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Evidence for the presence of CB_2-like cannabinoid receptors on peripheral nerve terminals

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Abstract

We have investigated whether there are cannabinoid CB_2 receptors that can mediate cannabinoid-induced inhibition of electrically evoked contractions in the mouse vas deferens or guinea-pig myenteric plexus-longitudinal muscle preparation. Our results showed that mouse vas deferens and guinea-pig whole gut contain cannabinoid CB_1 and CB_2-like mRNA whereas the myenteric plexus preparation seemed to contain only cannabinoid CB_1 mRNA. JWH-015 (1-propyl-2-methyl-3-(1-naphthoyl)indole) and JWH-051 (1-deoxy-11-hydroxy-Δ^8-tetrahydrocannabinol–dimethylheptyl), which have higher affinities for CB_2 than CB_1 cannabinoid binding sites, inhibited electrically evoked contractions of both tissues in a concentration related manner. This inhibition was attenuated by 31.62 nM of the cannabinoid CB_1 receptor selective antagonist SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] only in the myenteric plexus preparation. Vasa deferentia from Δ^9-tetrahydrocannabinol-pretreated mice (20 mg/kg i.p. once daily for two days) showed reduced sensitivity to JWH-015 and JWH-051. The results suggest that these compounds exert their inhibitory effects through cannabinoid CB_1 receptors in the myenteric plexus preparation, but mainly through CB_2-like cannabinoid receptors in the vas deferens. © 1997 Elsevier Science B.V.

Keywords: Cannabinoids; Cannabinoid CB_1 receptors; Cannabinoid CB_2 receptors; SR141716A; Vas deferens, mouse; Myenteric plexus-longitudinal muscle preparation, guinea-pig small intestine

1. Introduction

Two types of cannabinoid receptor have so far been identified, CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). Cannabinoid CB_1 receptors are present both within and outside the central nervous system whereas cannabinoid CB_2 receptors have as yet only been detected in peripheral tissues (Galiègue et al., 1995). Two peripheral tissues that appear to contain cannabinoid receptors are the mouse vas deferens and the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine. These are tissues in which cannabinoid receptor agonists show high potency and remarkable stereoselectivity and structure dependence as inhibitors of electrically evoked contractions (Pacheco et al., 1991; Pertwee et al., 1992, 1995a, 1996a; Kuster et al., 1993). This inhibitory effect is readily prevented by the selective cannabinoid CB_1 receptor antagonist, SR141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride], suggesting that it can be mediated by cannabinoid CB_1 receptors (Rinaldi-Carmona et al., 1994; Pertwee et al., 1995a, 1996a).

The present experiments addressed the question of whether the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparation also contain cannabinoid CB_2 receptors that can mediate inhibition of electrically evoked contractions. Two approaches were used. The first, was to investigate the inhibitory effect of two cannabinoid receptor agonists which, unlike cannabinoids used in previous experiments with the mouse vas deferens or guinea-pig myenteric plexus-longitudinal mus-

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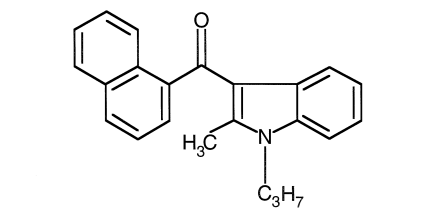
cle preparation, are known to bind significantly more readily to CB_2 than CB_1 cannabinoid receptors. These agonists, JWH-015 (1 propyl-2-methyl-3-(1-naphthoyl)indole) and JWH-051 (1-deoxy-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl) (Fig. 1), have respectively 27.8 and 37.5 times greater affinity for CB_2 than CB_1 cannabinoid binding sites (Huffman et al., 1996; Showalter et al., 1996). The experiments were carried out both in the presence and absence of cannabinoid receptor antagonists. These were either SR141716A (see above) or AM630 (6-iodopravadoline), an agent which behaves as a cannabinoid receptor antagonist in the mouse vas deferens, but as an agonist in the myenteric plexus-longitudinal muscle preparation (Pertwee et al., 1995b, 1996a). Our second approach was to search for the presence of cannabinoid CB_1 and CB_2 mRNA in the mouse vas deferens and guinea-pig myenteric plexus-longitudinal muscle preparation using reverse transcription coupled to the polymerase chain reaction.

