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6″-Azidohex-2″-yne-cannabidiol: a potential neutral, competitive cannabinoid CB_1 receptor antagonist

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Abstract

Previous experiments with the mouse vas deferens have shown that cannabidiol produces surmountable antagonism of cannabinoid CB_1 receptor agonists at concentrations well below those at which it binds to cannabinoid CB_1 receptors and antagonizes α_1-adrenoceptor agonists insurmountably. It also enhances electrically evoked contractions of this tissue. We have now found that subtle changes in the structure of cannabidiol markedly influence its ability to produce each of these effects, suggesting the presence of specific pharmacological targets for this non-psychoactive cannabinoid. Our experiments were performed with cannabidiol, 6″-azidohex-2″-yne-cannabidiol, abnormal-cannabidiol and 2′-monomethoxy- and 2′,6′-dimethoxy-cannabidiol. Of these, 6″-azidohex-2″-yne-cannabidiol was as potent as cannabidiol in producing surmountable antagonism of (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (*R*-(+)-WIN55212) in vasa deferentia. However, it produced this antagonism with a potency that matched its cannabinoid CB_1 receptor affinity, suggesting that, unlike cannabidiol, it is a competitive cannabinoid CB_1 receptor antagonist. Moreover, since it did not enhance the amplitude of electrically evoked contractions, it may be a neutral cannabinoid CB_1 receptor antagonist.

Keywords: Cannabidiol; Abnormal-cannabidiol; WIN55212; Cannabinoid CB_1 receptor; Novel cannabinoid receptor antagonist; Mouse vas deferens

1. Introduction

In the past few years, research into the pharmacology of cannabinoids has focused particularly on ligands for established cannabinoid receptors (Howlett et al., 2002). As a result, the pharmacology of non-psychoactive plant cannabinoids (phytocannabinoids) that lack significant affinity for these receptors has been largely ignored. However, interest is now growing in one non-psychoactive phytocannabinoid, cannabidiol ((−)-cannabidiol; Fig. 1), as this compound has therapeutic potential, for example for the management of epilepsy, as an anti-inflammatory agent and for attenuating unwanted effects of the psychoactive phytocannabinoid, Δ^9-tetrahydrocannabinol (see Pertwee, 2004). Moreover, cannabidiol is already widely taken in cannabis, when this is used for either medical (Consroe et al., 1997) or recreational purposes, and new cannabidiol-containing medicines are in development (Whittle et al., 2001).

Recently, we investigated the ability of cannabidiol to antagonize the cannabinoid receptor agonists, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (*R*-(+)-WIN55212) and (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), in the mouse isolated vas deferens (Pertwee et al., 2002). This is a tissue that contains neurones that express cannabinoid CB_1 receptors and it is generally accepted that when activated, these receptors inhibit electrically evoked contractions of the vas deferens by mediating inhibition of the evoked release of the contractile neurotransmitters, ATP, acting on P2X purinoceptors, and noradrenaline, acting mainly on α_1-adrenoceptors (Trendelenburg et al., 2000; Von Kügelgen and Starke, 1991; see also Pertwee, 1997; Schlicker and Kathman, 2001).

Our previous experiments with cannabidiol provided evidence that this cannabinoid shares the ability of the

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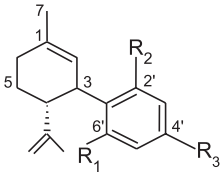

	R_1	R_2	R_3
(-)-CBD	OH	OH	C_5H_{11}
O-2654	OH	OH	$CH_2C{\equiv}C(CH_2)_2CH_2N_3$
O-2796	OH	OCH_3	C_5H_{11}
O-2797	OCH_3	OCH_3	C_5H_{11}
(-)-Abnormal-CBD	OH	C_5H_{11}	OH

Fig. 1. Structures of (−)-cannabidiol (CBD), (−)-6″-azidohex-2″-yne-cannabidiol (O-2654), (−)-2′-monomethoxy-cannabidiol (O-2796), (−)-2′,6′-dimethoxy-cannabidiol (O-2797) and (−)-abnormal-cannabidiol.

cannabinoid CB_1 receptor-selective antagonist, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A), to antagonize *R*-(+)-WIN55212- and CP55940-induced inhibition of electrically evoked contractions of the mouse vas deferens in a competitive, surmountable manner (Pertwee et al., 1995, 2002). However, cannabidiol produced this antagonism at concentrations well below those at which it binds to cannabinoid CB_1 (or CB_2) receptors, suggesting that it was competing with *R*-(+)-WIN55212 and CP55940 for an as yet uncharacterized cannabinoid non-CB_1 pharmacological target on nerve terminals. We also found that cannabidiol opposed the ability of the α_1-adrenoceptor agonist, phenylephrine, to produce contractions of electrically unstimulated mouse vasa deferentia (Pertwee et al., 2002). This antagonism was insurmountable, suggesting that it may be allosteric in nature.

Cannabidiol is a highly lipophilic molecule, raising the possibility that it acts in a non-specific manner, for example by perturbing membrane phospholipids, as was once proposed for Δ^9-tetrahydrocannabinol (Pertwee, 1988). The experiments described in this paper were directed at testing the hypothesis that cannabidiol antagonizes *R*-(+)-WIN55212 and phenylephrine through specific mechanisms. This we did by investigating whether the ability of cannabidiol to produce this antagonism could be affected by making minor changes to the phenol ring of this molecule. Some of these experiments were carried out with (−)-6″-azidohex-2″-yne-cannabidiol (O-2654), an analogue in which the 4′-pentyl group of cannabidiol has been replaced by a 6″-azido-2″-hexyne side chain (Fig. 1). This molecule was of potential interest since the same structural modification greatly affects the pharmacological properties of Δ^8-tetrahydrocannabinol, for example by markedly reducing the cannabinoid CB_1 receptor efficacy of this cannabinoid without affecting its affinity for cannabinoid CB_1 receptors (Ross et al., 1999). Experiments were also carried out both with (−)-abnormal-cannabidiol (Fig. 1), which has been reported to oppose phenylephrine-induced contractions of endothelium-intact and endothelium-denuded isolated segments of mesenteric arteries (Offertáler et al., 2003), and with (−)-2′-monomethoxy- and (−)-2′,6′-dimethoxy-cannabidiol (Fig. 1). As well as performing experiments with isolated vasa deferentia, we also determined the K_i values of cannabidiol and some of its analogues for displacement of [^{3}H]CP55940 from cannabinoid CB_1 receptors on mouse brain membranes. Some of the results described in this paper have been presented to the International Cannabinoid Research Society (Thomas et al., 2003).

