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Pharmacological characterisation of cannabinoid CB_1 receptors in the rat and mouse

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Abstract

The role of cannabinoid CB_1 receptors in sympathetic neurotransmission was characterised in nerve-mediated responses of isolated right atria, vasa deferentia and small mesenteric resistance arteries using the cannabinoid CB_1 receptor agonists Δ^9-tetrahydrocannabinol, CP 55,940 and anandamide and the cannabinoid CB_1-selective antagonist SR 141716A. In the mouse vas deferens, the twitch response was completely inhibited by each of the putative cannabinoid receptor agonists with pIC_{50} values of CP 55,940, 9.2 ± 0.1; Δ^9-tetrahydrocannabinol, 8.4 ± 0.1; anandamide, 7.1 ± 0.1. SR 141716A 10–100 nM was a competitive antagonist of all three agonists with a pK_B value of 8.4–8.6, consistent with an interaction at the cannabinoid CB_1 receptor. In the rat vas deferens CP 55,940 (0.01–10 μM) inhibited the contractions to a significant extent ($88.5 \pm 0.5\%$ at 10 μM; pIC_{50} of 7.1 ± 0.1) while Δ^9-tetrahydrocannabinol and anandamide (both up to 10 μM) were inactive. CP 55,940 exhibited low potency in rat compared with mouse vas deferens and the rat concentration–response curve was not competitively antagonised by SR 141716A (100 nM) or SR 144528 (10 nM–10 μM), suggesting an interaction at a receptor(s) distinct from cannabinoid CB_1 or CB_2. Sympathetic nerve-induced tachycardia in rat and mouse atria, and rat mesenteric artery smooth muscle contractile responses to perivascular nerve stimulation, were not inhibited by Δ^9-tetrahydrocannabinol, CP 55,940 or anandamide up to 1 μM. These data indicate that cannabinoid CB_1 receptor activation inhibits sympathetic neurotransmission only in the mouse vas deferens and thus point to species and regional differences in cannabinoid CB_1 receptor involvement in pre-synaptic inhibition of sympathetic neurotransmission and CP 55,940 may have inhibitory actions in rat vas deferens unrelated to cannabinoid receptor activity.

Keywords: Cannabinoid CB_1 receptor; Δ^9-Tetrahydrocannabinol; CP 55,940; Anandamide; SR 141716A; SR 144528; Sympathetic neurotransmission

1. Introduction

Interest in cannabinoid pharmacology has developed since the discovery of both cannabinoid receptors and the endogenous ligand, anandamide (Devane et al., 1988, 1992; Munro et al., 1993). Two cannabinoid receptor subtypes, the CB_1 and CB_2 have been characterised. Cannabinoid CB_1 receptors are located predominantly in the central nervous system but are also present pre-junctionally on some peripheral autonomic nerves where they inhibit neurotransmitter release in the urogenital, gastrointestinal and cardiovascular systems (Mackie and Hille, 1992; Ishac et al., 1996; Pertwee and Fernando, 1996).

Both Δ^9-tetrahydrocannabinol and an endogenous mammalian cannabinoid, anandamide, are naturally occurring cannabinoid receptor agonists. Anandamide is found both in the brain (porcine and rat) and in peripheral tissues such as the rat kidney vasculature (Devane et al., 1992; Deutsch et al., 1997) and is physiologically regulated by fatty acid amide hydrolase activity (Cravatt et al., 1996). To date, many synthetic cannabinoid receptor agonists have been synthesised, including, CP 55,940, a potent, bicyclic cannabinoid receptor agonist (Pertwee et al., 1993).

Most in vitro bioassays of the effects of cannabinoids have been conducted in the mouse or guinea pig (Ishac et al., 1996; Pertwee and Fernando, 1996; Pertwee et al., 1996a,b). However, there is higher, albeit small, conservation between rat and human cannabinoid CB_1 mRNA than between mouse and human (Chakrabarti et al., 1995; Shire et al., 1996). Therefore, it was of interest to compare functional responses caused by cannabinoid CB_1 receptor

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activation in tissues isolated from rat and mouse. Considerable data on cannabinoid CB_1 receptor agonist effects on electrically evoked contractions of the mouse vas deferens have been published (Pertwee and Griffin, 1995; Pertwee et al., 1992; Pertwee et al., 1995a,c, 1996b). The cannabinoid CB_1 mRNA has been found in both the mouse and rat vas deferens (Ishac et al., 1996; Pertwee et al., 1996b). Ishac et al. (1996) reported that Δ^9-tetrahydrocannabinol and anandamide inhibited electrically evoked release of [^{3}H]noradrenaline from rat isolated atria. Whether this translates to direct cannabinoid-induced chronotropic changes has not yet been established. In addition, a number of recent papers have suggested that anandamide may have a role in cardiovascular control. Deutsch et al. (1997) demonstrated that anandamide exerts significant vasorelaxant and neuromodulatory effects. Zygmunt et al. (1997) suggested that anandamide relaxes rat hepatic artery via smooth muscle cell membrane hyperpolarisation in endothelium-intact vessels and, more importantly, by direct inhibition of Ca^{2+} release from stores in the myocyte.

Therefore, the aim of this study was to investigate the role of cannabinoid CB_1 receptors in sympathetic neurotransmission in the vas deferens, right atrium and small mesenteric artery from both the rat and mouse. Preliminary data have been previously published in abstract form (Lay et al., 1998).

2. Materials and methods

Swiss white mice (35–40 g) and Sprague–Dawley rats (250–300 g) were killed by exposure to 80% CO_2 in O_2 and exsanguination. Atria and mesenteric arteries were bathed in a physiological salt solution (PSS) comprising (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylene-diaminetetracetic acid (EDTA) 0.026, glucose 11 and saturated with 95% O_2 and 5% CO_2, during dissection, mounting and the experiment.

2.1. Rat isolated vasa deferentia

Rat vasa deferentia were dissected with capsular connective tissue intact and set up in 5 ml organ baths at 37°C in Mg^{2+}-free physiological salt solution. The upper (epididymal) end was attached to an isometric force transducer (Grass FTO3C, Grass Instruments, Quincy, MA, USA) and the lower (prostatic) end tied to a fixed support between two parallel platinum field electrodes (5 mm apart, 5 mm long). The tissue was initially stretched by 2 g force and allowed to equilibrate for 20 min. The vas deferens was continuously stimulated (Grass S88 stimulator) to contract (twitch) with a single submaximal electrical field pulse (150 V, 0.5 ms duration) delivered every 20 s. Output from the transducer amplifier was recorded on a flat bed recorder (Linearcorder WR3300, Graphtec, Tokyo, Japan). All drugs were dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA). SR 141716A (100 nM), SR144528 (10 nM–10 μM), CP 55,940 (10 nM–10 μM), Δ^9-tetrahydrocannabinol (1 μM) and anandamide (10 nM–10 μM) were allowed to equilibrate for 30 min before the responses to field stimulation were assessed. Agonist concentration–response curves were constructed cumulatively with half log unit increments.