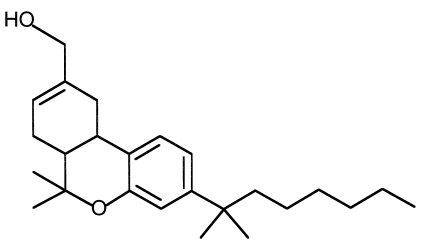
2. Materials and methods

2.1. Drugs and chemicals

Δ^9-tetrahydrocannabinol was obtained from the National Institute on Drug Abuse, SR141716A from Sanofi-Winthrop and AM630 from Dr Alexandros Makriyannis, University of Connecticut. JWH-015 and JWH-051 (Fig. 1) were synthesized in Dr. Huffman's laboratory. All the above drugs were mixed with 2 parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) as described previously for Δ^9-tetrahydrocannabinol (Pertwee et al., 1992). Other drugs were dissolved in saline and were supplied by Endo Laboratories (naloxone HCl), Sigma (yohimbine HCl), Koch Light Laboratories (noradrenaline bitartrate) or Research Biochemicals (β,γ-methylene-L-adenosine triphosphate). All drug additions were made in a volume of 10 μl. RNA X-Cell was supplied by Biogenes. Superscript II reverse transcriptase, oligo dT primer and deoxynucleotides (dNTPs) were supplied by Life Technologies, UK. Recombinant DNase-1 was purchased from Ambion. Taq DNA polymerase, amplification buffer and RNasin were obtained from Promega UK. Oligonucleotides were synthesised by the Department of Medical Microbiology (University of Aberdeen).



1-propyl-2-methyl-3-(1-naphthoyl)indole)
(JWH-015)



1-deoxy-11-OH-Δ^8-THC-dimethylheptyl
(JWH-051)

Fig. 1. Structures of JWH-015 and JWH-051.

2.2. Electrically evoked contractions

Experiments were performed with strips of myenteric plexus-longitudinal muscle or with isolated vasa deferentia. Each tissue was mounted in a 4 ml organ bath at an initial tension of 0.5 g using the methods described by Pertwee et al. (1992, 1995a, 1996a)). The baths contained Krebs solution which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (in mM): NaCl 118.2, KCl 4.75, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.0 and $CaCl_2 \cdot 6H_2O$ 2.54. For experiments using the myenteric plexus preparation, $MgSO_4 \cdot 7H_2O$ was also present in this solution, at a concentration of 1.29 mM. Isometric contractions were evoked by electrical field stimulation through a platinum electrode attached to the upper end and a stainless steel electrode attached to the lower end of each bath. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter). Contractions were monitored by computer (Apple Macintosh LC or Performa 475) using a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls). It was not possible to reverse the inhibitory effect of cannabinoids on the twitch response by washing them out of the organ bath. Consequently only one concentration–response curve was constructed per tissue. In control experiments, Tween 80 was added instead of a drug. The control dose of Tween 80 was the same as the dose added in combination with the highest dose of drug used.

Strips of myenteric plexus-longitudinal muscle were dissected from the small intestine of male albino Dunkin–Hartley guinea-pigs (320–737 g) using the method of Paton and Zar (1968). Contractions were evoked by single bipolar rectangular pulses of 110% maximal voltage, 0.5 ms duration and 0.1 Hz frequency. Drugs were added only after the twitch amplitude had become constant. Agonists were added cumulatively, JWH-015 every 30 min and JWH-051, which had a slower onset of action, every 45 min. When used, SR141716A was administered 15 min before the first addition of an agonist. Once a drug had been added, tissues were incubated for several hours without replacing the fluid in the bath.

Vasa deferentia were obtained from albino MF1 mice weighing 33 to 50 g. Contractions were evoked with 500 ms trains (0.1 Hz repetition rate) of three pulses at 5 Hz (110% maximal voltage and 0.5 ms pulse duration). Each vas deferens was subjected to more than one period of stimulation. The first of these began after the tissue had equilibrated but before drug administration and continued for 11 min. The stimulator was then switched off for a predetermined period after which tissues were subjected to further periods of stimulation, each lasting for 5 min. Baths were washed out by overflow at the end of each stimulation period. Drug additions were made immediately after the 11 min stimulation period (time zero) and also after each bath wash. The duration of the stimulation free period that followed each addition of an agonist varied depending on the compound being used (25 min for JWH-015 and 55 min for JWH-051). Noradrenaline was added every 5 min and β,γ-methylene-L-ATP every 20 min. When antagonists were used, these were added at time zero, the first addition of agonist being made 15 min later, after the bath wash at the end of the first 5 min stimulation period.

2.3. In vivo experiments

Mice were subjected to a drug treatment shown previously to render the vasa deferentia of these animals cannabinoid tolerant (Pertwee et al., 1993). The treatment consisted of two intraperitoneal injections of Δ^9-tetrahydrocannabinol, given 24 h apart at a dose of 20 mg/kg. Animals were killed 24 h after the second injection and their vasa deferentia removed. Control animals received intraperitoneal injections of 40 mg/kg Tween 80. The injection volume was 0.25 ml/25 g.