2. Materials and methods

2.1. Materials

Phenylephrine hydrochloride and β,γ-methyleneadenosine 5′-triphosphate (β,γ-methylene-ATP) were purchased from Sigma-Aldrich (Poole, Dorset, UK) and *R*-(+)-WIN55212 mesylate from Tocris (Bristol, UK). Cannabidiol was obtained from GW Pharmaceuticals (Porton Down, Wiltshire, UK) and from the National Institute on Drug Abuse (Bethesda, MD, USA) and cannabidiol analogues were synthesised by Dr. Raj Razdan (O-2654, abnormal-cannabidiol, 2′-monomethoxy-cannabidiol and 2′,6′-dimethoxy-cannabidiol). Cannabidiol and its analogues were dissolved in dimethyl sulphoxide (DMSO). They were all (−)-enantiomers. *R*-(+)-WIN55212 was dissolved in a 50% aqueous solution of DMSO (v/v) and both phenylephrine and β,γ-methylene-ATP were dissolved in a 0.9% aqueous solution of NaCl (saline). Drugs were added to organ baths in a volume of 10 μl. For the binding experiments, [^{3}H]CP55940 (126 Ci mmol^{-1}) was obtained from APBiotech (Little Chalfont, UK) and from the National Institute on Drug Abuse and bovine serum albumin, TRIZMA hydrochloride and TRIZMA base from Sigma-Aldrich (St. Louis, MO).

2.2. Experimental procedure

The methods we used comply with the European Community guidelines for the use of experimental animals.

2.2.1. Vas deferens experiments

Vasa deferentia were obtained from albino MF1 mice weighing 30–48 g. As described previously (Pertwee et al., 2002), the tissues were mounted vertically in 4-ml organ baths and subjected to a period of electrical stimulation that included an equilibration procedure in which they were subjected to alternate periods of stimulation (2 min) and rest (10 min) until contractions with consistent amplitudes were obtained. These contractions were monophasic and isometric and were evoked by 0.5 s trains of pulses of 110% maximal voltage (train frequency 0.1 Hz, pulse frequency 5 Hz, pulse duration 0.5 ms).

In all experiments with the twitch inhibitor, *R*-(+)-WIN55212, an addition of cannabidiol, cannabidiol analogue or DMSO was made after the equilibration procedure. Vasa deferentia were then rested from stimulation for 88 min. They were then stimulated for 2 min before the first of a series of additions of *R*-(+)-WIN55212. Between each of these additions, the tissues were rested for 13 min and then stimulated for 2 min. This cycle of drug addition, 13 min rest and 2 min stimulation was repeated without washout to allow the construction of a cumulative concentration–response curve for *R*-(+)-WIN55212. Only one *R*-(+)-WIN55212 concentration–response curve was constructed per tissue (Pertwee et al., 1996).

In experiments with phenylephrine and β,γ-methylene-ATP, no electrical stimuli were applied after the equilibration procedure. Concentration–contractile response curves for β,γ-methylene-ATP were constructed cumulatively without washout, 30 min after the addition of cannabidiol, cannabidiol analogues or DMSO, as described previously (Pertwee et al., 2002). To investigate the effect of a single concentration of a cannabinoid on the contractile effect of 10 μM phenylephrine, a comparison was made between an initial contractile response to 10 μM phenylephrine in the absence of other drugs and a subsequent contractile response to the same concentration of phenylephrine, measured 30 min after the addition of cannabidiol, a cannabidiol analogue or DMSO to the organ baths. Organ baths were washed out after the first addition of phenylephrine to prevent tissue desensitisation, and vasa deferentia then rested for 30 min before the addition of a cannabinoid or DMSO. To construct a concentration–response curve for abnormal-cannabidiol against 10 μM phenylephrine, the contractile response to this concentration of phenylephrine was measured first in the absence of abnormal-cannabidiol and then in the presence of increasing concentrations of this cannabinoid. Baths were washed out after each addition of phenylephrine. Abnormal-cannabidiol was added immediately after each washout and phenylephrine added 30 min later.

2.2.2. Binding experiments

Binding assays were performed with mouse brain membranes. Whole mouse brains from eight adult male MF1 mice were suspended in centrifugation buffer (320 mM sucrose, 2 mM Tris·EDTA, 5 mM $MgCl_2$) and the tissue homogenized with an Ultra-Turrex homogenizer. Tissue homogenates were centrifuged at 1600 × *g* for 10 min and the resulting supernatant collected. The pellet was resuspended in centrifugation buffer, centrifuged as before and the supernatant collected. Supernatants were combined before undergoing further centrifugation at 28,000 × *g* for 20 min. The supernatant was discarded and the pellet resuspended in 40 ml of buffer A (50 mM Tris, 2 mM Tris·EDTA, 5 mM $MgCl_2$ at pH 7.0) and incubated at 37 °C for 10 min. Following the incubation, the suspension was centrifuged for 20 min at 23,000 × *g*. After resuspending the pellet in another 40 ml of buffer A, the suspension was incubated for 40 min at room temperature before a final centrifugation for 15 min at 11,000 × *g*. The final pellet was resuspended in a Tris buffer (50 mM Tris, 1 mM Tris·EDTA, 3 mM $MgCl_2$, pH 7) to give a protein concentration of 1 mg ml^{-1} and stored at −80 °C. All centrifugation procedures were carried out at 4 °C.

