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Electrophysiological studies on oxindole, a neurodepressant tryptophan metabolite

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1 The aim of the present work was to investigate the electrophysiological effects of oxindole, a tryptophan metabolite present in rat blood and brain, and recently proposed as a contributing factor in the pathogenesis of hepatic encephalopathy.

2 Using rat hippocampal slices *in vitro* and extra- or intracellular recordings, we evaluated oxindole effects on the neurotransmission of the CA1 region following orthodromic stimulation of the Schaffer collaterals.

3 Oxindole (0.3–3 mM) decreased the amplitude of population spikes extracellularly recorded at the somatic level and of the fEPSPs recorded at the dendritic level. In intracellular recordings, oxindole (0.1–3 mM) did not affect the resting membrane potential or the neuronal input resistance, but reduced the probability of firing action potentials upon either synaptic or direct activation of the pyramidal cells.

4 Oxindole (0.3–3 mM) increased the threshold and the latency of firing action potentials elicited by depolarizing steps without changing the duration or the peak amplitude of the spikes. It also significantly increased the spike frequency adaptation induced by long lasting (400 ms) depolarizing stimuli.

5 In separate experiments, performed by measuring AMPA or NMDA-induced responses in cortical slices, oxindole (1–3 mM) did not modify glutamate receptor agonist responses.

6 Our results show that concentrations of oxindole which may be reached in pathological conditions, significantly decrease neuronal excitability by modifying the threshold of action potential generation.

Keywords: Oxindole; hippocampus; Na^+ channels; synaptic potentials; tryptophan metabolism; CA1

Introduction

In the course of experiments performed to study the neurochemical effects of inhibitors of kynurenine metabolism (Chiarugi *et al*., 1995; Carpenedo *et al*., 1994) we obtained data suggesting that tryptophan may be metabolized into oxindole and that oxindole is physiologically present in blood and other biological fluids in concentrations ranging from 0.1–1 μM (Carpenedo *et al*., 1997).

Systemic administration of oxindole to rats, dogs or humans has been shown to cause profound sedation, decrease in blood pressure, decrease in muscular tone and loss of consciousness (Orcutt *et al*., 1964). Since rats treated with oxindole or having acute liver impairment share several common signs and since it is widely accepted that tryptophan metabolites are involved in the neurological signs associated to liver damage (Bengtsson & Bergqvist, 1996; Bengtsson *et al*., 1991), we measured brain oxindole content in rat models of acute liver impairment. As expected, we found a very large increase (10–100 fold) in blood and brain oxindole content (Moroni *et al*., 1997; Carpenedo *et al*., 1998) thus suggesting that the compound could play an important role in the neurological symptoms associated with liver failure.

Large increases in oxindole concentration have also been found in urine samples obtained from a subpopulation of patients affected by phenylchetonuria, a hereditary disorder of amino acid metabolism. Although the biochemical mechanisms responsible for such increases have not been studied, it has been suggested that the compound could have a role in the pathophysiology of the syndrome (Kochen *et al*., 1972). It seems, therefore, that oxindole is a neuroactive metabolite which should be added to the already long list of compounds originating from tryptophan and able to affect brain function in physiology or in pathology (Allegri Filippini *et al*., 1997; Stone, 1993).

The mechanism of the neurodepressant action of oxindole has not been previously studied. In prior experiments, we found that it does not displace the binding of [^{3}H]-GABA to either $GABA_A$ or $GABA_B$ recognition sites in brain membranes, [^{3}H]-flunitrazepam to the benzodiazepine recognition sites and [^{3}H]-glycine or [^{3}H]-glutamate to the NMDA receptor complex (Moroni *et al*., 1997).

In the present study, we attempted to obtain quantitative information on the concentrations of brain oxindole which are able to cause sedation or coma and to investigate the mechanism(s) of its actions. In pursuing this goal, we attempted to correlate, in rats, brain oxindole concentration with overall behaviour, and we studied oxindole effects on: (i) the electrically evoked synaptic potentials and cell excitability in the CA1 region of rat hippocampal slices using both extracellular and intracellular recordings and (ii) the responses to ionotrophic glutamate receptor agonists in cortical wedge preparations (Harrison & Simmonds, 1985).

Methods

Extraction and quantification of oxindole

Details on mass-spectrometric identification of oxindole have been previously published (Carpenedo *et al*., 1997). In most of

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the present experiments, approximately 1 g of tissue (or 1 ml of blood) was homogenized in 2 vol of 0.4 N $HClO_4$ (or 1 N $HClO_4$ for the blood). The mixture was centrifuged at $18{,}000 \times g$ for 20 min and the supernatant was collected. The procedure was repeated twice. The supernatants were then mixed with 8 ml of chloroform and agitated for 5 min. The chloroform layers were collected and evaporated under a stream of nitrogen. The residues were resuspended in 200 μl of 0.4 N $HClO_4$ and aliquots were injected into the HPLC apparatus which consisted of a Perkin-Elmer LC pump (Model 250), a syringe-loading sample injection valve (Rheodyne Model 7125), a C_{18} reverse-phase precolumn filter (0.5 cm long; Waters, Milford, MA, U.S.A.), and a 25-cm reverse-phase 18 SpheriSorb ODS-2 10 U column (Alltech, Deerfield, IL, U.S.A.). The detection was performed with a spectrophotometer (Perkin-Elmer Model LC90UV). The mobile phases were: 0.05 M acetate buffer (pH 3.24) and acetonitrile (15%). The recovery of a known amount of oxindole passed through the entire procedure was $75 \pm 2\%$ (mean $\pm$ s.e.mean of ten determinations).

Evaluation of the spontaneous locomotor activity

Spontaneous locomotor activity of rats was evaluated using an Animex activity meter (mod. LKB FARAD). The number of interruptions of photocells was monitored by individually placing the rats in clean cages for 20 min.

Preparation of hippocampal slices

Experiments were carried out using *in vitro* hippocampal slices as previously described (Pugliese *et al*., 1996). Male Wistar rats (100–200 g body weight, Charles River, Como, Italy) were anaesthetized with ether and decapitated. The hippocampi were rapidly removed and placed in ice cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1.4, $CaCl_2$ 2, $NaHCO_3$ 25, D-glucose 10 (pH 7.4). Slices (400 μm thick) were cut from the hippocampus using a McIlwain (Gomshall, U.K.) tissue chopper and kept in oxygenated aCSF for at least 1 h at room temperature (20–23°C). A single slice was then placed on nylon mesh and completely submerged in a small chamber and perfused with oxygenated aCSF (30–32°C) at a constant flow rate of 2–3 ml min^{-1}. Drugs were administered through a three-way tap and a complete exchange of the chamber volume occurred in 1 min.

