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Effects of DAU 6215, a novel 5-hydroxytryptamine$_3$ (5-HT$_3$) antagonist on electrophysiological properties of the rat hippocampus

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1 The aim of the present study was to test the effects of DAU 6215 (endo-*N*-(8-methyl-8-azabicyclo-[3.2.1]-octo-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride), a newly synthesized, selective 5-hydroxytryptamine$_3$ (5-HT$_3$) antagonist, on the cell membrane properties and on characterized 5-HT-mediated responses of pyramidal neurones in the hippocampal CA1 region.

2 Administration of DAU 6215, even at concentrations several hundred fold its K_i, did not affect the cell membrane properties of pyramidal neurones, nor modify extracellularly recorded synaptic potentials, evoked by stimulating the Schaffer's collaterals.

3 Micromolar concentrations (15–30 μM) of 5-HT elicited several responses in pyramidal neurones that are mediated by distinct 5-HT receptor subtypes. DAU 6215 did not antagonize the 5-HT$_{1A}$-induced membrane hyperpolarization and conductance increase, a response that was blocked by the selective 5-HT$_{1A}$ antagonist NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-phtalamido)butyl-piperazine). Similarly, DAU 6215 did not affect the membrane depolarization and decrease in amplitude of the afterhyperpolarization, elicited by the activation of putative 5-HT$_4$ receptors.

4 5-HT increased the frequency of spontaneous postsynaptic potentials (s.p.s.ps) recorded in pyramidal neurones loaded with chloride. In agreement with previous observations, most of the s.p.s.ps were reversed GABAergic events, produced by the activation of 5-HT$_3$ receptors on interneurones, because they persisted in the presence of the glutamate NMDA and non NMDA antagonists, D-aminophosphonovaleric acid (APV; 50 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 25 μM), and were elicited by the selective 5-HT$_3$ agonist, 2-methyl-5-HT (2-Me-5-HT, 50 μM).

5 The increase in frequency of s.p.s.ps induced by 5-HT was significantly antagonized by DAU 6215 in 70% of the cases, whereas the 5-HT$_3$ antagonist always suppressed the effect of 2-Me-5-HT, at concentrations as low as 60 nM.

6 The antagonistic effect of DAU 6215 was also tested on the 5-HT$_3$-mediated block of induction of long-term potentiation (LTP), elicited by a primed burst (PB) stimulation. Extracellular recordings showed that low concentrations (60 nM) of DAU 6215 suppressed the inhibitory action of 5-HT on PB-induced LTP, without affecting the 5-HT$_{1A}$-induced reduction in the amplitude of the population spike.

7 These results provide evidence that DAU 6215 is an effective antagonist of the 5-HT$_3$-mediated responses in the central nervous system and may offer a cellular correlate for the pharmacological effects of DAU 6215 as an anxiolytic and cognition enhancer.

Keywords: 5-HT (5-hydroxytryptamine); 5-HT$_3$ receptor; DAU 6215; spontaneous postsynaptic potentials; NAN-190; hippocampus; long-term potentiation (LTP); primed burst stimulation (PB)

Introduction

In the last few years a novel class of 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor antagonist has been synthesized (Turconi *et al.*, 1990) and the potency and selectivity of these new molecules have been tested in isolated preparations and *in vivo* models. Among these compounds, DAU 6215 (endo-*N*-(8-methyl-8-azabicyclo-[3.2.1]-octo-3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride), a benzimidazolone derivative, exhibits a high affinity for the [^{3}H]-ICS 205-930 binding site in rat brain tissue, and it is equipotent with ICS 205-930 in inhibiting 5-HT-induced bradycardia in rats (Turconi *et al.*, 1990; 1991a). DAU 6215 was also found to be a weak, partial agonist at the 5-HT$_4$ receptor, as demonstrated by the ability of this compound to stimulate adenosine 3':5'-cyclic monophosphate (cyclic AMP) production in colliculi neurones in cultures (Dumuis *et al.*, 1991). Several central pharmacological effects of DAU 6215 have been described: it is a potent antiemetic (Sagrada *et al.*, 1990; 1991), an anxiolytic in animal models (Borsini *et al.*, 1993), and it decreases the morphine-induced reward in animal models (Turconi *et al.*, 1991b). Recently, it was found that DAU 6215, also reduces scopolamine-induced behavioural deficits in rats (Brambilla *et al.*, 1993; Pitsikas *et al.*, 1994). Electrophysiological recordings *in vivo*, have shown that chronic administration of DAU 6215 reduces the number of spontaneously firing dopaminergic neurones in the rat ventral tegmental area, suggesting a potential antipsychotic activity of this compound (Prisco *et al.*, 1992). In spite of the biochemical evidence that DAU 6215 is a selective 5-HT$_3$ receptor antagonist, the effect of this ligand on the neuromodulatory action of 5-HT in the CNS has not yet been tested. We studied the pharmacological profile of DAU 6215 in the hippocampal slice preparation for the following reasons. The hippocampus displays different patterns of activity that have been correlated with long-term synaptic potentiation and some forms of learning and memory storage (Teyler *et al.*, 1987); it is a region enriched with 5-HT receptors (Köhler, 1984) and 5-hydroxytryptaminergic terminals originating in the raphe nuclei (Swanson *et al.*, 1987) and electrophysiological studies *in vivo* and *in vitro* have demonstrated that 5-HT has an inhibitory action on evoked synaptic potentials (Segal, 1975; 1976; 1980; Jahnsen, 1980; Ropert,

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1988) and long-term potentiation (LTP; Corradetti et al., 1992a). Applications of micromolar concentrations of 5-HT exert multiple and well characterized actions on CA1 pyramidal neurones through distinct 5-HT receptor subtypes (Anwyl, 1990). Postsynaptic actions of 5-HT on CA1 pyramidal neurones include hyperpolarization of the cell membrane associated with decreased input resistance, and a reduction of the afterhyperpolarization (AHP; Segal, 1980; Andrade & Nicoll, 1987; Colino & Halliwell, 1987; Ropert, 1988). Activation of an inwardly rectifying potassium channel mediates the membrane hyperpolarization and decrease in input resistance, through a 5-HT_{1A} receptor (Andrade & Nicoll, 1987; Colino & Halliwell, 1987) associated with a G protein (Andrade et al., 1986). The slow excitatory action and reduction of the amplitude of the AHP (Chaput et al., 1990; Andrade & Chaput, 1991) is due to the closing of calcium-dependent K^+ channels (I_{AHP}; Andrade & Nicoll, 1987; Colino & Halliwell, 1987), supposedly through the activation of 5-HT_4-like receptors (Chaput et al., 1990; Andrade & Chaput, 1991). Up till now, no direct effect of 5-HT on CA1 pyramidal neurones in hippocampal slices has been ascribed to 5-HT_3 receptor stimulation. Activation of 5-HT_3 receptors is apparently responsible for the 5-HT-induced increase in spontaneous post-synaptic potentials (s.p.s.ps); this effect has been attributed to enhanced activity of GABAergic interneurones (Ropert & Guy, 1991), although a non-5-HT_3 component has also been described (Van den Hooff & Galvan, 1991).

