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Direct inhibition by cannabinoids of human 5-HT_{3A} receptors: probable involvement of an allosteric modulatory site

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1 Excised outside-out patches from HEK293 cells stably transfected with the human (h) 5-HT_{3A} receptor cDNA were used to determine the effects of cannabinoid receptor ligands on the 5-HT-induced current using the patch clamp technique. In addition, binding studies with radioligands for 5-HT_3 as well as for cannabinoid CB_1 and CB_2 receptors were carried out.

2 The 5-HT-induced current was inhibited by the following cannabinoid receptor agonists (at decreasing order of potency): Δ^9-THC, WIN55,212-2, anandamide, JWH-015 and CP55940. The WIN55,212-2-induced inhibition was not altered by SR141716A, a CB_1 receptor antagonist. WIN55,212-3, an enantiomer of WIN55,212-2, did not affect the 5-HT-induced current.

3 WIN55,212-2 did not change the EC_{50} value of 5-HT in stimulating current, but reduced the maximum effect.

4 The CB_1 receptor ligand [^{3}H]-SR141716A and the CB_1/CB_2 receptor ligand [^{3}H]-CP55940 did not specifically bind to parental HEK293 cells. In competition experiments on membranes of HEK293 cells transfected with the h5-HT_{3A} receptor cDNA, WIN55,212-2, CP55940, anandamide and SR141716A did not affect [^{3}H]-GR65630 binding, but 5-HT caused a concentration dependent-inhibition.

5 In conclusion, cannabinoids stereoselectively inhibit currents through recombinant h5-HT_{3A} receptors independently of cannabinoid receptors. Probably the cannabinoids act allosterically at a modulatory site of the h5-HT_{3A} receptor. Thus the functional state of the receptor can be controlled by the endogenous ligand anandamide. This site is a potential target for new analgesic and antiemetic drugs.

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Abbreviations: Anandamide, ((all Z)-N-(2-hydroxyethyl)-5,8,11,14-eicosa-tetraenamide); CP55940, (−)-cis -3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; GR65630, (3-5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone); JWH-015, ([2-methyl-1-propyl-1H-indol-3-yl]-1-naphthalen-ylmethanone); 5-HT, 5-hydroxytryptamine creatinine sulphate; SR141716A, (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carbox-amide hydrochloride); Δ^9THC, Δ^9-tetrahydrocannabinol; WIN55,212-2, ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone) mesyalte; WIN55,212-3, ((S)-(−)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxaxin-6-yl]-1-naphthalenylmethanone) mesylate

Introduction

It is generally accepted that the effect of Δ^9-THC and other exogenous and endogenous cannabinoids are mediated *via* cannabinoid receptors. Two cannabinoid receptor subtypes CB_1 and CB_2 have so far been identified, characterized and cloned (for reviews, see Pertwee, 1997; 1999; 2000; Ameri, 1999). The CB_1 receptor occurs in brain and nervous tissue (Herkenham *et al*., 1990; Matsuda *et al*., 1990; Mailleux *et al*., 1992) whereas the CB_2 receptor is expressed mainly extraneuronally by immune cells (Munro *et al*., 1993). Accordingly, the effects of cannabinoids in the nervous system are generally believed to be mediated almost exclusively by CB_1 receptors. Cannabinoid receptor agonists do not only exert psychotropic effects (see reviews quoted above and Abood & Martin, 1992), but they also play a role in the control of pain and emesis (reviews quoted above and Noyes *et al*., 1975; Martin *et al*., 1999; Darmani, 2001; Tramer *et al*., 2001). The latter property is shared by serotonin$_3$ (5-HT_3) receptor antagonists.

The 5-HT_3 receptor is a ligand-gated ion channel (Peters *et al*., 1992; Boess & Martin, 1994; Costall & Naylor, 1997) putatively endowed with allosteric modulatory sites. Until now, two human (h) 5-HT_3 receptor subunits, h5-HT_{3A} (Miyake *et al*., 1995) and h5-HT_{3B} (Davies *et al*., 1999) and two h5-HT_{3A} subunit splice variants (Brüss *et al*., 2000) have been described. The h5-HT_{3A} subunit and its splice variants contain the 5-HT recognition site, whereas the h5-HT_{3B} subunit does not. Only the h5-HT_{3A} subunit can form homopentameric functional receptor channels, but its splice variants and the h5-HT_{3B} subunit increase or decrease the current through the channel when coexpressed with the h5-HT_{3A} subunit (Davies *et al*., 1999; Brüss *et al*., 2000). The pharmacological properties of heteromeric 5-HT_3 receptors consisting of the 5-HT_{3A} and 5-HT_{3B} subunits or of the 5-HT_{3A} subunit and one or both of its

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splice variants are not qualitatively modified (Davies *et al*., 1999; Brüss *et al*., 2000; Brady *et al*., 2001); at most, minor differences in affinity or potency of 5-HT_3 receptor ligands may exist (Dubin *et al*., 1999).

