British Journal of Pharmacology (1998) 124, 873–882
http://www.stockton-press.co.uk/bjp

Modulation of $GABA_A$ receptors and inhibitory synaptic currents by the endogenous CNS sleep regulator *cis*-9,10-octadecenoamide (cOA)

[1,2,4]George Lees, [2]Michelle D. Edwards, [2]Abdul A. Hassoni, [3]C. Robin Ganellin & [3]Dimitrious Galanakis

[1]School of Health Sciences, University of Sunderland, Wharncliffe Street, Sunderland, SR2 3SD; [2]Academic Department of Anaesthetics, Imperial College of Medicine, at St Mary's Hospital Medical School, London, W2 1NY and [3]Department of Chemistry, University College London, Christopher Ingold Laboratories, 20 Gordon Street, London, WC1H 0AJ

1 *Cis*-9,10-octadecenoamide (cOA) accumulates in the CSF of sleep-deprived cats and may represent a novel signalling molecule. Synthetic cOA has been shown to induce physiological sleep when injected into laboratory rats. Here we assess the cellular mode of action of cOA *in vitro*.

2 In all rat cultured cortical neurones (pyramidal cells) examined, the synthetic brain lipid (3.2–64 μM) enhanced the responses to subsaturating GABA concentrations (up to *circa* 2×) in a concentration-dependent manner (EC_{50}, *circa* 15 μM).

3 (20 μM) cOA significantly enhanced the affinity of exogenous GABA for its receptor without changing the Hill slope or the maximal response. These effects were not voltage-dependent or secondary to shifts in E_{Cl}.

4 In the absence of GABA, cOA directly evoked small inhibitory currents in a subpopulation (<7%) of sensitive cells.

5 20 μM cOA reversibly enhanced the duration of spontaneous inhibitory post synaptic currents (*circa* 2 fold) without significantly altering their amplitude.

6 At 32–64 μM, cOA reversibly reduced the incidence and amplitude of both inhibitory post synaptic currents (i.p.s.cs) and excitatory post synaptic currents (e.p.s.cs) in the cultured neuronal circuits in common with other depressant drugs acting at the $GABA_A$ receptor.

7 32 μM Oleic acid did not modulate exogenous GABA currents or synaptic activity suggesting that cOAs actions are mediated through a specific receptor.

8 A specific, protein-dependent interaction with $GABA_A$ receptors was confirmed in *Xenopus* oocytes. Recombinant human receptors were modulated by 10 μM cOA (and diazepam) only when a γ_2 subunit was co-expressed with $\alpha_1\beta_2$: the cOA response was not sensitive to the specific benzodiazepine antagonist flumazenil (1 μM).

8 cOA may represent an endogenous ligand for allosteric modulatory sites on isoforms of $GABA_A$ receptors which are crucial for the regulation of arousal and have recently been implicated in the circadian control of physiological sleep.

Keywords: *Cis*-9,10-octadecenoamide(oleamide)/oleic acid; $GABA_A$ receptor; flumazenil/benzodiazepine; rat cultured cortical neurones; human recombinant receptors; *Xenopus* oocytes; electrophysiology/patch clamp; endogenous sleep regulator; inhibitory postsynaptic potentials

Introduction

Fatty-acid primary amides from rat, cat and human cerebrospinal fluid (CSF) may represent a novel class of biological signalling molecule. *Cis*-9,10-octadecenoamide (cOA) was purified from sleep-deprived cats and a specific hydrolytic enzyme was detected in rat brain membrane fractions which may regulate its concentration. Synthetic cOA induced physiological sleep when injected, i.p. or i.c.v., into laboratory rats. Several chemical analogues were also depressant: structural requirements for activity and potency *in vivo* have been examined. Neither the ethanolic saline vehicle used or oleic acid modified sleep-wake cycles. Varying the position of the olefin, extending the alkyl chain and injection of the *trans* isomer were all associated with a reduced degree or duration of effect (all the above results were derived from the pivotal study by Cravatt *et al*., 1995). Few studies addressing the cellular mode of action of cOA have been published. However, nM concentrations of cOA have been shown to enhance currents evoked by activation of 5-hydroxytryptamine $(5\text{-HT})_{2a/c}$ receptors, possibly by reducing their net desensitization (Huidobro-Toro & Harris, 1996). In contrast, another study showed that the binding of GTPγS to rat brain membranes is not modulated by 0.1–1 mM cOA and concludes that the hypnogenic effects *in vivo* are probably not secondary to activation of G-proteins (Boring *et al*., 1996). A low-affinity antagonistic action on human recombinant γ-aminobutyric $acid_A$ ($GABA_A$) receptors (composed of only $\alpha1 + \beta1$ subunits) has been demonstrated (Huidobro-Toro & Harris, 1996). These authors did not examine $\alpha_1\beta_2\gamma$ $GABA_A$ receptor isoforms which are the most prevalent ($\geqslant$40%) in mammalian CNS (McKernan & Whiting, 1996), where GABA is thought to be used at *circa* 30% of all synapses (Bloom & Iversen, 1977). These widespread inhibitory channel complexes have an acknowledged role in regulating arousal and as selective targets for a broad range of depressant drugs (Tanelian *et al*., 1993; Johnston, 1996), including hypnotics (Costa & Guidotti, 1996). In this study we addressed the intriguing possibility that cOA may represent an endogenous and chemically-specific mod-

[4]Author for correspondence at: School of Health Sciences, Wharncliffe Street, Sunderland SR2 3SD.

ulator of native, functional synaptic and recombinant $GABA_A$ receptor/chloride channel complexes.

Methods

Cell-culture

Neuronal cultures were prepared from cerebral cortices of 17–18 day old rat embryos (Lees & Leach, 1993). Cells were plated onto poly-D-lysine coated coverslips (200,000 cells ml^{-1}) in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 100 units μg^{-1} ml^{-1} penicillin/streptomycin. After 12–24 h the plating medium was replaced by a maintenance medium comprising, Neurobasal medium, with 2% B27 supplement, 1% glutamax (Gibco, Paisley, U.K.) and 100 units μg^{-1} ml^{-1} penicillin/streptomycin. Cells were used in experiments after 14–35 days *in vitro*.

Recombinant receptors in oocytes

Stage V–VI oocytes were isolated from adult female *Xenopus laevis* then de-folliculated and prepared for injection using established techniques (Edwards & Lees, 1997). cDNAs in the pCDM8 eukaryotic expression vector were injected blind into the nuclear pole in sterile buffer (0.2–0.3 ng of each cDNA, mixed to a final volume of 20 nl per oocyte). Cells were incubated at 18–22°C.

Electrophysiology

Cultured networks on coverslips were placed in a 5 mm perspex trench on the stage of an inverted microscope. The extracellular saline contained (mM): NaCl 142, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, D-glucose 10, pH 7.4. Whole-cell pipette saline contained (mM): K-gluconate 142, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10, EGTA 11, pH 7.4. All potentials cited are those based on the pre-amplifier null potential and take no account of the liquid junction offset inherent in the use of these asymmetrical solutions. Borosilicate patch pipettes (3–5 MΩ) were used: 60–80% series resistance compensation was applied at the List EP7 pre-amplifier. Pyramidal neurones, identified under phase-contrast optics, with input resistances of $\geqslant$100 MΩ were selected for study. Whole cell currents were filtered at 1–3 KHz before digitization and monitored on a Gould chart recorder or analysed using WCP (John Dempster, Strathclyde University) or CED software.

Agar-filled (1% in 2 M KCl) oocyte electrodes were broken back to between 0.5 and 3 MΩ. Frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES and 1.8 mM $CaCl_2$, pH 7.2) with, or without, GABA and modulatory drugs was superfused at *circa* 10 ml min^{-1}. Two-electrode voltage clamp was used (Axon GeneClamp 500). Results were measured either as a current or as change from baseline conductance in response to a −20 mV, 200 ms voltage jump, applied at 2.5 Hz. Real-time recordings were made on a pen recorder: upward deflections represent inward currents. GABA was applied for long enough to elicit peak responses (5–30 s). Modulatory drug responses were assessed at equilibrium: peak conductance being presented as a percentage of control GABA response. All experiments were conducted at 22–24°C.