Extracellular recordings

Test pulses (80–110 μs duration; 0.017–0.05 Hz) were delivered through bipolar nichrome electrodes positioned in the CA1 stratum radiatum. Evoked extracellular potentials were recorded with glass microelectrodes (2–10 MΩ) filled with 3 M NaCl, either from the pyramidal cell layer of CA1 (population spike, PS) or from stratum radiatum (field excitatory postsynaptic potential, fEPSP) in the CA1 area. Responses were amplified (Neurolog NL 104, Digitimer Ltd), displayed on a computer video, digitized (sample rate 33 kHz), and stored on floppy disks for later analysis using the software pClamp 6 (Axon Instruments Ltd.).

Stimulus-response relationships were investigated at the beginning of each experiment, and at defined times during the experiment by gradually increasing the stimulus strength when required. The test stimulus pulse was adjusted to produce a population spike whose amplitude was 40–50% (2–3 mV) of the maximum and, unless otherwise stated, was kept constant throughout the experiment. After 30 min of equilibration, a control period (10–15 min) was recorded and used to generate baseline values before experimental tests.

In the recordings from the pyramidal cell layer, the population spike of the evoked responses was measured as the peak-to-peak amplitude of the first negative phase of the population spike, whereas the slope of the positive-going phase of the somatic response was used to estimate excitatory synaptic input (Pugliese *et al*., 1994). In recordings from the stratum radiatum, the amplitude of both fEPSP and afferent volley were measured. The initial slope of fEPSP was also measured but since the changes induced by drugs were qualitatively consistent, only the measurements of the amplitude are here reported. Parameters were evaluated before, during and after drug treatment. Oxindole was applied for 10 min.

Intracellular recordings

CA1 pyramidal neurones were recorded in current-clamp mode with 3 M KCl-filled electrodes (35–50 MΩ). Electrical signals were amplified with an Axoclamp 2A (Axon Instruments, Foster City, U.S.A.) and displayed on an oscilloscope and chart recorder (2800 Gould, Valley View, U.S.A.). Traces were stored on a digital tape (DTR 1200, BioLogic, Claix, France; sampling frequency 48 kHz) and on a computer using pClamp6 software (Axon Instruments) for off-line analysis. Several criteria were used to select cells for the experiments: stable resting membrane potential (r.m.p.) of at least -60 mV and no spontaneous firing of action potentials; no sudden drops in the neuronal input resistance (R_{in}), indicating cell damage; constant amplitude of the action potential (>80 mV), obtained by direct activation of the cell. Modifications in synaptic efficacy by drugs were evaluated by measuring the changes in amplitude of the evoked EPSP and in probability of firing an action potential upon stimulation of afferent pathway. Stimulus response curves were constructed at the beginning and during the experiment by gradually increasing the stimulus strength.

To evaluate the effects of oxindole on EPSPs not contaminated by the early phase of GABA-mediated IPSPs, most of the experiments were carried out in the presence of a $GABA_A$ receptor blocker (bicuculline 10 μM or picrotoxin 25 μM). To keep neurones below firing threshold when investigating stimulus response relationships, cells were hyperpolarized relative to their r.m.p. by injecting a constant negative current (range from $-$ 50 to -200 pA) through the recording electrode. Since preliminary experiments ($n = 3$) and *post-hoc* analysis of results indicated that the presence of $GABA_A$ receptor blockers and/or hyperpolarization of the neurones did not change cell responses to oxindole, data obtained in the presence of $GABA_A$ receptor blockers were pooled with those in aCSF.

Probability of firing (p.f.) upon synaptic or direct depolarization of the neurone was calculated on five or ten responses. To study the effects of oxindole on cell discharge, direct activation of the impaled neurone was obtained by injection of short (40 ms, to elicit a probability of firing from 0.8–1 in control conditions) or long (400 ms, for action potential frequency adaptation) depolarizing current steps through the recording electrode. The intensity of the depolarizing current step was kept constant during the experiment. The slow afterhyperpolarization (AHP) which follows repetitive firing elicited by injection of depolarizing currents (from $+300$ to $+500$ pA, 400 ms) through the

recording electrode was measured either at the peak (peak AHP) or 150 ms (late AHP) after cessation of the depolarizing step. A series of current steps (400 ms, range from −900 to +500 pA) were injected to monitor cell R_{in} and to construct I/V plots.

Preparation of cortical wedges

The cortical wedge preparation described by Harrison and Simmonds (Harrison & Simmonds, 1985) and modified by Burton *et al*. (Burton *et al*., 1988) was used as previously described (Carlà & Moroni, 1992). Briefly, wedges obtained from white swiss mice (male 15/25 g) were placed in a two-compartment bath and silicone grease was placed between the two portions of the bath. The wedges were incubated at room temperature and perfused with Krebs solution (mM: NaCl 135; $CaCl_2$ 2.4; KH_2PO_4 1.3; $MgCl_2$ 1.2; $NaHCO_3$ 16.3 and glucose 7.7), gassed with 95% O_2 and 5% CO_2 at a flow rate of 2 ml min^{-1}. After stabilization, the gray matter was perfused with a Mg^{2+} free medium. NMDA and AMPA were repeatedly applied for 2 min every 15 min whereas the other compounds between the two compartments were monitored *via* Ag/AgCl electrodes and displayed on a chart recorder. The preparations were initially stabilized by repeated application of 10 μM NMDA, a concentration which gave a sub-maximal response but which did not significantly reduce the response of subsequent applications of the agonist (Moroni *et al*., 1995; Mannaioni *et al*., 1994).

Materials

Oxindole, obtained from Aldrich Sigma (Milan, Italy), was dissolved in DMSO. The maximal concentrations of DMSO used in electrophysiological studies was 0.003% (V/V) and had no measurable effects on evoked potentials. Picrotoxin was from RBI (Natick, MA, U.S.A.); (−)bicuculline methiodide was from Sigma-Aldrich (Milan, Italy); NMDA and AMPA were from Tocris (Bristol, U.K.). Solvents (acetonitrile and chloroform) were from Merck (Darmstadt, Germany).

Data analysis

All numberical data are expressed as means±s.e.mean. Data were analysed statistically by Student's *t*-test or by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

Brain oxidole content and behaviour

We previously showed that rat brain oxindole content is 0.05±0.01 nmol g^{-1} (Carpenedo *et al*., 1997). One h after intraperitoneal administration of the compound at 10 or 100 mg kg^{-1}, oxindole brain content was 8.1±1.7 or 103±15 nmol g^{-1}, respectively. When the lower dose was administered (10 mg kg^{-1}, i.p.), the rats were calm, easy to manage and had reduced locomotor activity for at least 30 min. After the larger dose (100 mg kg^{-1}, i.p.), the animals went into coma, the righting reflex was absent, muscular tone was clearly reduced (Table 1) and most of them died approximately 2 h later.

