British Journal of Pharmacology (2001) 132, 1281–1291 

www.nature.com/bjp

# Pharmacological analysis of cannabinoid receptor activity in the rat vas deferens

*,[1]Arthur Christopoulos, [1]Peter Coles, [1]Lucy Lay, [1]Michael J. Lew & [1]James A. Angus

[1]Department of Pharmacology, University of Melbourne, Grattan St., Parkville, Victoria, 3010, Australia

**1** The interaction between the cannabinoid agonists, WIN 55,212-2 or CP 55,940 with the $CB_1$ receptor-selective antagonists, SR141716A or LY320135 was investigated using the rat electrically-stimulated vas deferens bioassay.

**2** Tissues were stimulated by single-field pulses (150 V, 0.5 ms) delivered every 30 mins. In the presence of nifedipine (3 $\mu$M), agonists elicited a concentration-dependent inhibition of the contractile response, with $pEC_{50}$ values of 7.93 and 6.84 for WIN 55,212-2 and CP 55,940, respectively.

**3** SR141716A and LY320135 caused parallel dextral displacements of the agonist concentration-response curves. However, the shift of the agonist curves by either antagonist was accompanied by a concentration-dependent enhancement of basal (agonist-independent) tissue contraction.

**4** Addition of the amidase inhibitor, phenylmethylsulphonylfluoride (200 $\mu$M), resulted in a significant reduction of the basal twitch response, an effect consistent with the presence of tonic receptor activation mediated by the endogenous cannabinoid, anandamide.

**5** In light of these findings, we propose a theoretical model of competitive agonist-antagonist interaction in the presence of endogenous agonist tone that was used to derive an optimized analytical approach for the determination of antagonist potency estimates under conditions of tonic receptor activation.

**6** This approach yielded $pK_B$ estimates for SR141716A and LY320135 that were in good agreement with their activity at cannabinoid $CB_1$ receptors.

**7** It is concluded that the rat vas deferens contains prejunctional cannabinoid $CB_1$ receptors that are under tonic activation from endogenous substances; under these conditions our analytical approach is preferable to the standard methods for the determination of antagonist potency.
*British Journal of Pharmacology* (2001) **132,** 1281–1291

**Keywords:** Cannabinoid receptors; constitutive activity; endogenous agonist tone; inverse agonist; LY320135; rat vas deferens; receptor theory; SR141716A

**Abbreviations:** CP 55,940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; DMSO, dimethylsulphoxide; LY320135, [6-methoxy-2-(4-methoxyphenyl)benzo[*b*][thien-3-yl][4-cyanophenyl]methanone; PMSF, phenylmethylsulphonylfluoride; SR141716A, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; WIN 55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3,-de]-1,4-benzoxazin-yl]-(1-napthalenyl)-methanone mesylate

## Introduction

Over the last decade, there has been a significant growth in the field of cannabinoid research, primarily due to the identification of specific receptor binding sites for cannabinoid ligands (Devane *et al*., 1988), the subsequent cloning of two distinct cannabinoid receptor subtypes (Matsuda *et al*., 1990; Munro *et al*., 1993) and the discovery of endogenous cannabinoid signalling molecules, exemplified by the arachidonic acid derivative, anandamide (Devane *et al*., 1992). Currently, cannabinoid receptor research aims to elucidate the role of the endocannabinoid system in both physiological and pathophysiological states, as well as in designing selective cannabinoid receptor agonists and antagonists for therapeutic purposes (see reviews by Pertwee, 1997; 1999).