According to the involvement of both cannabinoid (for references, see above) and 5-HT_3 receptors (reviews quoted above and Aapro, 1991; Mitchelson, 1992; Karim *et al*., 1996; Voog *et al*., 2000; Simpson *et al*., 2000) in pain and emesis it is conceivable that cannabinoids act not only *via* CB_1 receptors, but also at an allosteric modulatory site of the 5-HT_3 receptor. This possibility has been supported by experiments on the rat nodose ganglion neurone (Fan, 1995) which suggested that neuronal cannabinoid receptors interact with the 5-HT_3 receptor ion channel and that the latter may be a direct target of cannabinoid receptor agonists. Cannabinoid receptor-independent effects of cannabinoids, in particular the endogenous agonist anandamide, have been reported for other ion channels such as vanilloid VR1 receptors (Zygmunt *et al*., 1999; Malinowska *et al*., 2001), TASK-1 K^+ channels (Maingret *et al*., 2001), Shaker-related Kv1.2 K^+ channels (Poling *et al*., 1996) and T-type calcium channels (Chemin *et al*., 2001).

In view of the putative physiological and therapeutic significance in humans, the aim of the present study was to clarify whether cannabinoids directly inhibit 5-HT-induced currents through the h5-HT_{3A} receptor channels and, if so, to analyse the underlying site and mechanism of action. The experiments were carried out on HEK 293 cells stably transfected with the h5-HT_{3A} receptor cDNA. The patch-clamp technique on excised outside-out patches and standard radioligand binding were used. The recombinant homomeric h5-HT_{3A} receptor channels in this system have previously been characterized (Barann *et al*., 2000). Preliminary results of the present investigation have been published elsewhere in abstract form (Barann *et al*., 2001; Godlewski *et al*., 2002).

Methods

Cell culture

HEK 293 cells were grown as monolayers on culture plates (Nunc) in DMEM Nutrient Mix F12 (1 : 1; v v^{-1}) medium containing 10% heat inactivated foetal calf serum, penicillin (100 IU ml^{-1}), streptomycin (100 μg ml^{-1}), geneticine (0.75 mg ml^{-1}) and glutamine (292 μg ml^{-1}). The cells were cultured at 37°C in a humidified atmosphere (5% CO_2). Two days before an experiment cells were subcultured in monodishes (NUNC, 35 mm diameter).

Transfection

For stable transfection 20% confluent HEK 293 cells were transfected by the modified calcium phosphate method with the h5-HT_{3A} receptor cDNA (according to Chen & Okayama, 1987) subcloned into the mammalian expression vector pcDNA3 (Invitrogen) under control of the human cytomegalo virus promoter. Two days after transfection, stably transfected cells were selected by the addition of geneticine (800 μg ml^{-1}) to the culture medium. The medium was changed every 2 days. After occurrence of single cell colonies these were separated by usage of cloning cylinders (Sigma) and further subcultured in 24 well plates (Falcon) until confluence. About 20 to 40 colonies from each transfection experiment were tested for stable expression of the particular cDNA by 5-HT-induced [^{14}C]-guanidinium influx and binding of the selective 5-HT_3 receptor antagonist [^{3}H]-GR65630. Colonies with the highest receptor expression were used for further experiments.

Electrophysiology

In a standard voltage-clamp experiment, 30 μM 5-HT was applied for 1–2 s at −100 mV on an excised outside-out patch using a fast solution exchange system (exchange time 1–2 ms; patch resistance: 1–10 gigaohm; Liu & Dilger, 1991). The external solution applied on the patch was of the following composition (mM): NaCl 150, KCl 5.6, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 10, pH 7.4. Patch pipettes with resistances of 3–6 MΩ were filled with 'intracellular' solution containing (mM) KCl 140, EGTA 10, $MgCl_2$ 5, HEPES 10, pH 7.4. For current measurements, we used a patch-clamp amplifier (EPC-7; List Electronic, Darmstadt, Germany) with the output filter set at 1 kHz and pClamp software (Axon Instruments, Foster City, CA, U.S.A.). The sampling rate varied between 200 and 2000 Hz; within this range, no significant differences were seen with regard to fitted time constants. The drug application system was equipped with inert materials like teflon tubing and glass to avoid losses of lipophilic drugs.

