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Pharmacological characterization of 5-HT_{1B} receptor-mediated inhibition of local excitatory synaptic transmission in the CA1 region of rat hippocampus

[1]Boris Mlinar, [1]Chiara Falsini & *,[1]Renato Corradetti

[1]Dipartimento di Farmacologia Preclinica e Clinica 'Mario Aiazzi-Mancini', Università di Firenze, V.le G. Pieraccini 6, 50139 Firenze, Italy

1 In the hippocampus, axon collaterals of CA1 pyramidal cells project locally onto neighbouring CA1 pyramidal cells and interneurones, forming a local excitatory network which, in disinhibited conditions, feeds polysynaptic epscs (poly-epscs). 5-hydroxytryptamine (5-HT) has been shown to inhibit poly-epscs through activation of a presynaptic receptor. The aim of the present work was the pharmacological characterization of the 5-HT receptor involved in this 5-HT action.

2 Poly-epscs, evoked by electrical stimulation of the stratum radiatum and recorded in whole-cell voltage-clamp from CA1 pyramidal neurones, were studied in mini-slices of the CA1 region under pharmacological block of $GABA_A$, $GABA_B$, and 5-HT_{1A} receptors.

3 The 5-HT_{1B} receptor selective agonist 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one dihydrochloride (CP 93129) inhibited poly-epscs ($EC_{50}=55$ nM), an effect mimicked by the 5-HT_{1B} ligands 5-carboxamidotryptamine (5-CT; $EC_{50}=14$ nM) and methylergometrine ($EC_{50}=78$ nM), but not by 1-(3-chlorophenyl)piperazine dihydrochloride (mCPP; 10 μM) or 7-trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline dimaleate (CGS 12066B; 10 μM).

4 The effects of CP 93129 and 5-CT were blocked by the selective 5-HT_{1B} receptor antagonist 3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride (GR 55562; $K_B \approx 100$ nM) and by cyanopindolol ($K_B=6$ nM); methiothepin (10 μM) and dihydroergotamine (1 μM). For both GR 55562 and methiothepin, application times of at least two hours were required in order to achieve their full antagonistic effects.

5 Our results demonstrate that 5-HT_{1B} receptors are responsible for the presynaptic inhibition of neurotransmission at CA1/CA1 local excitatory synapses exerted by 5-HT.
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Abbreviations: 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide; a.p., Action potential; CGP 35348, *P*-[3-aminopropyl]-*P*-diethoxymethylphosphinic acid; CGP 55845A, 3-*N*[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-*P*-benzyl-phosphinic acid; CGS 12066B, 7-trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline dimaleate; CP 93129, 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one dihydrochloride; GR 55562, 3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride; mCPP, 1-(3-chlorophenyl)piperazine dihydrochloride; R_{IN}, Input resistance; r.m.p., Resting membrane potential; R_S, Series resistance; WAY 100635, N-(2-(-4(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide

Introduction

The CA1 region of the hippocampus receives dense innervation originating from the raphe nuclei (Segal, 1975) and the released 5-hydroxytryptamine (5-HT) exerts multiple actions on CA1 neurones, acting through a number of distinct receptor types located at pre- and/or postsynaptic levels (for review, see Hoyer *et al*. (1994) and Barnes & Sharp (1999)). The resulting changes in synaptic transmission produced by endogenous 5-HT are a likely consequence of simultaneous activation of more than one 5-HT receptor.

Direct postsynaptic actions exerted by 5-HT through changes in somatodendritic excitability on CA1 neurones are fairly well known. In CA1 pyramidal cells, the 5-HT_{1A} receptor-mediated increase and the 5-HT_4 receptor-mediated block in K^+ conductances produce inhibitory and excitatory effects, respectively (Andrade & Nicoll, 1987; Colino & Halliwell, 1987). Furthermore, CA1 interneurones may be excited by activation of their somatodendritic 5-HT_2 (Shen & Andrade, 1998) and 5-HT_3 (Ropert & Guy, 1991) receptors.

Possible presynaptic modulation of CA1 neurotransmission by 5-HT remains still largely unexplored, in spite of evidence that 5-HT inhibits CA1 synaptic transmission at concentrations lower than those required for eliciting changes in cell excitability. In particular, stimulation of the principal excita-

*Author for correspondence at: Dipartimento di Farmacologia Preclinica e Clinica 'Mario Aiazzi-Mancini', Università di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy; E-mail: corradet@unifi.it

tory input to the CA1 region, Schaffer collaterals (CA3/CA1), evokes polysynaptic responses in the CA1 region, which are potently inhibited by 5-HT (Segal, 1990; Mlinar *et al*., 2001).

Both inhibitory and excitatory polysynaptic responses in the CA1 region are generated by the activation of a local excitatory network formed by collaterals of axonal branches of CA1 pyramidal neurones which projects to basal dendrites of other CA1 pyramidal cells as well as of CA1 interneurones (Knowles & Schwartzkroin, 1981; Freund & Buzsaki, 1996). These local excitatory recurrent connections between pyramidal neurones have been electrophysiologically characterized (Christian & Dudek, 1988; Thomson & Radpour, 1991; Deuchars & Thomson, 1996) and local polysynaptic responses have been elicited following blockade of A_1 adenosine receptors (Klishin *et al*., 1995) and $GABA_A$ receptors (Crepel *et al*., 1997; Mlinar *et al*., 2001). This local CA1/CA1 network appears to be implicated in physiological CA1 signal processing (Thomson & Radpour, 1991; Radpour & Thomson, 1991).

We have recently demonstrated (Mlinar *et al*., 2001) that 5-HT decreases the polysynaptic excitatory and inhibitory responses by presynaptic modulation of local excitatory connections formed by axon collaterals of CA1 pyramidal cells. In that study, an unexpected lack of sensitivity to several broad spectrum 5-HT receptor antagonists, together with the ineffectiveness of some agonists (i.e.: CGS 12066B), suggested unconventional pharmacological properties of the 5-HT receptor involved in the presynaptic inhibition of CA1 axon collateral transmission.

From the pharmacological profile of the effective agonists, a putative receptor, named provisionally 5-HT$_{1G}$, with nM affinity for 5-HT, 5-CT, and 5-MeOT and insensitive to several antagonists (Castro *et al*., 1997) could not be dismissed. Furthermore, an 'atypical' 5-HT$_{1A}$ receptor showing high-affinity for 5-CT and 8-OH-DPAT, while relatively insensitive to antagonists has been described in the CA1 region of the rat hippocampus (Waeber & Moskowitz, 1995). Finally, the anatomical consideration that 5-HT$_{1B}$ were likely to be expressed on axons of pyramidal cells in CA1 (Bruinvels *et al*., 1994; Boschert *et al*., 1994) prompted us to re-appraise the issue regardless of the ineffectiveness of the putative 5-HT$_{1B}$ receptor agonist CGS 12066B.

