mammalian CNS. Our results suggest that the hypnogenic isomer has a similar molecular mode of action to therapeutically important and widely prescribed drugs used globally for the treatment of anxiety, sleep disorders, epilepsy, and even to induce anaesthesia in the operating room.^{14,40} The pioneering work of Hughes *et al.*⁴¹ unequivocally established the presence of endogenous ligands for opiate pathways in mammalian nervous systems. Several candidates for benzodiazepine sites have been proposed, but flumazenil does not have marked anxiogenic or stimulatory effects in animals or patients, suggesting that we may not have a constitutively active endogenous benzodiazepine ligand.⁴² Increasingly selective receptor agonists and antagonists are important tools in the quest for endogenous neurohumoral agents and have recently been used to identify anandamide as an endogenous cannabinoid.⁴³ Our studies demonstrate that, like barbiturates, general anesthetics, and anticonvulsants, oleamide can concurrently exert depressant effects on voltage-gated Na⁺ channels and the GABA_A receptor family. In the absence of selective antagonists for such drugs (or for oleamide), careful pharmacologic analysis both *in vitro* and *in vivo* will be required to examine the exciting proposal that fatty acid amides can physiologically activate depressant sites on these crucial ion channels and directly regulate sleep and arousal.

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