Binding experiments were carried out with [^{3}H]CP55 940, 1 mg ml^{-1} bovine serum albumin and 50 mM Tris buffer, total assay volume 500 μl, using a modification of the filtration procedure of Compton et al. (1993) as described previously (Ross et al., 1999). Binding was initiated by the addition of mouse brain membranes (100 μg protein per tube). All assays were performed at 37 °C for 60 min before termination by addition of ice-cold wash buffer (50 mM Tris buffer, 1 mg ml^{-1} bovine serum albumin, pH 7.4) and vacuum filtration using a 12-well sampling manifold (cell harvester; Brandel, Gaitherburg, MD) and GF/B filters (Whatman, Maidstone, UK) that had been soaked in wash buffer at 4 °C for 24 h. Each reaction tube was washed six times with a 1.5-ml aliquot of wash buffer. The filters were oven-dried for 60 min and then placed in 5 ml of scintillation fluid (Ultima Gold XR, Packard). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabelled CP55940. The concentration of [^{3}H]CP55940 used in our displacement assays was 0.7 nM. Each unlabelled cannabinoid was stored as a stock solution of 10 mM in ethanol, the vehicle concentration in all assay tubes being 0.1% ethanol. Protein assays were performed using a Bio-Rad Dc kit (Bio-Rad, Hercules, CA). The binding parameters for [^{3}H]CP55940 in mouse brain membranes were determined by saturation experiments and the data were fitted to a one-site saturation plot using GraphPad Prism (GraphPad Software, San Diego, CA). This yielded a B_{max} value of 2336 fmol mg^{-1} protein and K_d value of 2.31 nM.

2.3. Analysis of data

Values have been expressed as means ± S.E.M. or with 95% confidence limits. The concentrations of cannabinoids that produced a 50% displacement of radioligand from specific binding sites (IC_{50} values) were calculated using GraphPad Prism. Dissociation constants (K_i values) were calculated using the equation of Cheng and Prusoff (1973).

In the vas deferens experiments, relaxant responses to drugs have been expressed as decreases in tension (g) or as percentages of the contraction measured in the absence of test compounds. Inhibition of the electrically evoked twitch response has been expressed in percentage terms (Pertwee et al., 2002). Mean values have been compared using Student's two-tailed *t*-test for paired or unpaired data or one-way analysis of variance (ANOVA) followed by Dunnett's test (GraphPad Prism). A P-value < 0.05 was considered to be significant. Values for pEC_{50}, for maximal effects (E_{max})

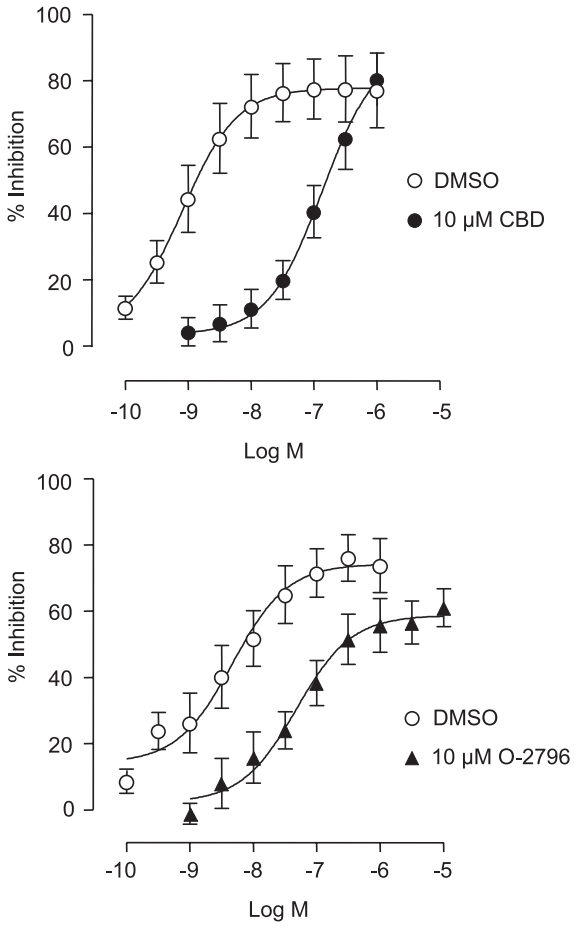

Fig. 2. The effect of pretreatment with 10 μM cannabidiol (CBD, upper panel) and 10 μM 2′-monomethoxy-cannabidiol (O-2796, lower panel) on the mean log concentration–response curve of *R*-(+)-WIN55212 in the mouse isolated vas deferens. Each symbol represents the mean value ± S.E.M. for inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of *R*-(+)-WIN55212 to the organ bath. Cannabidiol, O-2796 or DMSO was added 90 min before the first addition of *R*-(+)-WIN55212, further additions of which were made at 15 min intervals. Each log concentration–response curve was constructed cumulatively without washout. Mean E_{max} values of *R*-(+)-WIN55212 with 95% confidence limits shown in brackets were 77.8% (69.5% and 86.0%) after DMSO and 89.9% (72.7% and 107.1%) after cannabidiol (upper panel, $n = 6$) and 74.3% (65.6% and 83.1%) after DMSO and 58.8% (51.2% and 66.4%) after O-2796 (lower panel, $n = 8$).

and for the S.E.M. or 95% confidence limits of these values have been calculated by non-linear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism). As described previously (Pertwee et al., 2002), dissociation constant (K_B) values for antagonism of *R*-(+)-WIN55212 have been calculated by Schild analysis from values of the concentration ratio, defined as the concentration of a twitch inhibitor that produces a particular degree of inhibition in the presence of a competitive reversible antagonist at a concentration, *B*, divided by the concentration of the same twitch inhibitor that produces an identical degree of inhibition in the absence of the antagonist. The methods used to determine concentration ratios and to establish whether log concentration–response plots deviated significantly from parallelism are described elsewhere (Pertwee et al., 2002).