Pharmacology

cOA was dissolved in ethanol (only used in preliminary experiments on cultured cells) or dimethylsulphoxide (DMSO: cultures and oocytes) then diluted 1000× into saline: 0.1% ethanol or DMSO produced no effect on the parameters described here and was present in pre- and post-treatment phases of all experiments. To facilitate further dissolution of the modulator, all extracellular salines were supplemented with 0.033–0.1% bovine serum albumin (fraction V, Sigma) which was present during all phases of the experiments presented. cOA was formulated daily and perfused from glass reservoirs via teflon lines. GABA was rapidly and quantitatively delivered to cultured cells using the Y-tube technique (Murase *et al*., 1989) either alone (to obtain pretreatment, control responses) or co-applied with cOA/modulatory drugs (at the same concentration applied to the cell by bath perfusion). Agonist pulse duration was sufficiently long (>200 ms) to permit the current response to 1 or 3.2 μM GABA (the lowest concentration examined in any given experiment) to attain its maximal value in any given cell. Log concentration-response curves were fit to the Hill equation by non-linear regression (Graphpad Prism software, San Diego, CA.). Logarithmic values of EC_{50} and associated standard error were used for statistical comparisons. Student's *t* tests (two-tailed throughout, and paired where appropriate) or ANOVA were used as indicated in the text. Significant effects were indicated by $P<0.05$ throughout. Data are expressed as mean ± s.e.mean.

Chemical synthesis and suppliers

The acid chloride of oleic acid was prepared by treatment with oxalyl chloride in CH_2Cl_2. The amide was formed by treatment with saturated NH_4OH and purified as a single band by column chromotography on silica gel using chloroform for elution. Two batches of the white crystalline solid (m.p. 74.8–75.2°C) were prepared for use in our pilot studies: identical results have been generated using both batches. Unless otherwise stated, all chemicals and drugs were obtained from Sigma Aldrich Chemical Co. (Poole, Dorset, U.K.) or (Merck Ltd, Poole, Dorset, U.K.).

Results

cOA, but not oleic acid, modulates ionotropic receptors activated by GABA on pyramidal cortical neurones in culture

Neurones in cultured monolayers from rat embryonic cortex have previously been shown to bear $GABA_A$ receptors which are sensitive to modulation by diazepam and blocked by bicuculline or picrotoxinin (Lees & Leach, 1993; Foden & Lees, 1997). In this study, brief pulses of GABA from a Y-tube adjacent to whole-cell clamped pyramidal cells, V_h −45 mV, evoked outward currents at concentrations $\geqslant$1 μM. The peak currents saturated between 0.32 and 1 mM (EC_{50}: 26.5 μM, 95% C.I. 22.7–31.0, $n=46$) with a Hill slope of *circa* 1 (see Figure 2c). Estimation of the amount of potentiation of $GABA_A$ receptor isoforms and their affinity for modulatory drugs is complicated considerably by a strong dependence on the extent of occupation of the GABA site (e.g. Harris *et al*., 1995). Initially, we examined the actions of cOA using GABA concentrations below its EC_{50}. Peak currents evoked by 0.5–2 s pulses of 10 μM exogenous GABA were invariably enhanced by 20 μM cOA ($n=5$, Figure 1a). Repeated measures ANOVA on the raw peak currents (Dunnett's *post* test) indicated that control currents were significantly ($P<0.01$) smaller than those evoked in the presence of 20 μM cOA and that the effect was fully reversible ($P>0.05$: control *vs*

washout: Figure 1b). 32 μM, Oleic acid a chemical homologue of cOA with similar physical properties, which was not hypnogenic *in vivo* (Cravatt *et al*., 1995), was unable to modulate significantly the response to 3.2 μM GABA in cells demonstrably sensitive to cOA (32 μM, $n=5$, Figure 1c and d). At holding potentials of -30 or -45 mV, modulation of exogenous GABA by cOA was found to be dose-dependent in a further 35 cells (Figure 2a and b). The data were fitted by non-linear regression to a sigmoid curve (indicating either a saturable population of receptors or limiting aqueous solubility) with an apparent threshold of 3.2 μM and an EC_{50} of 15.3 μM (95% C.I. 5.9–39.8 μM).

cOA enhances the affinity of agonist for the $GABA_A$ receptor but its modulatory effects are not voltage-dependent or secondary to shifts in E_{Cl}

At 20 μM, cOA enhanced responses to exogenous GABA by increasing agonist affinity, as shown in Figure 2c (GABA EC_{50} was reduced, relative potency 0.64, to 17.0 μM, 95% C.I. 11.5–25.2 μM). A paired Student's *t* test on log value of EC_{50} and associated log standard error demonstrated that the reduction in EC_{50} was significant ($P<0.05$). To analyse the maximal responses statistically the normalization of data to peak pretreatment current was inappropriate (control data has no variance). The maximal current evoked by GABA, determined from curve fits to the raw data after normalizing for cellular capacitance (to correct for variations in cell size), was not significantly altered (see Figure 2c inset, for sample responses to a saturating agonist concentration). The Hill coefficient for GABA was not significantly altered by cOA, indicating a parallel shift in the log concentration-response curve. The maximal enhancement (*circa* 2 fold) was observed using agonist GABA at 3.2 μM. Under these conditions, in 4 cells clamped between -90 and 0 mV (30 mV increments), the modulatory effect of 20 μM cOA was not voltage-dependent (one-way ANOVA, Figure 3) and the reversal potential for the outwardly-rectifying, presumptive chloride-current was not shifted in any of the replicates from pretreatment values (Figure 3b). Therefore, cOA appears to enhance affinity of agonist GABA for its receptor without changing the concentration gradient for the permeant chloride ion.

cOA prolongs the decay of inhibitory post synaptic currents (i.p.s.cs) in cultured neurones

The cortical cultures (after 7–28 days *in vitro*) possess many functional synaptic contacts and are spontaneously active in physiological saline at 22–24°C. Bath superfusion of 50 nM tetrodotoxin (TTX) revealed that the majority of the large (0.1–1.0 nA) inhibitory and excitatory currents (i.p.s.cs and e.p.s.cs, respectively) are evoked (secondary to action