Recently, it was demonstrated that the simultaneous activation of both 5-HT_{1A} and 5-HT_3 receptors is responsible for the inhibitory action of 5-HT on the induction of LTP produced by a primed burst (PB) stimulation *in vitro*, (Corradetti et al., 1992a).

These properties make the hippocampal slice a suitable preparation to study the action of DAU 6215 on well characterized electrophysiological responses of CA1 pyramidal neurones to 5-HT and also its antagonistic effects on the neuromodulatory action of 5-HT on synaptic activity, mediated by 5-HT_3 receptors. Part of the results described here have been published previously (Corradetti et al., 1992b).

Methods

Preparation of hippocampal slices

Experiments were carried out on *in vitro* hippocampal preparations as previously described (Corradetti et al., 1992a). Charles River male Wistar rats (100–200 g body weight) 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 3.33, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2.5, $NaHCO_3$ 25, D-glucose 10 (pH 7.4). Slices (400 μm thick) were cut with a McIlwain tissue chopper from the dorsal half of the hippocampus and kept in oxygenated aCSF for at least 1 h at room temperature (20–23°C). A single slice was then placed on a nylon mesh and completely submerged in a small chamber and superfused 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 complete exchange of the chamber volume occurred in 1 min.

Intracellular recordings

CA1 pyramidal neurones were recorded in current clamp mode with either 3 M potassium-methylsulphate- (50–100 MΩ) or KCl- (35–50 MΩ) filled electrodes. Electrical signals were filtered at 30 kHz and amplified with an Axoclamp 2A (Axon Instruments, Foster City, CA, U.S.A.) and displayed on an oscilloscope and chart recorder (2800 Gould Instruments, Cleveland OH, U.S.A.). Traces were stored on a digital tape (DTR 1200, Biologic, France; 48 kHz sampling frequency) and on computer using a pClamp programme (Axon Instruments) for off-line measurements (sampling frequency = 3–10 kHz). Only neurones with stable resting membrane potentials (r.m.p.; range − 54/−80 mV) and input resistances (R_{in}; range 31–86 MΩ) throughout the recording were included in the analysis. When cells appeared to have reached a stable membrane potential, pulses of hyperpolarizing current (200–400 pA, 400 ms) were delivered through the recording electrode to monitor changes in input resistance during drug application. To study the s.p.s.ps, 3 M KCl-filled electrodes were used with a resistance not exceeding 50 MΩ. Low resistance electrodes were used to obtain a good diffusion of chloride within the impaled cells. Neurones were maintained at a negative potential (− 90/− 100 mV) by current injection to load the cell with chloride and prevent them from firing action potentials due to reversed inhibitory postsynaptic potentials. The cells were allowed to load with chloride for 15–20 min and to reach a stable membrane potential before results were recorded. Experiments aimed at studying the effects of DAU 6215 on the AHP were carried out in the presence of 1 μM tetrodotoxin (TTX), 5 mM tetraethylammonium chloride (TEA) and 1 μM NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-phthalamido)butil piperazine), a 5-HT_{1A} antagonist, to allow for the generation of calcium spikes (Andrade & Nicoll, 1987) and eliminate the 5-HT_{1A} component of the response. These compounds were perfused for at least 30 min before testing 5-HT. Under these conditions, injections of positive current pulses (100 ms) through the recording electrode elicited a calcium spike, followed by a large calcium-dependent K^+-activated AHP (Schwartzkroin & Slawsky, 1977; Madison & Nicoll, 1986, Andrade & Nicoll, 1987). This procedure was used, as opposed to examining the AHP following a burst of spikes, because the large amplitude, reproducible AHP produced by the calcium spike, enables the systematic examination of the effects of 5-HT and its antagonists (Chaput et al., 1990).

Extracellular recordings

Experiments were performed in 41 slice preparations. The Schaffer's collateral/commissural fibres were stimulated through bipolar nichrome electrodes with 80–110 μs, 0.017 Hz test pulses. Evoked potentials were recorded with 3 M NaCl-filled electrodes (3–6 MΩ) placed in the stratum pyramidale of the CA1 region. Responses were amplified (Neurolog NL104, Digitimer Ltd., England), displayed on an oscilloscope, digitized and stored on floppy disks for later analysis (sample rate 33 kHz; DATA 6000, Analogic, Danvers, MA, U.S.A.). Input-output curves were obtained at the beginning of each experiment by gradually increasing the stimulus strength. The test stimulus pulse was then adjusted at an intensity that elicited population spikes of an amplitude 30–40% of the maximum, and was maintained constant for the duration of the experiment. After 30 min equilibrium, a 25 min control period was used to generate baseline values. LTP was induced with a primed burst (PB) stimulation, consisting of a pulse followed by a burst of five pulses at 100 Hz, after a 170 ms interval. The strength of the pulses during the high frequency burst was kept at the same intensity as the test pulse. 5-HT was applied for 5 min and the PB stimulation delivered during the fifth min of application of the drug, which was washed out immediately after the patterned stimulation. DAU 6215 was allowed to equilibrate for at least 15 min before adding 5-HT and was left for at least 15 min after the PB stimulation and wash out of the agonist.

Data analysis

The instantaneous frequency of occurrence of spontaneous postsynaptic events was measured with a DATA 6000 (sampling frequency 3–5 kHz). A reference level was manually

chosen to detect only events of an amplitude exceeding background noise (0.5–1 mV). The programme then, automatically calculated the instantaneous average frequency, measured as the reciprocal of the average of the time intervals between 'paired' crossings of the reference y-level on the time axis. Paired crossings were defined as adjacent crossings of the same polarity with one crossing of opposite polarity between the two. Although the reference level used probably cut off most of miniature s.p.s.ps, it is possible that in some cells these were included in our measurements. The occasional inclusion of miniature potentials in the measurements, though, should not be relevant to our results, since 5-HT does not affect either the frequency or the amplitude of miniature i.p.s.p. (Ropert & Guy, 1991). The sampling was performed on 20 s periods and the average frequencies grouped in 3–4 min bins.