2.4. RNA extraction, reverse transcription and polymerase chain reaction (PCR)

Total cytoplasmic RNA was extracted from four pooled mouse vasa deferentia using RNA X-cell (1 ml) and following the manufacturer's recommended procedure to give total RNA. Contaminating genomic DNA was removed by DNAase-1 (10 U) treatment of the total RNA in the presence of RNasin (40 U). Complementary DNA (cDNA) was synthesised from 50 μg of total cytoplasmic RNA by reverse transcription with an oligo dT primer using standard methods. The resultant cDNA was precipitated with 100% ethanol and resuspended in 100 μl of sterile water. Either 5 μl of this resuspended cDNA, 5 μl of cDNA from non-DNase-1 treated RNA, 2.5 μg total RNA or 5 μl of sterile water as a negative control reaction were amplified by PCR in a final volume of 25 μl. A positive control reaction was carried out using primers specific for cyclophylin. Each PCR reaction contained final concentrations of 0.5 μM of each primer, 1.25 U of Taq polymerase, 1.5 mM (cannabinoid CB_2) or 3 mM (cannabinoid CB_1) $MgCl_2$ and 1 × reaction buffer as well as 0.2 μM deoxyadenosine-5′-triphosphate, 0.2 μM deoxycytosine-5′-triphosphate, 0.2 μM deoxythymidine-5′-triphosphate and 0.2 μM deoxyguanosine-5′-triphosphate. Amplification was carried out in a Perkin–Elmer GeneAmp model 2400 thermal cycler for 35 cycles. The cycles were: 94°C for 30 s, 66°C for 30 s and 72°C for 1 min for cannabinoid CB_1 and 94°C for 30 s, 58°C for 30 s and 72°C for 1 min for cannabinoid CB_2.

Primers were designed to conserved regions in the mouse, human and rat cannabinoid CB_1 sequences (GENEbank accession numbers U22948, X81120 and U40395, respectively) and mouse and human cannabinoid CB_2 sequences (GENEbank accession numbers U21681 and X74328 respectively). The primer sequences were 5′-GCCTCTCCTGGGCTGGAACT-3′ and 5′-TGGAGTTCAGCAGGCAGAGC-3′ corresponding to a 422 bp region between nucleotides 841 and 1262 of human cannabinoid CB_1 and 5′-CTCTGAGCTTTTCCCACTGA-3′ and 5′-CGGATCTCTCCACTCCGTAG-3′ corresponding to a 384 bp region between nucleotides 663 and 1046 of mouse cannabinoid CB_2. Amplified DNA was separated on 2% agarose gels containing ethidium bromide and visualised under ultra-violet light.

Cannabinoid CB_2 PCR products from the vas deferens were cloned and sequenced using the Invitrogen TOPO TA Cloning Kit. 1 μl of fresh PCR product was cloned into PCR TOPO vector, transformed into 'One Shot' competent cells and transformants selected by growth on LB plates containing 50 μg/ml ampicillin and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal). Plasmid DNA was recovered from positive colonies by standard alkaline lysis and the presence of insert sequence confirmed by restriction mapping. Colonies containing the cannabinoid CB_2 insert were sequenced by the Department of Medical Microbiology (University of Aberdeen) using an applied biosystems autosequencer. Data were collected and analysed using a Macintosh computer. The sequence obtained was compared with the sequences lodged in the GENEBank database.

Total RNA was extracted from guinea-pig whole gut and guinea-pig myenteric plexus-longitudinal muscle, care being taken to avoid Peyer's patches as these areas of lymphoid tissue contain cannabinoid receptors (Lynn and Herkenham, 1994). The extracted total RNA was then treated as already described for the mouse vas deferens. PCR was carried out under the same amplification conditions used for the vas deferens, employing the same primer sets.

2.5. Analysis of data

Values are expressed as means and limits of error as standard errors. Inhibition of electrically evoked contractions is usually expressed in percentage terms and has been calculated by comparing the amplitude of the twitch re-

sponse after each addition of an agonist with its amplitude immediately before the first addition of the agonist. In experiments using antagonists, mean log concentration–response curves of the agonist were constructed in the presence of a single concentration of the antagonist and the dissociation constant (K_d) for the interaction between antagonist and cannabinoid receptors has been calculated by substituting a single dose ratio value into the following equation $(x-1)=B/K_d$, where x (the 'dose ratio') is the dose of agonist that produces a particular degree of inhibition in the presence of the antagonist at a concentration, B, divided by the dose of agonist that produces an identical degree of inhibition in the absence of antagonist (Tallarida et al., 1979). Dose ratio and potency ratio values and their 95% confidence limits have been determined by symmetrical $(2+2)$ dose parallel line assays (Colquhoun, 1971) using responses to pairs of agonist concentrations located on the steepest part of each log concentration–response curve. In none of these assays did pairs of log concentration–response curves show significant deviation from parallelism ($P>0.05$). Values with their 95% confidence limits for agonist concentrations producing 50% of their maximum inhibitory effect on the twitch response (EC_{50} values), have been calculated by non-linear regression analysis using GraphPad Inplot (GraphPad Software, San Diego).