Radioligand binding experiments

All steps of the preparation procedure were performed on ice. Freshly harvested HEK 293 cells (parental or stably transfected with the human 5-HT_{3A} cDNA) were suspended in 40 ml of a buffer solution containing (mM): HEPES-Na^+ 5, EGTA 0.5, $MgCl_2$ 0.5, ascorbic acid 0.1, and phenylmethylsulphonylfluoride 0.3, pH 7.4 (buffer I), and homogenized using a glass-Teflon homogenizer (3 × 30 s). The homogenates were centrifuged (5 min, 1200 × *g*, 4°C). The supernatant was poured off, diluted to 420 ml with buffer I and recentrifuged (20 min, 40,000 × *g*, 4°C). The pellet was washed twice, resuspended in buffer II (mM): (HEPES-Na+ 5, EGTA 0.5, $MgCl_2$ 0.5, ascorbic acid 0.1, pH 7.4), homogenized, and diluted to give a protein concentration of about 1 mg ml^{-1} and stored at −80°C until use. Before the membranes were added to the incubation assay, they were centrifuged (20 min, 40,000 × *g*, 4°C), resuspended in the incubation buffer (buffer II), homogenized by ultrasonication and diluted to a final protein concentration of about 0.6 mg ml^{-1}.

A 400 μl aliquot of membranes was incubated for 60 min with [^{3}H]-GR65630 (1 nM) or [^{3}H]-SR141716A (3 nM) at room temperature in a final volume of 0.5 ml of a solution containing buffer II. The reaction was stopped by rapid vacuum filtration with a Brandel cell harvester through Whatman GF/C glass fibre filters presoaked with polyethyleneimine 0.5 M followed by rapid washing of the incubation tubes and filters with 10 ml ice-cold buffer II. The radioactivity retained in the filters was determined by liquid scintillation counting. Non-specific binding of [^{3}H]-GR65630 was defined by addition of 100 μM MDL72222 and as [^{3}H]-SR141716A binding in the presence of 3 μM CP55940.

Competition studies were carried out using [^{3}H]-GR65630 (10 nM) and up to 13 different concentrations of the unlabelled ligand under study, ranging from 1 nM up to 100 μM. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard.

To test parental HEK 293 cells for the expression of endogenous CB_1-/CB_2-receptors, radioligand binding was performed as described above with 5 nM [^{3}H]-CP55940 and specific binding was determined by competition with 3 μM unlabelled CP55,940.

Data analysis

The currents were digitized with an interface (Digidata 1200, Axon Instruments) and stored on an IBM 586-compatible PC. Data analysis was performed with pClamp^R 7 software (Axon). Graph Pad Prism^R software (Graph Pad, CA, U.S.A.) was used to create graphics. The concentration-response curves for 5-HT were fitted by the Hill equation, $i = c^n/(c^n + EC^n_{50})$: i is the immediate peak current as fraction of the maximal (control) current, c is the 5-HT concentration, n is the Hill coefficient and EC_{50} is the 5-HT concentration inducing the half-maximal effect. This equation was analogously applied to determine the cannabinoid inhibition of the 5-HT response.

Drugs used

3-Tropanyl-3,5-dichlorobenzoate (MDL72222) was from RBI (Natick, U.S.A.). (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carbox-amide hydrochloride (SR141716A) from Sanofi (Montpellier, France); 5-hydroxytryptamine creatine sulphate (5-HT), ([2-methyl-1-propyl-1H-indol-3-yl]-1-naphthalen-ylmethanone) (JWH-015), Δ^9-tetrahydrocannabinol (Δ^9-THC), (S)-(−)-[2,3-dihydro-5-methyl-3-(4-morpholinyl methyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone-mesylate (WIN55,212-3) were obtained from Sigma (Munich, Germany); (all Z)-N-(2-hydroxyethyl)-5,8,11,14-eicosa-tetraenamide (anandamide), (−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP55940) and ((R)-(+)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55,212-2) were obtained from Tocris/Biotrend (Cologne, Germany); [6-methoxy-2-(4-methoxyphenyl)benzo[b]furane-3-yl][4-cyanophenyl]methanone(LY320135) was a present from Lilly, Indianapolis, Indiana, U.S.A.; [^{3}H](−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol ([^{3}H]-CP55940 specific activity 101 Ci mmol^{-1}) from NEN, Zaventem, Belgium; [^{3}H]-(3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone) ([^{3}H]-GR65630) specific activity 64.8 Ci mmol^{-1} was obtained from NEN DuPont (Dreieich, Germany); [^{3}H]-(N-piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carbox-amide hydrochloride ([^{3}H]-SR141716A, specific activity 44.0 Ci nmol^{-1}) from Amersham (Braunschweig, Germany).

Solutions

5-HT solutions were prepared daily from 50 mM aqueous stocks (stored at −20°C). Cannabinoid solutions were prepared daily from 10 mM stocks in DMSO (stored at −20°C; final DMSO concentration $\leqslant$0.01%). MDL72222 was dissolved daily in methanesulphonic acid (final concentration $\leqslant$0.001%). The vehicles in the concentrations used, had no effect on radioligand binding or current response.