3. Results

3.1. Effects on R-(+)-WIN55212-induced inhibition of electrically evoked contractions of the vas deferens

At 10 μM, cannabidiol and 2′-monomethoxy-cannabidiol both produced rightward shifts in the log concentration response curve of *R*-(+)-WIN55212 (Fig. 2). The mean dextral shifts with 95% confidence limits shown in brackets were 30.4 (5.4 and 345.4) and 141.1 (56 and 322.3),

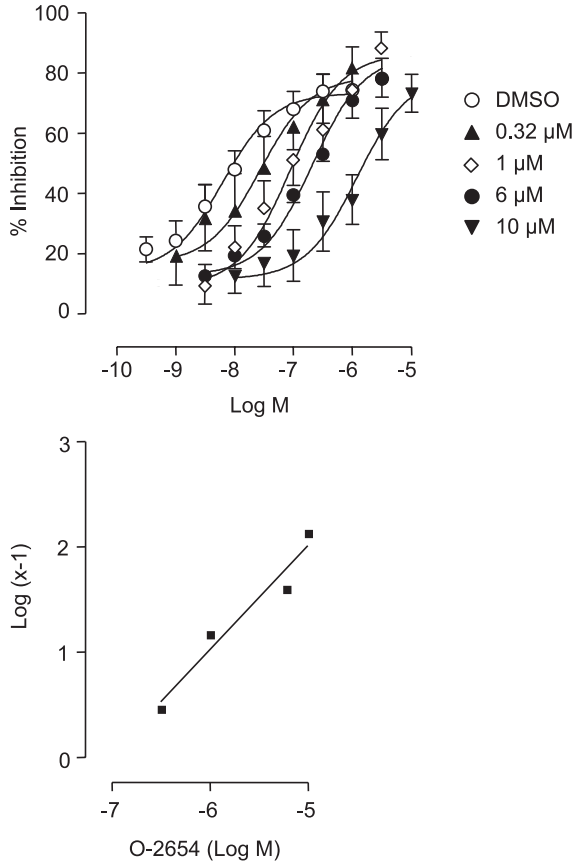

Fig. 3. Upper panel: The effect of pretreatment with O-2654 on the mean log concentration–response curve of *R*-(+)-WIN55212 in the mouse isolated vas deferens. Each symbol represents the mean value ± S.E.M. for inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of *R*-(+)-WIN55212 to the organ bath. O-2654 or DMSO was added 90 min before the first addition of *R*-(+)-WIN55212, further additions of which were made at 15-min intervals. Each log concentration–response curve was constructed cumulatively without washout ($n = 7–12$). Lower panel: Schild plot for antagonism of *R*-(+)-WIN55212 by 0.32–10 μM O-2654 in which values for log $x - 1$ were calculated from the data shown in the upper panel (x = concentration ratio). The slope of this plot is 0.99 ± 0.16 and this does not differ significantly from unity ($P > 0.05$, one-sample *t*-test).

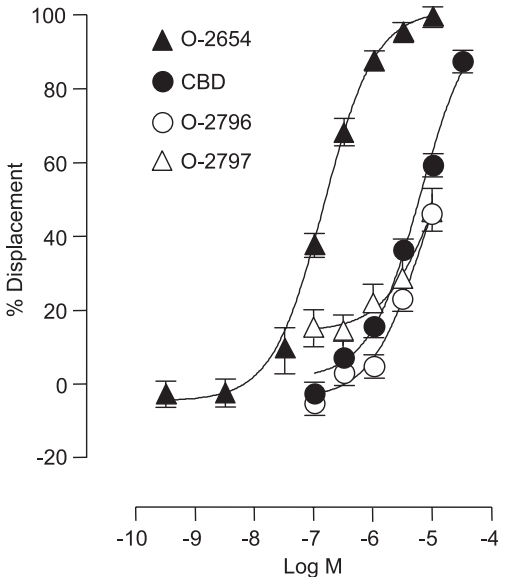

Fig. 4. Displacement of [^{3}H]CP55940 by cannabidiol (CBD, $n=5-12$), O-2654 ($n=5-11$), 2′-monomethoxy-cannabidiol (O-2796, $n=6$) and 2′,6′-dimethoxy-cannabidiol (O-2797, $n=6-8$) in mouse brain membranes. Each symbol represents the mean percent displacement ± S.E.M. Mean K_i values for displacement of [^{3}H]CP55940 with 95% confidence limits shown in brackets were calculated by the Cheng-Prusoff equation to be 114 nM (96 and 137 nM) for O-2654 and 4.9 μM (2.1 and 11.3 μM) for cannabidiol. The corresponding K_i values of O-2796 and O-2797 were >10 μM.

respectively. Corresponding K_B values, calculated from these dextral shifts using the Schild equation, were 340.6 nM (29 and 2265 nM) for the 2′-monomethoxy analogue and 71.4 nM (31.1 and 181.7 nM) for cannabidiol. This K_B value for cannabidiol does not differ significantly from the K_B value for this cannabinoid (120.3 nM) that we obtained in our previous experiments when cannabidiol was administered 30 min instead of 90 min before the first addition of *R*-(+)-WIN55212.