3. Results

3.1. Effects on evoked contractions in the mouse vas deferens

JWH-015 and JWH-051 each caused a concentration-related inhibition of electrically evoked contractions. The

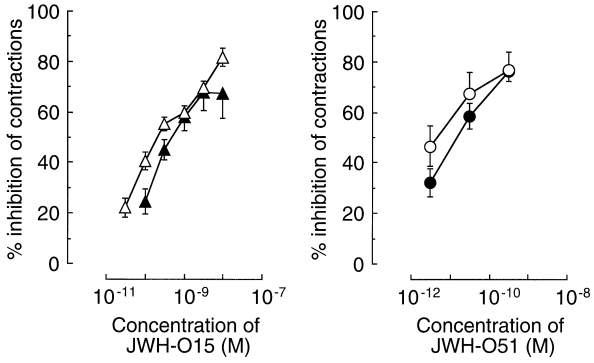


Fig. 2. Mean concentration–response curves for JWH-015 (triangles) and JWH-051 (circles) in mouse vasa deferentia constructed in the presence of 31.62 nM SR141716A (filled symbols) or in the presence of its vehicle, Tween 80 (open symbols). Each symbol represents the mean value ± S.E. of inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath ($n=7$ or 8 different vasa deferentia).

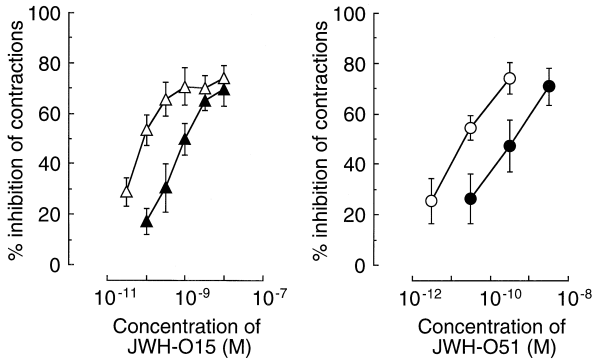


Fig. 3. Mean concentration–response curves for JWH-015 (triangles) and JWH-051 (circles) in vasa deferentia obtained from mice pretreated once daily for 2 days with Δ^9-tetrahydrocannabinol at a dose of 20 mg/kg i.p. (filled symbols) or with Tween 80 (open symbols) at a dose of 40 mg/kg i.p. Each symbol represents the mean value ± S.E. of inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath ($n=6$ or 7 different vasa deferentia). The ratio of the potency of JWH-015 in Tween pretreated tissues to that in Δ^9-tetrahydrocannabinol pretreated tissues is 10.8 (3.2 and 37.9) (mean with 95% confidence limits shown in brackets). The corresponding values for JWH-051 are 11.3 (1.9 and 69.3).

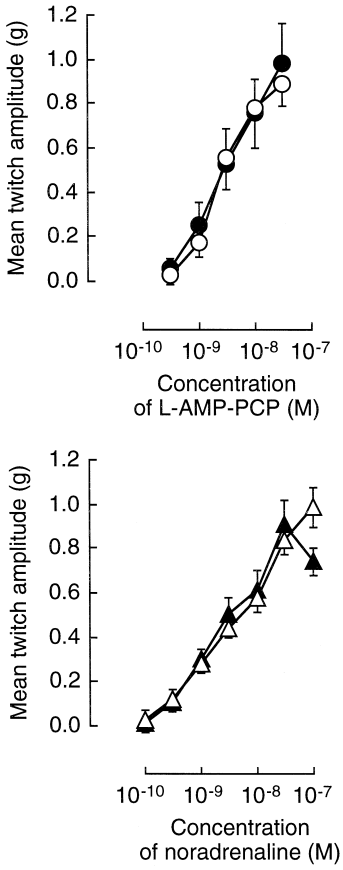


Fig. 4. Mean concentration–response curves for β,γ-methylene-L-ATP (L-AMP-PCP) (circles) and noradrenaline (triangles) in mouse isolated vasa deferentia constructed in the presence of 31.62 nM JWH-015 (filled symbols) or in its absence (open symbols). Each symbol represents mean tension ± S.E. ($n=6$ different vasa deferentia).

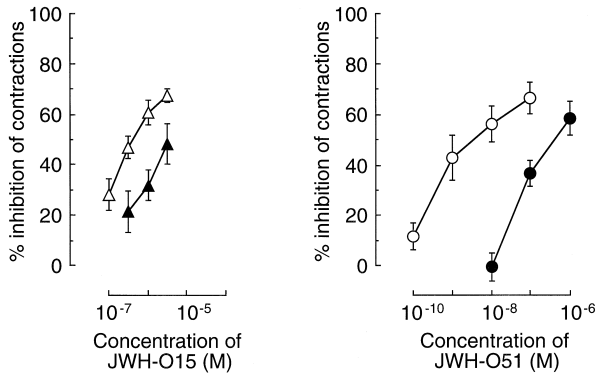


Fig. 5. Mean concentration–response curves for JWH-015 (triangles) and JWH-051 (circles) in myenteric plexus-longitudinal muscle preparations constructed in the presence of 31.62 nM SR141716A (closed symbols) or in the presence of Tween 80 (open symbols). Each symbol represents the mean value ± S.E of inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of an agonist to the organ bath ($n = 6$ to 7 different preparations). SR141716A and Tween 80 were added 15 min before the first addition of agonist. K_d values for SR141716A in the JWH-015 and JWH-051 experiments were 4.26 nM (1.65 and 18.14 nM) and 0.17 nM (0.05 and 0.43 nM) respectively (mean with 95% confidence limits shown in brackets).