Central to the development of better cannabinoid ligands is the need for a variety of bioassay systems that can reveal the full spectrum of cannabinoid effects. Although *in vivo* bioassays have long played an important role in characterizing the pharmacology of the cannabinoids (Pertwee, 1999), most exploratory research now relies on a variety of cell-based systems as well as a number of standard *in vitro* bioassays. Two common examples of the latter type of assay exploit the ability of cannabinoid agonists to inhibit the electrically-evoked contractions of the mouse vas deferens or the guinea-pig myenteric plexus longitudinal muscle (Pertwee *et al*., 1992). In both cases, the observed effects are mediated by prejunctional $CB_1$ receptors located on nerve terminals involved in neurotransmitter release (Pertwee, 1997). Interestingly, far fewer studies have been conducted on the rat vas deferens, although this tissue has also been identified as possessing prejunctional $CB_1$ receptors (Pertwee *et al*., 1993; Ishac *et al*., 1996). Given the slightly higher conservation between rat and human $CB_1$ mRNA compared to that between mouse and human (Chakrabarti *et al*., 1995; Shire *et*

*Author for correspondence; E-mail: arthurc1@unimelb.edu.au

*al*., 1996), the use of the rat as a common *in vivo* model of cannabinoid effects (Dewey, 1986; Martin, 1986) and the suggestion that the expression of cannabinoid activity can be highly species- (and even tissue-) dependent (Pertwee *et al*., 1995c), it is important that the rat vas deferens be explored in terms of its viability as a robust *in vitro* cannabinoid bioassay, in addition to the assays described above.

Recently, we reported an initial characterization of *in vitro* cannabinoid effects in the rat vas deferens in comparison to the mouse (Lay *et al*., 2000a), but that study was limited in nature and some of the findings may have been confounded by factors such as a differential contribution of multiple effector pathways to the observed tissue response, or the possible presence of endogenous cannabinoid agonist tone. Indeed, it is known that the twitch response of the rat vas deferens consists of two phases, the first being attributed to the release of ATP and the second to the release of noradrenaline (Ventura, 1998). Furthermore, tonic activation of cannabinoid receptors has already been suggested in a number of studies (see Discussion). Thus, the aim of the current study was to undertake a more rigorous pharmacological exploration of the effects of two standard cannabinoid agonists on the noradrenaline-mediated (second phase) twitch response in the rat vas deferens and to assess the nature of the interaction between the agonists and two $CB_1$-selective antagonists. Our findings validate the use of the rat vas deferens as a viable bioassay for cannabinoid receptor activity and suggest an optimized method for the analysis of agonist-antagonist interactions under conditions of endogenous agonist tone. Preliminary accounts of these data have been published in abstract form (Lay *et al*., 1999; Christopoulos & Lew, 2000).

## Methods

### *Electrical stimulation*

Male Sprague Dawley rats, 250–350 g were anaesthetized in a mixture of $CO_2$ (80%) and $O_2$ (20%) and subsequently killed by decapitation. Whole vasa deferentia were excised and bathed in $Mg^{2+}$-free Krebs' physiological salt solution (PSS), composition (mM); NaCl 119, KCl 4.69, $KH_2PO_4$ 1.18, glucose 11, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026, oxygenated with $O_2$ 95%: $CO_2$ 5% at pH 7.4.

Tissues were pinned and tied at either end before fixing the prostatic end to a static platinum hook, embedded in an acrylic organ bath leg between two parallel platinum field electrodes approximately 5 mm long and 5 mm apart. The epididymal end was tied to a stainless steel hook suspended by an inelastic cord ('Spiderwire', Safariland, Ontario, CA, U.S.A.) terminating at a Grass FTO3C isometric force transducer (Grass Instruments, Quincey, MA, U.S.A.). Electrode legs were fixed to a laterally constrained stage that was raised or lowered vertically by a micrometer (Mitutoyo Manufacturing Co., Japan). Tissues were suspended in a 5 ml jacketed organ bath containing PSS oxygenated with $O_2$ 95%: $CO_2$ 5% at 37°C. They were then stretched once by 2 g followed by two changes of bathing solution. A single square wave sympathetic nerve stimulation (150 V, 0.5 ms duration) was delivered by a Grass S88 stimulator *via* a Grass stimulus isolation unit (SIU-5) or by an externally (S88) triggered Grass SD9 stimulator (stimulus isolated, 100 V, 0.5 ms duration) to elicit a tetrodotoxin (0.1 $\mu$M)-sensitive twitch contraction that could be observed as biphasic at a fast time base.