Results

Effects of cannabinoid receptor ligands on 5-HT-induced currents

In excised outside-out patches of HEK 293 cells stably transfected with h5-HT$_{3A}$ receptors, 5-HT (present for 1–2 s) evoked currents in a concentration dependent manner (Figure 1; EC_{50} = 8.9 μM, Hill coefficient = 1.4; n = 3–6; see also Figure 2 for original traces).

In most of the experiments in which the effects of cannabinoid receptor ligands were determined, 5-HT was applied at a standard concentration of 30 μM and the cannabinoids were present 3 min before and during (and after) the 5-HT stimulus. In experiments with all cannabinoid receptor ligands investigated (at concentrations approximately corresponding to the respective EC_{50} values n = 3–6), this preexposure time has been shown to be sufficient to obtain an equilibrium effect (results not shown).

WIN55,212-2 inhibited the 5-HT-induced current (Figure 2). This inhibition was concentration-dependent (Figure 3a) and not voltage-dependent between −100 mV and +100 mV (n = 3, not shown). The CB_1 receptor antagonist SR141716A at the concentration of 1 μM did not modify the WIN55,212-2-induced inhibition (Figure 3a). In contrast to WIN55,212-2, its enantiomer WIN55,212-3 even at the high concentration of 1 μM did not change the 5-HT-evoked current (Figures 2 and 3a); at this concentration WIN55,212-2 almost abolished the 5-HT-induced current.

The cannabinoid receptor agonists Δ^9-THC, JWH-015 and CP55940, the endogenous cannabinoid receptor agonist anandamide and the CB_1 receptor antagonist LY320135

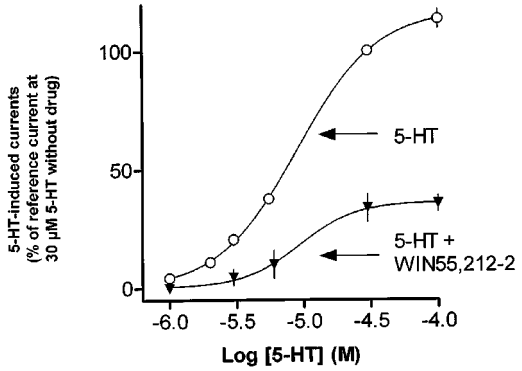

Figure 1 Influence of WIN55,212-2 on the 5-HT-induced currents (−100 mV holding potential) in excised outside-out patches of HEK 293 cells stably transfected with the h5-HT$_{3A}$ receptor cDNA. Concentration-response curve of 5-HT in the absence (open symbols) and in the presence (filled symbols) of WIN55,212-2 (300 nM; present 3 min before, during and after the 2 s 5-HT pulse). The currents in the absence and presence of the drug, respectively, are expressed as percentages of the currents evoked by the standard concentration of 30 μM 5-HT without additional drug (determined in all experiments as a reference effect). Shown are means±s.e.m. of 3–10 different patches.

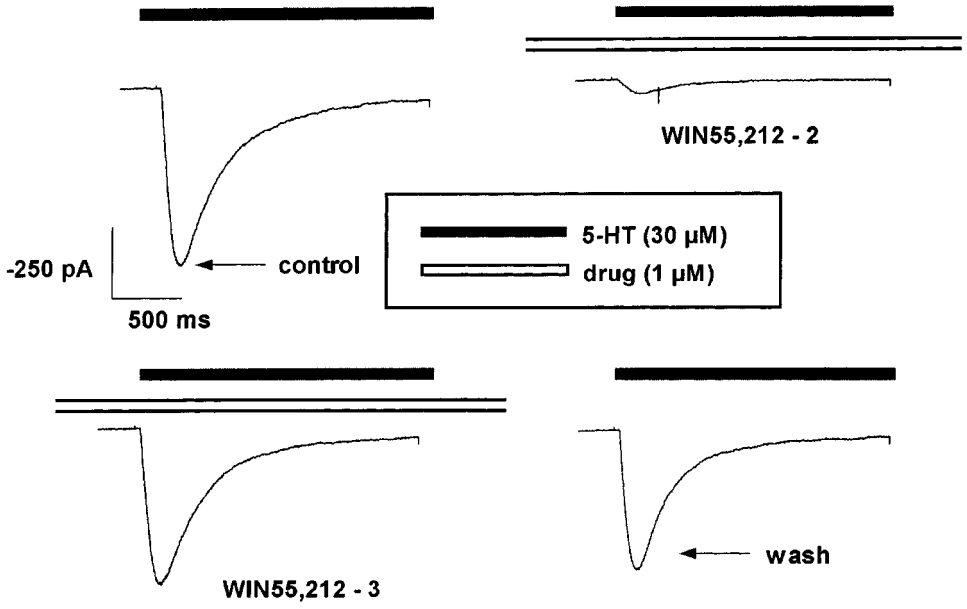

Figure 2 Effects of the enantiomers WIN55,212-2 (upper right panel) and WIN55,212-3 (lower left panel), both at 1 μM, on 5-HT (30 μM; -100 mV)-induced currents obtained in one outside-out patch of a HEK 293 cell stably transfected with the h5-HT$_{3A}$ receptor cDNA. Drugs were applied for 3 min before, during and after stimulation with 5-HT (upper right and lower left panels). 5-HT (30 μM; -100 mV)-induced currents under control conditions (i.e. absence of a further drug; upper left panel); 'wash' refers to the control response to 30 μM 5-HT after omission of the drug for 3 min at the end of the experiment (lower right panel).