Table 1 Effects of oxindole administration on locomotor activity and brain oxindole content

	Brain concentration (nmol g^{-1})	*Locomotor activity* (counts/ 20 min)	*Righting reflex*
Saline	0.05±0.01	580±15	normal
Oxindole 10 mg kg^{-1}	8.1±1.7	387±42*	normal
Oxindole 100 mg kg^{-1}	103±15	5±2**	absent

In order to measure locomotor activity, oxindole was injected 20 min before placing the animals in clean cages. The animals were killed 1 h after treatment. Doses are mg kg^{-1} i.p. Values are means±s.e.mean of at least six animals; *$P<0.05$ vs saline; **$P<0.01$ vs saline (ANOVA and Dunnett's *t*-test).

Extracellular recordings in rat hippocampal slices

In a first series of experiments, we tested the effects of several concentrations (0.3, 1 and 3 mM, 10 min application) of oxindole on evoked synaptic potentials recorded extracellularly from the CA1 region. Oxindole decreased the amplitude of somatic potentials in a concentration-dependent manner. The time-course of the effects of the drug in a typical experiment in which three concentrations of oxindole were tested is shown in Figure 1. The decrease in amplitude of synaptic responses had a rapid onset (<2 min) and was completely reversible within 15 min of drug washout (see Figures 1b and 3a). In most of the experiments, a transient rebound increase in the amplitude of the population spike was observed during washout (see Figures 1b and 3a). As shown in Figure 1a, the application of oxindole affected mainly the population spike component of evoked synaptic responses (i.e. the compound action potential generated by synchronous discharge of CA1 pyramidal cells). When oxindole was used at 1 mM, the population spike amplitude decreased by 40±10% ($n=5$, $P<0.05$) and at 3 mM, decreased by 85±9% ($n=5$, $P<0.05$). Figure 2 shows the results of a typical experiment in which the evoked fEPSP was recorded in the dendritic region of CA1 pyramidal cells. In these experiments, the oxindole-induced decrease in the fEPSP amplitude was statistically significant only at 3 mM, a concentration which also decreased the afferent volley amplitude (from 117±45 to 80±37 μV; $P<0.05$). When the effects of oxindole (1 and 3 mM) were evaluated in the stimulus-response relationship, the curves were displaced towards the right, but the maximal responses were not decreased (see Figure 2b).

Intracellular recording from pyramidal CA1 neurones

Intracellular recordings were carried out in 18 slices taken from 18 rats. Neurones ($n=18$) had a mean r.m.p. of −64±1 mV, R_{in} of 45±6 MΩ and an amplitude of action potentials greater than 80 mV (89±2 mV).

We studied the effects of oxindole (0.1–3 mM) on r.m.p., R_{in}, synaptic responses and cell excitability of pyramidal cells. Oxindole concentrations of up to 3 mM did not significantly affect cell r.m.p. or R_{in} (see Table 2).

Effects of oxindole on EPSPs and probability of firing an action potential upon synaptic stimulation

Figure 4 shows the effects of oxindole on EPSP amplitude and on the probability of firing an action potential upon synaptic activation of pyramidal cells. Stimulation of the

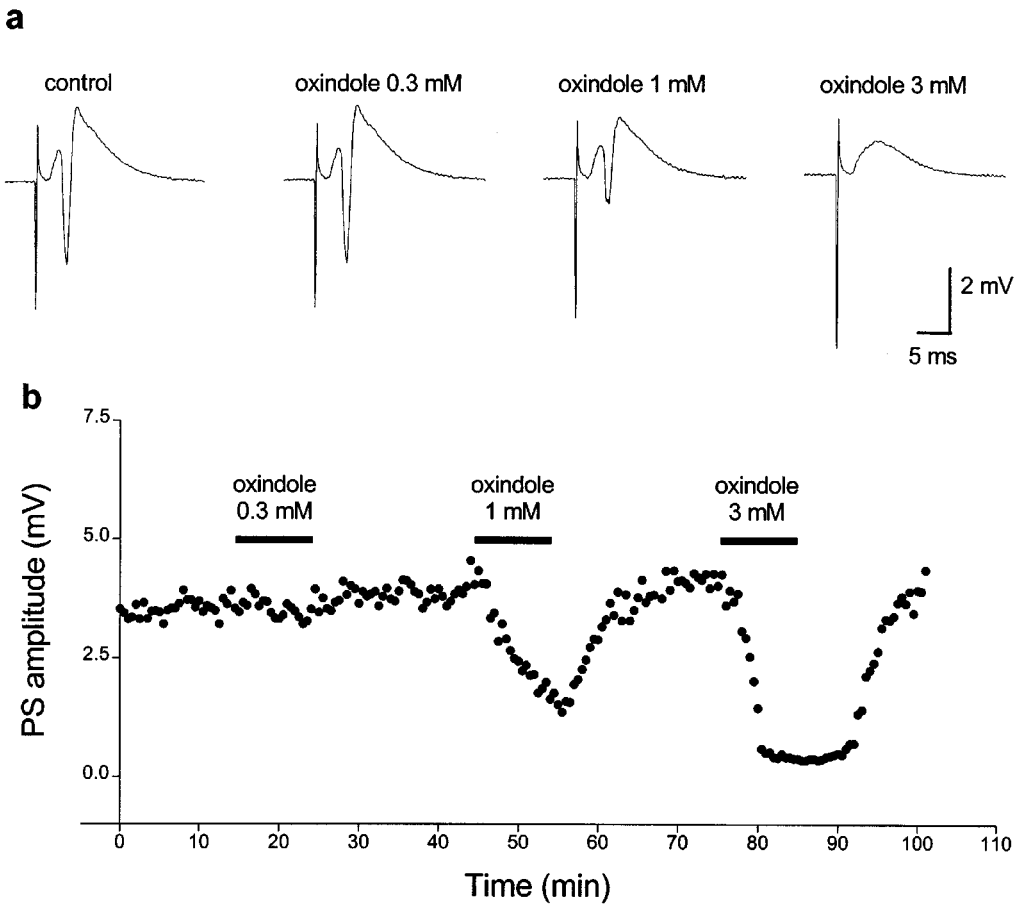

Figure 1 Effects of oxindole on the amplitude of somatic field potentials in the CA1 hippocampal region. (a) Superfusion of oxindole decreased the amplitude of the somatic potentials in a concentration-dependent manner. Traces are single responses evoked by constant strength (14 V) stimuli (biphasic fast transients) in control and at the end of drug application. (b) Time-course of the population spike (PS) amplitude in a typical experiment before and after drug application. Points represent the amplitude of the population spike expressed in mV. Oxindole reversibly decreased the amplitude of the somatic potential. Filled bars indicate the time of drug applications.