The effect of Δ^9-tetrahydrocannabinol pre-incubation (1 μM, 1 h) before construction of the CP 55,940 concentration–response curve in the rat vas deferens was examined to determine whether Δ^9-tetrahydrocannabinol was behaving as an antagonist in this assay. In other experiments, responses to anandamide were obtained in the absence and presence of 50 μM phenylmethylsulfonyl fluoride (in DMSO vehicle, pre-incubation for 30 min) to determine whether the presence of an amidase inhibitor affected the potency of this putative endogenous cannabinoid.

To determine whether the effect of CP 55,940 on sympathetic nerve-mediated twitch contractions of the rat vas deferens was due to inhibition at a post-junctional site, the effect of CP 55,940 on αβ-methyleneATP concentration–response curves was examined. Whole vasa deferentia were stretched to 2 g, then equilibrated for 30 min. To minimise desensitisation, non-cumulative sequential concentrations of αβ-methyleneATP were added to the bath and immediately removed once the peak contraction was obtained (3–5 min). Concentration–response curves to αβ-methyleneATP were conducted after 1 h incubation with CP 55,940 (1 μM) or DMSO vehicle in separate tissues.

2.2. Mouse isolated vasa deferentia

Mouse vasa deferentia were dissected with capsular connective tissue intact and set up in 20 ml organ baths at 37°C in Mg^{2+}-free PSS. The upper (epididymal) end was attached to an isometric force transducer (Grass FTO3C) and the lower (prostatic) end tied to a fixed support between two parallel platinum field electrodes (5 mm apart, 5 mm long). The tissue was initially stretched by 0.5 g force and allowed to equilibrate 10 min. The tissues were stimulated (Grass S88 stimulator) to contract using trains of electrical field stimulation of 3 pulses (4 Hz), 0.5 ms duration, 100 V (80% maximal voltage) every 20 s for 10 min. This electrical stimulation period was applied before and after both antagonist and agonist addition. The antagonist SR 141716A (or vehicle, DMSO) was incubated for 30 min, followed by a single concentration of anandamide or CP 55,940 for 30 min, or Δ^9-tetrahydrocannabinol for 1 h (or vehicle, Tween 80 (Sigma) suspensions in MilliQ water (Millipore, Sydney, Australia)). Output from the transducer amplifier was recorded on a flat bed recorder (Gould BS 272, Cleveland, OH, USA). The effects of the antagonist or agonists were measured as the percentage decrease of the pre-drug twitch force.

The effect of phenylmethylsulfonyl fluoride on the anandamide concentration–response curve was examined, as outlined above.

2.3. Rat and mouse isolated right atria

The rat or mouse isolated right atrium was placed vertically on stainless steel S-shaped hooks mounted on an acrylic leg in a physiological salt solution-filled 10 ml glass-jacketed organ bath heated to 37°C. The partially stretched atrium rested against two punctate platinum electrodes protruding from the acrylic leg 3 mm apart that recorded the spontaneous surface electrogram. The signal was amplified (Baker Medical Research Institute amplifier Model 108, Prahran, Victoria, Australia) and used to trigger a Maclab data acquisition system (chart version 3.5.2; AD Instruments, Castle Hill, N.S.W., Australia). Atrial period was continuously recorded on the Maclab. To examine the sympathetic response in the absence of bradycardia, the atria were equilibrated for 1 h in the presence of atropine (1 μM). One control group of electrical field pulse stimuli (one, two, four or eight pulses in one train) at 50 V, 2 ms duration at 2 or 3 Hz were applied to rat and mouse atria, respectively (Grass S88 stimulator) (Angus and Harvey, 1981). The responses to the control stimuli were compared to the responses following 1 h incubation with 1 μM of the cannabinoid receptor agonist.

2.4. Rat isolated mesenteric resistance arteries

Small arteries (300–400 μm internal diameter) were dissected from the mesenteric bed and mounted in physiological salt solution at 37°C as 2 mm ring preparations in dual 6 ml chamber myographs to record changes in isometric force (J.P. Trading, Aarhus, Denmark). Following a 30 min equilibration period, the endothelium-intact vessels were stretched to an internal diameter equivalent to 90% of a transmural pressure of 100 mm Hg (L_{100}; Mulvany and Halpern, 1977). Platinum electrodes (0.5 mm thick) were contained in the mounting supports of the myograph to deliver square wave field stimulation (Grass S88 stimulator). Output from the transducer amplifier was recorded on a flat bed recorder (Model 320, W and W Scientific Instruments, Basle, Switzerland).

After normalisation (Mulvany and Halpern, 1977), vessels were equilibrated for 30 min and then contracted to their maximum levels of active force with high K^+ PSS for 2 min (standard PSS with an equimolar exchange of KCl for NaCl, i.e., K^+ 124 mM, hereafter termed KPSS). Following return to baseline, noradrenaline (10 μM) was applied for 2 min and then washed out. Electrical field stimulation was applied at 30 V, 0.25 ms duration, 25 Hz for a 3 s train every minute. This stimulus train has been shown to cause contractions 40–60% of that to KPSS (Angus et al., 1988). Three trains of control field stimulation were applied and repeated 30 min later to obtain control values. Following this, a concentration (1 μM) of a cannabinoid CB_1 receptor agonist (Δ^9-tetrahydrocannabinol, CP 55,940 or anandamide) or vehicle was added and allowed to incubate for 30 min and nerve stimulation retested. Curves were constructed from drugs added cumulatively.

Attempts were made to repeat these experiments using mouse small mesenteric artery. However, due to very small and inconsistent contractions, no further experiments were conducted.