In addition to 8-OH-DPAT, to investigate the possibility of the involvement of an atypical 5-HT$_{1A}$ receptor, we used a wider range of 5-HT receptor ligands, including rodent 5-HT$_{1B}$ receptor selective agonist CP 93129 (Macor *et al*., 1990) or antagonists such as CR 55562 (Walsh *et al*., 1995; Lamothe *et al*., 1997) and cyanopindolol (Maura *et al*., 1987; Hoyer *et al*., 1994).

In our investigation, we used whole cell voltage-clamp recording of the polysynaptic excitatory response evoked in CA1 pyramidal cells by electrical stimulation of the stratum radiatum of rat dorsal hippocampal slices under block of GABA receptor-mediated responses and ablation of the CA3 region. The effects of 5-HT at the presynaptic level within the CA1/CA1 local network may be difficult to study in isolation. However, a favourable feature of the CA1 local network arrangement and of the selective sensitivity of CA1/CA1 synapses to 5-HT is that the poly-epscs originating from the temporal summation of CA1/CA1 excitatory connections can be easily separated from the monosynaptic component of epscs (mono-epsc) originating from CA3 afferents. This allows for quantitative measurement of the action of 5-HT agonists on CA1 pyramidal cell axons.

Methods

Experiments were carried out as previously described (Mlinar *et al*., 2001). All animal manipulations were performed according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use of the University of Florence.

Experimental procedure

Twenty- to 28-day old Wistar rats (Harlan Italy, Udine, Italy) were anaesthetised with ether and decapitated with a guillotine. The brain was quickly removed and cooled in partially frozen oxygenated artificial cerebrospinal fluid (aCFS) which consisted of (in mM): NaCl 126; KCl 1.5; KH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1.5; $CaCl_2$ 2; D-glucose 10 and was bubbled with 95% O_2/5% CO_2 gas mixture (pH 7.4). Transverse dorsal hippocampus slices of 350 μm nominal thickness were cut with a vibroslicer (VSL, WPI, Sarasota, FL, U.S.A.), and transferred to an incubation chamber containing aCFS at room temperature. After at least 1 h of incubation, one slice was transferred to a Petri dish, where, under the magnifying glass, the CA1 region was disconnected from the CA3 region by surgical cut. Following this procedure, the slice was returned to the incubation chamber and after at least one additional hour of recovery, was transferred to a recording chamber (volume 0.7 ml). Slices were maintained submerged with a U-shaped platinum wire and were continuously superfused with oxygenated aCFS at a rate of 2 ml·min^{-1}. All experiments were performed at room temperature (21–24°C).

In all experiments bath solutions contained: (−)-bicuculline methiodide or (−)-bicuculline methochloride (10 μM) to block $GABA_A$ receptors, 3-*N*[1-(S)-(3,4-dichlorophenyl)ethyl] amino-2-(S)-hydroxypropyl-*P*-benzyl-phosphinic acid (CGP 55845A; 1.5 μM) to block $GABA_B$ receptors (Davies *et al*., 1993), and N-(2-(-4(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide (WAY 100635; 10 nM), to block 5-HT$_{1A}$ receptors. In some early experiments *P*-[3-aminopropyl]-*P*-diethoxymethylphosphinic acid (CGP 35348, 300 μM) was used instead of CGP 55845A as $GABA_B$ receptor antagonist. Epileptiform activity was not observed under these experimental conditions.

In experiments where 5-HT$_{1B}$ antagonists were applied for 2 h or longer, slices were exposed to the antagonist for the required time in an incubation chamber containing oxygenated aCFS at room temperature. After this pre-incubation, the slice was transferred to the recording chamber, in which the antagonist was present throughout the experiment.

Experiments were made on an upright microscope (Axioskop; Zeiss, Göttingen, Germany) with a ×40 water immersion objective lens. Visual guidance was achieved by infrared differential interference contrast videomicroscopy (Stuart *et al*., 1993), utilizing a Newvicon camera (C2400-07; Hamamatsu, Hamamatsu City, Japan). All acquisition was done with the use of an Axopatch 200B amplifier (Axon

Instruments, Foster City, CA, U.S.A.) controlled with Clampex 6 software (Axon) through a PC equipped with a Digidata 1200 interface (Axon). Patch pipettes were pulled from borosilicate glass (1.5 mm O.D., 1.17 mm I.D.; Clark Electromedical Instruments, Reading, U.K.) and had resistance of 1.8 to 2.6 MΩ. The pipette solution contained (in mM): KCH_3SO_3 130; KCl 2; NaCl 2.5; $MgCl_2$ 1; MgATP 3; Na_3GTP 0.5; HEPES 10 and NaOH ≈5 to pH 7.3, ≈295 mosmol l^{-1}.

The pipette capacitive transient was compensated before obtaining the whole-cell configuration with the compensation circuits of Axopatch amplifier. The cell membrane capacitance and the series resistance (R_S), which typically ranged from 5 to 10 MΩ, were not compensated. The input resistance (R_{IN}) of recorded cells was in the range of 90 to 200 MΩ. The stability of recording conditions was monitored through the experiments by applying a 5 mV hyperpolarising pulse shortly before each stimulation pulse. Filtering was at 2–5 kHz and sampling was 20–40 kHz. Data traces were acquired every 10 s.

In most experiments, shortly after establishing the whole-cell configuration, we switched in current clamp mode to determine the resting membrane potential (r.m.p.) and voltage-current relationship to obtain a 'signature' of the recorded neurone. Neurones included in this study had r.m.p. more negative than −60 mV, did not spontaneously fire action potentials (a.p.-s) and had overall typical appearance of CA1 pyramidal neurone current-clamp recordings.

Following this procedure, about 5 min after the whole-cell configuration was established, the amplifier was switched back to voltage clamp mode and holding potential was set to −75 mV or −80 mV. epscs were evoked by stimulation of the stratum radiatum with computer driven stimulus isolation unit (DS2, Digitimer, Welwyn Garden City, U.K.) through a concentric bipolar electrode (Clark Electromedical Instruments, Pangbourne, U.K.) positioned in the middle of the layer 1–1.2 mm from the recorded cell. Stimulation intensity was selected to give epsc amplitude from 50 to 200 pA. Following several minutes of settling time, synaptic currents were routinely stable throughout recording (up to 100 min). Slow run-down of polysynaptic currents, likely due to the degradation of slices, was rarely observed and these experiments were discarded.

With these stimulation parameters almost 'pure' poly-epscs were obtained in approximately 20% of the cases (e.g. Figure 6A) and compound epscs in which mono- and poly-epscs were kinetically separable in the rest of experiments. Low resistance of patch electrodes and consequently good access to the cell interior were crucial for separating mono- and poly-epsc components. Poly-epscs were measured as average currents over a 30–50 ms wide time window, beginning at least 30 ms after the stimulus (see Figure 1B). The preparations showing a detectable residual mono-epsc component of the response in the region of poly-epsc measurement (⩾30 ms following the stimulus artefact) were discarded.