CA1 stratum radiatum evoked EPSPs which, according to the stimulus strength, eventually reached the threshold for cell discharge (Figure 4a,b). Application of oxindole (0.3 mM) significantly decreased the probability of cell firing from 0.4 to 0 ($n=4$). By increasing the stimulus strength, it was possible to elicit action potentials whose amplitude was unchanged in comparison with the controls. The stimulus-response relationships shown in Figure 4a demonstrate that the p.f. decrease was not due to a reduction in the amplitude of the EPSP. This is further illustrated by Figure 4b where, under control conditions, the stimuli-evoked EPSPs elicited cell firing in two of five trials (p.f. 0.4). In the presence of the drug, the cell never fired an action potential while the EPSP was not affected (see also Figure 4a). In addition, when the p.f. in the presence of oxindole was restored by increasing the stimulus strength, the mean amplitude of the EPSPs which failed to elicit cell firing was greater than that of controls (Figure 4c). Thus it appears that the decrease in p.f. was not due solely to a reduction in synaptic input but to an impairment of the mechanisms leading to the generation of action potentials. This was further confirmed by the observation that oxindole (0.3 mM) decreased the probability of eliciting an action potential by direct excitation of the cell produced by injection of constant current steps through the recording electrode (Figure 5). Similar results were obtained in a total of 7 cells (p.f. 0.34 ± 0.14 *vs* 0.90 ± 0.05 in controls, $n=7$; $P<0.05$). The amplitude and duration of the action potentials elicited by current injection were not affected by the drug (Figure 5, inset). In agreement with the results obtained in extracellular recordings, however, a significant decrease in the amplitude of the EPSP occurred when higher concentrations of oxindole were used: $-30\pm6\%$ ($n=3$) and $-69\pm8\%$ ($n=3$) after 1 or 3 mM respectively.

Effects of oxindole on action potential frequency adaptation and excitability of CA1 pyramidal cells

To further study the effects of oxindole on hippocampal cell excitability, changes of cell membrane potential were induced by injecting current steps through the recording electrode and we measured: (i) the threshold for action potential generation defined as the cell membrane potential at which a spike is generated, (ii) the latency of firing the first action potential; (iii) the action potential frequency adaptation (accommodation), measured as the number of action potentials elicited by 400 ms positive current steps, (iiii) the amplitude of the late AHP (AHP_L) which develops at the end of the depolarizing step.

As illustrated in Figure 6 oxindole (0.1–3 mM) increased the threshold and the latency of firing, elicited by depolarizing

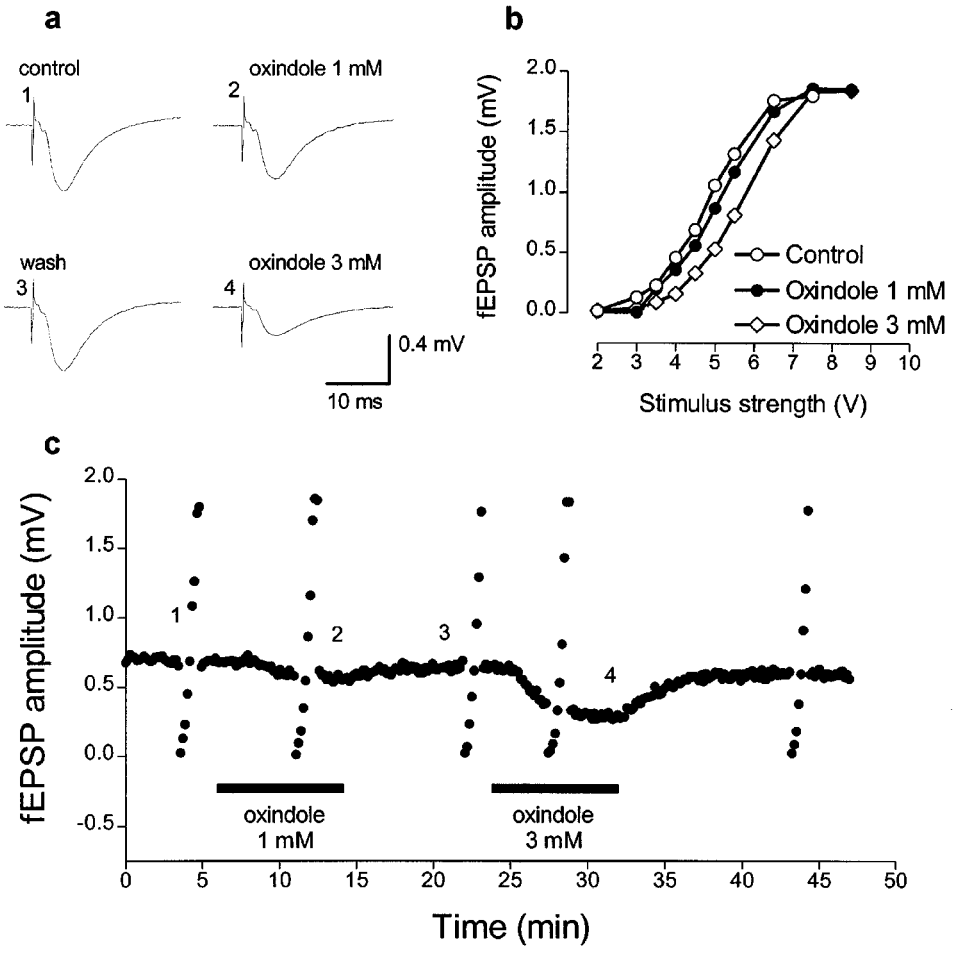

Figure 2 Effects of oxindole on the amplitude of field EPSP in the CA1 hippocampal region. (a) Bath applications of oxindole (1 and 3 mM) reversibly decreased field EPSP (fEPSP) amplitude in a concentration-dependent manner. Traces are single responses evoked by constant strength stimuli (4.5 V) by the corresponding numbers in c. (b) Stimulus-response curves were constructed by gradually increasing the stimulus strength. This protocol was repeated during the application of 1 mM and 3 mM of oxindole. (c) Time-course of the changes of fEPSP amplitude during a typical experiment. Each point represents the amplitude of the fEPSP. Each ascending series of points shows the measure of fEPSPs (from absent to maximal) obtained by gradual increases in stimulus strength (see b). Data shown are obtained from the same experiment which was repeated five times with similar results.

steps, but did not change the action potential amplitude or duration (Table 2).

Figure 7 shows the effects of oxindole (0.3, 1 and 3 mM) on the action potential frequency adaptation. Under control conditions, the repetitive firing, produced by depolarizing current steps of 400 ms duration, showed accommodation and at the end of the current injection an AHP_L was clearly evident. Oxindole decreased the number of action potentials discharged by the neurone (enhanced the accommodation). Furthermore, oxindole reduced the amplitude of the AHP_L (Table 2). Figure 7 also shows the lack of changes in R_{in} monitored by the injection of negative steps (-900 pA, 400 ms) when oxindole (up to 3 mM) was applied.

In separate experiments, we studied the role of $GABA_A$ receptors, by using picrotoxin (25 μM; $n=3$), a $GABA_A$ channel blocker. Oxindole (1 mM) still decreased the number of action potentials elicited by depolarizing current steps (from seven in controls to one in 1 mM oxindole).