2.5. Drugs

Drugs used and suppliers were: anandamide ((all Z)-*N*-(2-hydroxyethyl)-5,8,11,14-eicosatetraenamide; Tocris Cookson, Bristol, UK), atropine sulphate (Sigma), CP 55,940 ((−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; Tocris Cookson), (−)-noradrenaline bitartrate (Arterenol, Sigma), phenylmethylsulfonylfluoride (Sigma) and Δ^9-tetrahydrocannabinol (Research Biochemicals International, Natick, MA, USA). SR 141716A (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide and SR 144528 (*N*-[1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-2-methylphenyl)-1-(4-methylbenzyl0-pyrazole-3-carboxamide) were gifts from Sanofi-Recherche, Montpellier, France. Solutions of drugs were made up fresh daily. Δ^9-Tetrahydrocannabinol, CP 55,940, anandamide, SR 141716A and SR 144528 were made up in DMSO (dimethyl sulphoxide) for experiments in the rat vas deferens and rat mesenteric artery as these data were completed first and Tween 80 caused considerable inhibition of contractions of the rat mesenteric resistance artery. For all other experiments, the cannabinoid receptor agonists were made up in ethanol and equivalent volume of Tween 80 (polyoxyethylenesorbitan monooleate; Sigma), after which the ethanol was evaporated off and MilliQ water (Millipore, Lane Cove, N.S.W., Australia) added to dilute the drug.

2.6. Analysis and statistical methods

Data are presented as mean ± 1 standard error of the mean (S.E.M.). In the vasa deferentia, the effects of the antagonist or agonists were measured as a percentage of the baseline twitch contraction. Sympathetic responses to electrical field stimulation of right atria are expressed as absolute rate and change in atrial rate. Mesenteric artery responses are expressed as a percentage of those obtained to the second set of control field stimulation (C2). Data obtained from the mouse vasa deferentia (single concentration per tissue) were fitted to a logistic curve and constrained to fewer parameters when required (Stone and Angus, 1978; Lew and Angus, 1995). From these logistic curve fits rat or mouse pIC_{50} values (the −log concentration of cannabinoid receptor agonist required to cause 50%

of the maximal agonist-induced inhibition of the contractions) were compared in the absence (control) and presence of SR 141716A (100 nM) using an unpaired Student's *t* test. For all other preparations, within and between drug treatment groups, responses to electrical field stimulation were assessed by repeated measures analysis of variance (ANOVA) with Greenhouse–Geisser correction for correlation (Ludbrook, 1994), calculated by means of statistical program SuperANOVA 1.11 for Macintosh. The average S.E.M. within rat tissues was calculated from repeated measures ANOVA using the pooled estimate of error from the residual mean square as (error mean square/number of tissues)$^{0.5}$ after sums of squares between tissues and between cannabinoid concentrations (or, in atria, number of electrical field pulses) had been subtracted from the total sums of squares for each treatment group (Snedecor and Cochran, 1989). These average S.E.M.s are located on the lines shown in Figs. 2, 3 and 5 (Wright et al., 1987).

The pK_B value of SR 141716A in the mouse vas deferens for each of the three cannabinoid CB_1 receptor agonists was determined from a non-linear regression method of all the pIC_{50} values of the agonist in the absence or presence of 10–100 nM SR 141716A from the equation $pIC_{50} = -\log([B] + 10^{-pK_B} - \log c$, where pK_B is a fitted parameter and the constant log c is the difference between the antagonist pK_B and the agonist control curve pIC_{50} in the absence of antagonist (Lew and Angus, 1995). The values of agonist pIC_{50} (y axis) and the corresponding values of antagonist concentration (SR 141716A, $\log(B + K_B)$, (x-axis) were displayed in a 'Clark Plot' (see Stone and Angus, 1978).

In all cases, P values < 0.05 were considered significant.

3. Results

3.1. Vasa deferentia

3.1.1. Effect of vehicle

In rat preparations stimulated with single pulses, there was a decrease of $19 \pm 5\%$ ($n = 6$) in contraction of the tissue in response to electrical stimulation in the vehicle (increasing DMSO concentrations) group over time, however, this did not reach statistical significance ($P > 0.05$). In the mouse preparation stimulated with intermittent trains of stimuli, there was no significant decrease in twitch ($9 \pm 1\%$; $n = 4$; $P > 0.05$) in the presence of vehicle (Tween 80). We examined whether DMSO or Tween 80 vehicle affected the potency of Δ^9-tetrahydrocannabinol in inhibiting the contractions in the mouse or rat vas deferens assay (data not shown). The pIC_{50} for Δ^9-tetrahydrocannabinol dissolved in DMSO vehicle was 8.0 ± 0.3 ($n = 4$); Δ^9-tetrahydrocannabinol dissolved in Tween 80 vehicle (pIC_{50} of 8.5 ± 0.2; $n = 4$) was 3.1 ± 0.2-fold more potent ($P < 0.05$) in the mouse vas deferens. In addition, Δ^9-tetrahydrocannabinol dissolved in Tween 80 (10 nM–10 μM) was also ineffective in inhibiting the contractions of the rat vas deferens.

3.1.2. Δ^9-Tetrahydrocannabinol

In tissues from the rat, Δ^9-tetrahydrocannabinol (0.01–10 μM) did not significantly inhibit the twitch response

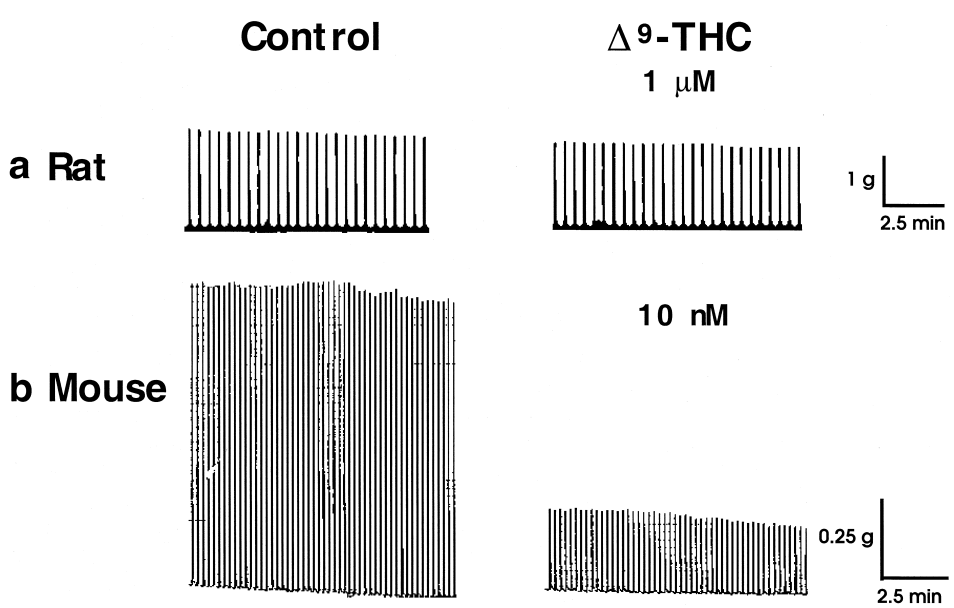

Fig. 1. Computer scan of representative traces of electrically evoked contractions of the vas deferens isolated from (a) rat and (b) mouse. Left panels show control responses and right panels show the effect of 1 h incubation with Δ^9-tetrahydrocannabinol at (a) 1 μM and (b) 10 nM. The rat vas deferens was continuously stimulated with single pulses (150 V, 0.5 ms duration) delivered every 20 s, whereas the mouse vas deferens was stimulated intermittently for 10 min intervals (trains of three pulses at 4 Hz, 100 V, every 20 s) (see Section 2).