Chemical substances

The following drugs were applied in the perfusion solution: 5-hydroxtryptamine (5-HT) creatinine sulphate or hydrochloride salts, 2-methyl-5-hydroxytryptamine maleate (2-Me-5-HT), 1-(2-methoxyphenyl)-4-[4-(2-phthalamido)butil-piperazine (NAN-190), all from Research Biomedical Inc. Wayland, U.S.A.: endo-*N*-(8-methyl-8-azabicyclo-[3.2.1]-octo -3-yl)-2,3-dihydro-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride (DAU 6215-Cl; gift of Dr Borsini, Boehringer Ingelheim, Milan, Italy); D-aminophosphonovaleric acid (D-APV) and 6,7-dinitroquinoxaline-2,3-dione (DNQX), both from Tocris Neuramin, (Essex, England); tetrodotoxin (TTX) and tetraethylammonium chloride (TEA), both from Sigma, St Louis MO, U.S.A.

Statistics

All numerical data are given as means ± s.e.mean; Student's paired *t* test and multiple comparison ANOVA, followed by Fisher LSD *post hoc* test, were used where appropriate; a value of $P<0.05$ was considered statistically significant.

Results

In a first series of experiments we investigated the selectivity of the antagonistic action of DAU 6215 on well characterized 5-HT responses of hippocampal neurones, mediated by different receptor subtypes.

DAU 6215 does not block 5-HT$_{1A}$-mediated responses

In 14 pyramidal neurones (resting membrane potential: r.m.p. = − 68 ± 1.5 mV; input resistance: R_{in} = 52 ± 4.9 MΩ), 3–5 min applications of 30 μM 5-HT, a concentration that elicits a maximal effect, consistently induced a membrane hyperpolarization (7 ± 1 mV) and reduced the input resistance by an average of 30 ± 2.9% (Figure 1a). The decrease in membrane resistance was still observable when the membrane potential was manually clamped at the control resting potential during 5-HT application, which eliminated the possibility that the change in resistance was due to non-linearities of the current/voltage relationship. DAU 6215 up to micromolar concentrations, did not significantly change the cell membrane potential and input resistance and was ineffective in antagonizing the 5-HT-induced responses (Figures 1a,b; Table 1). On the other hand, 1 μM NAN-190, a 5-HT$_{1A}$ ligand (Glennon *et al.*, 1988a,b) with antagonist effects in the hippocampus (Fletcher *et al.*, 1993; see also Discussion), significantly antagonized the membrane potential change and reduction in input resistance induced by 30 μM 5-HT (n = 4; Figure 1a). NAN-190, *per se*, had no effect on either the input resistance or the resting membrane potential (R_{in} and r.m.p. in control medium were 41.8 ± 3.9 MΩ and − 67 ± 2 mV, respectively; in 1 μM NAN-

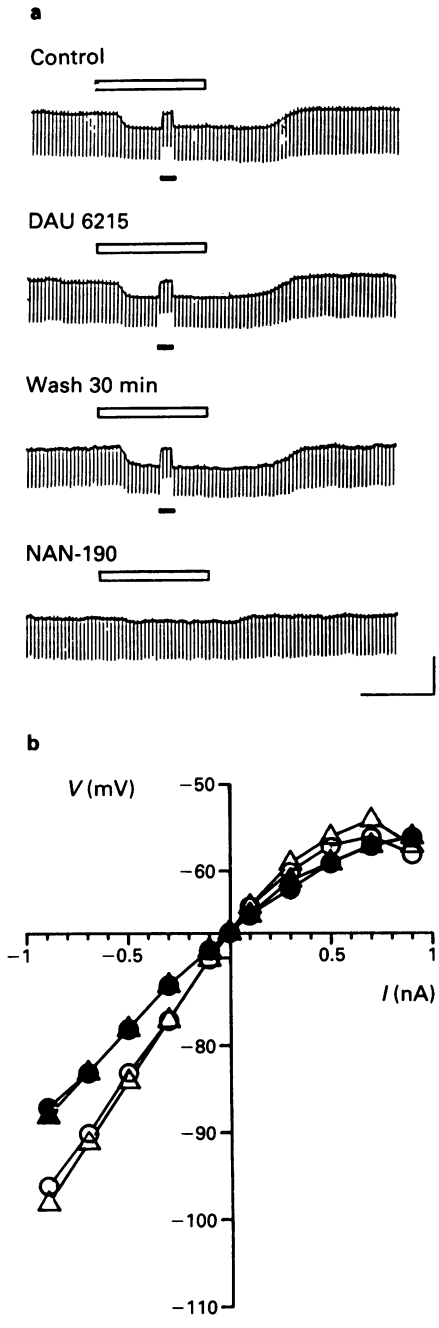

Figure 1 The hyperpolarization and conductance increase produced by 5-hydroxytryptamine (5-HT) are not antagonized by DAU 6215. (a) Chart records of resting membrane potential of a CA1 pyramidal cell in which 5-HT (30 μM, open bars) was applied (from top trace): in normal aCSF, in the presence of DAU 6215 (3 μM, 15 min), after 30 min washout of DAU 6215; and in the presence of the 5-HT$_{1A}$-receptor antagonist NAN-190 (1 μM, 20 min). Downward deflections are electrotonic cell membrane responses to constant current pulses (0.3 nA, 400 ms) injected through the recording electrode to monitor total input resistance. During 5-HT application, the membrane potential was manually clamped at the control value (− 67 mV) by injecting + 0.18 nA through the recording electrode (filled bars) to rule out the possibility that the decrease in input resistance was only apparent and was due to cell hyperpolarization. Note that the response to 5-HT was almost completely blocked by NAN-190, but was not affected by DAU 6215. (b) Plot of current-voltage relationships (I/V curves) recorded in a pyramidal neurone bathed in tetrodotoxin (1 μM) to prevent cell firing at positive potentials: control (Δ), 30 μM 5-HT (▲), 3 μM DAU 6215 (○), 5-HT in the presence of DAU 6215 (●). During the application of 5-HT, membrane potential was manually clamped at the control value by injecting positive current (d.c. = + 0.42 nA). Note that 5-HT both in control aCSF and in the presence of DAU 6215, produced similar changes of the I/V relationship, whereas DAU 6215 by itself did not affect the I/V curve. Calibration bars: 10 mV, 3 min.