Cortical wedge preparations

In order to evaluate the possibility that the oxindole-induced decrease of EPSP was mediated by glutamate receptor antagonism, we tested the effects of oxindole on AMPA/kainate or NMDA receptor responses using 'cortical wedges' a technique which allows the study of excitatory amino acid agonists and antagonists (Mannaioni *et al*., 1996). As depicted in Figure 8, neither the responses to AMPA (4 μM) nor that to NMDA (10 μM) were decreased by oxindole (1 mM).

Discussion

Oxindole is a neurodepressant tryptophan metabolite physiologically present in mammalian brain and blood. When administered to rats at a dose of 10 mg kg^{-1} (i.p.) it had anticonvulsant and sedative properties while larger doses (100 mg kg^{-1}, i.p.) caused loss of the righting reflex and coma.

We used brain slices and studied oxindole's mechanism of action in rat hippocampal neurones. The results obtained showed that concentrations of oxindole in the range of those found in the CNS in pathological conditions were able to reduce neuronal excitability. In a first series of experiments performed with extracellular recordings of electrically evoked synaptic potentials in CA1 pyramidal cells, we observed that oxindole decreased the PS amplitude. This represents the synchronous discharge of neurone action potentials located close to the recording electrode and is proportional to the

number of discharging cells (Lynch & Schubert, 1980). A decrease in the population spike amplitude could be due to a reduction in the afferent excitatory input, a decrease neurone excitability or a combination of both. Our recordings at somatic and dendritic levels converge in showing that oxindole (1–3 mM) reduced fEPSPs, and that 3 mM oxindole caused a significant decrease in the volley amplitude (i.e. the extent of afferent presynaptic fibres excitation). These data could therefore suggest that the reduction in the fEPSP amplitude is due to a decrease in presynaptic excitation. We previously showed that it is possible to obtain a significant decrease in fEPSP by applying adenosine agonists to hippocampal slices and that this decrease is due to a significant reduction in the stimulation induced transmitter output (Corradetti *et al*., 1984a,b). Adenosine agonists, however, did not affect the afferent volley, suggesting that oxindole acts through a different mechanism of action. It is also possible to reduce EPSP amplitude by antagonizing glutamate receptors of AMPA or NMDA types which are present in CA1 neurones (Collingridge *et al*., 1983). Since neither AMPA nor NMDA responses were affected by large concentrations of oxindole (see Figure 8) a direct inhibitory action on excitatory amino acid receptors is unlikely.

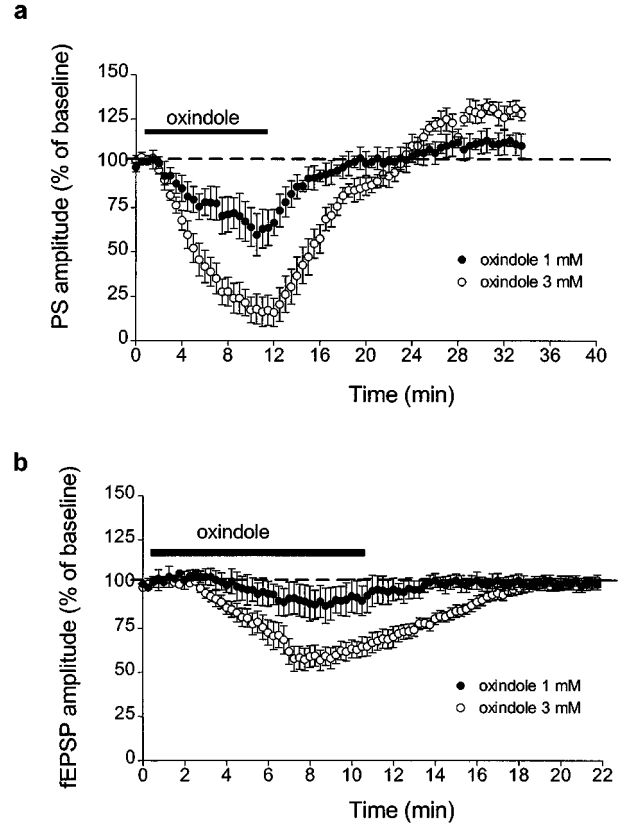

Figure 3 Oxindole decreases synaptic transmission in the CA1 hippocampal region. Time-course of the effects of oxindole on the amplitude of the population spike (PS; a) or of the fEPSP (b). Data, expressed as percentage of controls (broken lines), are the means ±s.e.mean of at least five experiments. Filled bars represent the duration time of oxindole application.

In order to explore in further detail the effects of oxindole both at pre- and post-synaptic levels, we performed a series of experiments using intracellular recordings and synaptic or direct stimulation of the pyramidal cells. The voltage-dependent activation of Na^+ channels which leads to generation of action potentials is due to cell depolarization physiologically produced by the EPSP. The depolarizing effects of EPSPs leading to generation of action potentials are also mimicked by injecting positive current steps through the recording electrode. Low concentrations of oxindole (0.1–0.3 mM) reduced the probability of firing, induced by both synaptic or direct activation of the neurones, without affecting EPSP. Larger concentrations (>1 mM), however, also affected the EPSPs. The analysis of the relationship stimulus-response carried out in the controls and in oxindole treated slices showed that the decrease in EPSP amplitude was not sufficient to explain the decrease in synaptically-induced firing. Higher stimulus strength had to be used in oxindole treated slices as compared to controls in order to obtain an equal probability of firing. Furthermore, EPSPs of a larger amplitude were needed for generating action potentials in the presence of oxindole. Consistent with these findings, oxindole increased the threshold and the latency of firing action potentials when the neurones were directly activated. This suggests that oxindole affects the mechanism(s) of action potential generation and points to the voltage-operated Na^+ channels as one of its sites of action.

Previous experiments with the use of tetrodotoxin (Hu *et al*., 1992) and felbamate (Pugliese *et al*., 1996) have suggested that CA1 pyramidal cells express Na^+ channels which are

Table 2 Effects of oxindole on main properties, synaptic potentials and excitability of CA1 hippocampal pyramidal neurones

	control	*oxindole* 0.3 (mM)	*oxindole* 1 (mM)	*oxindole* 3 (mM)
r.m.p. (mV)	−63.8±2	−61.7±2	−61.1±1	−63.1±2
R_{in} (MΩ)	47±5	50±5	41±8	40.1±3
AP amplitude (mV)	102±3	99±3	103±3	101±2
AP half width (ms)	0.82±0.03	0.81±0.02	0.85±0.08	0.77±0.03
p.f. (synaptic)	0.80±0.14	0.10±0.05*	0.10±0.05*	0
p.f. (direct)	0.97±0.02	0.30±0.12*	0	0
peak AHP (mV)	−4.97±0.5	−4.2±0.4*	−3.8±0.6*	−3.1±0.4*
late AHP (mV)	−3.19±0.6	−2.84±0.6	−1.8±0.4*	−0.9±0.4*
Accommodation (n° of AP)	9.6±1	4.8±1*	4.1±2*	0.4±0.2*