Concentration-response relationships

To obtain data for concentration-response relationships, for each preparation, 4–5 concentrations of an agonist were applied with a cumulative protocol. Only one concentration-response curve, either in the presence or in the absence of an antagonist, was constructed per slice. The duration of the individual agonist concentrations applied was chosen on the basis of the 5-HT action time-course studied in a previous work (see Figure 5D, Mlinar *et al*., 2001), and of the preliminary results obtained with 5-HT$_{1B}$ agonists. Thus, in order to reach at least 95% of the steady-state (maximum) effect for each concentration applied, the low concentrations (10–30 nM) of agonists were applied for 7–8 min and higher concentrations for at least 5 min. For each concentration of agonist, steady-state values were obtained by averaging seven consecutive responses taken at the last minute of the application.

Data analysis

Data were analysed using Clampfit (Axon) and Prism 3.02 softwares (GraphPad Software, San Diego, CA, U.S.A.). Fitting of data points to functions was done by non-linear least squares algorithms. All values are expressed as mean ± s.e.mean. Data were tested for statistical significance with one-sample, unpaired or paired two-tailed Student *t*-test, as appropriate. A value of $P<0.05$ was considered significant.

For the analysis of the action of competitive antagonists the Gaddum-Schild equation, based on the assumption that equal responses rely on equal fractional occupancy of a given receptor, was applied. Under this assumption, the ratio (dose ratio : dr) between the concentration of agonist A in the presence of antagonist B ([A′]) and that giving the same response obtained in the absence of antagonist ([A]) is related to the affinity constant of the antagonist (K_B) by the equation:

$$dr = [A']/[A] = 1 + ([B]/K_B) \quad (1)$$

Alternatively, double reciprocal plots of equiactive concentrations of agonist in the absence and the presence of antagonist were used. This type of calculation does not assume any particular type of non-competitive antagonism and may be applied in almost all cases with the exclusion of irreversible antagonism (Gaddum *et al*., 1955; Kenakin, 1997).

It can be demonstrated (Kenakin, 1997) that, when the antagonist can completely block the response to agonist, equiactive concentrations of the agonist in the absence ([A]) and in the presence ([A′]) of antagonist (B) are related to each other in the following double reciprocal relationship:

$$1/[A] = 1/[A'] \cdot (1 + [B]/K_B + \alpha[B]/K_B \cdot K_A) \quad (2)$$

where K_B and K_A are the equilibrium dissociation constants of the antagonist- and agonist-receptor complex, respectively.

The slope of the straight line resulting from the double reciprocal plot is related to K_B by the equation:

$$K_B = [B]/(\text{slope} - 1) \quad (3)$$

and therefore K_B can be calculated from the slope of the line obtained with regression of the equiactive concentrations obtained experimentally.

It should be noted that while equation 2 also takes into account possible changes in agonist affinity produced by the antagonist (factor α), the calculation of K_B is not affected by the allosteric effects of the antagonist on agonist affinity, since the slope term does not contain α. Double reciprocal plots were constructed with values of equiactive concentrations obtained from nonlinear regression of concentration-response curves.

Drugs

(−)-bicuculline methiodide, (−)-bicuculline methchloride, methiothepin maleate, GR 55562, cyanopindolol hemifumarate, CP 93129, methylergometrine maleate and 8-OH-DPAT were from Tocris (Bristol, U.K.). 5-CT, CGS 12066B, mCPP and dihydroergotamine methanesulphonate were from Sigma-RBI (Milano, Italy). Inorganic salts were Aristar grade (BDH, Poole, U.K.) and all other drugs were from Sigma-Aldrich (Steinheim, Germany) or Fluka (Buchs, Switzerland). CGP 55845A and CGP 35348 were kindly provided by Dr Wolfgang Froestl (Novartis, Basel, Switzerland) and WAY 100635 was a gift of Dr Michel Hamon (INSERM U-288, Paris, France).

Serotonergic drugs were prepared as stock solutions in distilled water or in up to 0.1 M HCl, at one thousand times the highest experimental concentration, aliquoted and stored at −20°C until use. Methiothepin (10 mM) was dissolved by gentle heating in equimolar HCl, and was used on the same day to avoid precipitation. Cyanopindolol was prepared as 20 mM stock solutions in DMSO.

Results

The experiments were carried out on 104 slices taken from 23 rats. In all experiments the GABAergic inhibition was blocked by 10 μM bicuculline and 1.5 μM CGP 55845A. 5-HT$_{1A}$ receptors were blocked by 10 nM WAY 100635.

The stimulation of the stratum radiatum evoked compound epscs comprising an early monosynaptic (mono-epsc) component and a late polysynaptic (poly-epsc) response (Figure 1A,B). In similar experimental conditions we have previously demonstrated that the poly-epscs evoked by the stimulation of the stratum radiatum are the summation of epscs originating from CA1 axon collaterals (Mlinar *et al*., 2001).

Effects of 5-HT$_{1B}$ receptor agonists

As illustrated in Figure 1, application of the selective 5-HT$_{1B}$ receptor agonist CP 93129, decreased the amplitude of poly-epscs in a concentration-dependent manner without affecting membrane conductance of the recorded cell. In contrast, the mono-epscs and the membrane current at the holding potential were unaffected (Figure 1D).

Similar results were obtained with 5-CT (Figure 2A,B) and with methylergometrine (Figure 2C,D), two non-selective, 5-HT$_{1B}$ agonists. The concentration-response curves obtained with 5-CT and CP 93129 are shown in Figure 2E. The resulting EC$_{50}$ values were 55 nM (95% Confidence Limits: 38–59 nM) for CP 93129, 14 nM (95% C.L.: 12–16 nM) for 5-CT, and 78 nM (95% C.L.: 64–95 nM) for methylergometrine (not shown).

The putative 5-HT$_{1B}$ receptor agonist CGS 12066B did not significantly affect poly-epsc responses at a concentration (10 μM) which should have ensured high occupancy of 5-HT$_{1B}$ receptors (Figure 3A,B). In addition, CGS 12066B did not prevent the effect of 5-CT on poly-epscs (Figure 3A,B). Similarly, m-CPP (10 μM) and dihydroergotamine (1 μM), known to bind to 5-HT$_{1B}$ receptors, were ineffective in decreasing the poly-epsc response (Figure 3C). Figure 3C also shows that superfusion with 1 μM 8-OH-DPAT, a 5-HT$_{1A}$ receptor ligand, did not significantly affect the epscs, indicating that the effect of 5-CT could not be ascribed to 5-HT$_{1A}$ receptor activation.