Table 1 DAU 6215 has no direct effect on cell membrane properties, nor does it antagonize the changes in membranes potential (Δ MP), input resistance (ΔR_{in}) and population spike (PS) amplitude produced by 5-HT

Treatment	*ΔMP* (MV)	(n)	*ΔR_{in}* (%)	(n)	*PS amplitude* (% control)	(n)
DAU 6215 60 nM	0 ± 0	(3)	2 ± 1.4	(3)	106 ± 6	(11)
DAU 6215 300 nM	−2.5 ± 1.1	(5)	−4 ± 3.5	(5)	97 ± 1	(6)
DAU 6215 1 μM	NT		NT		104 ± 4	(6)
DAU 6215 3 μM	0.8 ± 0.7	(6)	1.3 ± 2.5	(6)	NT	
5-HT 30 μM	−4.3 ± 0.4	(3)	−40 ± 4.2	(3)	34 ± 6	(5)
+ DAU 6215 60 nM	−4.7 ± 0.3	(3)	−42 ± 6.9	(3)	29 ± 7	(5)
5-HT 30 μM	−10 ± 2.4	(5)	−33 ± 5.4	(5)	44 ± 14	(4)
+ DAU 6215 300 nM	−9.4 ± 2.4	(5)	−29 ± 13	(5)	45 ± 12	(4)
5-HT 30 μM	NT		NT		9 ± 7	(4)
+ DAU 6215 1 μM	NT		NT		12 ± 9	(4)
5-HT 30 μM	−5.8 ± 0.7	(6)	−23 ± 3	(6)	NT	
+ DAU 6215 3 μM	−6.7 ± 0.6	(6)	−23 ± 3	(6)	NT	

Number of experiments are indicated in parentheses (*n*). NT, not tested.

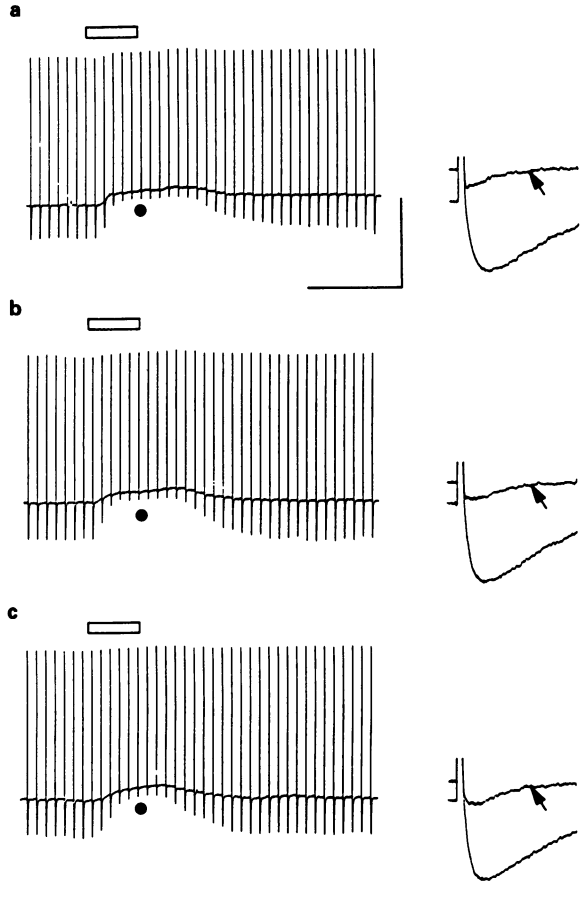

Figure 2 DAU 6215 does not antagonize the 5-hydroxytryptamine (5-HT)-induced reduction of the afterhyperpolarization (AHP) in hippocampal pyramidal cells. Chart record of calcium spikes evoked by depolarizing current pulses (+ 300 pA; 100 ms) delivered every 30 s in the presence of tetrodotoxin (1 μM), tetraethylammonium (5 mM) and NAN-190 (1 μM, see Methods). The calcium spike was followed by a large AHP (downward deflections). (a) 5-HT (15 μM, open bar) elicited a decrease in the amplitude of the AHP (− 71%) and a depolarization of the membrane potential (6 mV). (b) The action of 5-HT persisted in the presence of 1 μM DAU 6215 and (c) after 15 min washout of DAU 6215. R.m.p. = − 54 mV. Calibration bars: 30 mV, 5 min. In the panels at the right, a control response before application of 5-HT and the response taken at time indicated by the spot in the corresponding left panel, are amplified at different voltage gain and timebase (calibrations: 15 mV, 1 s) and superimposed to show the effect of 5-HT (arrows) on AHP more clearly.

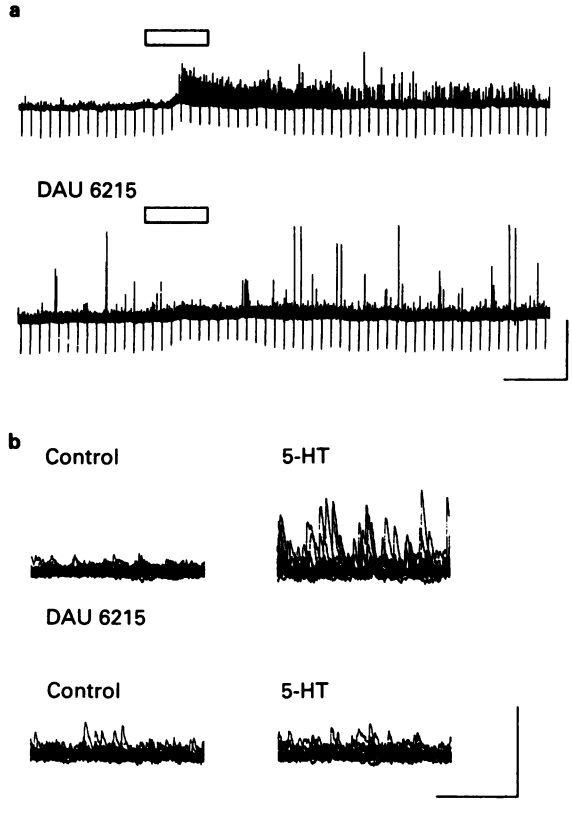

Figure 3 DAU 6215 antagonizes 5-hydroxytryptamine (5-HT) induced increase in the frequency of GABA-mediated spontaneous postsynaptic potentials (s.p.s.ps). (a) Voltage chart recording of a pyramidal neurone maintained at − 95 mV by injecting − 0.9 nA of current through the recording electrode filled with KCl. Upper trace: superfusion of 30 μM 5-HT (open bar) augmented the frequency and amplitude of s.p.s.ps, an effect that outlasted the application of the amine. Lower trace: DAU 6215 (300 nM) antagonized the effect of 5-HT. Downward deflections are electrotonic responses to constant current pulses (− 0.3 nA, 400 ms). Note the reduction of input resistance during 5-HT applications. Calibration bars: 20 mV, 3 min. (b) Voltage chart recording of the same neurone as in (a) plotted on a different time scale and voltage gain (sampling rate, 3.3 kHz). Each panel shows 14 superimposed sweeps taken in control conditions immediately before (upper left panel), and during 5-HT application (upper right panel). In the lower panels, s.p.s.ps were recorded in the presence of DAU 6215 alone (left) and during application of 30 μM 5-HT in the presence of the antagonist. Calibration bars: 10 mV, 300 ms.