The data were obtained in at least ten cells for the control group and four cells for each of oxindole treated groups. They are means±s.e.mean. Oxindole was applied for 10 min at the indicated concentrations; r.m.p.: resting membrane potential; R_{in}: neuronal input resistance; action potential (AP) amplitude was measured from r.m.p. AP half width was the time between 50% of the peak on the rising phase and 50% of the peak on the falling phase; p.f.: probability of firing. Peak AHP: peak amplitude of the AHP which followed repetitive discharge cells elicited by positive step (range from +300 to +500 pA). Late AHP: amplitude of the slow AHP measured at 150 ms after cessation of the depolarizing step. *Significantly ($P<0.05$) different from the control (*t*-test). Cells recovered from all oxindole effects after a 20 min wash.

differentially able to respond to drugs. Both compounds decreased synaptically-driven cell firing without changing the action potential elicited by direct neuronal activation suggesting that they were affecting subpopulations of Na^+ channels different than those affected by oxindole. Na^+ channel β-subunits may play an important role in modulating the threshold for channel activation and/or time of channel inactivation (Catterall, 1992). When Na^+ channel α-subunits alone are expressed in Xenopus oocytes, the properties of the resulting functional channel are quite peculiar due to the slowness of the channel inactivation and the shifting of the voltage-dependence to more positive membrane potentials (Messner *et al*., 1986). Coexpression in the same cells of both α and β-subunits modify the inactivation kinetics and shift the voltage dependence of the channel toward more negative membrane potentials (Krafte *et al*., 1990; Trimmer *et al*., 1989; Auld *et al*., 1988).

Oxindole shifted the threshold for channel activation towards positive potentials but once the threshold was reached, the resulting spikes were unchanged in duration and in peak amplitude. This pattern of activity could suggest that oxindole may interact with Na^+ channel β-subunits. Although appealing, this hypothesis needs further investigation.

The density and/or subunit composition of the voltage-operated Na^+ channels located in different regions of the pyramidal cells may differ (Catterall, 1981). In particular, a high density of Na^+ channels is located at the axon hillock, where the action potentials are generated. This region seems important in the regulation of cell discharge because it receives the tonic inhibition exerted by a population of GABAergic interneurones named 'chandelier' (Li *et al*., 1992). In our experiments, oxindole-mediated potentiation of $GABA_A$ inhibitory responses was considered unlikely because oxindole effects on cell discharge were still present when bicuculline or pictrotoxin, two $GABA_A$ receptor blockers, were added to the perfusion medium.

Oxindole was found to increase the action potential frequency adaptation which limits the frequency of cell

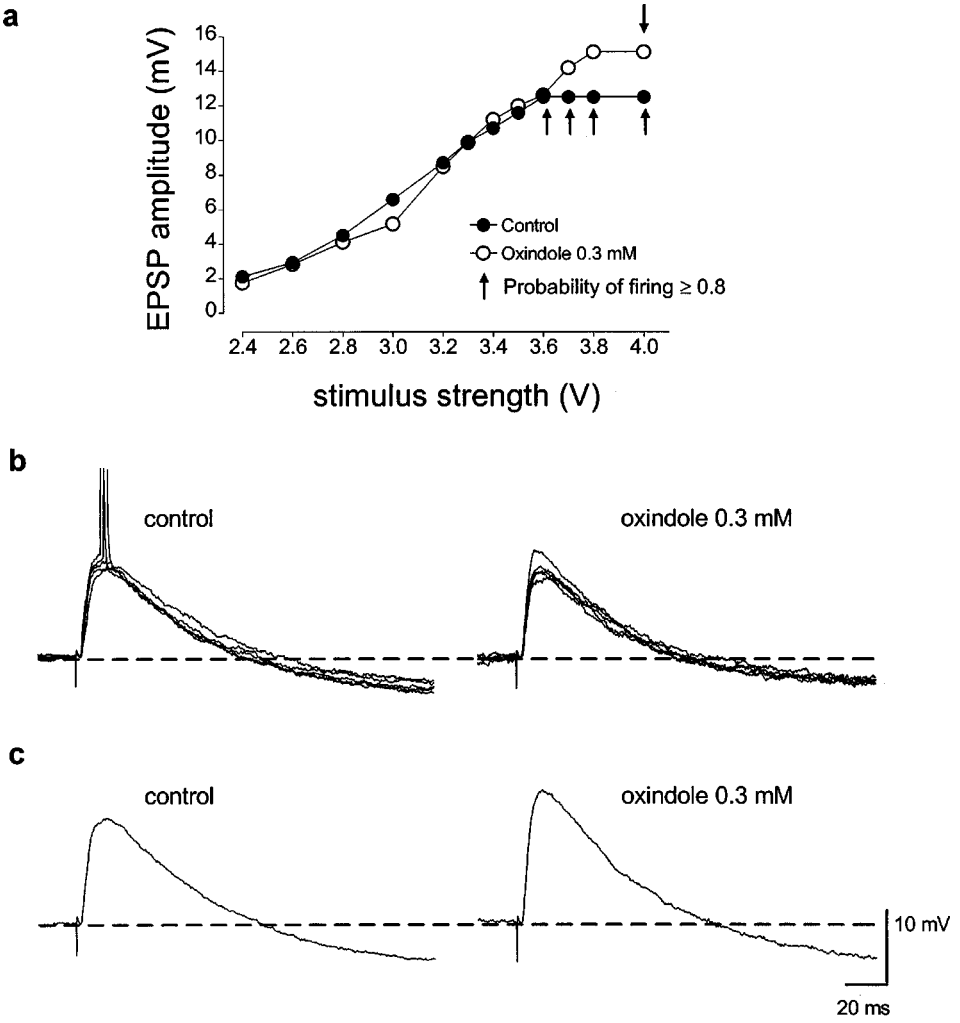

Figure 4 Oxindole reduction of synaptic firing elicited by electrical stimulation of the stratum radiatum in the CA1 hippocampal region. (a) Effects of oxindole on the relationship between the stimulus strength and the EPSP amplitude in control conditions and under 0.3 mM of oxindol. No significant changes in EPSP amplitude occurred during oxindole treatment. Note that the EPSP amplitude needed to generate an action potential was increased in the presence of oxindole. (b) Traces are five superimposed responses evoked by stimulation of the stratum radiatum (3.5 V, downward transients) either in control (left) or after a 10 min application of 0.3 mM oxindole (right). Note that the decrease in p.f. (from 0.4 to 0) induced by oxindole was not accompanied by changes in EPSP amplitude. (c) Responses obtained in the same cell before and during oxindole (0.3 mM) treatment. In order to have a p.f. = 0.4, the stimulus strength was 3.5 V in controls and 3.8 V in the presence of oxindole (see a). Each trace is the average of the three responses not followed by AP evoked by stimulation of the stratum radiatum. The amplitude of the EPSPs which failed to elicit firing was greater in the presence of oxindole than in controls. R_{in}: 35 MΩ; r.m.p.: −73 mV; 3 M KCl-filled electrodes in the presence of 10 μM bicuculline.