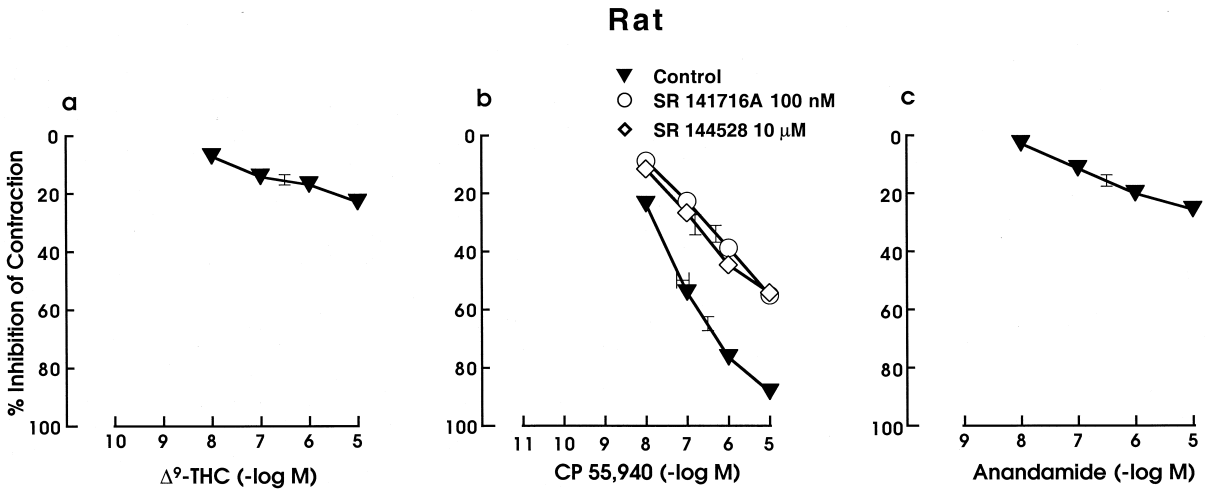

Fig. 2. Effects of the cannabinoid CB_1 agonists (a) Δ^9-tetrahydrocannabinol, (b) CP 55,940, and (c) anandamide, in rat vas deferens. Increasing concentrations of the agonist were added cumulatively ($n = 6$ tissues per line). Panel (b) displays the effects of SR 141716A (100 nM) and SR 144528 (10 μM) on the CP 55,940 concentration–response curve. The vertical error bar on the line corresponds to the average S.E.M. from ANOVA (see Section 2). Horizontal error bars correspond to the respective $pIC_{50} \pm$ S.E.M. values (where applicable).

(Figs. 1a, 2a), and responses were similar to those in the vehicle group ($n = 6$; $P > 0.05$). In contrast, Δ^9-tetrahydrocannabinol at 10 nM markedly decreased the twitch (Fig. 1b) and completely inhibited the twitch response at 100 nM in the mouse vas deferens ($n = 4$; pIC_{50} of 8.4 ± 0.1; Fig. 3a).

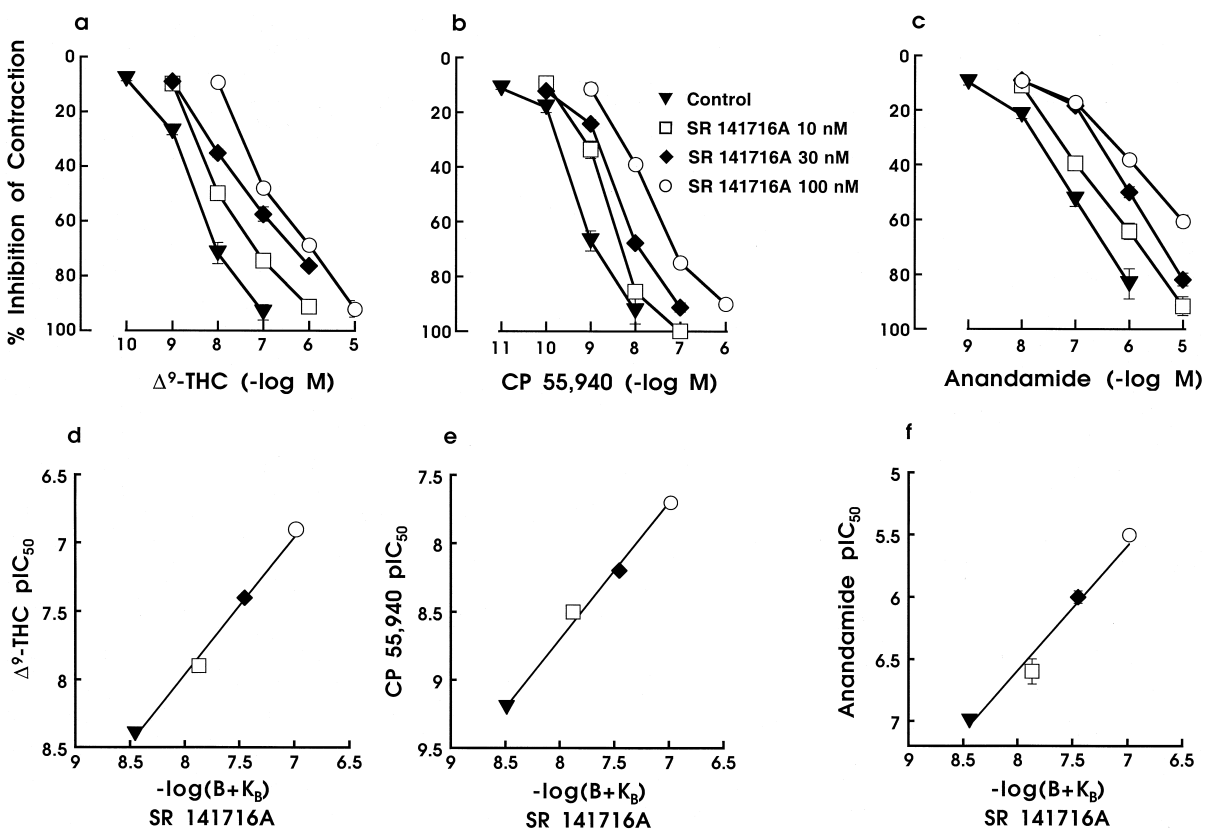

Fig. 3. Effect of the cannabinoid agonists on electrically evoked contractions of the mouse vas deferens (a–c) in the absence and presence of 10, 30 and 100 nM SR 141716A. Each point corresponds to four different tissues, a total of 16 tissues making up each line. Vertical error bars are ±S.E.M.. Lower panel shows the corresponding Clark Plots (see Section 2) displaying the effect of SR 141716A on the pIC_{50} value of (d) Δ^9-tetrahydrocannabinol, (e) CP 55,940 and (f) anandamide.