shared the concentration-dependent inhibitory effect of WIN55,212-2 (Figure 3b). The IC_{50} values of all compounds are listed in Table 1: The rank order of potency derived from the concentration-response curves shown in Figures 3a,b was as follows Δ^9-THC > WIN55,212-2 $\geqslant$ anandamide $\geqslant$ JWH-015 > LY320135 $\geqslant$ CP55940.

In a further series of experiments, the effect of WIN55,212-2 on the concentration-response curve of 5-HT was determined. When applied at a concentration (300 nM) three times higher than that which produced half maximal inhibition of 5-HT-induced currents through h5-HT$_{3A}$ receptors (compare Table 1), WIN55,212-2 did not change the EC_{50} value of 5-HT in stimulating currents (8.9 versus 9.3 μM), but reduced the maximum of the 5-HT concentration-response curve (Figure 1).

Finally the effect of 1 μM WIN55,212-2 on the 5-HT-evoked current was studied at different time schedules of application (Figure 4). Under the standard conditions, i.e. application of the drug 3 min before, during (and after) the 2 s stimulation with 5-HT, WIN55,212-2 produced an almost complete inhibition of current (lower left panel). Virtually the same degree of inhibition of 5-HT-induced current was obtained when the cannabinoid was present only 3 min before (and after), but not during stimulation with 5-HT (lower right panel). In contrast, application of the drug exclusively during the 2 s 5-HT pulse (upper right panel) was ineffective. Analogous results were obtained with all cannabinoids investigated (at least $n=3$ in each series of experiments; results not shown).

Radioligand binding experiments

In contrast to the clear-cut specific binding of the 5-HT$_3$ receptor radioligand [^{3}H]-GR65630 to membranes of transfected HEK 293 cells (Figure 5), no such binding was detectable in parental cells ($n=5$, not shown). In membranes of parental HEK 293 cells, we did not find any specific binding of the CB_1 receptor ligand [^{3}H]-SR141716A nor of the CB_1/CB_2 receptor ligand [^{3}H]-CP55940 at concentrations of 3 and 5 nM, respectively, which are sufficient to label cannabinoid receptors ($n=8$ and 3, respectively; results not shown).

In competition experiments, 5-HT concentration-dependently inhibited the specific binding of the selective 5-HT$_{3A}$ receptor ligand [^{3}H]-GR65630 (Figure 5a), whereas the cannabinoid receptor agonists WIN55,212-2, CP55940 and anandamide (Figures 5a–c) as well as the CB_1 receptor antagonist SR141716A (results not shown) did not reduce specific [^{3}H]-GR65630 binding to membranes of HEK 293 cells stably transfected with the h5-HT$_{3A}$ receptor cDNA. The concentration-dependent inhibition by 5-HT of specific [^{3}H]-GR65630 binding was not altered by CP55940 (Figure 5a). Furthermore, CP55940 (10 μM, 1 h incubation time) did also neither affect the association of [^{3}H]-GR65630 to ($n=5$, compared to five controls), nor its dissociation from ($n=4$, compared to four controls) membranes of transfected HEK 293 cells (results not shown).

Discussion

The aim of the present study was to examine whether cannabinoid receptor ligands produce an inhibition of currents through recombinant homomeric h5-HT$_{3A}$ receptors by an action at the receptor itself. The possibility that cannabinoid receptor agonists might directly influence the 5-HT$_3$ receptor was derived from an electrophysiological study on rat nodose ganglion neurones (Fan, 1995). In that