mean EC_{50} values of these drugs with their 95% confidence limits shown in brackets are respectively 0.24 nM (0.16 and 0.36 nM) and 4.3 pM (0.02 and 860 pM). At a concentration of 31.62 nM, SR141716A did not antagonise the inhibitory effect of either compound (Fig. 2). Vasa deferentia obtained from mice that had been pretreated with Δ^9-tetrahydrocannabinol (20 mg/kg i.p. once daily for two days) showed a significant decrease in sensitivity to the inhibitory effects of both agonists (Fig. 3). In vivo pretreatment with Tween 80 did not affect the inhibitory potencies of JWH-015 or JWH-051. However, as reported previously (Pertwee et al., 1996b), in vitro addition of Tween 80 by itself was followed by a small but statistically significant decrease in amplitude (data not shown).

Further experiments with JWH-015 showed that it can be antagonized by SR141716A at a concentration of 10 μM. The degree of this antagonism was small. Thus the mean dextral shift in the log concentration–response curve of JWH-015 with its 95% confidence limits shown in brackets was 2.36 (1.37 and 4.48). The mean K_d value of SR141716A calculated from these data was 7359.49 nM, its 95% confidence limits being 26887.17 and 2876.76 nM. JWH-015 was not antagonized by 316.2 nM AM630, 300 nM naloxone or 1 μM yohimbine (data not shown). Nor did JWH-015 (31.62 nM) attenuate the ability of noradrenaline or β,γ-methylene-L-ATP to induce contractions of the vas deferens (Fig. 4).

3.2. Effects on evoked contractions in the myenteric-plexus longitudinal muscle preparation

JWH-015 and JWH-051 each produced a concentration-related inhibition of electrically evoked contractions of this preparation. The mean EC_{50} values of these drugs with their 95% confidence limits shown in brackets are respectively 264.8 nM (126.8 and 553.0 nM)

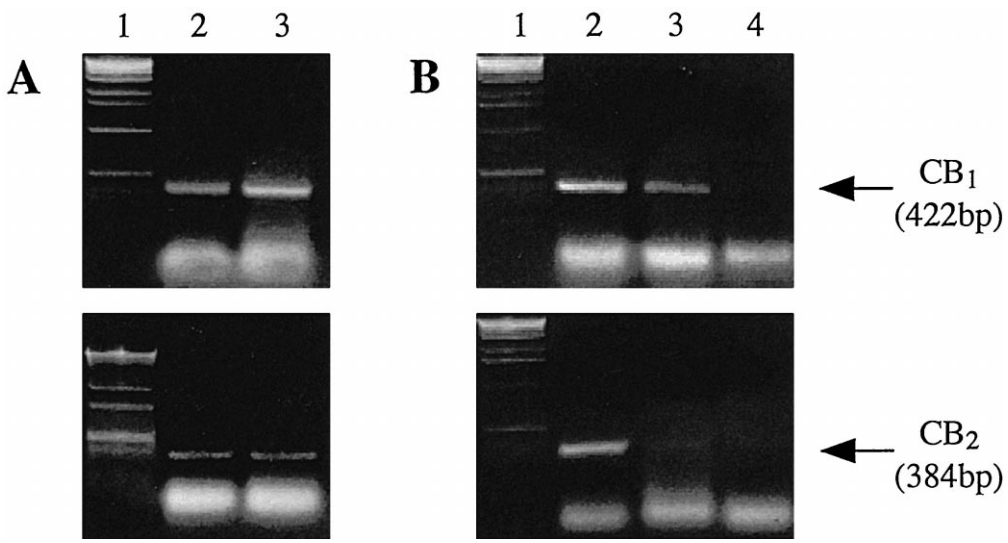


Fig. 6. Polymerase chain reaction amplifications of mouse vas deferens and guinea-pig whole gut. Following polymerase chain reaction amplifications, DNA products were separated on a 2% agarose gel containing ethidium bromide. Panel (A) shows the 1 kb marker (lane 1) and cannabinoid CB_1 and CB_2 amplicons (of expected sizes of 422 bp and 384 bp, respectively) obtained after the amplification of cDNA prepared from DNAase-1 treated RNA prepared from mouse vas deferens (lane 2) and guinea-pig whole gut (lane 3). Controls with primers and no template were negative (data not shown). Panel (B) shows the 1 kb marker (lane 1) and cannabinoid CB_1 and CB_2 amplicons (of expected sizes 422 bp and 384 bp, respectively) obtained after the amplification of cDNA prepared from DNAase-1 treated vas deferens RNA (lane 2), vas deferens total RNA (no reverse transcription) with no DNAase-1 treatment (lane 3) and vas deferens total RNA following DNAase-1 treatment (lane 4). Controls with primers and a water template gave no bands (data not shown). Similar results to those shown in lanes 3 and 4 were obtained in control experiments with guinea-pig whole gut (data not shown).