Tissues were subsequently equilibrated for 30 min with nifedipine (3 $\mu$M) to inhibit the ATP-mediated first phase of the twitch response, leaving the remaining second phase that is a prazosin-sensitive noradrenergic twitch. A second single pulse twitch was then established before incubation with various concentrations of the cannabinoid receptor antagonists, SR141716A, LY320135 or dimethylsulphoxide (DMSO) vehicle equivalent for 60 min. Single pulse twitches were then repeated at 30 min intervals prior to additions of cannabinoid agonists WIN 55,212-2 or CP 55,940 in half log unit increments, to construct an inhibitory cumulative concentration-response curve for each agonist. Pilot experiments indicated a slight fade in tissue response over time ($14 \pm 5\%$), but this did not reach statistical significance.

In some experiments, after initial equilibration with nifedipine and subsequent test stimulation (as above), tissues were incubated with the amidase inhibitor, phenylmethylsulphonyl fluoride (PMSF; 200 $\mu$M), or DMSO vehicle equivalent volumes for 60 min prior to nerve stimulation.

### *Drugs*

Drugs used were CP 55,940 ((−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; purchased from Tocris Cookson, Bristol, U.K.), LY320135 ([6-methoxy-2-(4-methoxyphenyl)benzo[*b*][thien-3-yl][4-cyanophenyl]methanone; generous gift from Eli Lilly research Laboratories, Indianapolis, U.S.A.), SR141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; generous gift from Sanofi-Recherche, Montpellier, France) and WIN 55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl]-pyrrolo[1,2,3,-de]-1,4-benzoxazin-yl]-(1-napthalenyl)-methanone mesylate; purchased from Research Biochemicals Incorporated, Natick, U.S.A.). All other chemicals were purchased from Sigma Chemical Company (St. Louis, U.S.A.).

### *Data analysis*

Individual agonist concentration-response curves, in the absence and presence of antagonist, were fitted *via* nonlinear regression to the following four-parameter Hill equation, using PRISM 3.0 (GraphPad Software, San Diego, CA, USA):

$$E = \text{Basal} + \frac{E_{max} - \text{Basal}}{1 + 10^{(\text{LogEC}_{50} - \text{Log[A]})^{n_H}}} \quad (1)$$

where E denotes effect, Log[A] the logarithm of the concentration of agonist, $n_H$ the midpoint slope, $\text{LogEC}_{50}$ the logarithm of the midpoint location parameter, and $E_{max}$ and Basal the upper and lower asymptotes, respectively. When required, simultaneous model-fitting with parameter-sharing across datasets was performed using SCIENTIST 2.0 (MicroMath Software, UT, U.S.A.).

Because a significant change in the basal parameter was observed in the presence of antagonist (see Results), antagonist potency estimates were determined using two

approaches. The first represents the standard method of fitting agonist $pEC_{50}$ values obtained in the absence or presence of antagonist *via* nonlinear regression to the following equation (Lew & Angus, 1995):

$$pEC_{50} = -\log([B]^s + 10^{-pK}) - \log c \quad (2)$$

where $pEC_{50}$ denotes the negative logarithm of the $EC_{50}$, [B] denotes antagonist concentration, *pK* and logc are fitting constants, and s is a measure of the molecularity of the antagonist-receptor interaction, directly equivalent to the Schild slope factor (Arunlakshana & Schild, 1959). Values of s not significantly different from unity suggest that the spacings of the agonist concentration-response curves in the absence and presence of antagonist are consistent with a simple competitive interaction. Under this circumstance, the parameter s is constrained to unity and the resulting *pK* value is then equivalent to the $pK_B$. The parameter log c equals the logarithm of the ratio of control curve $EC_{50}$ to the $K_B$. The use of equation (2) for the determination of antagonist potency *via* nonlinear regression has a number of advantages over the standard Schild method. First, the control curve $pEC_{50}$ values (absence of antagonist) are included in the analysis, increasing the degrees of freedom. Second, the data weighting is more even because the control $pEC_{50}$ values are treated equally to all other $pEC_{50}$ values; in contrast, the Schild method relies heavily on the determination of the concentration-ratio, which could be overly and incorrectly biased by a poorly defined control agonist curve.