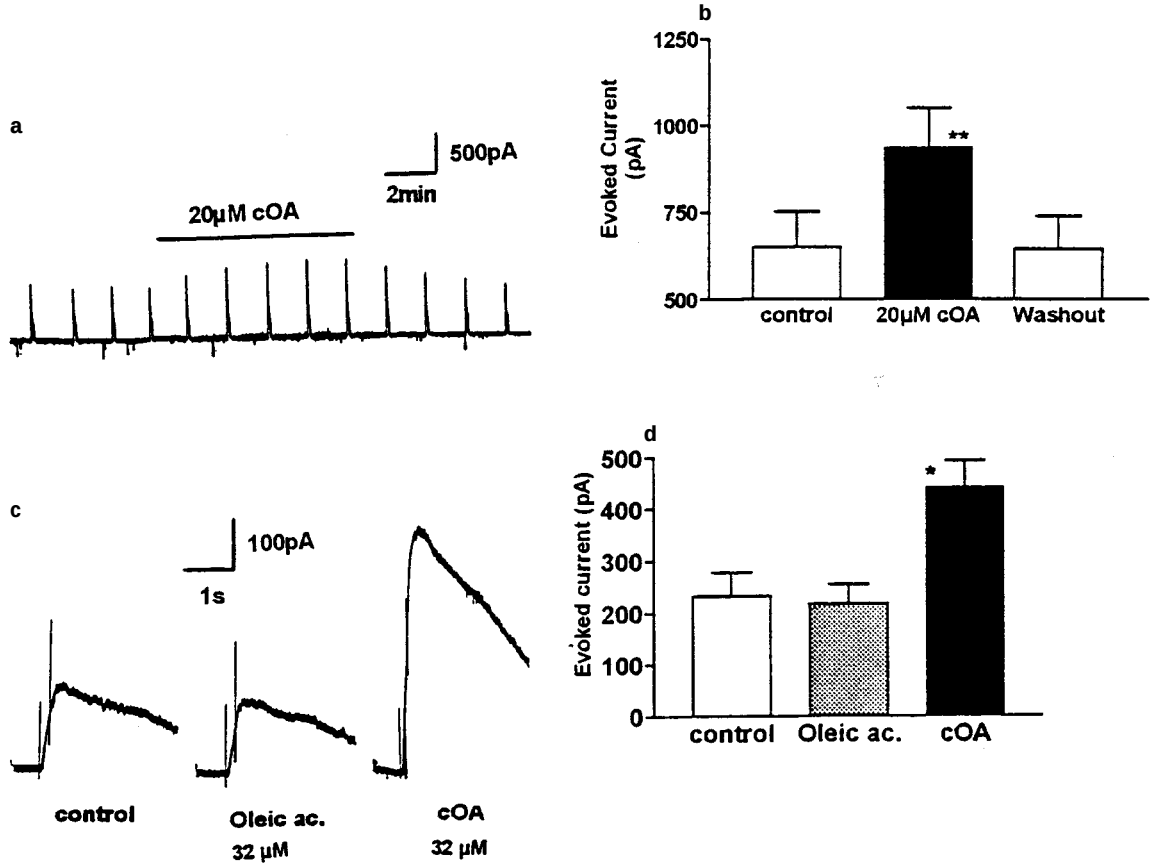

Figure 1 cOA, but not oleic acid, modulated responses to exogenous GABA. (a) Responses to 10 μM GABA expelled from a Y-tube for 5 s onto a neurone whole-cell clamped at -45 mV. 20 μM cOA (horizontal bar) reversibly enhanced peak outward current. Note that the modulatory action took 10–12 min to attain steady-state: all subsequent data reported at equilibrium. (b) Compounded data from 5 cells (exposed to 500 ms–5 s pulses of 10 μM GABA, V_h -45 mV) showing the peak GABA evoked-response in control saline or in the presence of 20 μM cOA. Data were analysed by repeated measures ANOVA: each column was compared with control data using Dunnett's *post*-test (see Motulsky, 1995; $^{**}P<0.01$). (c) Responses from a single cell, clamped at -45 mV, to 3.2 μM GABA (200 ms pulses) in control saline (left) and after 10 min exposure to 32 μM oleic acid (centre); and 32 μM cOA (right). (d) Data on peak responses from 5 identical experiments. The results were analysed by repeated measures ANOVA with Dunnett's *post* test ($^{*}P<0.05$). Oleic acid did not significantly alter the amplitude of the peak GABA-evoked current.

potentials in presynaptic cells: $n=6$, Figure 4a). Details of the pharmacology and reversal potentials of these events have been published (Lees & Leach, 1993). In our pilots, at −30 to −45 mV, an arbitrary concentration of 20 μM cOA marginally, but reversibly, reduced the frequency of these spontaneous synaptic currents (16 of 19 cells tested, Figure 4b). By logging and averaging (50–500 events) the outward synaptic currents in a further 8 neurones before, during and after treatment with the brain lipid (20 μM), a marked, reversible prolongation in the kinetics of inhibitory current decay was

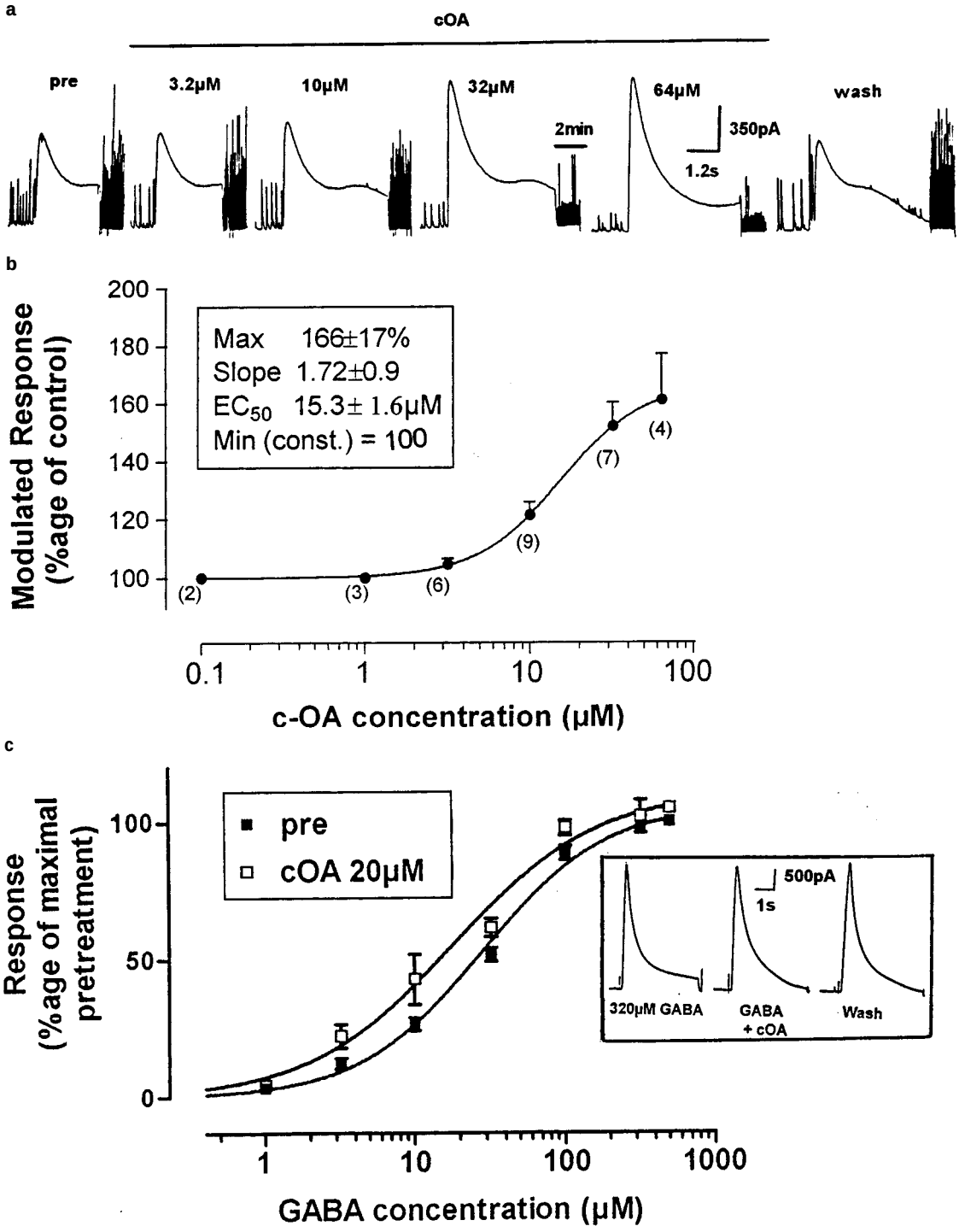

Figure 2 The actions of cOA were concentration-dependent and saturable: the affinity of GABA for its receptor was enhanced by the brain lipid. (a) Discontinuous chart records of responses to repeated challenge with 400 ms pulses of 3.2 μM GABA in the presence of the indicated concentration of cOA (steady-state response). The cell was clamped at −45 mV throughout. Note the different time bases and the marked reversible depressant effect of the higher concentrations on spontaneous multiquantal synaptic responses. Expanded data on left of each sweep: compressed data on right. (b) Data on peak currents (mean ± s.e. of mean) from the concentration-response experiments on cells clamped at −30 to −45 mV (numbers in parentheses denote the number of cells examined). (c) Normalized concentration-response curves to GABA in the absence and presence of 20 μM cOA (superfused for 10–12 min) in cells clamped at −30 mV. Data points represent mean and vertical lines ± s.e.mean ($n \geqslant 5$ per point). The inset shows a response to a high concentration of GABA before, during and after equilibration with cOA: at 20 μM cOA had no significant effect on the maximal response to GABA.

observed (Figure 4c and d). cOA did not significantly alter the amplitude of the averaged i.p.s.cs but enhanced total charge transfer ($P<0.05$, not shown) and increased time for 50% decay ($P<0.01$) and 90% decay ($P<0.005$) of spontaneous inhibitory currents.

cOA directly evoked inhibitory currents in a subpopulation of cells

In 3 of 46 cells exposed to 20 μM cOA (by superfusion) at -45 mV, a small reversible outward shift in current (mean 0.61 ± 0.2 pA/pF, not shown) was observed upon application of the compound: the mean response to a saturating concentration of exogenous GABA in these cells was almost two orders of magnitude greater at 48.9 ± 2.9 pA/pF (Figure 5). Because of the low incidence of directly evoked inhibitory currents the pharmacology and reversal potential have not yet been characterized and we have not quantified the incidence of these responses at higher concentrations.