As shown in Fig. 3, experiments with O-2654 indicated that it also antagonized *R*-(+)-WIN55212. This it did in a manner that was concentration-related and not accompanied by any significant change in the maximum effect (E_{max}) of *R*-(+)-WIN55212 ($P>0.05$, ANOVA followed by Dunnett's test, $n=7-12$). The dextral shifts produced by O-2654 in the log concentration response curve of *R*-(+)-WIN55212 do not deviate significantly from parallelism and yield a Schild plot with a slope that is not significantly different from unity (Fig. 3). The mean K_B value of O-2654 was calculated by the Tallarida method (see Pertwee et al., 2002) to be 85.7 nM with 95% confidence limits of 58.4 and 160.8 nM, a value that does not differ significantly from the mean K_B value that we obtained for cannabidiol (see above).

Mean twitch amplitude increased significantly (paired *t*-test) during a 90-min exposure to 10 μM cannabidiol (217.1 ± 79.6%, $P<0.05$, $n=6$), to 10 μM 2′-monomethoxy-cannabidiol (93.0 ± 31.3%, $P=0.01$, $n=8$) and to 10 μM 2′,6′-dimethoxy-cannabidiol (36.5 ± 7.5%, $P<0.01$, $n=9$). However, exposure for 90 min to DMSO or to 0.32, 1, 6 or 10 μM O-2654 was not accompanied by any significant increase in mean twitch amplitude ($P>0.05$, paired *t*-test, $n=8-12$).

At 10 μM, 2′,6′-dimethoxy-cannabidiol ($n=9$) and abnormal-cannabidiol ($n=8$) did not produce statistically significant dextral shifts in the log concentration response curve of *R*-(+)-WIN55212 (data not shown). Nor when administered alone at 10 μM did abnormal-cannabidiol increase the amplitude of evoked contractions at +90 min (2.9 ± 9.3%, $n=8$, paired *t*-test).

3.2. Radioligand binding studies

Since 2′-monomethoxy-cannabidiol and O-2654 antagonized *R*-(+)-WIN55212, it was important to establish their affinities for cannabinoid CB_1 receptors and to compare these values with the cannabinoid CB_1 receptor affinity of cannabidiol, determined under the same conditions. As shown in Fig. 4, O-2654 was markedly more potent than cannabidiol in displacing [^{3}H]CP55940 from specific binding sites in mouse brain membranes. In contrast, 2′-monomethoxy-cannabidiol did not show greater potency than cannabidiol in this assay (Fig. 4). Nor, indeed, did 2′,6′-dimethoxy-cannabidiol.

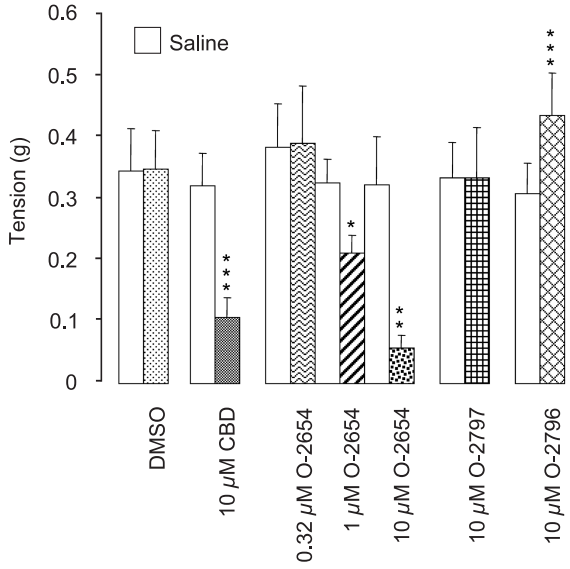

Fig. 5. The effect of DMSO ($n=14$), 10 μM cannabidiol (CBD, $n=7$), 0.32 μM O-2654 ($n=7$), 1 μM O-2654 ($n=6$), 10 μM O-2654 ($n=6$), 2′-monomethoxy-cannabidiol (O-2796, $n=9$) and 2′,6′-dimethoxy-cannabidiol (O-2797, $n=7$) on the amplitude of contractions of the mouse isolated vas deferens induced by 10 μM phenylephrine. The columns represent mean values ± S.E.M. of phenylephrine-induced increases in tension expressed in grams. Each tissue was exposed to phenylephrine twice, first before the administration of any other compound (open columns) and then 30 min after the administration of DMSO or a cannabinoid. Organ baths were washed out after the first addition of phenylephrine and tissues rested for 30 min before administration of DMSO or a cannabinoid. The asterisks indicate significant differences between responses to the first and second additions of phenylephrine (*$P<0.05$, **$P<0.02$, ***$P<0.01$, paired *t*-test).

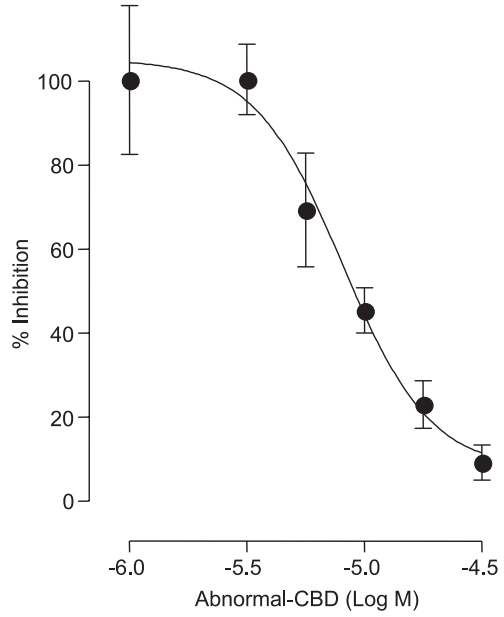

Fig. 6. The effect of abnormal-cannabidiol (abnormal-CBD) on the amplitudes of contractions of the mouse isolated vas deferens induced by 10 μM phenylephrine. Each symbol represents a mean value ± S.E.M. ($n = 9–22$) for the attenuation of the contractile response to phenylephrine expressed as a percentage of the contraction produced by 10 μM phenylephrine before the first addition of abnormal-cannabidiol to the organ bath. Phenylephrine was added repetitively and each organ bath washed out immediately after the response to each of these additions had been measured. Abnormal-cannabidiol was added immediately after each washout, 30 min before the next addition of phenylephrine. The mean pEC_{50} of abnormal-cannabidiol was 5.1 ± 0.07. Its mean E_{max} was 7.5 ± 11.1%, a value that did not differ significantly from zero ($P > 0.05$, one-sample *t*-test).