190 R_{in} = 41.9 ± 5 MΩ and r.m.p. = − 67 ± 1.1 mV). In the presence of NAN-190, the hyperpolarizing response to 5-HT was greatly reduced (− 1.8 ± 1 mV versus − 5.3 ± 0.8 mV; $P<0.05$), as well as the percentage decrease in input resistance (7.3 ± 4.3% versus 24 ± 4%; $P<0.01$). In these cells, 30 μM 5-HT never elicited a depolarizing response. The small hyperpolarization still present in NAN-190 suggests an incomplete block of the 5-HT$_{1A}$ response that hindered the 5-HT$_4$-mediated depolarization. However, when a lower concentration (15 μM) of 5-HT was used, the 5-HT$_{1A}$ effect was completely antagonized and the 5-HT$_4$-mediated response was revealed (see below). The effects of 5-HT and DAU 6215 were also studied on the input/voltage relationship in four cells (r.m.p. = − 58 ± 1.8 mV; R_{in} = 45 ± 4.6 MΩ) bathed in 1 μM TTX to prevent cell firing at positive potentials. 5-HT (30 μM) induced a reduction of the slope of the *I/V* curve (Figure 1b). DAU 6215 (3 μM) *per se* did not affect the *I/V* curve and it did not modify the action of 5-HT on the slope resistance (Figure 1b).

DAU 6215 does not antagonize the 5-HT-induced depolarization and AHP amplitude decrease

We tested DAU 6215 on the slow depolarization and reduction of the AHP produced by 5-HT through a putative 5-HT$_4$ receptor subtype. In five pyramidal neurones (r.m.p. = − 54 ± 2 mV; R_{in} = 65 ± 4.8 mΩ), the AHP amplitude following a calcium spike was 13.1 ± 2 mV. In the presence of 15 μM 5-HT the reduction of the AHP amplitude, measured at the peak of the effect, was 78 ± 2.7%, accompanied in all cells by a depolarization (4.5 ± 1.1 mV; Figure 2a). The calcium spike amplitude did not significantly change during the treatment (62.7 ± 5.4 mV in control conditions and 60.5 ± 5.5 mV during 5-HT application). Fifteen to twenty min superfusion with 1 μM DAU 6215 did not affect the calcium spike amplitude, nor antagonize the effect of 5-HT on the membrane potential and AHP amplitude. In the

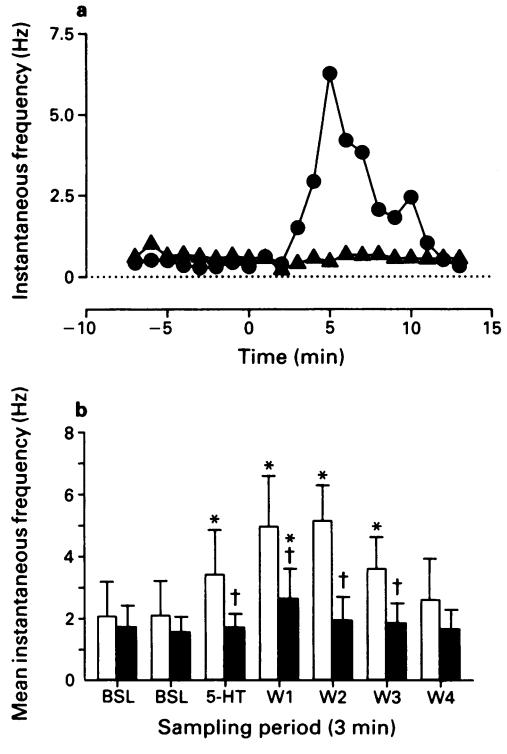

Figure 4 Analysis of the effects of DAU 6215 on 5-hydroxytrytamine (5-HT)-elicited spontaneous postsynaptic potentials (s.p.s.ps). (a) Effect of 5-HT (30 μM) on the instantaneous frequency of s.p.s.ps (e.g., see Figure 3) in the absence (●) or in the presence of 300 nM DAU 6215 (▲). 5-HT was bath-applied at time 0 for 3 min. Each point represents the average instantaneous frequency measured in 1 min recordings from a CA1 pyramidal cell maintained at − 98 mV by − 0.68 nA of steady current. (b) Effect of 5-HT in the absence (open columns) and in the presence of 300 nM DAU 6215, (solid columns). Each column is the mean ± s.e.mean of values from 5 pyramidal cells. Values were obtained measuring the average instantaneous frequency during 3 min recordings. Abbreviations: BSL, baseline; W1–W4, washout. *Values are statistically significant when compared to corresponding BSL values ($P<0.05$, Student's one-tailed paired *t* test): †Values are significantly different ($P<0.05$, multiple comparison ANOVA followed by Fisher LSD *post-hoc* test) from corresponding periods in the absence of DAU 6215.