High concentrations of cOA (but not oleic acid) indiscriminately suppress excitatory and inhibitory synaptic activity in the cultured networks

At higher concentrations, 32–64 μM, cOA depressed both the frequency and the amplitude of the spontaneous inhibitory and excitatory synaptic currents (Figure 6): this overt depressant effect on the cultured circuits was fully reversible with extensive washing over several minutes (see also Figure 2a). It is noteworthy that the kinetics of onset (6–12 min to attain steady state at 32 μM cOA) and recovery for this response were similar to those for the modulation of exogenous GABA in any given cell. Throughout this study, cOA responses equilibrated much more slowly than those seen with isoflurane (eg Edwards & Lees, 1997) or barbiturate anaesthetics (not shown) in equivalent experiments in our laboratory (the anaesthetics fully equilibrate in <1 min). Oleic acid at 32 μM was unable to mimic this effect in cOA responsive cells ($n=5$, Figure 6). The evoked release of GABA at central inhibitory synapses is thought to generate a transient peak concentration of transmitter approaching 1 mM (Clements, 1996). We studied the response of a saturating GABA concentration (1 mM) for modulation by high concentrations (50 μM) of cOA to seek a mechanism for the graded depression of i.p.s.c. amplitude. Even at this high concentration, cOA did not depress the maximal GABA currents ($n=3$, not shown) suggesting that depression of the i.p.s.cs did not reflect a postsynaptic change in receptor function.

Subunit-dependent modulation of recombinant human $GABA_A$ receptors was not sensitive to the specific benzodiazepine receptor antagonist flumazenil

The effects of depressant drugs on $GABA_A$ receptor function are notably subunit-dependent: e.g. benzodiazepines (Farrant & Cull-Candy, 1993; Mihic *et al*., 1994), loreclazole (Wingrove

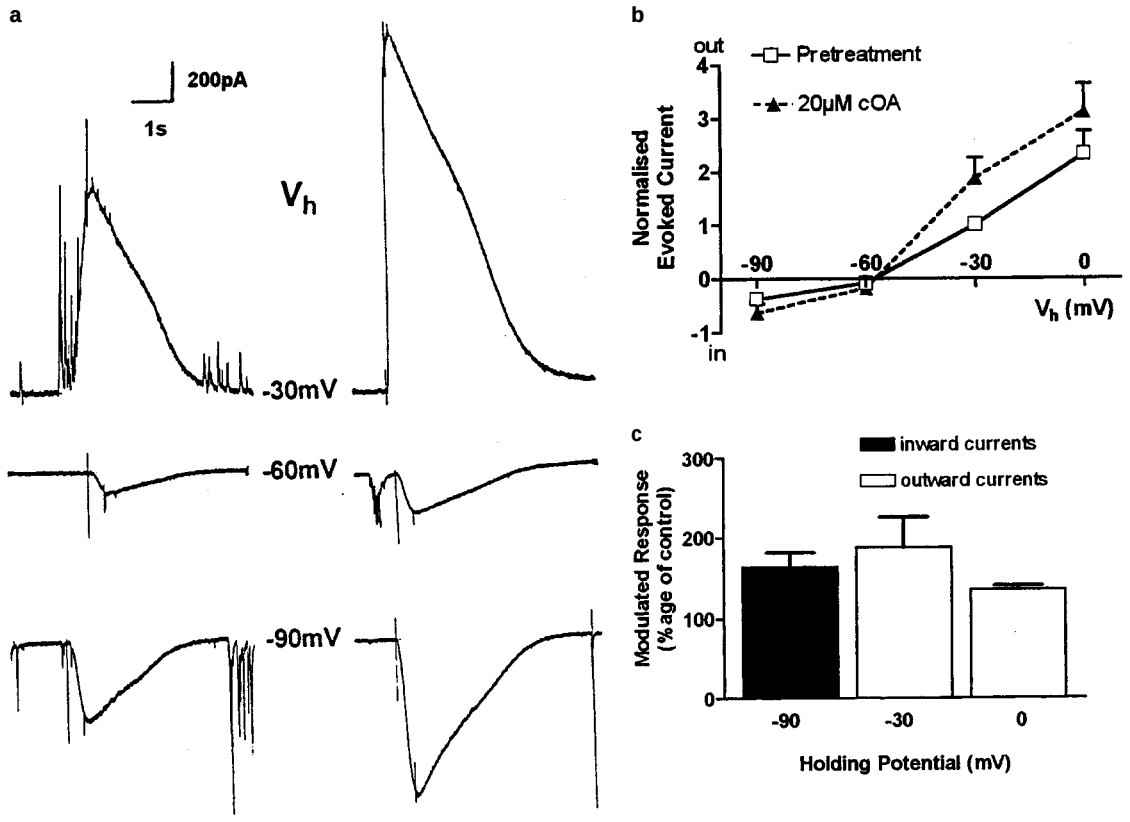

Figure 3 Modulatory actions of cOA were not voltage-dependent or secondary to shifts in E_{Cl}. (a) Pretreatment currents (left column) depicted alongside those evoked after equilibration with 20 μM cOA (right) at the indicated holding voltages from a representative single cell: 400 ms pulses of 3.2 μM GABA were used. (b) *I*/V curves derived from 4 identical experiments confirmed that inward and outward currents, normalized to pretreatment values for current at -30 mV, were enhanced by cOA but the reversal potential (approximating to E_{Cl}) was not significantly altered. Mean values were 57.8 ± 1.6 mV in control saline and 60.0 ± 1.3 mV in cOA. (c) Compounded data indicating that the effects of cOA were not voltage-dependent. This was confirmed by ANOVA ($P>0.05$). All data presented as mean$\pm$s.e. of mean.

et al., 1994) or ethanol which is more potent on a γ subunit splice variant (Wafford *et al*., 1991; Wafford & Whiting, 1992). Human $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2L}$ subunit combinations were injected into *Xenopus laevis* oocytes and characterized by two-electrode voltage clamp after 2–4 days. The apparent EC_{20} for GABA in any given batch of oocytes was determined and this was applied before, during and after cOA at 10 μM (a saturating dose of 3 mM was applied to all cells: if the test GABA concentration used was outwith the EC_{10-35}, in any single cell, the data generated was not included in the subsequent analysis). cOA enhanced the responses from $\alpha_1\beta_2\gamma_{2L}$ receptors in all 12 cells examined ($P<0.001$, paired two-tailed t test) but reduced GABA-evoked conductance in $\alpha_1\beta_2$ receptors ($P<0.01$, $n=9$) (Figure 7). Neither response was significantly