3.3. Effects on phenylephrine-induced contractions of the vas deferens

The ability of 10 μM phenylephrine to induce contractions of the vas deferens was opposed by 10 μM cannabidiol, as shown previously (Pertwee et al., 2002), and by O-2654 at concentrations of 1 and 10 μM (Fig. 5). However, the amplitude of phenylephrine-induced contractions was unaffected by DMSO, 0.32 μM O-2654 or 10 μM 2′,6′-dimethoxy-cannabidiol, and was slightly but significantly enhanced by 10 μM 2′-monomethoxy-cannabidiol (Fig. 5). We also investigated the effect of abnormal-cannabidiol on phenylephrine-induced contractions of the vas deferens. This cannabinoid has been reported to relax isolated segments of phenylephrine-precontracted rat endothelium-denuded and endothelium-intact mesenteric arteries (Offertáler et al., 2003). Since cumulative concentration response curves for abnormal-cannabidiol were constructed in these mesenteric artery experiments, we attempted to construct such a curve in our vas deferens experiments as we wished to compare the relaxant potency of this cannabinoid in these two tissues. The results we obtained indicate that as in arteries (Offertáler et al., 2003) so too in vasa deferentia (Fig. 6), abnormal-cannabidiol can oppose the contractile effect of phenylephrine in a concentration-related manner. Moreover, its pEC_{50} in the vas deferens was calculated to be 5.1 ± 0.07, a value that is close to the pEC_{50} values obtained by Offertáler et al. (2003) in their abnormal-cannabidiol experiments with endothelium-denuded (4.81 ± 0.15) and endothelium-intact (5.67 ± 0.07) arterial segments.

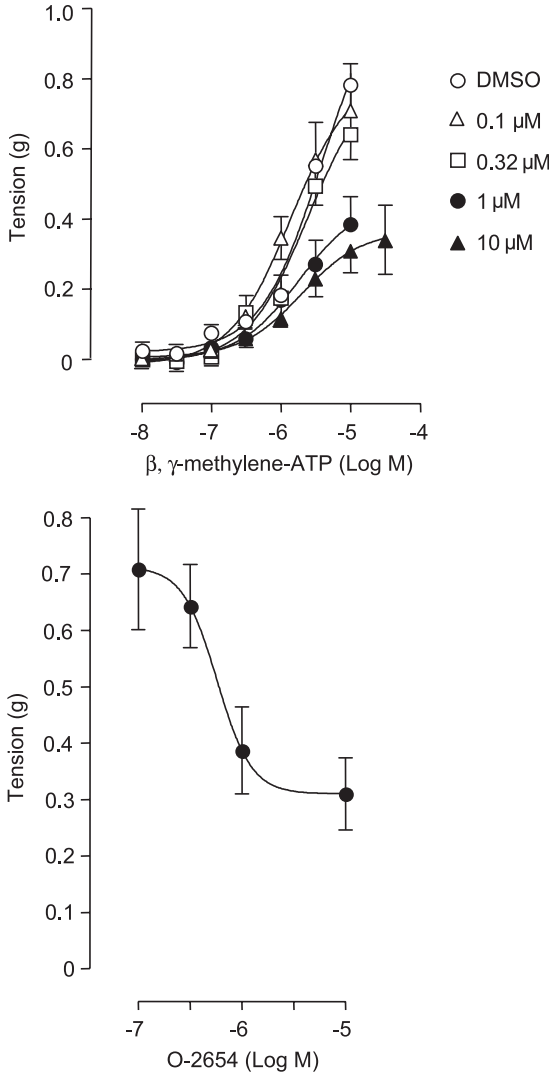

Fig. 7. The effect of O-2654 on the amplitude of contractions of the mouse isolated vas deferens induced by β,γ-methylene-ATP. The symbols represent mean values ± S.E.M. of β,γ-methylene-ATP-induced increases in tension expressed in grams. Upper panel: DMSO ($n = 11$) or 0.1, 0.32, 1 or 10 μM O-2654 ($n = 7–12$) was added 30 min before the first addition of β,γ-methylene-ATP. A further addition of β,γ-methylene-ATP was made immediately after the response to the previous addition had peaked. The mean response to 10 μM β,γ-methylene-ATP was significantly less in the presence of 1 μM O-2654 ($P < 0.05$) or 10 μM O-2654 ($P < 0.001$) than in the presence of DMSO, as was the mean response to 3.2 μM β,γ-methylene-ATP in the presence of 10 μM O-2654 ($P < 0.05$) (ANOVA followed by Dunnett's test). Lower panel: The mean log concentration–response curve of O-2654 for the attenuation of contractions induced by 10 μM β,γ-methylene-ATP, constructed from the data shown in the upper panel. The mean pEC_{50} of O-2654 was 6.24 ± 0.25. Its mean E_{max} was 0.31 ± 0.08 g, a value that was significantly greater than zero ($P < 0.05$, one-sample *t*-test).

3.4. Effects on β,γ-methylene-ATP-induced contractions of the vas deferens

In contrast to results obtained previously with 10 μM cannabidiol (Pertwee et al., 2002), the contractile response of the vas deferens to 10 μM β,γ-methylene-ATP was significantly attenuated by O-2654, both at 1 and 10 μM, the higher of these concentrations also producing significant antagonism of 3.2 μM β,γ-methylene-ATP (Fig. 7). However, contractions induced by 3.2 or 10 μM β,γ-methylene-ATP were not attenuated by 2′-monomethoxy-cannabidiol, 2′,6′-dimethoxy-cannabidiol or abnormal-cannabidiol at 10 μM ($n = 6$ or 12, data not shown).