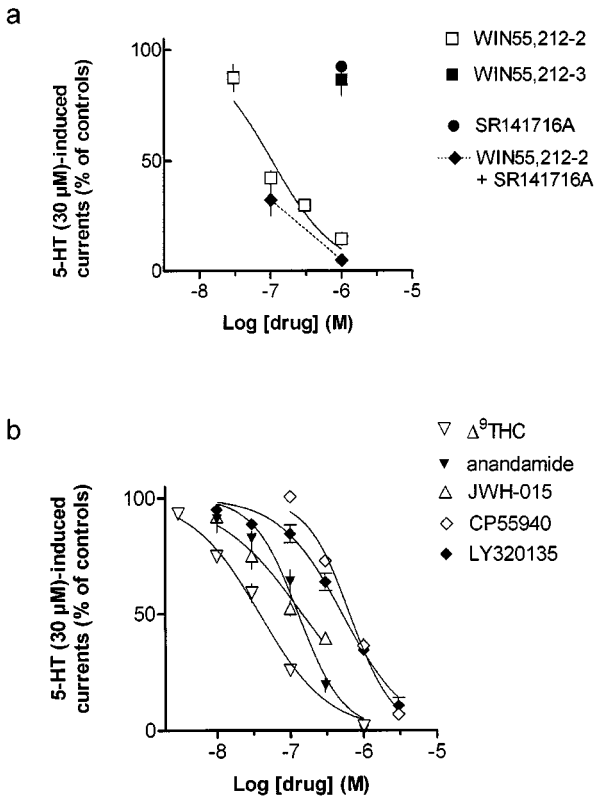

Figure 3 Influence of cannabinoid receptor ligands on 5-HT (30 μM; -100 mV)-induced currents in patches of HEK 293 cells stably transfected with the h5-HT$_{3A}$ receptor cDNA. (**a**) Concentration-dependent inhibition by the cannabinoid receptor agonist WIN55,212-2 in the absence and presence of the CB$_1$ receptor antagonist SR141716A (at 1 μM) and lack of inhibition by the enantiomer WIN55,212-3 and by SR141716A alone. (**b**) Concentration-dependent inhibition of 5-HT (30 μM; -100 mV)-induced currents by various cannabinoid receptor ligands. Drugs were present 3 min before, during and after the 2 s 5-HT pulse. Shown are means$\pm$s.e.m. of 3–6 different patches.

investigation on whole cells, Δ^9-THC, anandamide, WIN55,212-2, CP55940 and CP56667 inhibited the 5-HT-evoked current. CP55940 and CP56667 are enantiomers, the latter compound being clearly less potent (by a factor of more than 10). The anandamide-induced inhibition was noncompetitive and was not affected by nonhydrolyzable cAMP and GTP analogues. These results were compatible with an action at the 5-HT$_3$ receptor itself, but in view of the putative presence of CB$_1$ receptors in addition to 5-HT$_3$ receptors in these cells, an interaction of both receptors could not be ruled out, in particular because interaction experiments with cannabinoid receptor antagonists were not performed.

The present investigation with cannabinoid receptor ligands on excised outside-out patches from HEK 293 cells expressing the human 5-HT$_{3A}$ receptor, which also revealed an inhibition of 5-HT-induced currents, provides an answer to this open question. The rank order of potencies of cannabinoids in inhibiting h5-HT$_{3A}$ receptors was clearly different from the affinities of these drugs for hCB$_1$ or hCB$_2$ receptors (Table 1), arguing against an involvement of the latter receptor types in this effect. This view is strongly supported by our finding that the selective CB$_1$ receptor

Table 1 Inhibition of h5-HT$_{3A}$ receptor-mediated currents and affinities for hCB$_1$ and hCB$_2$ receptors

	h5-HT$_{3A}$ IC$_{50}$ (nM)	*Hill-coefficient*	*hCB$_1$* K$_i$ (nM)	*hCB$_2$* K$_i$ (nM)
Δ^9-THC	38.4	-1.0	33.6*	44.9*
WIN55,212-2	103.5	-1.0	17.4*	0.14*
Anandamide	129.6	-1.5	321.0*	371.0*
JWH-015	146.5	-0.8	383.0†	14.0†
LY320135	523.2	-1.1	141.0††	>10,000††
CP55940	647.6	-1.5	1.21*	0.88*
SR141617A	>1,000	–	0.68*	>10,000††

Potencies of cannabinoid receptor ligands in inhibiting 5-HT-induced currents in patches of HEK 293 cells stably transfected with the h5-HT$_{3A}$ receptor cDNA; IC$_{50}$ values and Hill coefficients for their (noncompetitive) inhibitory action mediated by the postulated allosteric modulatory site (see Figure 1 for the effect of WIN55,312-2); the values shown in the table were determined from the concentration-response curves shown in Figure 3a,b. K_i values for inhibition of radioligand binding to human cannabinoid receptors in membrane preparations were taken from the literature: *Tao & Abood, 1998; †Showalter *et al*., 1996; ††Felder *et al*., 1998.

antagonist SR141716A (Thomas *et al*., 1990) did not antagonize the inhibitory effect of WIN55,212-2 on the 5-HT-evoked current (Figure 3a). In addition, the inhibition was stereoselective, which argues against the possibility that the inhibitory effect of the cannabinoids might be exclusively related to their lipophilicity, although a hydrophobic component in the mechanism of action cannot be excluded.

The effect of WIN55,212-2 on the concentration-response curve of 5-HT was typical for a non-competitive inhibitor. This makes a direct interaction with the agonist recognition site unlikely. This conclusion is supported by our radioligand binding experiments, since (1) three cannabinoid receptor agonists did not reduce the specific binding of a selective 5-HT$_{3A}$ receptor ligand and (2) CP55940 did not alter the concentration-dependent inhibition of specific [^{3}H]-GR65630 binding by 5-HT.