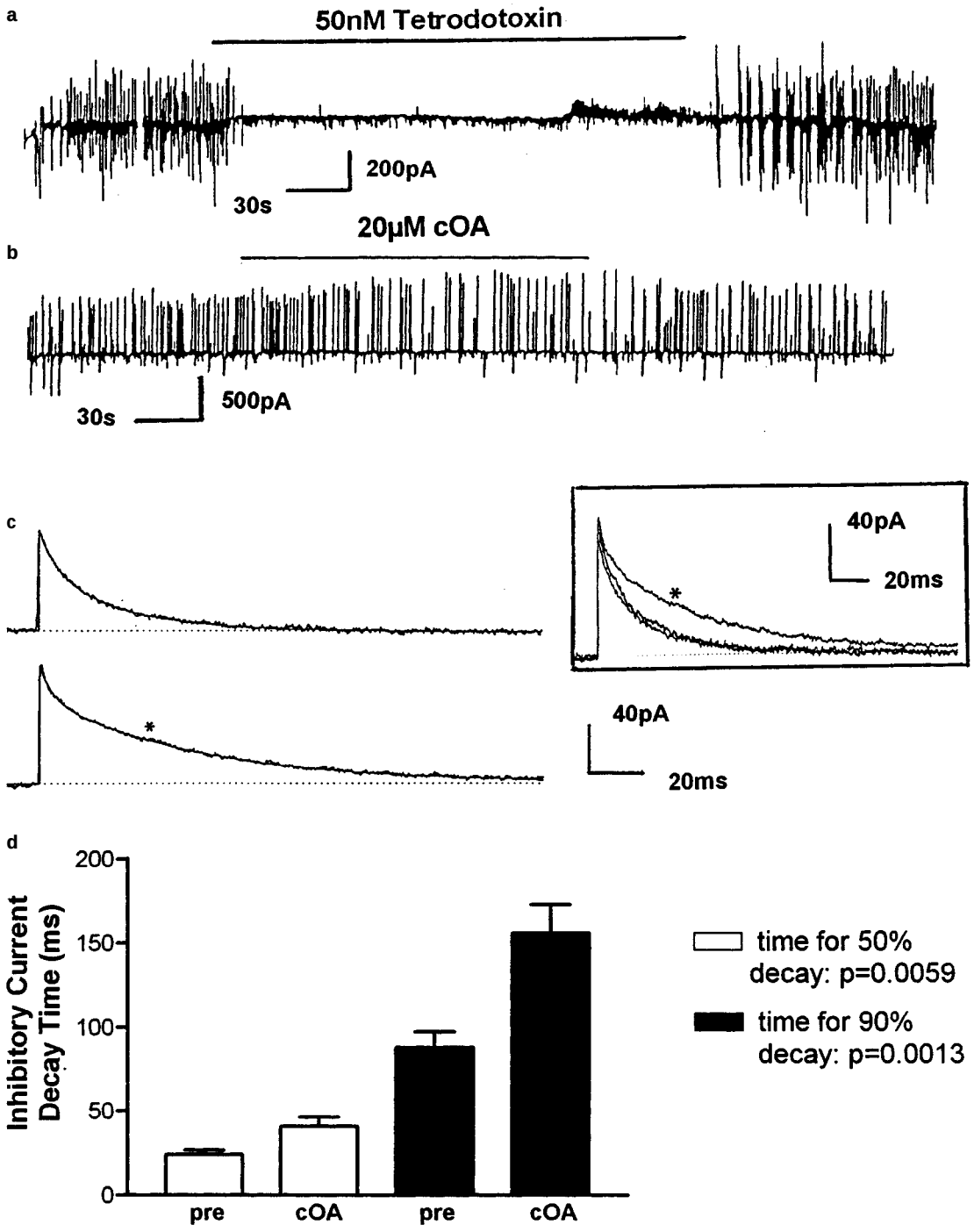

Figure 4 cOA modulated spontaneous inhibitory synaptic currents. (a) Effect of 50 nM tetrodotoxin on spontaneous synaptic currents (synaptic activity is observed in >80% of all cells impaled). Note the residual miniature post synaptic currents. Inhibitory currents (upwards deflections): excitatory currents (downward). (b) cOA 20 μM (bar) marginally reduced the frequency of synaptic currents. The apparent increase in i.p.s.c. amplitude can be attributed to the limited frequency response of the chart recorder and prolongation of the i.p.s.c. by cOA (below). (c) Average of 100 inhibitory synaptic currents (aligned at the mid-point of the rising phase) before and during (*) exposure to 20 μM cOA superimposed on bi-exponential fits to the decaying currents. Inset: traces superimposed with the response after washout demonstrated reversibility. In this and one other cell cOA selectively prolonged the slow component of decay (time constants were 5.8 ± 0.4 ms and 27.0 ± 0.7 ms pretreatment: 4.4 ± 0.3 ms and 56.4 ± 1.2 ms). In the other replicates both τ_{fast} and τ_{slow} were enhanced. The cells were exposed to cOA for at least 10 min before sampling data for kinetic analysis. (d) Compounded data from 8 replicated experiments; means$\pm$s.e.mean are shown.

altered by concurrent treatment with 1 μM flumazenil ($n=5$ for each subunit combination). In contrast, the positive modulatory effect of diazepam which was significant ($P<0.001$) only in $\alpha_1\beta_2\gamma_{2L}$ receptors, was completely blocked by the benzodiazepine antagonist flumazenil (EC_{50} *circa* 112 nM determined using cumulative application of 6 concentrations in 4 cells, not shown) at the same concentration (Figure 7).

Discussion

Cellular mode of action of fatty-acid ethanolamides

The data presented here are consistent with previous *in vitro* studies on cOA where a low-affinity antagonistic action on

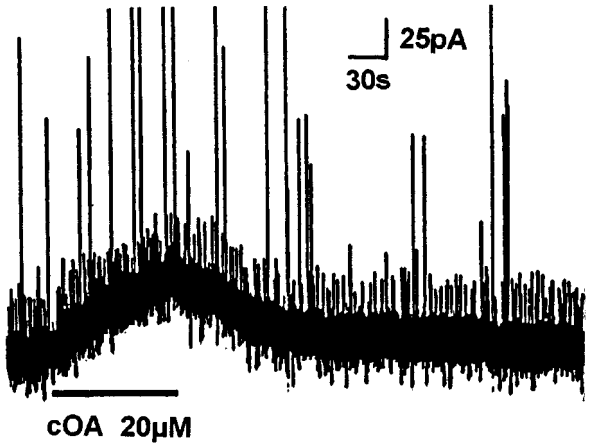

Figure 5 cOA directly activated small outward current shifts in a sub-population of neurones. 20 μM cOA (horizontal bar) directly-evoked outward currents from a holding potential of -45 mV in only 3 of 46 cells examined. Note the small size of these currents.

$GABA_A\alpha\beta$ receptors was reported (Huidobro-Toro & Harris, 1996). Crucially, these authors did not examine $\alpha_1\beta_2\gamma$ $GABA_A$ receptor isoforms: the most prevalent in mammalian CNS (McKernan & Whiting, 1996). Benzodiazepine sensitivity is conferred by the γ subunit in such heteromeric receptors. The enhancement of receptor function by cOA also clearly requires the co-expression of a γ subunit but was not blocked by the specific benzodiazepine antagonist. Huidoboro-Toro and Harris (1996) reported that cOA at nM concentrations (maximal at 100 nM) was able to enhance currents evoked by 5-$HT_{2a/c}$ receptors. The affinity of this modulatory response for the metabotropic 5-HT receptors is greater than two orders of magnitude higher than that found in our study for ionotropic GABA receptors. In spite of the high affinity and specificity, the functional significance of this result has yet to be demonstrated *in vivo* or at a systems level (see Cadogan *et al*., 1998). Because receptors sharing common second-messenger pathways were not affected by cOA, this earlier study concluded that either 5-HT_2 receptor desensitization was impaired or that activation of coupled G-proteins was promoted. More recent data suggest that G-proteins in rat brain are not activated by 0.1–1 mM cOA (Boring *et al*., 1996).

However, the relatively slow onset kinetics of cOA are consistent with a second-messenger coupled pathway or even cleavage of the molecule chemically or via a specific enzymic pathway (e.g. Maurelli *et al*., 1995; Sugiura *et al*., 1996). Both the biosynthetic (Cravatt *et al*., 1995) and amidohydrolytic enzymes for cOA are distributed in membrane fractions: no soluble activity was demonstrable in supernates from cell homogenates (Maurelli *et al*., 1995). Substrate diffusion into such sites may underpin the slow onset of observed responses. One potential breakdown product, ammonia (NH_4^+), has been shown to enhance $GABA_A$ currents in cortical neurones without shifting reversal potentials, by enhancing GABA affinity (Takahashi *et al*., 1993). The effects of ammonia qualitatively mimic those of cOA described here and were also flumazenil insensitive.