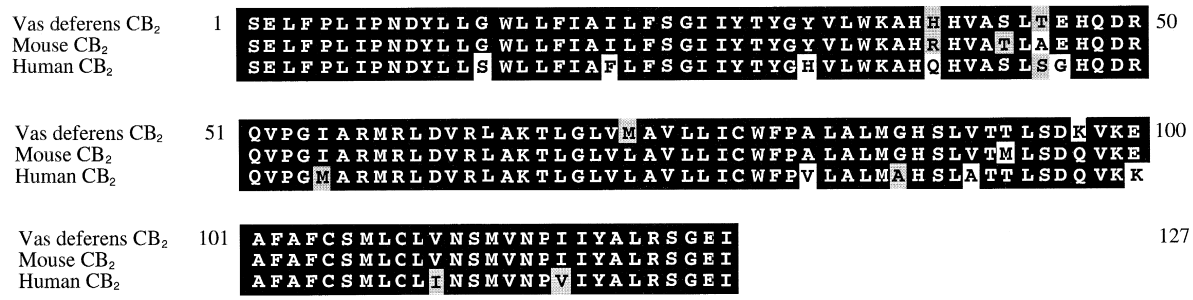


Fig. 7. Predicted cannabinoid CB_2 amino acid sequence in the mouse vas deferens and its alignment with published mouse and human cannabinoid CB_2 sequences. Top line: PCR amplicon obtained from the vas deferens. Middle line: mouse cannabinoid CB_2 sequence (GeneBank accession number U21681). Bottom line: human cannabinoid CB_2 sequence (GeneBank accession number X74328). The black background indicates the degree of homology between sequences (vas deferens versus published mouse sequence $> 95\%$; vas deferens versus human $> 88\%$). The shaded background indicates amino acids at the same position that are chemically similar and the white background chemically dissimilar amino acids.

and 4.56 nM (2.61 and 7.99 nM). At a concentration of 31.62 nM, SR141716A behaved as a competitive surmountable antagonist of both agonists, producing a parallel rightward shift in each of their mean log concentration response curves (Fig. 5). The mean rightward shifts with their 95% confidence limits shown in brackets are respectively 8.4 (2.7 and 20.2) for JWH-015 and 189.5 (74.1 and 621.7) for JWH-051. By itself, Tween 80 did not significantly affect the amplitude of the twitch response of the myenteric-plexus longitudinal muscle preparation.

3.3. Cannabinoid CB_1 and CB_2 mRNA from mouse vas deferens

Amplification and agarose gel electrophoresis of cDNA synthesised from RNA isolated from mouse vas deferens showed the presence of bands of the expected size for both cannabinoid CB_1 and CB_2 (Fig. 6A). The faint cannabinoid CB_1 and CB_2 bands visible in untreated RNA were no longer present after incubation with DNase-1, confirming the removal by this treatment of all genomic DNA (Fig. 6B). No amplification of the negative (water) control was seen (data not shown). The predicted amino acid sequence of the cloned cannabinoid CB_2 product showed $> 95\%$ homology to that previously published for mouse cannabinoid CB_2 and $> 88\%$ homology with the published human sequence (Fig. 7).

3.4. Cannabinoid CB_1 and CB_2 mRNA from guinea-pig whole small intestine and myenteric plexus-longitudinal muscle

PCR was performed on cDNA prepared from whole small intestine and myenteric plexus-longitudinal muscle RNA using the same primers and conditions as described previously. In RNA isolated from whole intestine, bands for both cannabinoid CB_1 and CB_2 were visible after PCR and agarose gel electrophoresis (Fig. 6). Using the myenteric plexus longitudinal muscle RNA, a faint signal was obtained for cannabinoid CB_1 but not for cannabinoid CB_2 (data not shown).