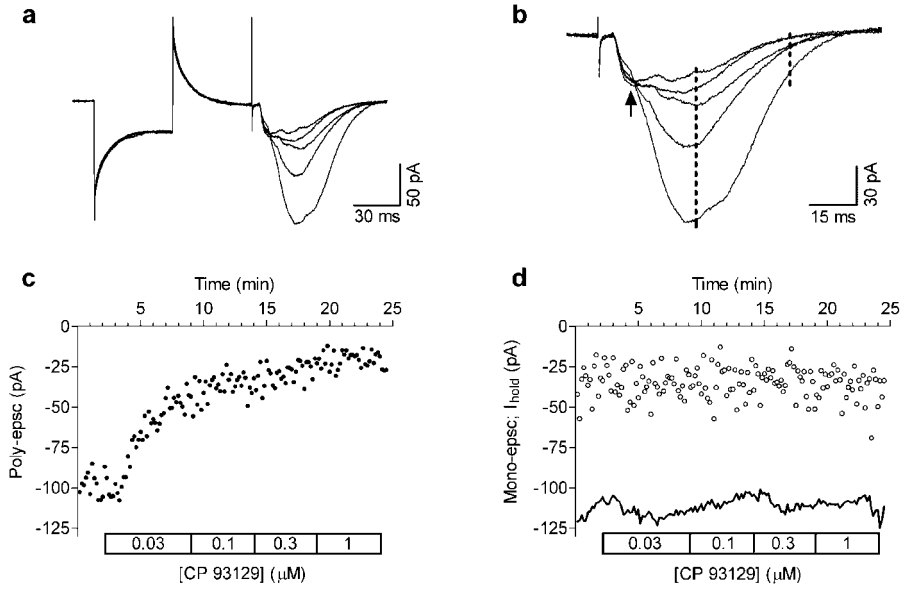

Figure 1 Activation of 5-HT$_{1B}$ receptors inhibits poly-epscs evoked by stimulation of stratum radiatum in the CA1 hippocampal region. (A) Whole-cell recordings show the concentration-dependent decrease in poly-epscs produced by the selective 5-HT$_{1B}$ agonist CP 93129 (30 nM–1 μM; see D). In this and in the following figures the initial part of traces is the response evoked by 5 mV hyperpolarising pulses used to monitor stability of the recording. The following fast, transient, upward deflections are stimulus artefacts. Traces shown are the average of seven consecutive responses obtained in control and at the end of application of each concentration of CP 93129. (B) Same traces as in (A) are shown with expanded time base to illustrate the separation between mono-epscs (arrow) and poly-epscs. Broken lines indicate the time-window used for measuring the amplitude of poly-epscs (see Methods). (C) Time-course of the effects of increasing concentrations of CP 93129 on the amplitude of poly-epsc. (D) Mono-epscs (open circles) were not significantly affected by CP 93129. Continuous line shows the current at holding potential (I$_{hold}$) during the experiment. All experiments were performed under block of GABA$_A$, GABA$_B$, and 5-HT$_{1A}$ receptors.

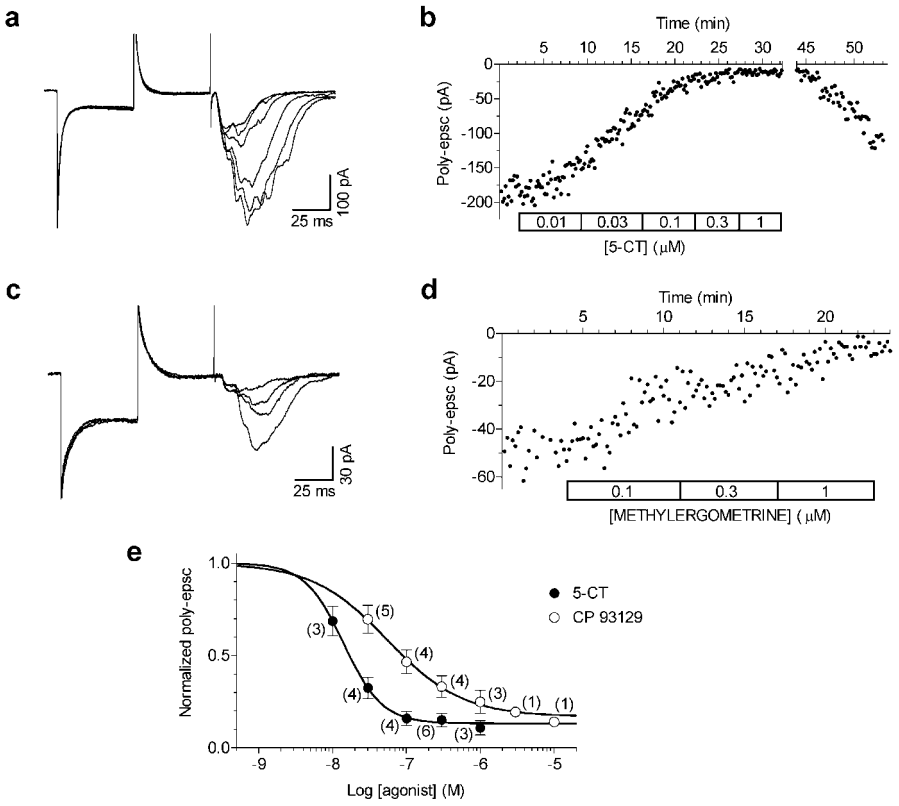

Figure 2 5-CT and methylergometrine inhibition of poly-epsc. (A) Whole-cell recordings show the concentration-dependent decrease in poly-epscs produced by 5-CT (10 nM – 1 μM; see B). (B) Time-course of the effects of increasing concentrations of 5-CT on the amplitude of poly-epscs. Note the gradual recovery of responses after the washout. Whole-cell recordings (C) and the time-course (D) of the effects of increasing concentrations of methylergometrine (100 nM – 1 μM) on the amplitude of poly-epscs. (E) Individual points correspond to mean values (mean $\pm$ s.e.mean) of poly-epsc amplitudes normalised by taking the control as unity. Numbers of experiment replication are given in parentheses. The solid lines are the best least squares fits to the logistic equation, $b+(1\text{-}b)/(1+(EC_{50}/[A])^{n}{}_{H})$, where EC_{50} is the half-maximally effective concentration, [A] is the agonist concentration, n_H is the Hill coefficient, and b is the fraction of poly-epsc remaining at the maximal agonist effect.

5-HT$_{1B}$ receptor antagonists

In a first set of experiments we tested the effectiveness of the selective 5-HT$_{1B}$ antagonist GR 55562 on the inhibition of poly-epscs exerted by CP 93129 or 5-CT. As shown in Figure 4, application of GR 55562 (10 μM) for 30 – 60 min did not greatly decrease the responses to CP 93129 (300 nM) and to 5-CT (300 nM). Nevertheless, the antagonist appeared to hasten the recovery from CP 93129 action indicating that GR 55562 was not completely ineffective. Taking into account the possibility of a slow onset of antagonist action, we preincubated the slices for longer than 2 h before application of the agonists. As illustrated in Figure 4C, when applied for a long time GR 55562 was able to significantly antagonise the effects of both CP 93129 (300 nM) and 5-CT (300 nM).