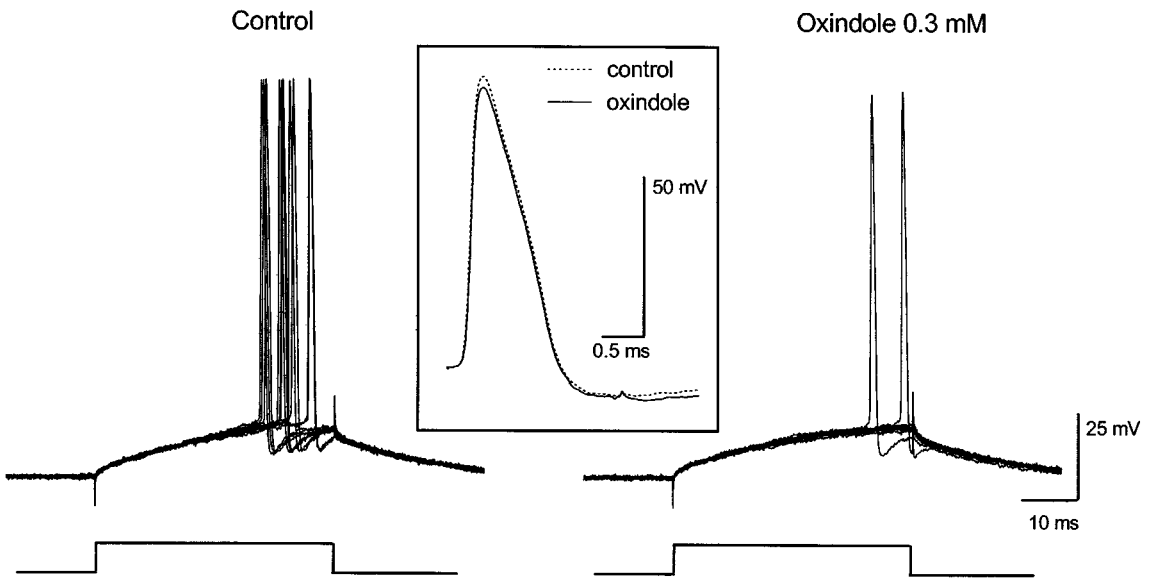

Figure 5 Effect of oxindole on cell firing elicited by direct depolarization of a CA1 pyramidal neurone. Each panel shows ten superimposed responses evoked by a depolarizing current injection (+200 pA, 40 ms, bottom trace) through the recording electrode; under control (left) conditions and in the presence of 0.3 mM oxindole (right). Note that the probability to generate an action potential is decreased (from 1 to 0.2) during application of oxindole (0.3 mM). The amplitude of the action potentials under control conditions and under oxindole treatment was not changed. Inset, the average of the two larger action potentials in the control (dotted line) is superimposed over the average of the two residual action potentials in oxindole to show the lack of significant effect of the drug on the action potential shape. R_{in}: 45 MΩ; r.m.p.: −71 mV; 3 M KC1-filled electrode in the presence of 10 μM bicuculline.

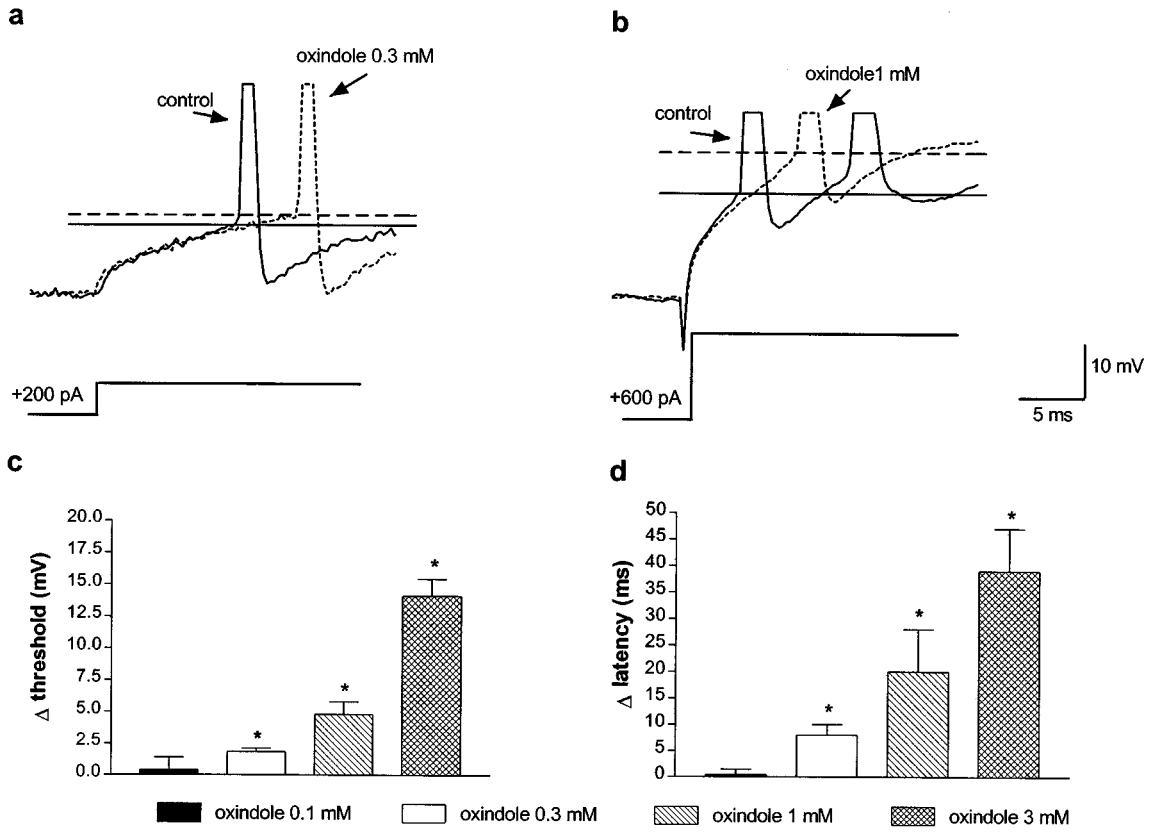

Figure 6 Oxindole increased the threshold and the latency of the action potentials induced by depolarizing current injections in CA1 pyramidal neurones. (a) Injection of depolarizing current steps (+200 pA, 400 ms) through the recording electrode elicited cell discharge in the control (continuous line) and in the presence of 0.3 mM oxindole (broken line). The corresponding horizontal lines show the shift of the action potential threshold towards a more positive membrane potential (ΔmV = 1.6 mV). R_{in}: 55 MΩ; r.m.p.: −64 mV. (b) In a different cell, the same protocol was applied in the control (continuous line) and in the presence of oxindole (1 mM). Note the larger shift of the action potential threshold (ΔmV = 6.1 mV). R_{in}: 60 MΩ; r.m.p.: −71 mV; both cells were recorded with KCl-filled electrodes in the presence of 10 μM bicuculline. In the lower graphs, each column represents the mean ± s.e.mean increase in threshold (Δ threshold: c) and in latency (Δ latency: d) measured in at least six experiments. *Significantly ($P<0.05$) different from the control (Student's t-test). Statistics were performed on absolute values.