4. Discussion

Our results indicate that when added to the mouse vas deferens, each of the cannabidiol analogues investigated exhibited a different pharmacological profile both from that of cannabidiol (Pertwee et al., 2002) and from that of the other analogues. More particularly, unlike cannabidiol, O-2654 did not enhance (or inhibit) the amplitude of electrically evoked contractions and attenuated the contractile response of the vas deferens to the P2 receptor agonist, β,γ-methylene-ATP. On the other hand, O-2654 was equipotent with cannabidiol as a surmountable antagonist of the cannabinoid receptor agonist, *R*-(+)-WIN55212, and shared the ability of cannabidiol to attenuate contractions induced by the α_1-adrenoceptor agonist, phenylephrine. At 10 μM, 2′-monomethoxy-cannabidiol differed from cannabidiol in that it enhanced the contractile response to phenylephrine. It did, however, resemble cannabidiol in antagonizing *R*-(+)-WIN55212, in increasing the amplitude of electrically evoked contractions and in not antagonizing β,γ-methylene-ATP. 2′,6′-Dimethoxy-cannabidiol resembled cannabidiol only in its ability to increase the amplitude of electrically evoked contractions, albeit less markedly than cannabidiol, and in its lack of activity against β,γ-methylene-ATP. Thus, at 10 μM, it antagonized neither *R*-(+)-WIN55212 nor phenylephrine. Finally, abnormal-cannabidiol differed from cannabidiol by not antagonizing *R*-(+)-WIN55212 or altering the amplitude of electrically evoked contractions. However, like cannabidiol, it did antagonize phenylephrine without also antagonizing β,γ-methylene-ATP. As to the ability of these analogues to bind to cannabinoid receptors, it has been reported previously that abnormal-cannabidiol lacks significant affinity for cannabinoid CB_1 receptors, at least at concentrations of up to 10, 30 or 100 μM (Járai et al., 1999; Offertáler et al., 2003; Showalter et al., 1996). In the present investigation we found that O-2654 exhibited greater affinity than cannabidiol for cannabinoid receptors on mouse brain membranes, whereas the mono- and dimethoxy-analogues did not. The cannabinoid CB_1 receptor affinity value for cannabidiol determined in our experiments approximates well to values obtained previously in [^{3}H]CP55940 binding experiments with rat brain membranes or with membranes from Chinese hamster ovary cells transfected with human cannabinoid CB_1 receptors (Showalter et al., 1996; Thomas et al., 1998).

Although O-2654 and cannabidiol antagonized *R*-(+)-WIN55212 with equal potency, it is likely that the mechanism underlying this antagonism is not the same for these two compounds. The K_B value of cannabidiol is well below its K_i value for displacing [^{3}H]CP55940 from cannabinoid CB_1 binding sites suggesting that it does not antagonize *R*-(+)-WIN55212 by competing with this cannabinoid receptor agonist for the cannabinoid CB_1 receptor recognition site (Pertwee et al., 2002). However, O-2654 may well produce its antagonism of *R*-(+)-WIN55212 by competing for this site, as the cannabinoid CB_1 K_i value of O-2654 (114 nM) is not significantly different from its K_B value for antagonism of *R*-(+)-WIN55212 (85.7 nM). While this CB_1 K_i value of O-2654 is somewhat less than reported cannabinoid CB_1 K_i values of one established cannabinoid CB_1 receptor antagonist, SR141716A (1.98–12.3 nM), it is close to the cannabinoid CB_1 K_i value of another cannabinoid CB_1 receptor antagonist, LY320135 (141 nM) (Felder et al., 1998; Howlett et al., 2002).

If O-2654 does indeed antagonize *R*-(+)-WIN55212 by competing with this agonist for cannabinoid CB_1 receptors, our data suggest that it may be a neutral antagonist for these receptors and so differ from those ligands that are currently widely used as cannabinoid CB_1 receptor antagonists as these behave as inverse agonists (Howlett et al., 2002). Thus, for example, unlike O-2654, SR141716A has been found to enhance the amplitude of electrically evoked contractions when added by itself to mouse isolated vasa deferentia (Pertwee et al., 1996). There are also reports that SR141716A augments electrically evoked noradrenaline release in the mouse isolated vas deferens (Schlicker et al., 2003) and that it behaves as an inverse cannabinoid CB_1 receptor agonist in several other bioassay systems (Pertwee, 2003).

There may, however, be another reason why O-2654 does not share the ability of SR141716A to enhance the twitch response of the mouse vas deferens. Thus, the production of electrically evoked contractions of this tissue is thought to depend on the ability of co-released noradrenaline and ATP to activate α_1-adrenoceptors and P2X receptors (Von Kügelgen and Starke, 1991). Consequently, any increase in twitch amplitude produced by O-2654 through enhancement of evoked contractile transmitter release may well have been masked by its ability to attenuate contractile responses resulting both from α_1-adrenoceptor activation by noradrenaline and from P2X receptor activation by ATP. Conversely, it is possible that O-2654 did not reduce the amplitude of electrically evoked contractions of the vas deferens in spite of its antagonism of both α_1-adrenoceptors and P2X receptors because this antagonism was overcome by an O-2654-induced increase in the evoked neuronal

release of noradrenaline and ATP. It is also worth noting firstly that, under our stimulation conditions, the height of electrically evoked contractions of the mouse vas deferens seems to be determined far more by released ATP than released noradrenaline (Pertwee et al., 2002), and secondly, that what appears to be a maximal concentration of O-2654 for attenuating the contractile response to 10 μM β,γ-methylene-ATP produced only a partial blockade of this response (Fig. 7). Hence, it may be that at concentrations of up to 10 μM, O-2654 failed to reduce the amplitude of electrically evoked contractions because it did not produce sufficient blockade of P2X receptors. As to cannabidiol, it has already been postulated that, at 10 μM, this cannabinoid enhances the twitch response of the mouse vas deferens even though it markedly attenuates contractions induced by phenylephrine, noradrenaline or methoxamine, because on the one hand, it increases evoked contractile transmitter release and, on the other, it leaves β,γ-methylene-ATP-induced contractions unaffected (Pertwee et al., 2002).