A second approach (illustrated theoretically in the Appendix) to determining antagonist potency was also undertaken whereby equieffective agonist concentrations determined at a response level close to the tissue maximum were used in place of the $pEC_{50}$ in equation (2). This was necessary because a comparison of $EC_{50}$ values under conditions of varying basal responses violates the null-method approach of the traditional methods, which assume that the $EC_{50}$ values being compared represent equieffective concentrations. For the WIN 55,212-2 experiments, the response level yielding 0.4 g force was used, whereas for the CP 55,940 experiments, the response level yielding 0.5 g force was used. In each case, these response levels corresponded to approximately 80% of the maximal tissue response to each agonist. For presentation purposes, the relationship between the estimated antagonist potency and the shift of the agonist concentration-response curves in each instance was then displayed as a linear Clark plot (Stone & Angus, 1978; Lew & Angus, 1995).

Data are shown as mean ± s.e.mean. Estimates of agonist concentration-response curve maximal and basal response parameters were compared by one-way ANOVA. Hyperbolic and non-hyperbolic forms of the Hill equation fitted to all datasets were compared using an extra-sum-of-squares (*F* test). All other comparisons were by Student's *t*-test. $P<0.05$ was taken as significant.

## Results

### *Effect of agonists*

In the presence of nifedipine (3 $\mu$M), both WIN 55,212-2 and CP 55,940 caused a concentration-dependent reduction in the monophasic twitch response of the rat electrically-driven vas deferens. Figure 1A shows a representative trace of the electrically evoked contractions in the absence or presence of WIN 55,212-2. Of the two agonists, CP 55,940 was significantly less potent ($P=0.02$) than WIN 55,212-2, with nonlinear regression analysis yielding a $pEC_{50}$ of $6.84 \pm 0.26$ ($n=11$) for CP 55,940 and $7.93 \pm 0.34$ ($n=12$) for WIN 55,212-2 (Figure 2). In some experiments, the DMSO vehicle at the highest concentration employed caused a slight enhancement of the basal twitch response. This effect was not consistently observed and, in the most pronounced cases, resulted in approximately a $<10\%$ increase in basal response.

### *Effect of antagonists*

The presence of increasing concentrations of either SR141716A or LY320135 resulted in progressive degrees of dextral shift of the WIN 55,212-2 (Figure 3) or CP 55,940 (Figure 4) concentration-response curves. In all instances, however, the antagonist shifts were also associated with a progressive increase in the basal contractile response of the tissue to field stimulation that was statistically significant ($P<0.05$). Figure 1B shows a representative trace of the effects of 0.1 $\mu$M SR141716A on the twitch response prior to exposure of the tissue to increasing concentrations of WIN 55,212-2.

Each family of agonist concentration-response curves in the absence or presence of antagonist were subsequently re-fitted to equation (1) with the '$E_{max}$' parameter constrained to be shared, the 'Basal' parameter allowed to vary between treatments and the slope ($n_H$) parameter allowed to either (i) vary between each curve, (ii) be shared amongst curves or (iii) be fixed to a value of 1 for all curves (i.e., fitted to a hyperbola). Results of the simultaneous fitting were compared by an extra-sum-of-squares test and it was found that no significant improvement ($P>0.05$) in the sum-of-squares was achieved by either allowing the slope to vary between curves or be constrained to a value other than 1.

Although the concentration-response data did not conform to the standard criteria for simple competitive antagonism, the $pEC_{50}$ values derived from the hyperbolic curve fits described above were utilized in equation (2) to obtain approximate measures of antagonist $pK_B$ values and slope parameters (Table 1).

### *Possible mechanism of antagonist-mediated increase in basal tissue response*

An antagonist-mediated change in basal responses has been reported previously for cannabinoid ligands in various tissues from different species (see Discussion for references). Two common explanations that have been offered are the possible inverse agonist activity of cannabinoid antagonists, such as SR141716A, or the confounding influence of endogenous cannabinoid agonist tone. Inverse agonism has not yet been conclusively demonstrated in any receptor class in native tissue systems under physiological conditions. Although we cannot exclude this possibility, experiments were performed to address the second possibility, that is, the possible contribution of endogenous cannabinoid agonist tone to our findings.