Since, furthermore, there was no specific binding of the CB$_1$ receptor ligand [^{3}H]-SR141716A (Rinaldi-Carmona *et al*., 1996; for reviews, see Pertwee, 1997; 2000) nor of the CB$_1$/CB$_2$ receptor ligand [^{3}H]-CP55940 (for reviews, see Pertwee, 1997; 2000) to membranes of HEK 293 cells, the possibility that such receptors are expressed in the cell membrane of HEK 293 cells is excluded. The most plausible alternative is that WIN55,212-2 and the other cannabinoid receptor agonists act at a particular modulatory site of the h5-HT$_{3A}$ receptor itself. Since the possibility that this site may modify ligand association to, or dissociation from, the 5-HT recognition site of the h5-HT$_{3A}$ receptor was excluded by our binding experiments, the alternative was considered that cannabinoid binding to this site may inhibit currents through the receptor pore, e.g., either by an open channel block or by an inhibitory allosteric interaction leading to a change in channel gating or closure mechanisms.

Evidence as to whether or not the drugs produce an open channel block could be derived from experiments in which the time schedule of drug application was modified and in which the voltage-dependence of the inhibitory effect of WIN55,212-2 was investigated: obviously, the site involved in the inhibition of channel function is not easily accessible, as

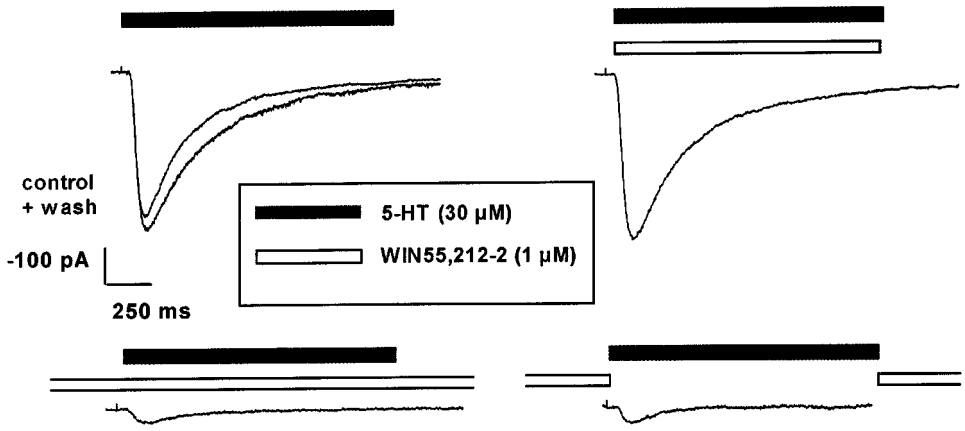

Figure 4 Effects of the cannabinoid receptor agonist WIN55,212-2 (1 μM) on 5-HT (30 μM, -100 mV)-induced currents obtained in one outside-out patch of a HEK 293 cell stably transfected with the h5-HT$_{3A}$ receptor cDNA at three different modes of drug application: exclusive co-application of the drug with 5-HT (without preexposure to the drug; upper right panel); application of the drug 3 min before, during and after stimulation with 5-HT (lower left panel); application exclusively for 3 min prior to and after, but not together with, 5-HT (lower right panel); 5-HT-induced current under control conditions (upper left panel); 'wash' refers to the response to 30 μM 5-HT after omission of the drug for 3 min at the end of the experiment.