Our finding that abnormal-cannabidiol attenuates phenylephrine-induced contractions of vasa deferentia is in line with previous reports that both this cannabinoid and cannabidiol relax mesenteric arteries precontracted with phenylephrine (Járai et al., 1999; Offertáler et al., 2003). The relaxant potency of abnormal-cannabidiol in the vas deferens is close to potency values for this cannabinoid obtained by Offertáler et al. (2003) in their experiments with endothelium-denuded and endothelium-intact rat mesenteric arterial segments (see Results). It is possible, therefore, that abnormal-cannabidiol and cannabidiol antagonize phenylephrine by acting on the same pharmacological target in the vas deferens as in endothelium-denuded and/or endothelium-intact mesenteric arteries. This may also be the site at which some or all of the other compounds we investigated acted to modulate phenylephrine-induced contractions of vasa deferentia.

Further experiments are required to establish how it is that cannabidiol antagonizes *R*-(+)-WIN55212 in the vas deferens in a manner that appears to be competitive and yet not to involve direct competition for CB_1 receptors. As discussed elsewhere (Pertwee et al., 2002), it is unlikely that this interaction takes place at novel neuronal receptors for *R*-(+)-WIN55212 of the type proposed by Breivogel et al. (2001). Nor is it likely that it occurs at the non-CB_1 receptors that have been detected on central glutamatergic neurones (Hájos and Freund, 2002). Thus, capsazepine antagonizes *R*-(+)-WIN55212 at these proposed receptors (Hájos and Freund, 2002) but does not attenuate the ability of this cannabinoid to inhibit electrically evoked contractions of the mouse vas deferens at a concentration at which it does oppose capsaicin-induced inhibition of these contractions (Ross et al., 2001). Indeed, it is improbable that *R*-(+)-WIN55212 inhibits electrically evoked contractions of the vas deferens by acting through any type of non-CB_1 receptor since it reduces noradrenaline release in vasa deferentia obtained from CB_1-expressing mice but not in tissues obtained from CB_1-deficient mice (Schlicker et al., 2003). There is still a need, however, for additional experiments directed at establishing the effect of genetic deletion of the CB_1 receptor on the ability of *R*-(+)-WIN55212 to inhibit electrically evoked contractions. One possibility is that cannabidiol is an allosteric CB_1 receptor antagonist. Thus, we have found that in the mouse vas deferens, the parallel dextral shift in the log concentration-response curve for *R*-(+)-WIN55212 produced by 31.6 μM cannabidiol is no greater than the shift produced by 3.2 μM cannabidiol, an observation that could reflect the presence of a saturable allosteric binding site for cannabidiol in the vas deferens (Kenakin, 1997). More specifically, the mean shifts with 95% confidence limits shown in brackets, were 23.2-fold (10.8 and 46.5, $n=9$) and 33.1-fold (24.3 and 45.3, $n=6$), respectively. Similar results have been obtained previously with gallamine, which is thought to be an allosteric muscarinic receptor antagonist and yet behaves as a competitive surmountable antagonist at these receptors over a limited concentration range (Clark and Mitchelson, 1976; Kenakin, 1997). Before this hypothesis can be accepted for cannabidiol, it will be important to rule out alternative hypotheses, for example that an additional action of this cannabinoid emerges at 31.6 μM that counteracts its ability to antagonize *R*-(+)-WIN55212 in the vas deferens. It will also be important to explore the possibility that the apparent competitive antagonism of *R*-(+)-WIN55212 produced by cannabidiol stems from its ability to enhance the amplitude of evoked contractions.

In conclusion, we have found that subtle changes in the structure of cannabidiol can markedly affect its ability to enhance electrically evoked contractions of the vas deferens and to antagonize agonists for cannabinoid CB_1 receptors or for α_1-adrenoceptors in this tissue, suggesting that cannabidiol produces these effects by acting through specific mechanisms and so pointing to the presence of specific pharmacological targets for this molecule. Our data also suggest that the relationship between structure and activity for each of these three effects is not the same, an indication that each effect may be produced through a different mechanism. It now remains for these mechanisms to be characterized, an objective that could be met in part by focusing on the presynaptic actions of cannabidiol and its analogues in experiments with the vas deferens in which the effects of these compounds on evoked contractile transmitter release and on *R*-(+)-WIN55212-induced inhibition of this release are measured directly. We also found that one cannabidiol analogue, O-2654, has markedly greater affinity than cannabidiol for cannabinoid CB_1 receptors and, unlike cannabidiol, behaves as a neutral cannabinoid CB_1 receptor antagonist in the vas deferens. However, while there is little doubt that O-2654 can act through cannabinoid CB_1 receptors to antagonize agonists for these receptors with reasonable potency, further experiments are needed to establish more conclusively whether or not it is indeed a neutral cannabinoid CB_1 receptor antagonist. Such an antagonist

would offer major advantages over present cannabinoid CB_1 receptor antagonists/inverse agonists as an experimental tool and perhaps also as a medicine. Since we found O-2654 to antagonize phenylephrine and β γ-methylene-ATP at concentrations at which it behaved as a cannabinoid CB_1 receptor antagonist, further research directed at improving its selectivity for cannabinoid CB_1 receptors is also required.

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