4. Discussion

Several similarities can be discerned between the results obtained in our experiments with JWH-015 and JWH-051 and those obtained in previous isolated tissue experiments with other cannabimimetic agents (Pertwee et al., 1992, 1995a, 1996a; Rinaldi-Carmona et al., 1994). First, JWH-015 and JWH-051 were found to be potent inhibitors of electrically evoked contractions of both the mouse isolated vas deferens and the guinea-pig myenteric plexus preparation. Second, the inhibitory effects of these agents were shown to be concentration-related. Third, their rank order of potency in both tissues (JWH-051 > JWH-015) was found to correspond to their rank order of affinity for both CB_1 and CB_2 cannabinoid binding sites (Huffman et al., 1996; Showalter et al., 1996). Fourth, the inhibitory effects of JWH-015 and JWH-051 on electrically evoked contractions of the myenteric plexus preparation were readily antagonized by submicromolar concentrations of the cannabinoid CB_1 receptor antagonist, SR141716A. This finding indicates that, like other cannabinoids, JWH-015 and JWH-051 probably exert their inhibitory effects on the myenteric plexus preparation by acting through cannabinoid CB_1 receptors. JWH-051 was significantly more susceptible to antagonism by SR141716A in this preparation than JWH-015. The reason for this remains to be determined.

One notable difference between our present results and those obtained in previous experiments with other cannabinoid receptor agonists (Rinaldi-Carmona et al., 1994; Pertwee et al., 1995a,b) is that the effects of JWH-015 and JWH-051 on electrically evoked contractions of the mouse vas deferens were not attenuated by submicromolar concentrations of either SR141716A or AM630. This finding makes it unlikely that the inhibitory effects of these compounds on the vas deferens are mediated by cannabinoid

CB_1 receptors. It is also unlikely that JWH-015 or JWH-051 acted through opioid receptors or α_2-adrenoceptors, both of which are known to inhibit the twitch response of the mouse vas deferens when activated (McCulloch and Pollock, 1985). Thus, the inhibitory effect of JWH-015 on electrically evoked contractions of the vas deferens was found not to be attenuated by naloxone or yohimbine. It remains to be established whether JWH-015 or JWH-051 can exert their inhibitory effects on the vas deferens by acting through other types of non-cannabinoid receptor, for example purinergic receptors or receptors for γ-aminobutyric acid, 5-hydroxytryptamine, dopamine or neuropeptide Y (Stone, 1981; Stjärne et al., 1986; Smith and Rowland, 1989; Von Kügelgen et al., 1989; Seong et al., 1990).

We found that in vivo pretreatment with Δ^9-tetrahydrocannabinol could render vasa deferentia tolerant to the inhibitory effects of JWH-015 and JWH-051. The degree of this tolerance was similar to that which has been observed in experiments in which Δ^9-tetrahydrocannabinol-pretreated vasa deferentia were challenged with established cannabinoid receptor agonists (Pertwee et al., 1993; Pertwee and Griffin, 1995). These findings constitute further evidence for the non-involvement of opioid receptors or of α_2-adrenoceptors in the inhibitory effects of JWH-015 or JWH-051 on the vas deferens as it has been shown previously that in vivo Δ^9-tetrahydrocannabinol pretreatment does not desensitize mouse vasa deferentia to opioid agonists or clonidine (Pertwee and Griffin, 1995).

Results obtained with JWH-015 make it unlikely that this cannabinoid inhibits electrically evoked contractions of the mouse vas deferens by acting postjunctionally to decrease the size of responses to neuronally released contractile transmitters. For the mouse vas deferens these transmitters are noradrenaline and adenosine triphosphate (Stjärne and Åstrand, 1985; Von Kügelgen et al., 1989). It was found that a concentration of JWH-015 effective in attenuating the amplitude of electrically evoked twitches did not prevent contractions elicited by exogenous administration of noradrenaline or of the stable adenosine triphosphate analogue, β,γ-methylene-L-adenosine triphosphate. Similar results have been obtained in previous experiments with Δ^9-tetrahydrocannabinol (Pertwee and Griffin, 1995).

Since JWH-015 and JWH-051 do not seem to inhibit electrically evoked contractions of the mouse vas deferens by acting through cannabinoid CB_1 receptors, opioid receptors or α_2-adrenoceptors and since they have significantly greater affinities for CB_2 than CB_1 cannabinoid binding sites (Huffman et al., 1996; Showalter et al., 1996), the possibility has to be considered that the mouse vas deferens contains cannabinoid CB_2 receptors through which these compounds can act to produce their inhibitory effects. One implication of this idea is that in vivo Δ^9-tetrahydrocannabinol pretreatment can produce tolerance to CB_2 as well as to CB_1 cannabinoid receptor-mediated effects. However, this is not unexpected as Δ^9-tetrahydrocannabinol binds equally well to both receptor types (Rinaldi-Carmona et al., 1994; Felder et al., 1995; Showalter et al., 1996).