Long preincubation times (>2 h) were therefore used for constructing concentration-response curves to agonist action in the presence of CR 55562 (10 μM). As shown in Figure 5, the concentration-response curve to CP 93129 in the presence of GR 55562 was displaced to the right in an apparently competitive manner. The apparent K_B of GR 55562 calculated from the shift to the right of CP 93129 concentration-response curve was 104 nM (see Methods). Similar results were obtained using 5-CT as agonist (Figure 6) and the calculated K_B of GR 55562 was 72 nM.

The same experimental protocol (i.e. >2 h drug application) was used to investigate the effects of other known 5-HT$_{1B}$ receptor antagonists (Figure 7). The potent 5-HT$_{1B}$ receptor antagonist cyanopindolol (10 μM) completely blocked the effects of CP 93129 and 5-CT. Similarly, methiothepin, a non-selective 5-HT$_{1B}$ antagonist (10 μM), significantly blocked the effects of CP 93129 and 5-CT. We also tested dihydroergotamine, a high affinity 5-HT$_{1B}$ ligand. At the concentration of 1 μM, which did not affect poly-epscs (Figure 3C), the drug significantly antagonised the effects of 5-CT within 30 min of superfusion (Figure 7C).

Figure 8A illustrates that, when applied in the presence of 1 μM CP 93129, cyanopindolol (10 μM) almost fully antagonised the effect of the agonist within 5 min of combined application. The effect of cyanopindolol reversed within 30 min of washout. This indicated that cyanopindolol was effective in antagonising the action of CP 93129 with faster onset action when compared with GR 55562.

In three cells, we tested the antagonism of cyanopindolol (200 nM) applied for longer than 2 h on increasing concentrations of CP 93129. As shown in Figure 8B, cyanopindolol shifted the concentration-response curve of CP 93129 to the right and decreased the maximal response to the agonist. Given the non-competitive behaviour of the antagonist, its apparent K_B was calculated using the double reciprocal plot of equieffective agonist concentrations (see Methods). Figure

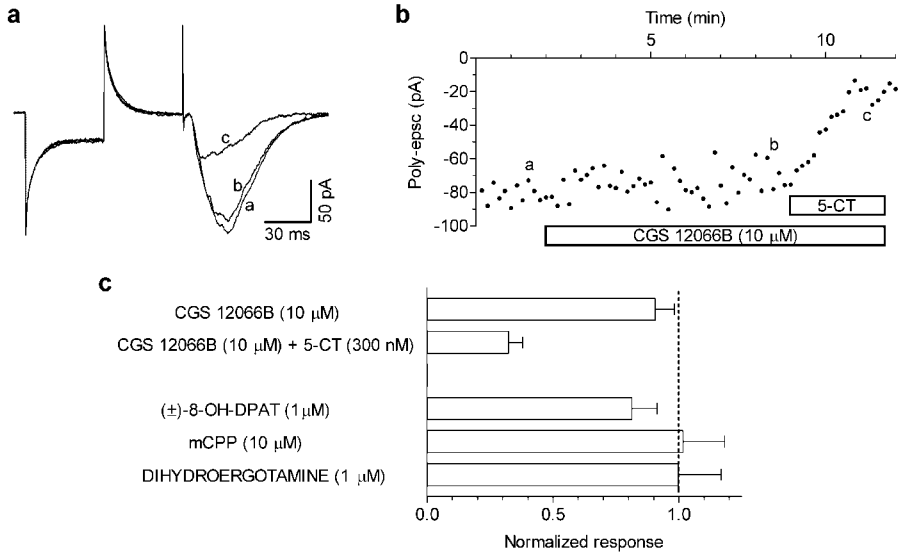

Figure 3 The selective 5-HT$_{1B}$ receptor ligand CGS 12066B neither mimicks nor antagonises the 5-CT-mediated inhibition of poly-epscs. (A) Whole-cell recordings in control (a), in the presence of 10 μM CGS 12066B (b), and of CGS 12066B + 300 nM 5-CT. Traces are averages of seven responses taken at times indicated by corresponding letters in (B). (B) Time-course of the poly-epsc amplitude in the presence of CGS 12066B and of CGS 12066B + 5-CT. (C) Responses to CGS 12066B ($n=7$), CGS 12066B + 5-CT ($n=4$), 8-OH-DPAT ($n=4$), mCPP ($n=6$), and dihydroergotamine ($n=3$) are normalised compared to the pre-drug values. All compounds were applied for 10 – 15 min.

8C shows the plot which gave a K_B value of 6 nM. Although the GR 55562 antagonism appeared to be competitive, a possible non competitive component cannot be ruled out completely. Therefore, Figure 8C also shows the double reciprocal plot for CP 93129 and 5-CT responses in the presence of GR 55562. The resulting K_B values of GR 55562 were 171 nM for CP 93129 and 152 nM for 5-CT effects.

Discussion

The major result of our investigation is the pharmacological identification of the receptor responsible for the inhibition of excitatory neurotransmission exerted by 5-HT at the level of axon collaterals of CA1 pyramidal cells. The inhibition of poly-epscs by the selective 5-HT$_{1B}$ agonist CP 93129 and its block by cyanopindolol and by the selective 5-HT$_{1B}$ antagonist GR 55562 identified 5-HT$_{1B}$ receptor as responsible receptor type. These findings, together with the ineffectiveness of 8-OH-DPAT to inhibit poly-epscs rule out the possible involvement of 'atypical' 5-HT$_{1A}$ receptors (Waeber & Moskowitz, 1995), and/or of the putative 5-HT$_{1G}$ receptors (Castro *et al*., 1997).

CP 93129 recognises the rat 5-HT$_{1B}$ receptor with high affinity and selectivity (Macor *et al*., 1990). In functional studies, CP 93129, acting on presynaptic 5-HT$_{1B}$ receptors, inhibited glutamatergic field potentials in subiculum (Boeijinga & Boddeke, 1993), as well as glutamatergic epscs in caudal raphe neurones (Li & Bayliss, 1998). In our study, CP 93129 mimicked the previously described effect of 5-HT on poly-epscs with an EC_{50} value (55 nM) consistent with the affinity of the compound for 5-HT$_{1B}$ receptors (Macor *et al*., 1990) and with a potency similar to that found for inhibition of glutamatergic epscs in caudal raphe neurones (Li & Bayliss, 1998). In addition, the non-selective agonists 5-CT and methylergometrine potently inhibited CA1 poly-epscs. The EC_{50} of 5-CT in the present investigation is closely consistent with that obtained with sharp-electrode recordings and reported in our previous work (i.e.: $\sim$15 nM; Mlinar *et al*., 2001) where also 5-HT ($EC_{50}=100-200$ nM) and 5-MeOT ($EC_{50}=320$ nM) were tested. The resulting rank of potency of the agonists active in inhibiting poly-epsc/ps in CA1 in similar conditions is 5-CT > CP 93129 $\geqslant$ methylergometrine $\geqslant$ 5-HT > 5-MeOT, which closely fits with the pharmacological profile of 5-HT$_{1B}$ receptors.