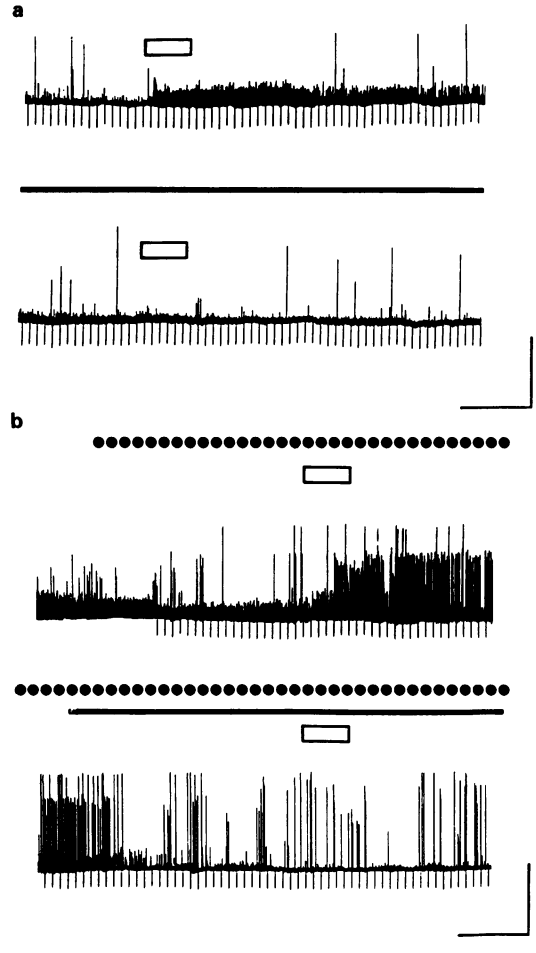

Figure 5 The 5-HT$_3$ selective agonist 2-methyl-5-hydroxytryptamine (2-Me-5-HT) mimics the effect of 5-HT on spontaneous postsynaptic potentials (s.p.s.ps) and its action is antagonized by DAU 6215. (a) Voltage chart recording of a pyramidal neurone held at − 92 mV by injecting − 0.84 nA of current through the recording electrode filled with KCl. Application of 50 μM 2-Me-5-HT (open bar, upper trace) produced an increase in the frequency of s.p.s.ps. This effect was abolished by 60 nM DAU 6215 (filled bar, lower trace). Downward deflections are electrotonic responses to current pulses (− 0.3 nA, 400 ms) to monitor input resistance. Note that 2-Me-5-HT did not change the input resistance, indicating the lack of 5-HT$_{1A}$ or 5-HT$_4$ mediated effects on cell conductance. (b) In a pyramidal cell held at − 93 mV membrane potential by injection of − 0.85 nA, the frequency of s.p.s.ps is only slightly decreased by application of NAN-190 (1 μM; upper trace, dotted line), which did not block the increase in s.p.s.ps elicted by 2-Me-5-HT (open bar). Note that the largest upward deflections are 30–35 mV, reversed i.p.s.ps that reached threshold for triggering action potentials (traces are clipped by chart record frequency response). In contrast, when DAU 6215 (300 nM, filled bar) was applied, still in the presence of NAN-190 (dotted line), a noticeable decrease in the frequency of s.p.s.ps occurred and the effect of 2-Me-5-HT was antagonized. Downward deflections represent electrotonic responses to constant current pulses. Calibration bars: 40 mV, 6 min for (a) and (b).

presence of DAU 6215, 5-HT elicited a 74 ± 2.7% reduction in the amplitude of the AHP and a 4.6 ± 1.5 mV depolarization, values not significantly different from those obtained in the presence of 5-HT alone (Figure 2b and c). Similar results were obtained with 30 μM 5-HT ($n = 2$; not shown).

DAU 6215 antagonizes the increase in frequency of spontaneous synaptic potentials elicited by 5-HT

Neurones impaled with 3 M KCl-filled electrodes and held at a negative potential by constant current injections exhibited depolarizing s.p.s.ps (Figure 3) that have been characterized as reversed $GABA_A$-mediated inhibitory postsynaptic potentials (Alger & Nicoll, 1980). As illustrated in Figure 3a, 5-HT (30 μM; $n = 17$) increased the frequency and amplitude of s.p.s.ps; the effect of 5-HT developed rapidly and outlasted the application of the drug by several minutes. The action of 5-HT did not decrease with time, since repeated applications to the same cell at intervals ranging between 15 and 60 min, produced comparable responses ($n = 6$; not shown). When the cells were maintained near the reversal potential for potassium (− 89 mV ± 1.7), the hyperpolarizing effect of 5-HT was not observed, and the only direct postsynaptic action of 5-HT on pyramidal neurones was a reduction in input resistance (Figure 3a). 5-HT increased the frequency of s.p.s.ps in the presence of 50 μM APV and 25 μM DNQX, compounds that at these concentrations block NMDA and non NMDA glutamate receptors ($n = 5$; not shown). Conversely, 5-HT had no effect in the presence of bicuculline (10 μM; $n = 3$) that abolished the reversed i.p.s.ps (not shown). These results are in agreement with previous observations indicating that these depolarizing events are GABA-mediated, reversed potentials (Ropert & Guy, 1991). Applications of DAU 6215 (300 nM) suppressed the 5-HT-mediated increase in spontaneous synaptic potentials in six out of nine cases (Figure 3). DAU 6215 required 20–30 min equilibration to exert its maximal effect, which was long-lasting since it persisted for over 1 h, after washing out the compound. For these reasons, recovery of the response to 5-HT was achieved only in 3 cases. The instantaneous frequency of the s.p.s.ps was analysed in five out of the six cells, chosen on the basis of a signal-to-noise ratio that allowed a reliable detection of synaptic events (see methods). In these preparations the instantaneous frequency of s.p.s.ps in control conditions varied from cell to cell (range 0.4–6.5 Hz) but was rather constant over time in individual cells, allowing good estimation of the effect of 5-HT on this parameter. Figure 4 illustrates the results of this analysis applied to responses in a pyramidal neurone. 5-HT elicited a 6 fold increase in instantaneous frequency of s.p.s.p. and this effect was antagonized by 300 nM DAU 6215. Figure 4b shows the means and the statistical significance of measures taken from the 5 similar experiments analysed.

As mentioned in the introduction, the 5-HT-induced increase in the frequency of s.p.s.ps presumably involved more than one 5-HT receptor subtype. In an attempt to isolate the 5-HT_3 component of the response to 5-HT, we applied 2-Me-5-HT, a selective 5-HT_3 agonist. Three-five min applications of 50 μM 2-Me-5-HT caused an increase in the frequency of synaptic events, in a manner comparable to the effect of 5-HT ($n = 4$, Figure 5). In all cases, DAU 6215 was effective in blocking the 2-Me-5-HT response at concentrations as low as 60 nM ($n = 3$, Figure 5a). The fact that in two cells DAU 6215 blocked the increase in instantaneous frequency of s.p.s.ps elicited by either 2-Me-5-HT (Figure 5b) or 5-HT in the presence of NAN-190 (not shown) confirms that in these cases the effect was not mediated by 5-HT_{1A} receptors. In two cases, 5-HT (30 μM) applied to neurones that had not responded to 2-Me-5-HT, elicited an increase in the frequency of spontaneous synaptic events. In these preparations,