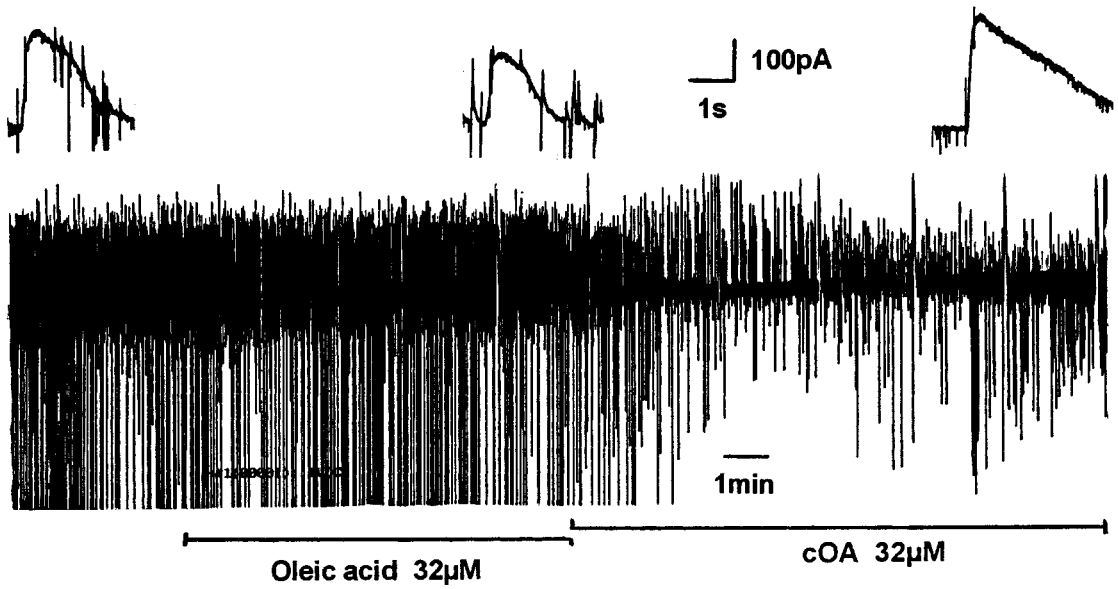

Figure 6 cOA at high concentrations, but not oleic acid, depressed the frequency and amplitude of both e.p.s.cs and i.p.s.cs in cultured networks. Compressed sweeps demonstrating that the incidence of spontaneous i.p.s.cs, e.p.s.cs and action currents (large downward deflections), in a cell clamped at -45 mV, were only depressed in frequency and/or amplitude by treatment with 32 μM cOA (upper bar) but not by 32 μM oleic acid (lower bar, $n=5$). The upper traces depict (at higher gain) the control response to a 400 ms pulse of 3.2 μM exogenous GABA (left), the steady-state response in oleic acid (centre) and the modulated response with cOA (right) in the same cell.

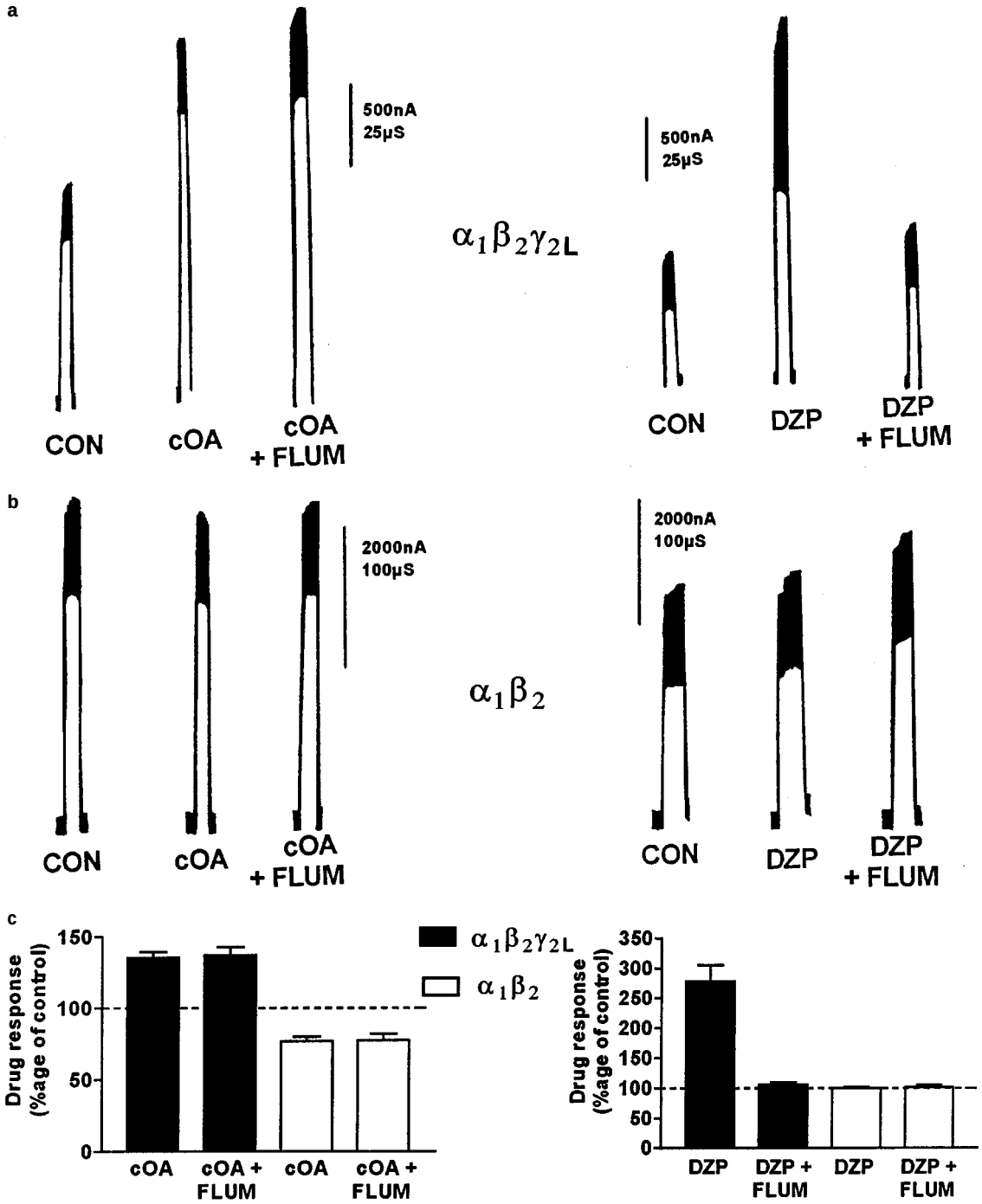

Figure 7 The actions of cOA on recombinant receptors in oocytes were subunit selective. All cells were clamped at −35 mV. Membrane conductance in response to brief hyperpolarizing steps (details in Methods) was used to quantify the evoked responses. (a) Representative effects of 10 μM cOA (left) and 333 nM diazepam (DZP, right) on the response to a fixed concentration of GABA (*circa* the EC_{20} for the indicated subunit combination). Note that both molecules markedly enhanced the evoked response with $\alpha_1\beta_2\gamma_{2L}$ receptors but that only DZP was antagonized by 1 μM flumazenil (Flum). (b) In identical experiments with $\alpha_1\beta_2$ receptors, DZP produced no significant effect. Both currents and conductance changes (difference between dense black bars at the top and bottom of each trace) evoked by GABA were marginally depressed by cOA. (c) Compounded data on conductance changes (normalized to pretreatment responses) from a total of 5 cells for each modulator. Neither the positive- or negative-modulatory effects of cOA were significantly attenuated by the benzodiazepine antagonist.