Our finding that the inhibitory effect of JWH-015 on the vas deferens is attenuated by 10 μM but not 31.62 nM SR141716A is consistent with the hypothesis that this effect is mediated largely by cannabinoid CB_2 receptors. This is because it is known that at the higher of these concentrations, SR141716A binds to CB_2 as well as CB_1 cannabinoid receptors (Felder et al., 1995; Showalter et al., 1996). Nor is this hypothesis weakened by our evidence that JWH-015 and JWH-051 act through cannabinoid CB_1 receptors in the myenteric plexus preparation (see above). Thus, although both these compounds are selective for cannabinoid CB_2 receptors, they still undergo significant binding to cannabinoid CB_1 receptors with dissociation constants in the submicromolar range (Huffman et al., 1996; Showalter et al., 1996). Indeed, the EC_{50} values of JWH-015 and JWH-051 for inhibition of the twitch response of the myenteric plexus (264.8 and 4.56 nM, respectively) are of the same order as their cannabinoid CB_1 receptor dissociation constants (383 and 1.2 nM, respectively) (Huffman et al., 1996; Showalter et al., 1996).

Since JWH-015 and JWH-051 have higher affinities for CB_2 than for CB_1 cannabinoid receptors, our finding that both agents showed significantly greater potency in the vas deferens than in the myenteric plexus preparation lends further support to the hypothesis that they inhibit the twitch response of the vas deferens by acting through cannabinoid CB_2 receptors. It is noteworthy, however, that although the EC_{50} values of JWH-015 and JWH-051 in the myenteric plexus match their respective cannabinoid CB_1 receptor dissociation constants reasonably well (see above), the EC_{50} values of these compounds in the vas deferens (0.24 nM and 4.3 pM, respectively) are much lower than their cannabinoid CB_2 receptor dissociation constants (13.8 nM and 32 pM, respectively) (Huffman et al., 1996; Showalter et al., 1996). As a result, the ratio of the potency of JWH-015 in the vas deferens to that in the myenteric plexus preparation (1596) greatly exceeds the ratio of the affinity of this compound for transfected cannabinoid CB_2 receptors to that for transfected cannabinoid CB_1 receptors (27.8). This is also true for JWH-051 for which the corresponding ratios are 279 and 37.5, respectively. This discrepancy could reflect a significant cannabinoid CB_2 receptor reserve in the vas deferens. However, it could also be an indication that there are cannabinoid receptors in the vas deferens with an unusually high affinity for the JWH compounds that are neither CB_1 nor CB_2.

The hypothesis that in the vas deferens at least, cannabinoid CB_1 and CB_2 receptors can both mediate inhibition of electrically evoked contractions, is considerably strengthened by our observation that this tissue contains mRNA for these cannabinoid receptor types. The

amplification experiments were purely qualitative and the relative amounts of the two receptors cannot be deduced from the results obtained. Cannabinoid CB_1 receptor mRNA has also been detected in rat vas deferens (Ishac et al., 1996). However, the presence of cannabinoid CB_2 receptor mRNA in the vas deferens has not been reported before. Although our initial sequence data strongly support the presence of this type of mRNA in the mouse vas deferens, there is a discrepancy of 6 out of 127 amino acids between the vas deferens sequence and the previously reported mouse cannabinoid CB_2 sequence (Fig. 7). Consequently, we cannot exclude the possibility that this sequence may encode a variant of the previously reported mouse cannabinoid CB_2 receptor. The presence of this variant could also explain the significant discrepancy that exists between the cannabinoid CB_2 receptor binding affinities of JWH-015 and JWH-051 and the EC_{50} values of these compounds in the vas deferens.

In conclusion, we have obtained evidence that the mouse vas deferens contains CB_1 and CB_2-like cannabinoid receptors and that both these receptor types are capable of mediating inhibition of electrically evoked contractions. Cannabinoid receptor agonists which bind at least as well to CB_1 as to CB_2 cannabinoid receptors presumably exert their inhibitory effects on the vas deferens mainly through cannabinoid CB_1 receptors as such agonists are readily antagonized by SR141716A (see Section 1). On the other hand, cannabinoid receptor agonists with significant CB_2 selectivity may exert their inhibitory effects on the vas deferens mainly through the CB_2-like cannabinoid receptors. It is possible that SR141716A antagonizes JWH-015 and JWH-051 in the myenteric plexus preparation because this tissue contains cannabinoid CB_1 receptors but lacks any CB_2-like cannabinoid receptors capable of mediating inhibition of electrically evoked contractions. This hypothesis is supported by our finding that the myenteric plexus preparation contains CB_1 but not CB_2-like cannabinoid receptor mRNA. Interestingly, both types of mRNA were detected in guinea-pig whole gut, possibly indicating the presence in the whole tissue of resident macrophages and other immune cells that are known to contain cannabinoid CB_2 receptors (Munro et al., 1993; Galiègue et al., 1995). Further experiments are required to investigate the presence of CB_2 or CB_2-like cannabinoid receptors that mediate inhibition of evoked contractions in tissues other than the mouse vas deferens. The existence in a peripheral tissue of cannabinoid CB_2 receptors with this property would be fortuitous as there is still a need for a functional in vitro bioassay system for CB_2 receptor ligands that is both quantitative and sensitive and that contains 'natural' as opposed to transfected cannabinoid CB_2 receptors.

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