GR 55562, a selective 5-HT$_{1B}$ antagonist (Walsh *et al*., 1995; Lamothe *et al*., 1997), completely antagonised the actions of CP 93129 and 5-CT on poly-epscs. The GR 55562 antagonism appeared competitive and the calculated K_B values (104 – 171 nM for CP 93129 and 72 – 152 for 5-CT) are fairly well in agreement with those described in binding studies (39 – 54 nM; Lamothe *et al*., 1997; Pauwels *et al*., 1999). Moreover, the K_B values obtained using either CP 93129 or 5-CT as agonists were virtually the same. This independence of K_B from the agonist used further ensured that both compounds inhibited poly-epscs through activation of 5-HT$_{1B}$ receptors.

Cyanopindolol and methiothepin, two recognised 5-HT$_{1B}$ antagonists (Hoyer *et al*., 1994), also fully antagonised the actions of CP 93129 and 5-CT on poly-epscs. Interestingly, we found a non-competitive component in the antagonism exerted by cyanopindolol. It is becoming increasingly clear that substances showing fully competitive behaviour in binding studies may display additional non-competitive actions in functional assays (Kenakin, 1997). Given the paucity of functional data obtained using concentration-response curves in the presence of cyanopindolol it appears difficult to ascertain whether this is a general characteristic of the drug or if it depends on particular characteristics of the studied receptor.

It should be noted that methiothepin and GR 55562 showed an unusually slow onset of action (e.g. Figure 4). Importantly, this unexpected property of antagonists action

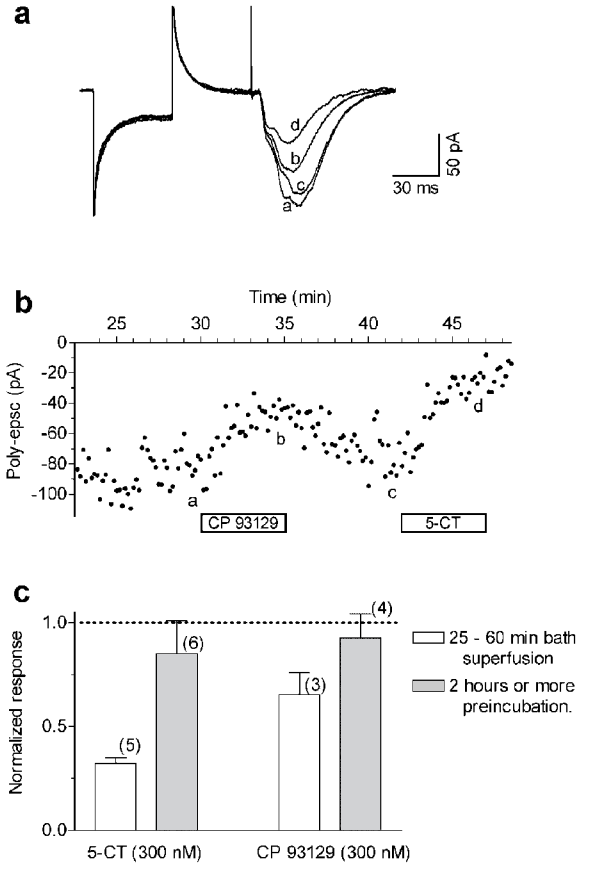

Figure 4 The antagonism of responses to CP 93129 or 5-CT by the selective 5-HT$_{1B}$ antagonist GR 55562 has slow onset. (A) Whole-cell recordings in the presence of GR 55562 (10 μM, 30 min application) (a), after the addition of CP 93129 (300 nM, 5 min) (b), after recovery from CP 93129 effect (c), and after the addition of 300 nM 5-CT (d). Note that 45 min of GR 55562 application did not fully antagonise the effect of 5-CT on poly-epscs (see Figure 6C). Traces are averages of seven responses taken at times indicated by corresponding letters in (B). (B) Time-course of the poly-epsc amplitude inhibition by CP 93129 and 5-CT in the presence of GR 55562. Times on ordinate are given in respect to the beginning GR 55562 application. (C) Superfusion of GR 55562 (10 μM) for $\geqslant$2 h substantially antagonised the action of both agonists. Values are mean$\pm$s.e.mean, responses are normalised compared to the pre-drug values. The number of neurones is given in parentheses.

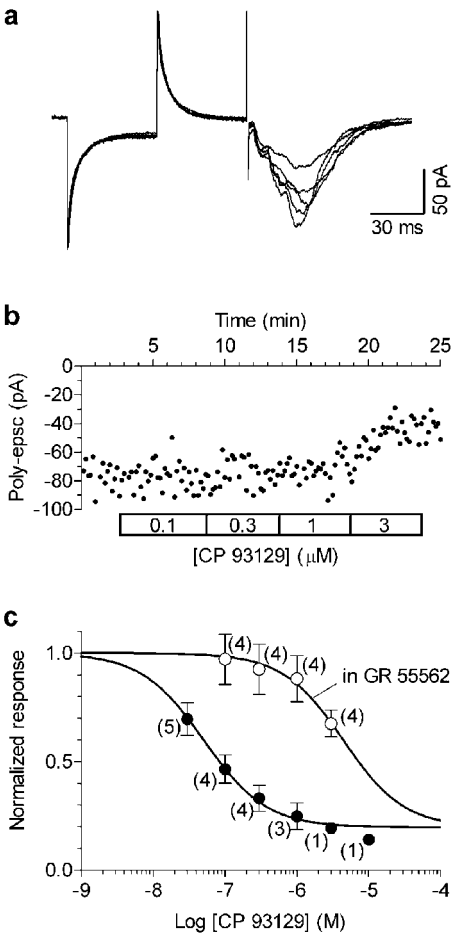

Figure 5 Long applications of GR 55562 antagonise the inhibitory effect of CP 93129. Slices were incubated in 10 μM GR 55562 for at least 2 h and were then transferred in the recording chamber where increasing concentrations (100 nM$-$3 μM, 5 min each) of CP 93129 were applied in the presence of the antagonist. Whole-cell recordings (A) and time-course of the effect on poly-epsc amplitudes (B) show that in the presence of GR 55562 the effect of CP 93129 was antagonised in an apparently surmountable manner. (C) Individual points correspond to mean values (mean$\pm$s.e.mean) of poly-epsc amplitudes normalised compared to values before agonist application. Solid lines are best least squares fits to the logistic equation (see Figure 2E). The data in the presence of GR 55562 were fitted with the bottom (b; fraction of poly-epsc remaining at the maximal agonist effect) constrained to 0.17 (the value determined from CP 93129 concentration-response curve in the absence of antagonist). In the presence of GR 55562 (open circles), the concentration-response curve of CP 93129 appeared shifted to the right in comparison with that obtained in control conditions (filled circles). The number of neurones is given in parentheses.

on 5-HT$_{1B}$ receptors expressed on axon collaterals of CA1 pyramidal cells appears sufficient to explain the lack of antagonist effects reported in our previous work, in which 25$-$30 min application of 10$-$100 μM methiothepin or 100 μM methysergide failed to antagonise the inhibition of polysynaptic responses produced by 5-CT or 5-HT, both in patch-clamp and sharp-electrode recordings (Mlinar *et al*., 2001).