Specificity of cOA action, hydrophobic interactions and relevance to hypnogenic actions in vivo

The slow onset of the action of cOA may merely reflect the time for it to partition into, and equilibrate with, the plasma membrane. The molecule is highly lipophilic with a calculated logP of >6.5 (C.R. Ganellin, unpublished data) which necessitates the use of organic solvents. Here we used both ethanol (an acknowledged $GABA_A$ modulator: Wafford *et al.*, 1991) and DMSO and obtained a similar cOA-dependent modulation, making a synergistic interaction with either solvent unlikely. The effects we have described on $GABA_A$ receptor function could be non-selective, reflecting a hydrophobic 'membrane perturbing action'. However, oleic acid is equally hydrophobic (calculated logP of 7.5 C.R. Ganellin, unpublished data) and bears a conformationally flexible

unsaturated lipid chain but was unable to modulate the responses. Furthermore, in invariant oocyte membranes, the molecule showed a clear subunit/protein-dependence: more evidence in favour of a specific, receptor-mediated, response. The saturable nature of the response indicates a finite number of receptor sites for cOA (or limiting aqueous solubility). Other fatty-acid amides are increasingly emerging as important and selective modulators of neuronal function (Maurelli *et al*., 1995; Boring *et al*., 1996). A critical appraisal of the functional significance of our findings is difficult because of the lack of data on effective CSF concentrations of cOA in sleep-deprived or 'sleepy' animals. Cravatt *et al*. (1995) did not administer the compounds by normalization for bodyweight and did not indicate the sex, maturity or nutritional status of the rats used to demonstrate threshold for response or dose-dependent sleep times. Therefore, extracellular levels of synthetic cOA required for sleep induction can only be crudely extrapolated. Assuming that 350 g adults were used and that total and rapid distribution throughout the body was achieved for the >5 mg, i.p., doses (and that i.c.v. injections distributed evenly only throughout the CNS) then initial concentrations in brain could lie in the range of 6–60 μM (see Boring *et al*., 1996). The low-affinity effects described here are entirely consistent with many depressant molecules which interact directly with $GABA_A$ receptors. Before this could be accepted as a relevant causative mode of action, further studies are required (*in vivo*) to confirm both the efficacy of cOA (e.g. Dugovic *et al*., 1996; who failed to reproduce hypnotic actions) and to quantify accurately brain concentrations.

Analogies with neurodepressant drugs

Many depressant molecules are allosteric modulators of $GABA_A$ receptor function (Johnston, 1996): several of these are routinely exploited in clinical practice in the form of anxiolytics, sedatives, hypnotics and anaesthetics. Conversely the antagonism or blockade of such receptors by bicuculline, picrotoxin or halogenated insecticides leads to overt neuroexcitation in the form of epileptiform/convulsant symptoms in intoxicated animals (e.g. Lees & Calder, 1996). Our data are broadly consistent with the cellular actions of barbiturates (e.g. Macdonald *et al*., 1989), steroids (e.g.Cottrell *et al*., 1987) and inhalational anaesthetics (e.g. Jones & Harrison, 1993). All of these enhance GABA affinity, increase currents evoked by subsaturating agonist concentrations and increase the τ for decay of inhibitory synaptic currents and, at higher concentrations, can directly elicit inhibitory currents (Johnston, 1996; Sincoff *et al*., 1996). Benzodiazepines enhance exogenous currents by increasing the probability of channel opening (Barker & Owen, 1986) but also exert detectable effects on τ (eg Whittington *et al*., 1996). The hypnotic benzodiazepine zolpidem has been shown to prolong the decay of miniature i.p.s.cs in adult rat granule cells without affecting the peak amplitude (Soltesz & Mody, 1994): this modulatory action is developmentally regulated suggesting specific subunit-dependent interactions with the $GABA_A$ complex (Hollrigel & Soltesz, 1997). Interestingly many of these allosteric modulators result in bell-shaped dose-response profiles (eg Cottrell *et al*., 1987) to the extent that higher concentrations effectively block currents, possibly by interacting with a discrete site within the channel lumen (Rho *et al*., 1996; Edwards & Lees, 1997). Such a post-synaptic mechanism did not appear to underpin the apparent graded depression of both i.p.s.cs and e.p.s.cs noted at the higher concentrations of cOA (although the kinetics of GABA application in our experiments do not reflect the rapid and localized changes in the synaptic cleft). This effect on spontaneous synaptic traffic or action potentials is seen with other acknowledged $GABA_A$ effectors like pentobarbitone and inhalational anaesthetics, at or above clinical concentrations (Krnjevic, 1991; Lees unpublished observations). This may reflect the influence of enhanced inhibitory tone in polysynaptic pathways, tonic inhibition or summation of miniatures (as reported by Mody *et al*., 1991), or a discrete effect on presynaptic excitability or transmitter release (Foden *et al*., 1997). As oleic acid was unable to modulate $GABA_A$ receptors the depressant effect on synaptic traffic may indeed be a manifestation of enhanced inhibitory influences on the *in vitro* circuits.

Endogenous modulators of sleep and the $GABA_A$ receptor complex

Several compounds including interleukin-1 (Miller *et al*., 1990) δ-sleep-inducing peptide (Schoenberger, 1984) and prostaglandin (PG)D_2 (Ueno *et al*., 1982) have been suggested to play a role in sleep induction. Uridine and oxidized glutathione ('sleep promoting substance') have been proposed to facilitate GABAergic transmission and inhibit glutamatergic synapses respectively (Inoue *et al*., 1995). Melatonin and vitamin B12 (Ikeda *et al*., 1995) modulate circadian rhythms in general, not just sleep-wake cycles. Recent experiments have addressed the possibility that cholesterol and endogenous steroids interact with a single modulatory site on $GABA_A$ receptors: effects may reflect subunit composition (Bennett & Simmonds, 1995).

In summary, cOA may represent an endogenous humoral or, more likely, membrane-associated modulator of crucial and widespread inhibitory synapses/receptors. $GABA_A$ receptors appear to play a pivotal role in the circadian regulation of sleep by changing the excitability of neurones in the hypothalamic supra-chiasmatic nucleus (Wagner *et al*., 1997). cOA accumulation could conceivably amplify these diurnal trends. A greater understanding of the action of such endogenous modulators may facilitate the development of well-tolerated, non-addictive hypnotic drugs (Costa & Guidotti, 1996).

This work was supported by a Wellcome Trust grant to G.L. Thanks to Nigel Foden for preparing the cell-cultures and Jan Sadler for assistance with the manuscript. Merck Sharpe and Dohme (Neuroscience Research centre, Harlow, U.K.) and Roche (Basel, Switzerland) kindly supplied the subunit clones and flumazenil, respectively.

References

BARKER, J.L. & OWEN, D.G. (1986). Electrophysiological pharmacology of GABA and diazepam in cultured CNS neurons. In *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties,* pp. 135–165. New York: AR Liss.

BENNETT, P.J. & SIMMONDS, M.A. (1996). The influence of membrane cholesterol on the $GABA_A$ receptor. *Br. J. Pharmacol.,* **117,** 87–92.

BLOOM, F.E. & IVERSEN, L.L. (1971). Localizing [^{3}H]GABA in nerve terminals of cerebral cortex by electron microscopic autoradiography. *Nature* **229,** 628–630.

BORING, D.L., BERGLUND, B.A. & HOWLETT, A.C. (1996). Cerebrodiene, arachidonyl-ethanolamide, and hybrid structures: potential for interaction with brain cannabinoid receptors. *Prostaglandins, Leukotrienes and Essential Fatty-acids,* **55,** 207–210.

CADOGAN, C.A., FLETCHER, C., DEBANK, P., MARSDEN, C.A. & KENDALL, D.A. (1998). Differences in the *in vivo* effects of the endogenous sleep-inducing agent oleamide on $5HT_2$ receptor function. *Br. J. Pharmacol. Proc. Suppl.*

CLEMENTS, J.D. (1996). Transmitter timecourse in the synaptic cleft: it's role in central synaptic function. *Trends Neurosci.,* **19,** 163–171.

COSTA, E. & GUIDOTTI, A. (1996). Benzodiazepines on trial: a research strategy for their rehabilitation. *Trends Pharmacol. Sci.,* **17,** 192–200.

COTTRELL, G.A., LAMBERT, J.J. & PETERS, J.A. (1987). Modulation of $GABA_A$ receptor activity by alphaxalone. *Br. J. Pharmacol.,* **90,** 491–500.

CRAVATT, B.F., PROSPERO-GARCIA, O., SIUZDAK, G., GILULA, N.B., HENRIKSON, S.J., BOGER, D.L. & LERNER, R.A. (1995). Chemical characterisation of a family of brain lipids that induce sleep. *Science,* **268,** 1506–1509.

DUGOVIC, C., VAN DEN BROECK, W. & CLINCKE, G. (1996). Effects of cis-9,10-octadecenoamide on sleep-wakefulness states and EEG power spectra in rats. *J. Sleep. Res.,* **5** (Supplement 1) **54,** (P107).