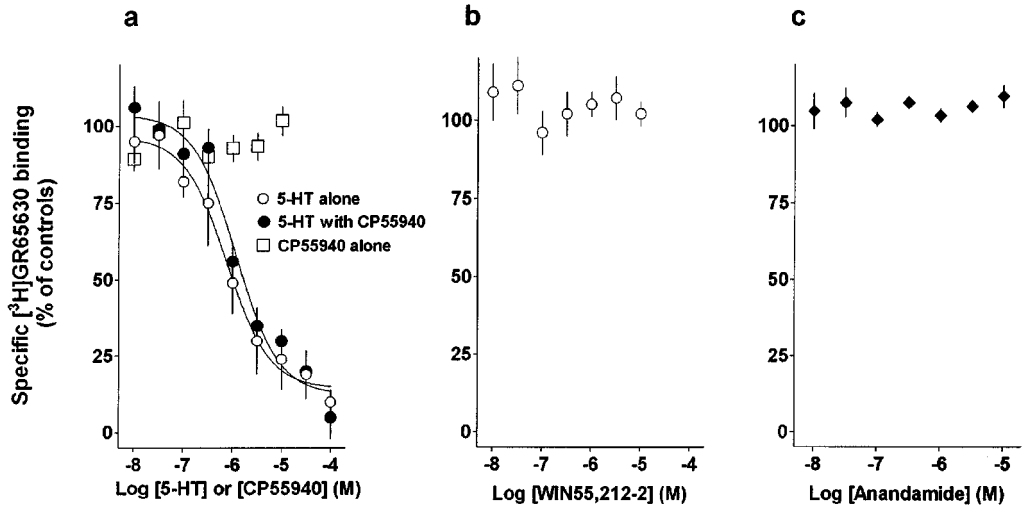

Figure 5 Influence of 5-HT and cannabinoid receptor agonists on specific binding of the selective 5-HT$_3$ receptor ligand [^{3}H-GR65630 to membranes of HEK 293 cells stably transfected with the h5-HT$_{3A}$ receptor cDNA. (**a**) Concentration-dependent inhibition by 5-HT of specific [^{3}H-GR65630 binding in the absence (open symbols) and presence of the cannabinoid receptor agonist CP55940 and lack of inhibition by CP55940 alone. (**b**) No inhibition by WIN55212-2 and (**c**) no inhibition by anandamide. All points are means$\pm$s.e.m., $n=3-6$ different binding experiments.

can be derived from the rather long equilibrium period of 3 min before stimulation which is necessary to establish the effect and from the failure of the drugs to produce an inhibition when applied exclusively during, but not before, stimulation with 5-HT. However, a location in transmembrane or cytosolic domains of the receptor protein rather than within the open pore would be compatible with the necessity of the presence of the cannabinoids before stimulation at such an equilibrium time and with the lack of voltage-dependence of the WIN55,212-2-induced inhibition of 5-HT-evoked current.

The location in the transmembrane domain or its immediate proximity would make this site easily accessible to the endogenous ligand anandamide (Devane *et al*., 1992; Fride & Mechoulam, 1993); this compound is produced by enzymatic cleavage from membrane lipid precursors (reviewed by Piomelli *et al*., 2000; Porter & Felder, 2001) and it inhibits 5-HT-evoked currents with remarkable potency (Table 1). It is an attractive hypothesis to postulate that the 5-HT$_3$ receptor is endowed with a motif which is recognized not only by all CB$_1$ and CB$_2$ receptor agonists, but also by the CB$_1$ receptor antagonist LY320135 (Felder *et al*., 1998). This drug mimics the inhibitory effect of the cannabinoid receptor agonists at the allosteric modulatory site of the 5-HT$_3$ receptor.

At this modulatory site which, thus, shares pharmacological properties with hCB$_1$ and hCB$_2$ receptors without being identical with them, the phytocannabinoid Δ^9-THC acts at a

potency similar to its affinity for hCB_1 and hCB_2 receptors, whereas synthetic cannabinoids are less potent at this site compared to their affinity for hCB_1 or hCB_2 or both receptors (Table 1). This property of Δ^9THC is compatible with the view that the inhibition of h5-HT_{3A} receptor function found here may contribute to the behavioural effects of this compound.

As already mentioned, the potency of the endogenous ligand anandamide at this site is rather high (Table 1). Accordingly, it is conceivable that the allosteric site of the h5-HT_{3A} receptor may be tonically activated by anandamide and, hence may play a physiological role by mediating a regulatory effect on the functional state of the 5-HT_3 receptor. The allosteric modulatory site of the 5-HT_3 receptor may be considered as a target for a new class of drugs used, e.g., for the control of pain and emesis.

A prerequisite for such effects is that the cannabinoid receptor-independent inhibitory effect of cannabinoids is also operative *in vivo*. Evidence for this can be derived from preliminary results obtained in a study which has been performed in anaesthetized rats pretreated with the CB_1 receptor antagonist SR141716A (Godlewski *et al*., 2002). In such animals, activation of the 5-HT_3 receptors on cardiac afferent vagal nerves by bolus injection of phenylbiguanide induced the Bezold-Jarisch reflex, i.e. a decrease in heart rate. This effect was inhibited by CP55940 and WIN55,212-2 but not by WIN55,212-3, whereas the vanilloid VR1 receptor-mediated Bezold-Jarisch reflex was unaffected.

Taken together, the present patch-clamp study on excised outside-out patches of HEK 293 cells expressing recombinant homomeric 5-HT_{3A} receptors revealed that cannabinoid receptor ligands including the phytocannabinoid Δ^9THC, the endocannabinoid anandamide and the CB_1 receptor antagonist LY320135 inhibit the 5-HT-induced currents. The synthetic cannabinoid WIN55,212-2, but not its enantiomer WIN55,212-3, shared the inhibitory effect. Since radioligand binding experiments excluded both the involvement of CB_1 and CB_2 receptors in this effect and an action of the cannabinoids at the 5-HT recognition site of the 5-HT_3 receptor, the most plausible site and mechanism of action is a direct modulatory effect on the h5-HT_{3A} receptor channel *via* an allosteric site.

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