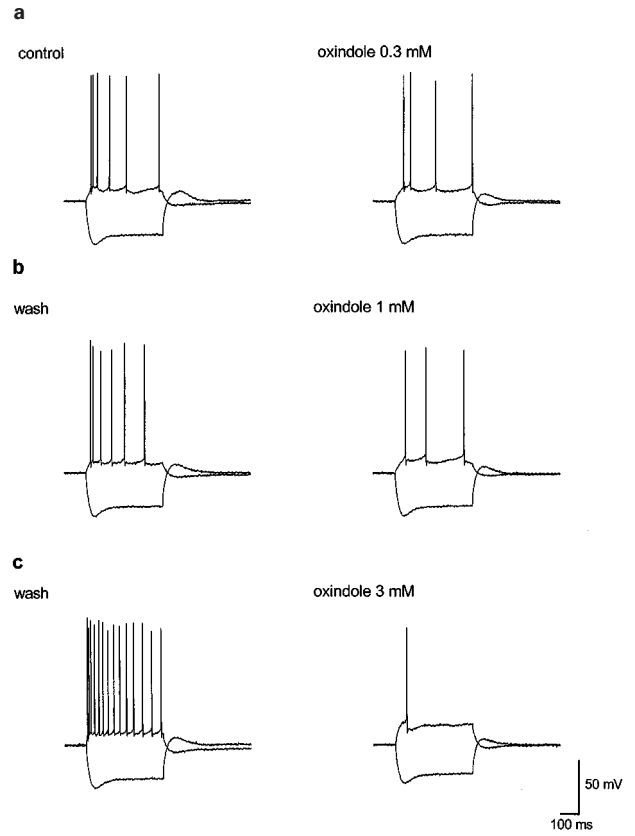

Figure 7 Effects of oxindole on the action potential frequency adaptation, AHP amplitude and input resistance in CA1 pyramidal cell. Different concentrations of oxindole were tested on cell responses to injection of current pulses (a from −900 to +200 pA, 400 ms; b from −900 to +200 pA, 400 ms; c from −900 to +500 pA, 400 ms). Under control conditions, a positive current step caused depolarization and discharge of the action potentials followed by AHP, whereas a negative current step produced membrane hyperpolarization; this hyperpolarization is useful in monitoring changes in the total input resistance. Note that in the presence of oxindole (0.3, 1 and 3 mM), the number of action potentials elicited by the depolarizing step was reduced in a reversibly and concentration-dependent manner, with no changes in the total input resistance. R_{in}: 40 MΩ; r.m.p.: −65 mV; 3 M KCl-filled electrodes.

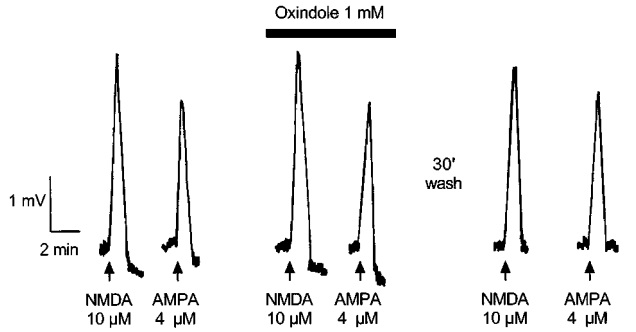

Figure 8 Oxindole does not affect NMDA or AMPA responses in wedges of mouse cerebral cortex. Records from a typical experiment show the response elicited by application (2 min, spaced by 15 min wash) of NMDA or AMPA in a Mg^{2+}-free aCSF (see Methods). The d.c. shifts produced by either agonist were not decreased in the presence of oxindole (1 mM). Similar results were obtained in a total of three experiments.

discharge, in CA1 pyramidal neurones, following depolarizing stimuli of long duration (400 ms). A number of pharmacological agents (for instance carbamazepine or phenytoin) are able to limit the high-frequency repetitive firing of the neurones by prolonging the time spent by Na^+ channels in the inactivated state. The above-mentioned agents, however, not only limit the firing rate of the neurones, but also cause a progressive decrease in the action potential amplitude (MacDonald *et al*., 1985). Since high concentrations of oxindole failed to affect the action potential amplitude, we assumed that oxindole did not facilitate the maintenance of the Na^+ channels in the inactive state. It is also possible to limit the high-frequency repetitive firing of hippocampal pyramidal cells by activating potassium currents. In CA1 pyramidal neurones, the slowing of the repetitive discharge is mediated by the activation of at least two voltage-dependent K^+ currents, I_A and I_D, and a set of Ca^{2+} dependent K^+ currents, including I_C and I_{AHP} (Storm, 1990; Rudy, 1988). Although implication of K^+ channels in the effects of oxindole cannot be discarded, the increase in the threshold of action potentials we observed suggests that the most relevant biological effects of oxindole may be mediated through an interaction with Na^+ channels.

We used concentrations of oxindole in which sedation was observed when whole brain oxindole levels reached 8.1 ± 1.7 nmol g^{-1} and coma when whole brain oxindole levels reached 103 ± 15 nmol g^{-1} (see Table 1). Since the compound is highly lypophilic, its concentration at the active sites of membranes may probably have reached the millimolar range. Furthermore it is reasonable to assume that neurones physiologically discharging at a high firing rate are more sensitive to the oxindole-induced increase in threshold than neurones discharging at a low firing rate.

Oxindole content in various brain areas and in blood are remarkably similar (0.05 ± 0.01 nmol g^{-1} or 0.06 ± 0.01 nmol ml^{-1} respectively). Since these concentrations are approximately two orders of magnitude lower than those found able to affect neuronal function, it is reasonable to rule out physiological roles for oxindole. In pathological situations, however, it is possible that blood and brain oxindole concentrations reach levels which are sufficient to depress the function of excitable cells. In hepatic encephalopathy oxindole could participate in the symptomatology in association with other neurodepressant compounds (Moroni *et al*., 1998; Basile *et al*., 1990; Olasmaa *et al*., 1989). Phenylketonuria (Kochen *et al*., 1972) is another example of a pathological condition in which the concentrations of oxindole in biological fluids reaches levels we found sufficient to reduce cellular excitability.

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