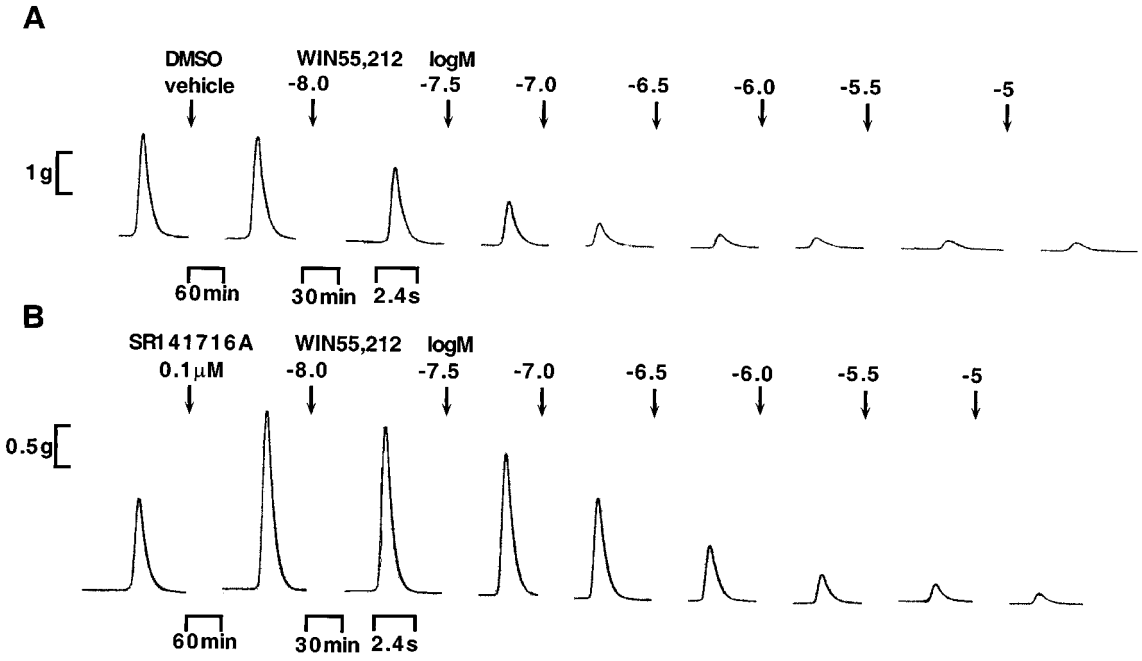



**Figure 1** Representative traces of the electrically-evoked contractions of the rat vas deferens. (A) Single pulse (150 V, 0.5 ms duration) twitch responses delivered every 30 min in the absence or presence of the cannabinoid agonist, WIN 55,212-2 (30 min incubation per concentration). (B) Concentration-response profile for WIN 55,212-2 in the presence of 0.1 $\mu$M SR141716A under identical stimulation parameters to (A). Also shown are the effects of DMSO vehicle (A) of SR141716A (B) prior to addition of agonist.

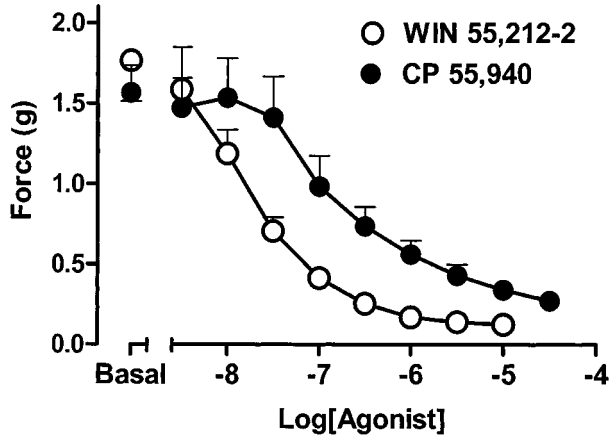



**Figure 2** Concentration-response curves for the inhibition of the electrically-evoked contractions of the rat vas deferens smooth muscle preparation by WIN 55,212-2 and CP 55,940. Data points are the mean$\pm$s.e.mean of 11–12 experiments per curve.