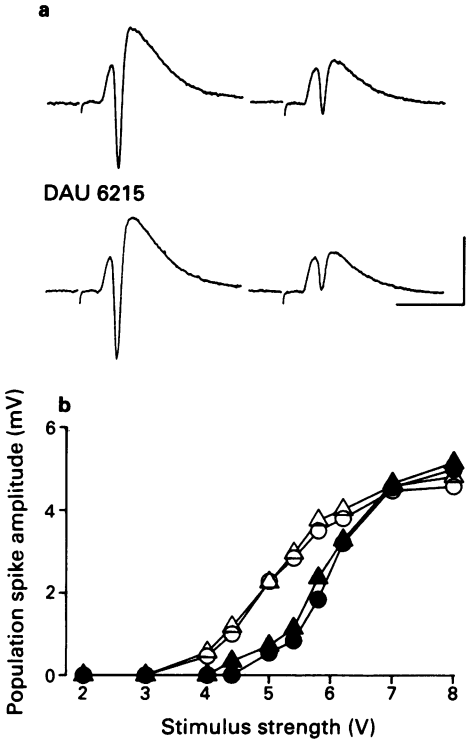

Figure 6 DAU 6215 does not modify the reduction of population spike amplitude induced by 5-hydroxytryptamine (5-HT). (a) Stimulation of the stratum radiatum evoked an excitatory postsynaptic potential and a population spike (upper left). Superfusion with 5-HT (30 μM; 15 min) decreased the amplitude of the population spike (upper right). DAU 6215 did not affect the evoked response (300 nM, 20 min; lower left), nor antagonize the action of 5-HT (lower right). Stimulus artifacts are partially blanked (first small downward deflection). Calibration bars: 2 mV, 10 ms. (b) Input/output curves were constructed in control conditions (○) by increasing the strength of the pulse delivered to the stratum radiatum. This was repeated starting from the fifth minute of application of 5-HT (30 μM; ●); after 20 min superfusion with DAU 6215 (1 μM, △); and at the fifth min of 5-HT application in the presence of DAU 6215 (▲).

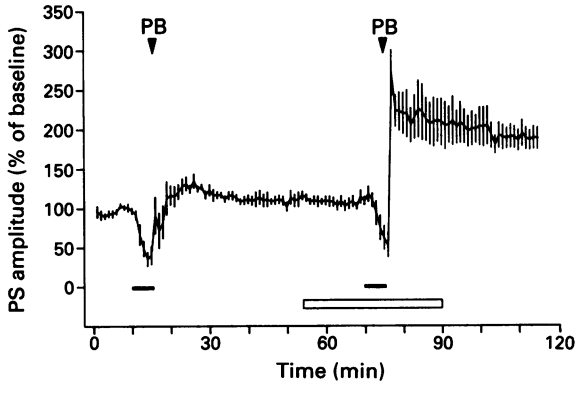

Figure 7 DAU 6215 antagonizes the 5-hydroxytryptamine (5-HT)-mediated block of long-term potentiation (LTP) produced by primed burst (PB) stimulation. The graph shows the mean amplitude (± s.e.mean; $n = 7$) of population spikes (PS) evoked by test pulses. For each experiment, the amplitude of the population spike was expressed as a percentage of the average of the population spike amplitudes obtained during a 10 min period before starting the experiment. Application of 5-HT (30 μM, filled bars) for 5 min decreased the amplitude of population spikes and blocked the induction of LTP by a PB, delivered at the fifth min of 5-HT application. When the PB was delivered at the fifth min of 5-HT application in the presence of DAU 6215 (60 nM, open bar), a long-lasting increase in the amplitude of the population spike was obtained.

300 nM DAU 6215 failed to antagonize the 5-HT-mediated response. In addition, in 4 experiments DAU 6215 (300 nM) failed to block the response elicited by 30 μM 5-HT in the presence of D-APV (50 μM), DNQX (25 μM) and 1 μM NAN-190 (not shown). These results suggest that in some preparations receptors other than the 5-HT$_3$ and 5-HT$_{1A}$ subtype participated in the 5-HT-mediated response.

DAU 6215 antagonizes the 5-HT-mediated block of LTP

In the following experiments we tested the effects of DAU 6215 on synaptic hippocampal potentials and LTP and its antagonistic action on the inhibitory effects that 5-HT exerts on the same parameters.

Micromolar concentrations of 5-HT reduced the amplitude of the population spike evoked by low frequency stimulation of the Schaffer's collaterals (Figure 6), a response apparently due to the activation of 5-HT$_{1A}$ receptors (Beck *et al.*, 1985; Corradetti *et al.*, 1992a). As reported previously (Segal, 1980; Corradetti *et al.*, 1992a), the effect of 5-HT decreased at higher intensities of stimulation (Figure 6b). Applications of DAU 6215 at concentration up to 1 μM, (about 250 times its K_i had, *per se*, no effect on the amplitude of the population spike evoked with different stimulus intensities, and the inhibitory action of 5-HT on the amplitude of the population spike was not antagonized by any of the concentrations of DAU 6215 tested (Figure 6b; Table 1). We than investigated the effect of DAU 6215 on the inhibition exerted by 5-HT on the LTP induced by PB stimulation (see methods). A PB stimulation delivered to the stratum radiatum evoked an LTP that was prevented by the application of 30 μM 5-HT (Figure 7). When the PB stimulation was repeated in the presence of both 30 μM 5-HT and DAU 6215 (60 nM; $n = 7$), a long-lasting increase in the amplitude of the evoked response was observed (Figure 7).

Discussion

The results of our investigation demonstrate that DAU 6215 exerts a powerful antagonism of the presumed 5-HT$_3$-mediated effects of 5-HT on synaptic activity in the hippocampal CA1 region. Our data indicate that DAU 6215 antagonizes the effect of 5-HT on GABA-mediated spontaneous potentials and on the induction of LTP, through the selective blockade of 5-HT$_3$ receptors. The biochemical evidence that DAU 6215 binds to 5-HT$_3$ receptor subtypes in the CNS (Turconi *et al.*, 1990), was confirmed by several of our results. Our intracellular recordings from pyramidal neurones showed that DAU 6215 does not exert any direct action on cell membrane properties; concentrations of DAU 6215 up to 3 μM did not change the membrane potential of pyramidal neurones, nor the *I/V* relationship. Consistently, extracellular recordings of evoked responses did not show any modification of the synaptic response.

Two receptor subtypes, 5-HT$_{1A}$ and 5-HT$_4$ mediate direct effects of 5-HT on pyramidal neurones (Beck *et al.*, 1985; 1993; Chaput *et al.*, 1990; Andrade & Chaput, 1991). DAU 6215 did not antagonize the 5-HT$_{1A}$-mediated hyperpolarization and shift of the *I/V* curve slope. On the other hand, NAN-190 that itself did not elicit any response, almost completely blocked the 5-HT changes in resting membrane potential and input resistance. Although NAN-190 may act as a partial agonist in the raphe, our results demonstrate that this compound exerts an effective antagonism of 5-HT$_{1A}$-mediated responses of hippocampal pyramidal cells. The different behaviour of the compound in these tissues may reside in the absence of a 5-HT$_{1A}$ receptor reserve in the hippocampus, as suggested by Fletcher *et al.* (1993).