A slow penetration of antagonists in the slice, due to their solubility into membrane lipids, seems not to be a convincing explanation, because cyanopindol, the most lipophilic among the antagonists tested, had faster action than the highly hydrophilic GR 55562.

Furthermore 30 min application of methiothepin (10 μM) exerted the expected block of 5-HT$_{1A}$ receptor-mediated hyperpolarisation in CA1 pyramidal cells produced by 100 nM 5-CT ($-$61.5$\pm$8.3%; n=4; Giachi & Corradetti, unpublished observation; see also Andrade & Nicoll, 1987). Although methiothepin and GR 55562 have been shown to behave as inverse agonists (Pauwels *et al*., 1999) it is difficult to ascertain whether the slow onset of the response could be ascribed to this property or is merely coincidental.

In conclusion, these antagonist peculiarities, while not challenging the selectivity of the 5-HT$_{1B}$ block, may deserve further investigation directed to full understanding of the mechanisms underlying the unexpectedly slow kinetics of effects shown by these substances. CGS 12066B has been claimed to be a selective 5-HT$_{1B}$ receptor agonist (Neale *et al*., 1987), although it was later demonstrated to have a higher affinity for 5-HT$_{1D}$ and 5-HT$_{1A}$ than for 5-HT$_{1B}$

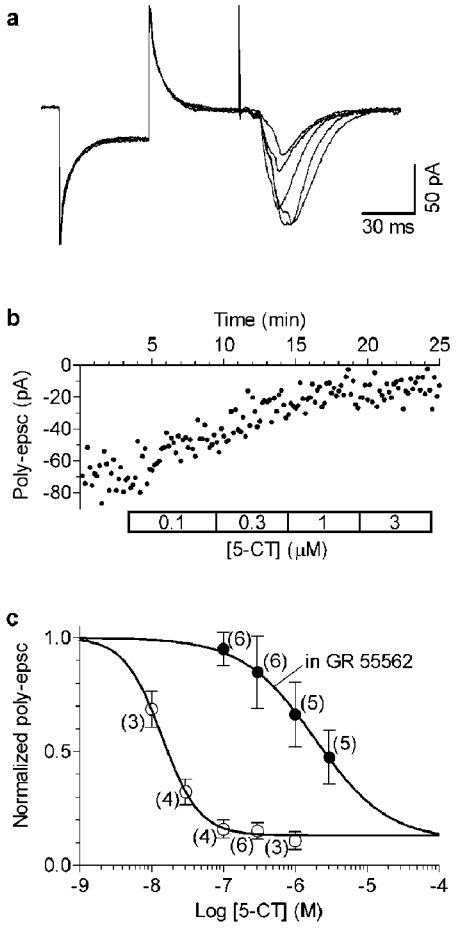

Figure 6 Long applications of GR 55562 antagonise the inhibitory effect of 5-CT. Slices were incubated in 10 μM GR 55562 for at least 2 h and then transferred in the recording chamber where increasing concentrations (100 nM – 3 μM, 5 min each) of 5-CT were applied in the presence of the antagonist. Whole-cell recordings (A) and time-course of the effect on poly-epsc amplitudes (B) show that in the presence of GR 55562 the effect of 5-CT was antagonised. (C) Individual points correspond to mean values of poly-epsc amplitudes normalised compared to values before agonist application. Solid lines are best least square fits to the logistic equation (see Figure 2E). The data in the presence of GR 55562 were fitted with the bottom (b; fraction of poly-epsc remaining at the maximal agonist effect) constrained to 0.13 (the value determined from CP 93129 concentration-response curve in the absence of antagonist). In the presence of GR 55562 (open circles), the concentration-response curve of 5-CT appeared shifted to the right in comparison with that obtained in control (filled circles). Note that the antagonism by GR 55562 was apparently surmountable. The number of neurones is given in parentheses.

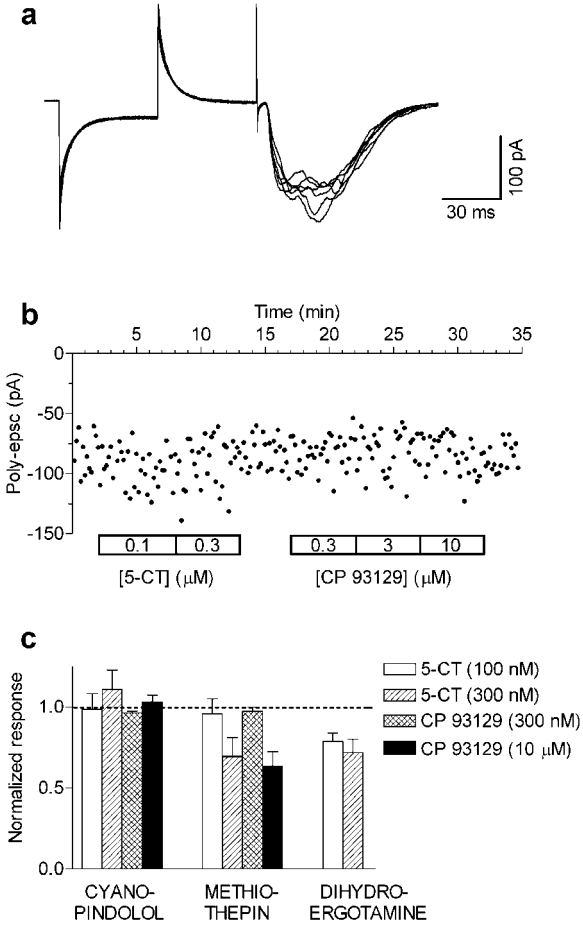

Figure 7 Long applications of cyanopindolol, methiothepin, or dihydroergotamine antagonise the effects of CP 93129 and 5-CT on poly-epscs. Whole-cell recordings (A) and time-course of the effect on poly-epsc amplitudes (B) show that in the presence of cyanopindolol (10 μM; $n=3$) applied for longer than 2 h the effects of 5-CT and of CP 93129 were fully antagonised. In similar conditions, also methiothepin (10 μM; $n=3$) or dihydroergotamine (1 μM; $n=4$) antagonised CP 93129 and/or 5-CT effects on poly-epscs. Bars correspond to mean values (mean $\pm$ s.e.mean) of poly-epsc amplitudes normalised to the respective values before agonist application.

receptors (Macor *et al*., 1990; Adham *et al*., 1992). Nevertheless, CGS 12066B was found to inhibit 5-HT release (Neale *et al*., 1987) and to act as a full 5-HT$_{1B}$ agonist in some functional assays *in vitro* (Schoeffter & Hoyer, 1989).