3.1.3. CP 55,940

CP 55,940 at 10 μM caused a pronounced inhibition ($88 \pm 1\%$; $n = 6$) of the rat vas deferens contractions. The concentration–response relationship was characterised by a pIC_{50} of 7.1 ± 0.1 (Fig. 2b). The CP 55,940 concentration–response curve was not shifted by pre-incubation with Δ^9-tetrahydrocannabinol (1 μM), suggesting that Δ^9-tetrahydrocannabinol is not acting at this CP 55,940-sensitive site ($n = 6$; $P > 0.05$). In the mouse vas deferens, CP 55,940 was able to cause complete inhibition of twitches at 10 nM ($n = 4$; pIC_{50} of 9.2 ± 0.1; Fig. 3b), i.e., some 250-fold more potent than in the rat tissue.

The range of contraction elicited by αβ-methyleneATP (1–1.3 g force) of the rat vas deferens was the same as that observed with electrical nerve stimulation (~ 1 g force). CP 55,940 (1 μM for 1 h in DMSO) had no significant effect at the P_{2x} post-junctional receptor stimulated by αβ-methyleneATP. The pEC_{50} and maximum contraction of the αβ-methyleneATP concentration–response curve were 5.6 ± 0.1 (−log M) and 1.4 ± 0.2 g in the presence of CP 55,940 and 5.7 ± 0.03 and 1.0 ± 0.2 g in the presence of vehicle (DMSO) (Fig. 4). CP 55,940 did not change the sensitivity or the maximum response ($n = 6$; $P > 0.05$), indicating that its site of action was not post-junctional.

3.1.4. Anandamide

Anandamide caused no significant decrease in twitch height in the rat vas deferens ($n = 6$; $P > 0.05$; Fig. 2c). However, anandamide caused a concentration-dependent inhibition of twitches in the mouse vas deferens (Fig. 3c). Anandamide was less potent (pIC_{50} of 7.1 ± 0.1) than both Δ^9-tetrahydrocannabinol and CP 55,940 ($n = 4$). Pre-incubation of the rat and mouse vas deferens with phenylmethylsulfonyl fluoride, an amidase inhibitor, did not affect the anandamide-induced inhibition ($n = 4$; $P > 0.05$; data not shown).

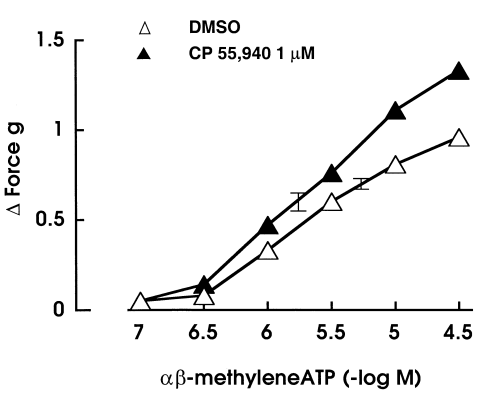

Fig. 4. Effect of CP 55,940 (1 μM; $n = 6$) or DMSO vehicle ($n = 6$) on αβ-methyleneATP concentration–contraction curves in the rat vas deferens. Sequential concentrations of αβ-methyleneATP were added to the bath and immediately removed once the peak contraction was obtained (3–5 min). The vertical error bar on the line corresponds to the average S.E.M. from ANOVA (see Section 2).

3.1.5. Antagonism by SR 141716A

Pre-incubation with increasing concentrations of SR 141716A (10–100 nM) caused corresponding parallel rightward shifts of the log concentration–response curve for Δ^9-tetrahydrocannabinol (Fig. 3a), CP 55,940 (Fig. 3b) and anandamide (Fig. 3c) in the mouse vas deferens. Logistic curve fitting and non-linear regression analysis (see Section 2) gave estimated pK_B values for SR 141716A with the three agonists as follows: 8.5 ± 0.1 (Δ^9-tetrahydrocannabinol), 8.6 ± 0.2 (CP 55,940) and 8.4 ± 0.2 (anandamide). The rightward displacement of the agonist concentration–response curves in the presence of the antagonist SR 141716A was consistent in each case with simple competitivity. This is displayed in the Clark Plots (Fig. 3d–f).

In contrast, SR 141716A (100 nM) shifted the log concentration–response curve of CP 55,940 in a non-parallel fashion in the rat vas deferens (Fig. 2b), indicating that SR 141716A was not acting in a simple competitive manner. This is reinforced by the shallowness of the curve, suggesting that CP 55,940 in the presence of SR 141716A at 100 nM may not completely abolish the contractions of the rat vas deferens.

3.1.6. Antagonism by SR 144528

Pre-incubation with a concentration of SR 144528 (10 nM), which is claimed to act exclusively at the cannabinoid CB_2 receptor (Rinaldi-Carmona et al., 1998) did not shift the CP 55,940 concentration–response curve (data not shown), suggesting there are no functional CB_2 receptors in the rat vas deferens. Pre-incubation with a higher concentration of SR 144528 (10 μM) did cause a small rightward shift in the CP 55,940 concentration–response curve similar to that observed with SR 141716A (Fig. 2b). However, this shift was not parallel to the CP 55,940 control curve, suggesting that SR 144528 was also not acting in a simple competitive manner at either the cannabinoid CB_1 or CB_2 receptor.