The absence of any antagonism by DAU 6215 at the 5-HT$_{1A}$ receptor was confirmed by the results obtained with extracellular recordings. The use of selective agonists and antagonists (Beck *et al.*, 1985) demonstrated that the inhibitory effect of 5-HT on evoked potentials is mediated by 5-HT$_{1A}$ receptors. DAU 6215 applied at concentrations as high as 1 μM, did not modify the inhibitory effect of 5-HT on evoked synaptic potentials.

It has been reported that DAU 6215 behaves as a weak partial agonist at the 5-HT$_4$ receptor of colliculi neurones (Dumuis *et al.*, 1991; Baxter *et al.*, 1992), although the affinity of DAU 6215 for the 5-HT$_3$ receptor is in the nM range (Turconi *et al.*, 1991a) and that for the 5-HT$_4$ receptor is in the μM range (Dumuis *et al.*, 1991). Our results, though, did not reveal any agonist or antagonist effect of DAU 6215 on 5-HT$_4$-mediated responses in CA1 pyramidal neurones.

Although 5-HT$_3$ receptors do not appear to mediate any direct action of 5-HT on pyramidal neurones, there is increasing evidence indicating that GABAergic interneurones may express 5-HT$_3$ receptors. Binding studies demonstrated the presence of 5-HT$_3$ receptors in the hippocampus (Kilpatrick *et al.*, 1987) and anatomical observations showed that 5-hydroxytryptaminergic projections from raphe nuclei impinge on hippocampal GABAergic interneurones (Kosofsky & Molliver, 1987; Hornung & Celio, 1992). Electrophysiological studies in hippocampal primary cultures showed the activation of a 5-HT$_3$ receptor-mediated current in a subset of neurones (Yakel & Jackson, 1988; Yakel *et al.*, 1988). Although the experiments were directed at studying pyramidal cells, it is possible that these were interneurones. Recently, Ropert & Guy (1991) demonstrated that 5-HT increases both frequency and amplitude of spontaneous GABA-mediated synaptic activity in the CA1 region. This action can be mimicked by the selective 5-HT$_3$ agonist 2-Me-5-HT and blocked by 5-HT$_3$ antagonists such as ICS 205-930 at nanomolar concentrations. The inhibitory potentials, that, when recorded with KCl-loaded electrodes in cells maintained at negative potentials appear as depolarizing events (Alger & Nicoll, 1980), are suppressed by bicuculline and persist when NMDA and non-NMDA receptors are blocked by selective antagonists (Ropert & Guy, 1991 and present results). Our results confirm that 5-HT increases the frequency and amplitude of s.p.s.ps. Although only the increase in frequency was quantified, a qualitative analysis of the responses showed that 5-HT augmented the amplitude of spontaneous events, as well. DAU 6215 blocked the 5-HT-mediated increase in synaptic activity at concentrations within the nM range. It was suggested that the activation of receptor subtypes other than 5-HT$_3$ may participate in this response (e.g., 5-HT$_{1A}$; Van den Hooff & Galvan, 1991). Our experiments conducted in the presence of a 5-HT$_{1A}$ antagonist (NAN-190) or using the selective agonist 2-Me-5-HT, also indicate that other components may be involved in the 5-HT-induced increase in s.p.s.p. frequency. In those cases in which the 5-HT$_3$ component was predominant (approximately 70%), DAU significantly antagonized the increase in synaptic activity produced by either 5-HT or 2-Me-5-HT. The participation of receptors other than 5-HT$_3$ would explain why in a few circumstances 2-Me-5-HT did not exert any effect, whereas 5-HT augmented the synaptic activity, a response not blocked by DAU 6215. Also, in a few cases DAU 6215 failed to block the response elicited by 5-HT in the presence of NAN-190, DNQX and D-APV, suggesting that other, still unidentified 5-HT receptor subtype(s) may be involved. Due to the intrinsic limitations of the protocol used, we were unable to construct a concentration-effect relationship for DAU 6215; however, the fact that antagonism of the 2-Me-5-HT induced response was obtained with concentrations as low as 60 nM indicates that DAU 6215 exerts its action within the range of concentrations consistent with its selective antagonism of 5-HT$_3$ receptors. Our results, therefore, confirm the presence of a 5-HT$_3$ component in the 5-HT induced increase in the frequency of inhibitory s.p.s.ps, and demonstrate that DAU 6215, at nM concentrations, significantly affects the modulatory action of 5-HT on the hippocampal synaptic activity, by blocking 5-

HT_3 receptors presumably located on GABAergic interneurones.

Our extracellular recordings show that DAU 6215 is able to antagonize the inhibitory effect of 5-HT on the induction of LTP produced by PB stimulation. In a previous study (Corradetti *et al.*, 1992a), we have demonstrated that the inhibition of PB-induced LTP can be achieved by the blockade of either 5-HT_{1A} or 5-HT_3 receptors, because the activation of both receptor subtypes seems to be necessary for the action of 5-HT. Since DAU 6215 is devoid of 5-HT_{1A} antagonistic effects at nM concentrations, the action of DAU 6215 in this experimental protocol appears to be mediated by antagonism at the 5-HT_3 receptor. Indeed, the effect of DAU 6215 was comparable to that obtained in the presence of other selective 5-HT_3 antagonists such as ICS 205-930 and ondansetron (Corradetti *et al.*, 1992a).

The hippocampal region is physiologically involved in emotional and cognitive functions, therefore the effects of DAU 6215 on the synaptic activity of this structure may be relevant for its pharmacological actions *in vivo*. In particular, DAU 6215 has been shown to act as an anxiolytic in animal models (Borsini *et al.*, 1993), and, like other 5-HT_3 antagonists (Barnes *et al.*, 1990; Chugh *et al.*, 1991; Domeney *et al.*, 1991; Carey *et al.*, 1992), it exerts a cognition enhancing effect (Brambilla *et al.*, 1993; Pitsikas *et al.*, 1993; 1994). The results of our investigation showing the antagonism of the 5-HT-mediated inhibition of LTP, a phenomenon of synaptic plasticity possibly involved in learning (Teyler & DiScenna 1987), may provide a cellular correlate for the pharmacological effects of DAU 6215 on cognition.

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