In our hands, CGS 12066B was ineffective in inhibiting polysynaptic responses both in sharp electrode (Mlinar *et al*., 2001) and in patch-clamp recordings (Figure 3). In addition, the agonist action of 300 nM 5-CT was not antagonised by 10 μM CGS 12066B, indicating lack of partial agonist/antagonist activity of CGS 12066B at 5-HT$_{1B}$ receptors in CA1 pyramidal cells. Another putative 5-HT$_{1B}$ agonist, mCPP (Schoeffter & Hoyer, 1989), was similarly ineffective in inhibiting poly-epscs (Figure 3C). The observed lack of agonism by CGS 12066B, together with its poor receptor selectivity, challenges the use of CGS 12066B as a tool in characterizing 5-HT$_{1B}$ receptor-mediated responses.

The characterization of 5-HT$_{1B}$ receptor-mediated responses has suffered for a long time from poor selectivity of compounds. Nevertheless, convincing evidence indicates that 5-HT$_{1B}$ receptors mediate presynaptic inhibition in many areas of the central nervous system including glutamatergic neurotransmission in the subiculum (Boeijinga & Boddeke, 1993), caudal raphe (Li & Bayliss, 1998) and nucleus accumbens (Muramatsu *et al*., 1998), serotonergic transmission in the dorsal raphe (Morikawa *et al*., 2000) and GABAergic transmission in the ventral midbrain (Johnson *et al*., 1992). However, the availability of novel selective agonists and antagonists, makes it increasingly clearer that additional variability in pharmacological sensitivity of 5-HT$_{1B}$ receptor-mediated responses may derive from the different characteristics of 5-HT$_{1B}$ receptors expressed in various neurone populations. For instance, in neurochemical assays 5-HT$_{1B}$ receptors have been shown to inhibit the

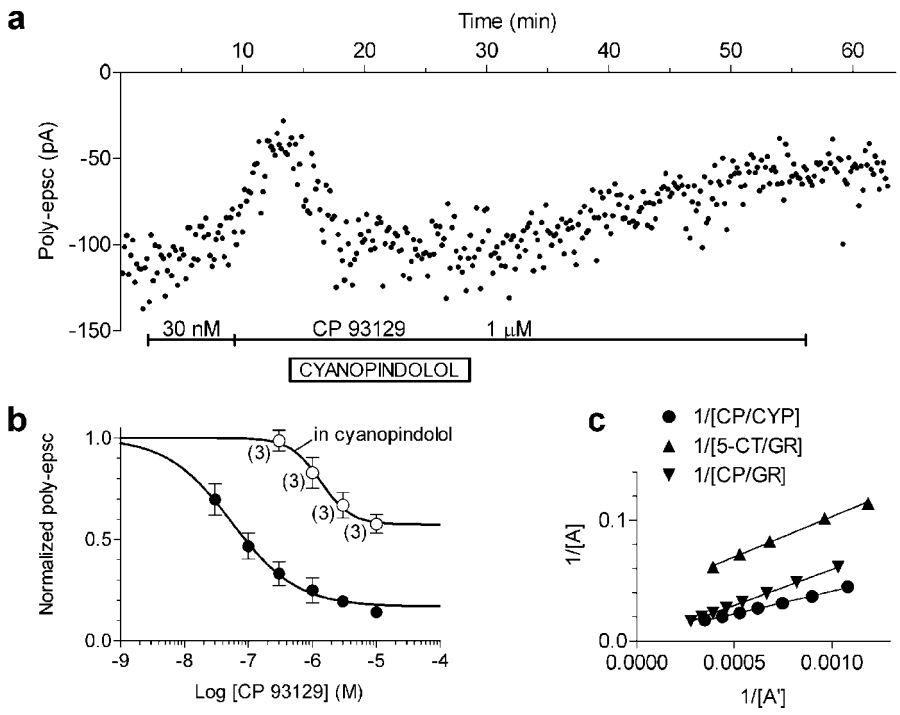

Figure 8 Properties of cyanopindolol antagonism of CP 93129. (A) The time-course of the amplitude of poly-epscs shows effects of 30 nM and 1 μM CP 93129 comparable to those expected from the control concentration-response curve. Addition of cyanopindolol 10 μM rapidly and reversibly antagonised the effect of CP 93129 1 μM. (B) In slices incubated in cyanopindolol 200 nM (open circles), the concentration-response curve of CP 93129 was shifted to the right and the maximum was depressed in comparison with control (filled circles; see Figure 5C), suggesting a non-competitive component of the antagonism. (C) Double reciprocal plot of equiactive concentrations of CP 93129 in the presence of cyanopindolol 200 nM. For comparison the same plot of CP 93129 and 5-CT in the presence of GR 55562 10 μM is shown (see text for details).

release of various neurotransmitters, e.g. acetylcholine (Maura & Raiteri, 1986), and 5-HT$_{1B}$ autoreceptors have been demonstrated to have higher sensitivity to 5-HT and CP 93129 than heteroreceptors on dopaminergic and cholinergic terminals (Sarhan & Fillion, 1999).

Additional pharmacological complexity may arise from the finding that 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors form homodimers when expressed alone, but form heterodimers when co-expressed (Xie *et al*., 1999). If physiological dimerisation occurs, 5-HT$_{1B}$ homodimers would be formed in neurones expressing only 5-HT$_{1B}$ receptor (CA1 pyramidal cells) and 5-HT$_{1B}$/5-HT$_{1D}$ heterodimers would be present in neurones expressing both receptors (e.g. dorsal raphe). Since there are differences in the pharmacology of rodent 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, responses to 5-HT$_{1B}$ agonists and antagonists in different assays may additionally depend on whether 5-HT$_{1B}$ homodimers or 5-HT$_{1B/D}$ heterodimers are present in the presynaptic terminals.

In the hippocampus, 5-HT$_{1B}$ receptors are abundant on axons of CA1 pyramidal cells (Boschert *et al*., 1994; Bruinvels *et al*., 1994) and 5-HT, acting through 5-HT$_{1B}$ receptors, potently inhibits both local excitatory transmission (Mlinar *et al*., 2001) and CA1 projections to subiculum (Boeijinga & Boddeke, 1993; 1996). Functionally, local CA1 excitatory connections are involved in the induction of long-term potentiation (Radpour & Thomson, 1991) and therefore 5-HT$_{1B}$ receptors on axon collaterals of CA1 pyramidal cells are suitably located for controlling hippocampus-dependent learning and/or memory. Moreover, the fact that 5-HT activates 5-HT$_{1B}$ receptors and reduces polysynaptic responses at concentrations lower than those required to produce its postsynaptic effects in hippocampus, together with strategic localisation of 5-HT$_{1B}$ receptors for control of CA1 output suggests that activation of these receptors represents an important physiological mechanism of 5-HT action in the hippocampal formation.

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