3.2. Rat and mouse right atria

In rat and mouse isolated atria in the presence of atropine (1 μM), vehicle (Tween 80) did not significantly alter atrial rate elicited by sympathetic nerve stimulation ($n = 6$; $P > 0.05$). Fig. 5, panels a and c, represent the effects of the cannabinoid receptor agonists on absolute atrial rate while panels b and d display the data as change from baseline. The rat atrial resting rate was not significantly different after drug treatment ($n = 6$; $P > 0.05$). The mouse pre-treatment resting right atrial rate (beats/min) was 410 ± 7 in the presence of vehicle compared to 368 ± 14, 381 ± 8 and 370 ± 14 in the presence of Δ^9-tetrahydrocannabinol, CP 55,940 and anandamide,

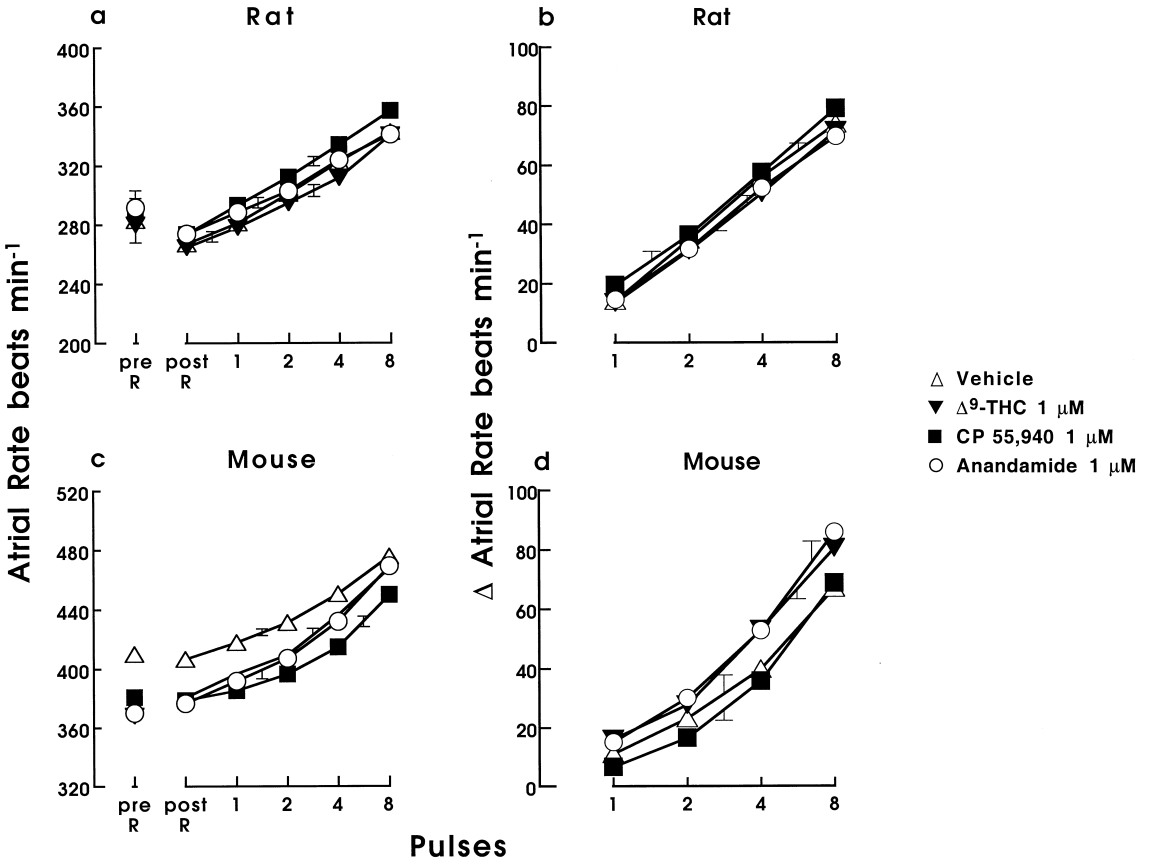

Fig. 5. Effect of sympathetic stimulation via trains of one to eight electrical field pulses on rat (a, b) and mouse (c, d) isolated right atrial rate (beats/min) in the presence of 1 μM atropine: responses are shown in both atrial rate (a, c) and change in rate from the baseline (b, d) in the absence and presence of 1 μM Δ^9-tetrahydrocannabinol ($n = 6$), CP 55,940 ($n = 6$) and anandamide ($n = 6$). Pre R: resting rate before drug treatment. Post R: resting rate after drug treatment. Error bars on the lines are average S.E.M. from ANOVA (see Section 2). Error bars on pre R correspond to ±S.E.M.; those not shown are within the symbol.

respectively ($n = 6$; $P > 0.05$). There was no significant difference between pre- and post-treatment resting rate for each of the respective treatment groups, indicating that there was no cannabinoid receptor agonist-induced changes in resting rate ($n = 6$; $P > 0.05$). Furthermore, neither Δ^9-tetrahydrocannabinol, CP 55,940 or anandamide caused significant inhibition of the sympathetically driven electrical stimulus–response curve in rat or mouse isolated right atria at 1 μM ($n = 6$; $P > 0.05$). When the data were expressed as change in atrial rate (Fig. 5b and d), it is clear that there was no inhibition of sympathetic tachycardia ($n = 6$; $P > 0.05$).

3.3. Rat isolated mesenteric resistance arteries

The average internal diameter of the rat mesenteric resistance arteries at L_{100} was 428 ± 34 μm ($n = 4$). Responses to the control field stimulation (C2) were similar in all drug treatment groups ($n = 4$; $P > 0.05$). Vehicle did not significantly affect responses to nerve stimulation ($85 \pm 6\%$ of C2; $n = 4$; $P > 0.05$). Δ^9-Tetrahydrocannabinol, CP 55,940 and anandamide, all at 1 μM, were ineffective in inhibiting intramural nerve-mediated contractions, with values of $100 \pm 2\%$, $89 \pm 5\%$ and $103 \pm 3\%$, respectively, of the control contraction ($n = 4$; $P > 0.05$).

4. Discussion

This work shows that putative cannabinoid CB_1 receptor agonists display tissue and species differences in the rat and mouse. Criteria for putative cannabinoid CB_1 receptor actions were (i) inhibition of sympathetic nerve-mediated responses to a range of cannabinoid CB_1 receptor agonists at low concentrations; and (ii) rightward parallel shift of the cannabinoid CB_1 receptor agonist concentration–response curve by at least ~10-fold by the selective antagonist SR 141716A at a concentration of 100 nM. Thus, in the mouse vas deferens, the sympathetically evoked contractions were sensitive to all three cannabinoid receptor agonists with a rank order of potency of CP 55,940 > Δ^9-tetrahydrocannabinol > anandamide, supporting previously published work with this tissue (Pertwee et al., 1992, 1995b, 1996b).