EDWARDS, M.D. & LEES, G. (1997). Modulation of a recombinant invertebrate γ-aminobutyric acid receptor-chloride channel complex by isoflurane: effects of a point mutation in the M2 domain. *Br. J. Pharmacol.,* **122,** 726–732.

FARRANT, F. & CULL-CANDY, S. (1993). GABA receptors, granule cells and genes. *Nature,* **361,** 302–303.

FODEN, N.D. & LEES, G. (1996). Chelation of intracellular calcium in rat cortical neurons: effects on $GABA_A$ receptor function. *J. Physiol.,* **494P,** 56P.

FODEN, N.D., LEES, G. & LI, Y. (1997). The volatile anaesthetic isoflurane supresses release of transmitters. *Soc. for Neurosci.,* abstract 23, 88.8 (New Orleans, Oct).

HARRIS, R.A., MIHIC, J.S., DILDY-MAYFIELD, J.E. & MACHU, T.K. (1995). Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition. *FASEB. J.,* **9,** 1454–1462.

HOLLRIGEL, G.S. & SOLTESZ, I. (1997). Slow kinetics of miniature IPSCs during early postnatal development in granule cells of the dentate gyrus. *J. Neurosci.,* **17,** 5119–5128.

HUIDOBRO-TORO, J.P. & HARRIS, R.A. (1996). Brain lipids that induce sleep are novel modulators of 5-hydroxytryptamine receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 8078–8082.

HUNKELER, W., MOHLER, M., PIERI, L., DOLE, P., BONETTI, E.P., CUMIN, R., SCHAFFNER, R. & HAFELY, W. (1981). Selective antagonists of benzodiazepines. *Nature,* **290,** 514–516.

IKEDA, M., HONDA, K. & INOUE, S. (1995). Circadian time-dependent modulation of sleep and brain temperature by methylcobalamine etc. *Biological Rythm Res.,* **26,** 521–531.

INOUE, S., HONDA, K. & KOMODA, Y. (1995). Sleep as neuronal detoxification and restitution. *Behavioural Brain Res.,* **69,** 91–96.

JOHNSTON, G.A.R. (1996). $GABA_A$ receptor pharmacology. *Pharmacol. Ther.,* **69,** 173–198.

JONES, M.V. & HARRISON, N.L. (1993). Effects of volatile anaesthetics on the kinetics of inhibitory postsynaptic currents in cultured rat hippocampal neurons. *J. Neurophysiol.,* **70,** 1339–1349.

KRNJEVIC, K. (1991). Cellular mechanisms of anesthesia. *Ann. New York. Acad. Sci.,* **625,** 155–173.

LEES, G. & CALDER, J.A.D. (1996). Interactions of lindane isomers with chloride currents in insect membranes: steric requirements for channel modulation and block. *Pest Biochem. Physiol.,* **55,** 40–48.

LEES, G. & LEACH, M.J. (1993). Studies on the mechanism of action of the novel anticonvulsant lamotrigine (lamictal) using primary neuroglial cultures from rat cortex. *Brain Res.,* **612,** 190–199.

MACDONALD, R.L., ROGERS, C.J. & TWYMAN, R.E. (1989). Barbiturate regulation of kinetic properties of the $GABA_A$ receptor channel of mouse spinal neurons in culture. *J. Physiol.,* **417,** 483–500.

MAURELLI, S., BISOGNO, T., DE PETROCELLIS, L., DI LUCCIA, A., MARINO, G. & DI MARZO, V. (1995). Two novel classes of neuroactive fatty-acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. *FEBS Lett.,* **377,** 82–86.

MCKERNAN, R.M. & WHITING, P.J. (1996). Which $GABA_A$ receptor subtypes really occur in the brain? *Trends Neurosci,* **19,** 139–143.

MIHIC, S.J., WHITING, P.J., KLEIN, R.L., WAFFORD, K.A. & HARRIS, R.A. (1994). A single amino acid of the human GABA type A receptor gamma2 subunit determines benzodiazepine efficacy. *J. Biol. Chem.,* **269,** 32768–32773.

MILLER, L.G., GALPERN, W.R., DUNLAP, K. DINARELLO, C.A. & TURNER, T.J. (1990). Interleukin-1 augments γ-aminobutyric $acid_A$ receptor function. *Mol. Pharmacol.,* **39,** 105–108.

MODY, I., TANELIAN, D.L. & MACIVER, M.B. (1991). Halothane enhances tonic neuronal inhibition by elevating intracellular calcium. *Brain Res.,* **538,** 319–323.

MOTULSKY, H. (1995). In *Intuitive Biostatistics.* pp. 258–261. New York/Oxford: Oxford University Press.

MURASE, K., RYU, P.D. & RANDIC, M. (1989). Excitatory and inhibitory amino acids and peptide-induced responses in acutely dissociated dorsal horn neurons. *Neurosci. Lett.,* **103,** 56–63.

RHO, J.M., DONEVAN, S.D. & ROGOWSKI, M.A. (1996). Direct activation of $GABA_A$ receptors by barbiturates in cultured rat hippocampal neurons. *J. Physiol.,* **497,** 509–522.

SCHOENENBERGER, G.A. (1984). Characterization, properties and multivariate functions of delta-sleep inducing peptide (DSIP). *Eur. Neurol.,* **23,** 321–345.

SINCOFF, R., TANGUY, J., HAMILTON, B., CARTER, D., BRUNNER, E.A. & YEH, J.Z. (1996). Halothane acts as a partial agonist of the $\alpha_6\beta_2\gamma_{2S}$ $GABA_A$ receptor. *FASEB J.,* **10,** 1539–1545.

SOLTESZ, I. & MODY, I. (1994). Patch-clamp recordings reveal powerful GABAergic inhibition in dentate hilar neurons. *J. Neurosci.,* **14,** 2365–2376.

SUGIURA, T., KONDO, S., KODAKA, T., TONEGAWA, T., NAKANE, S., YAMASHITA, A., ISHIMA, Y. & WAKU, K. (1996). Enzymatic synthesis of oleamide (*cis*- 9,10-octadecenoamide), an endogenous sleep inducing lipid, by rat brain microsomes. *Biochem. Mol. Biol. Int.,* **40,** 931–938.

TANELIAN, D.L., KOSEK, P., MODY, I. & MACIVER, M.B. (1993). The role of the $GABA_A$ receptor/chloride channel complex in anesthesia. *Anesthesiology,* **78,** 757–776.

TAKAHASHI, K., KAMEDA, H., KATAOKA, M., SANJOU, K., HARATA, N. & AKAIKE, N. (1993). Ammonia potentiates $GABA_A$ response in dissociated rat cortical neurons. *Neurosci. Lett.,* **151,** 51–54.

UENO, R., ISHIKAWA, Y., NAKAYAMA, T. & HAYAISHI. O. (1982). Prostoglandin D_2 induces sleep when microinjected into the preoptic area of conscious rats. *Biochem. Biophys. Res. Commun.,* **109,** 576–582.

WAFFORD, K.A., BURNETT, D.M., LEIDENHEIMER, N.J., BURT, D.R., WANG, J.B., KOFUJI, P., DUNWIDDIE, T.V., HARRIS, R.A. & SIKELA, J.M. (1991). Ethanol sensitivity of the $GABA_A$ receptor expressed in *Xenopus* oocytes requires 8 amino acids contained in the $gamma_{2L}$ subunit. *Neuron,* **7,** 27–33.

WAFFFORD, K.A. & WHITING, P.J. (1992). Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the $gamma_2$ subunit. *FEBS Lett.,* **313,** 113–117.

WAGNER, S., CASTEL, M., GAINER, H. & YAROM, S. (1997). GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. *Nature,* **387,** 598–603.

WINGROVE, P.B., WAFFORD, K.A., BAIN, C. & WHITING, P.J. (1994). The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β_2 and β_3 subunit. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 4569–4573.

WHITTINGTON, M., JEFFERYS, J.G.R. & TRAUB, R.D. (1996). Effects of intravenous anaesthetic agents on fast inhibitory oscillations in the rat hippocampus *in vitro*. *Br. J. Pharmacol.,* **118,** 1977–1987.

(Received December 23, 1997
Revised March 25, 1998
Accepted March 31, 1998)