Anandamide and plant-derived cannabinoids have been shown to inhibit the electrically evoked twitch response of

the mouse isolated vas deferens without affecting the contractile response to exogenous noradrenaline, and this effect is competitively antagonised by SR 141716A (Rinaldi-Carmona et al., 1994). Therefore, it is likely that in the mouse vas deferens, cannabinoid agonists inhibit the pre-junctional release of noradrenaline and ATP via cannabinoid receptors negatively coupled to N-type Ca^{2+} channels, thereby decreasing electrically stimulated contractions of this tissue. The lack of cannabinoid CB_1 receptor agonist-induced inhibition of electrically stimulated small mesenteric arteries and isolated right atria of the rat and mouse (responses sensitive to ω-conotoxin GVIA; (Wright and Angus, 1996), may suggest that there is an absence of cannabinoid CB_1 receptors in these tissues. It is also possible that the lack of response was due to very poor coupling between the receptor and transduction mechanism.

The protocol for intermittent stimulation of the mouse vas deferens was chosen after Pertwee et al. (1992) showed that the amplitude of the twitch response of vasa deferentia decreased significantly with time when trains of stimuli were applied continuously, but remained constant when tissues were subjected to intermittent 10 min periods of stimulation. Single agonist concentrations were applied to each mouse tissue because of the long incubation protocol. This necessitated the use of non-linear regression analysis to determine the pK_B value for SR 141716A. The Clark Plot is a display of the relationship between the theoretical predictions for spacing of agonist concentration–response curves in a competitive interaction and the experimental agonist curve spacing in the absence ($B = 0$) and presence of antagonist (B) concentrations, as well as the pK_B value (when $B = 0$) obtained from non-linear regression analysis (Stone and Angus, 1978). The points on the Clark Plot are the mean log IC_{50} values from the agonist concentration–response curves at each concentration of antagonist plotted against the log ([antagonist] + K_B). The line represents the ideal interaction between the agonist and antagonist for simple competitive antagonism. In this study, SR 141716A behaved as a simple competitive antagonist at the cannabinoid CB_1 receptor in mouse tissue. SR 141716A was able to competitively reverse the inhibition of contractions in the mouse vas deferens with a consistent, agonist-independent estimated pK_B value of 8.4–8.6 strongly supporting evidence for an interaction at the cannabinoid CB_1 receptor for all three cannabinoid receptor agonists (Pertwee et al., 1995c).

Interestingly, others have found that SR 141716A can behave as an inverse agonist. It should be noted, however, that this property of SR 141716A has been demonstrated in overexpressed receptor systems (Landsman et al., 1997; MacLennan et al., 1998; Pan et al., 1998). Furthermore, inverse agonist activity will not cause significant deviations in the analysis of agonist–inverse agonist interactions when the inverse agonist has little or no activity alone (Colquhoun, 1998). Under our physiological conditions, SR 141716A did not consistently cause potentiation of the contractions of the mouse vas deferens. Therefore, it was valid to use non-linear regression analysis to obtain pK_B values for SR 141716A as a competitive antagonist.

In contrast to the mouse vas deferens, the more robust rat vas deferens assay allowed cannabinoid receptor agonists to be added cumulatively to the continuously stimulated tissue. This continuous nerve stimulation protocol did not cause significant fade over the course of the experiment. The contractions of the rat vas deferens were insensitive to Δ^9-tetrahydrocannabinol and anandamide and only very high concentrations of CP 55,940 were effective. Even when correcting agonist pIC_{50} values for vehicle effect (DMSO vs. Tween 80), there is still a large discrepancy between the effects of the agonists in the rat and mouse. Pre-incubation with a high concentration of Δ^9-tetrahydrocannabinol (1 μM) did not shift the CP 55,940 concentration–response curve, suggesting that CP 55,940 acts at a site in the rat vas deferens that is not sensitive to Δ^9-tetrahydrocannabinol. In addition, SR 141716A did not cause a parallel shift of the CP 55,940 concentration–response curve; in fact the shallowness of the concentration–response curve in the presence of SR 141716A (100 nM) was suggestive of incomplete inhibition. The hypothesis that CP 55,940 was acting at cannabinoid CB_2 receptors in the rat vas deferens was refuted after the lack of shift by the antagonist SR 144528 at a low cannabinoid CB_2-selective concentration (10 nM), and the absence of a parallel, dextral shift at a higher, CB_1/CB_2 non-selective concentration (10 μM) (Rinaldi-Carmona et al., 1998). This lack of simple competitivity may suggest that there are confounding factors in the analysis of response of CP 55,940 acting at the cannabinoid CB_1 receptor in the rat vas deferens or that CP 55,940 is acting at a non-CB_1/CB_2 receptor. The similarity in the CP 55,940 concentration–response curves generated in the presence of either SR 141716A or SR 144528 suggests a common mechanism of action. The absence of cannabinoid CB_2 receptor-mediated responses is not surprising as negligible amounts of cannabinoid CB_2 mRNA have been located in this tissue (Galiègue et al., 1995).

The contractions of the vas deferens are biphasic; the first phase being attributed in the main to ATP acting at P_{2x} receptors and the slower onset second phase to noradrenaline acting at α_1-adrenoceptors. The contractions resulting from the nerve stimulation parameters (single field pulse every 20 s) used for the rat vas deferens in this study were predominantly ATP-mediated because high concentrations of αβ-methyleneATP caused receptor desensitisation and blockade of contraction. Therefore, to determine whether CP 55,940 was acting at a post-junctional site to cause an inhibition of the rat vas deferens, its effect on αβ-methyleneATP concentration–response curves was examined. Pre-incubation of the rat vas deferens with CP 55,940 did not, in fact, cause any change in sensitivity to the αβ-methyleneATP concentration–re-

sponse curve, indicating that the inhibitory actions of CP 55,940 on the nerve-mediated contractions of the rat vas deferens were most probably at a pre-junctional site.

The inhibition of the twitch by only one of the cannabinoid CB_1 receptor agonists (CP 55,940) in the rat vas deferens may indicate differences in efficacy at this receptor. Furthermore, this agonist displayed a 250-fold lower potency in the rat compared with the mouse vas deferens. Sim et al. (1995) found that there were regional differences in the efficiency of coupling between the cannabinoid CB_1 receptor and G-proteins in the brain. It is possible that a similar scenario exists in the periphery. However, pre-incubation of the rat vas deferens with Δ^9-tetrahydrocannabinol (1 μM) did not shift the CP 55,940 concentration–response curve significantly, suggesting that Δ^9-tetrahydrocannabinol did not have affinity for the CP 55,940 site of action. The presence of a particular receptor mRNA does not necessarily lead to expression of a functional receptor. Thus, the putative cannabinoid CB_1 receptor may be expressed in the rat vas deferens but is not functional because it is not expressed at a sufficient level. Another possible explanation, taking into account the SR 141716A-induced shift of the CP 55,940 concentration–response curve in the rat vas deferens, may be the presence of another cannabinoid receptor subtype that is also sensitive to SR 141716A. This may explain the 250-fold potency difference measured for CP 55,940 between the mouse and rat vas deferens. White and Hiley (1998) also suggest the possibility of an uncharacterised cannabinoid receptor subtype in the rat mesenteric artery. Other possibilities may include inadequate penetration of the cannabinoid compounds into the rat vas deferens, due to its greater thickness compared to the mouse vas deferens, or that the simple competitivity of SR 141716A and CP 55,940 was masked by confounding factors such as tonic activation of cannabinoid CB_1 receptors.

To date, only a cannabinoid CB_{1A} subtype has been found, taking the form of a truncated version of the cannabinoid CB_1 receptor (Shire et al., 1995). Shire et al. (1995) demonstrated that this splice-variant receptor is found throughout the human central nervous system and in the periphery where the cannabinoid CB_1, mRNA was also present, although the cannabinoid CB_1 mRNA remained the predominant form. Rinaldi-Carmona et al. (1996) showed that CP 55,940 was approximately 7-fold and 50-fold more potent than Δ^9-tetrahydrocannabinol and anandamide, respectively, at inhibiting forskolin-induced accumulation of cAMP via cannabinoid CB_1 and CB_{1A} receptors transiently expressed in Chinese Hamster Ovary cells. SR 141716A was able to completely block the inhibition induced by CP 55,940 on the cannabinoid CB_{1A} receptors, however, at a 5-fold lower potency than at the cannabinoid CB_1 receptor. Therefore, it is unlikely that CP 55,940 was acting at a cannabinoid CB_{1A} receptor in the rat vas deferens in this study because Δ^9-tetrahydrocannabinol and anandamide were still ineffective at concentrations up to 60-fold more than that corresponding to the pIC_{50} for CP 55,940. Furthermore, SR 141716A was ~80-fold less potent in shifting the CP 55,940 log concentration–response curve in the rat vas deferens compared to the mouse vas deferens. Perhaps, CP 55,940 is acting at another, yet unidentified, cannabinoid receptor subtype in the rat vas deferens. Another possible explanation is that CP 55,940 and SR 141716A are acting at putative pre-synaptic imidazoline receptors in the rat vas deferens as has been recently shown using evoked [^{3}H]noradrenaline release from post-ganglionic sympathetic nerve fibres in the human right atrial appendage (Molderings and Göthert, 1998; Molderings et al., 1999).

The cannabinoid receptor agonists did not affect sympathetically mediated responses in either the spontaneously beating right atria or mesenteric small resistance arteries. Although the baseline rate for vehicle was significantly different to that of the cannabinoid receptor agonists (1 μM), this effect was not drug-induced, as the pre- and post-treatment baseline rates were not significantly different for vehicle or either of the three cannabinoid receptor agonists (1 μM). Furthermore, the cannabinoid receptor agonists were also not able to affect sympathetically mediated tachycardia. In contrast, Ishac et al. (1996) demonstrated that both Δ^9-tetrahydrocannabinol and anandamide at high (> 10 μM) concentrations inhibited the electrically evoked exocytotic release of [^{3}H]noradrenaline from sympathetic neurons, in rat atria and vas deferens. Ishac's group also showed a minimal 3-fold rightward displacement of [^{3}H]noradrenaline release with 10 μM SR 141716A, which does not correlate with the known potency for SR 141716A at the cannabinoid CB_1 receptor. Previous work has shown that anandamide may cause a relaxation in rat isolated mesenteric artery (Randall and Kendall, 1997; Randall et al., 1996; White and Hiley, 1997, 1998) demonstrated that anandamide and CP 55,940 caused relaxation of methoxamine-induced tone, which was attenuated by SR 141716A, and also inhibited phasic contractions induced by methoxamine in calcium-free conditions. In light of this, our data may be negative because we focused on contraction rather than relaxation and our tissues were bathed in physiological solution containing calcium.

Anandamide is known to be rapidly degraded by amidases in some tissues such as the brain and guinea pig myenteric plexus, but to be stable in others (mouse vas deferens) (Deutsch and Chin, 1993; Pertwee et al., 1995b). Pertwee et al. (1995b) established that phenylmethylsulfonyl fluoride, an amidase inhibitor, enhanced the response of the guinea pig myenteric plexus to anandamide but did not affect the anandamide-induced inhibition of electrically evoked contractions of the mouse vas deferens. We were able to show that phenylmethylsulfonyl fluoride did not increase the potency of anandamide in either the mouse or rat vas deferens. This indicates that the mouse vas deferens may be a tissue that does not metabolise anandamide to

any significant extent and that the lack of response of the rat vas deferens to anandamide was not due to rapid anandamide metabolism.

A lot of cannabinoid in vitro bioassays have been carried out in mice. It was of interest to compare the respective tissues of the rat and mouse because of the higher cannabinoid CB_1 receptor amino acid sequence conservation between rat and humans as opposed to mouse and humans, and because both rats and mice are used in in vivo assays of cannabinoids (Ward et al., 1990; Ishac et al., 1996; Pertwee and Fernando, 1996; Pertwee et al., 1996a,b). There is a 97% sequence homology between the rat and human cannabinoid CB_1 receptor (they differ only in 13 residues), and this increases to 99% in the transmembrane regions. In contrast, the mouse cannabinoid CB_1 receptor has 90% similarity with the human cannabinoid CB_1 receptor; exclusively comparing the transmembrane region increases the homology to 97% (Chakrabarti et al., 1995; Shire et al., 1996). Although this difference between rat and mouse may be small, it may occur in an essential part of the receptor significant for agonist binding or coupling.

In conclusion, not only does there appear to be a species difference in the distribution of functional cannabinoid CB_1 receptors between the rat and mouse, there is also a regional difference in cannabinoid CB_1 receptor-induced modulation of the sympathetic nervous system. In the tissues examined in this study, the putative cannabinoid CB_1 receptor was confined to the mouse vas deferens. Furthermore, the examination of the inhibitory actions of CP 55,940 in the rat vas deferens was inconclusive, although a likely explanation is that there is a component of cannabinoid CB_1 receptor action in conjunction with additional, non-selective effects.

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