Figure 5 shows the effects of pretreating the vasa deferentia for 60 min with 200 $\mu$M PMSF, a fatty acid amide hydrolase inhibitor that has been shown to inhibit the breakdown of the endogenous cannabinoid agonist, anandamide. In contrast to DMSO vehicle alone, which resulted in a slight increase of the basal response (approximately 8%), PMSF pretreatment caused a significant reduction ($P<0.01$) in the basal response to stimulation, thus indicating that endogenous anandamide is probably present in our preparation and likely to contribute to a tonic activation of cannabinoid receptors.

### *Determination of antagonist potency*

A theoretical model was constructed in order to explore the consequences of endogenous agonist tone on the determination of antagonist potency, and this procedure is outlined in detail in the Appendix. The salient feature of the model is its prediction that the use of $pEC_{50}$ values in nonlinear regression analysis will underestimate antagonist potency in the presence of endogenous agonist tone. In contrast, the use of truly equieffective agonist concentrations would be expected to overestimate the antagonist $pK_B$ unless response levels approaching the agonist maximum were used in the determination of equiactivity. This approach is illustrated in Figure 6 for the WIN 55,212-2 versus SR141716A datasets, where the curves through the points represent the best-fit of the hyperbolic concentration-response model and the dashed line shows the 0.4 g response level chosen for the determination of equieffective agonist concentrations. Analysis of these data according to equation (2) yielded the estimates of the SR141716A $pK_B$ and slope factor shown in Table 1. A similar approach was used for the remaining agonist/antagonist datasets. Figure 7 shows the results of the analysis in the form of Clark plots and the data are summarized in Table 1. In all instances, the slope factors were not significantly different from unity (as assessed by an extra-sum-of-squares test) and were constrained to 1 for the determination of the $pK_B$ value. It can be seen that the

$pK_B$ value was similar for either antagonist irrespective of the agonist that was used and that the estimates obtained using equieffective agonist concentrations were higher than the $pK_B$ estimates obtained using the $pEC_{50}$ values. Importantly, the potency estimates for each antagonist were consistent with their previously-reported activity at the $CB_1$ receptor.

## Discussion

To our knowledge, this is the first study to present a rigorous pharmacological characterization of cannabinoid receptor activity in the rat electrically-stimulated vas deferens smooth muscle bioassay. Our findings provide evidence of tonic cannabinoid receptor activation that may be due to basal

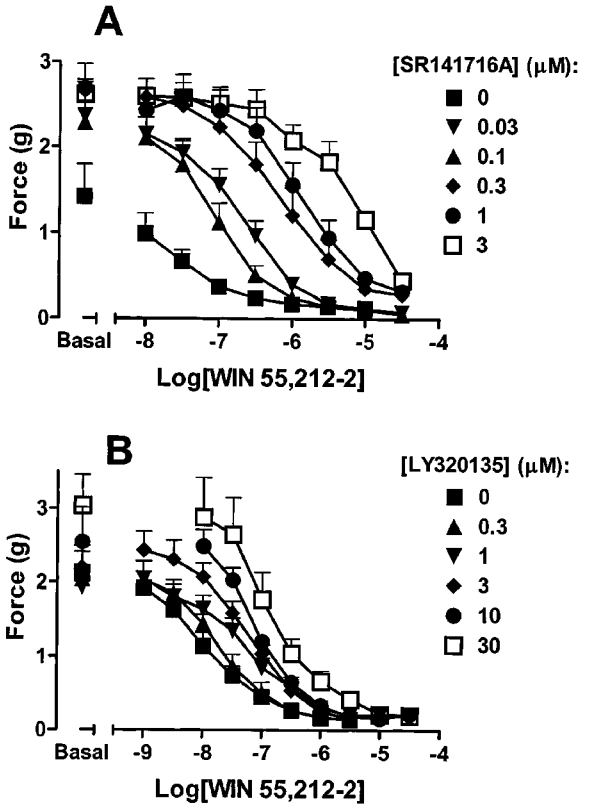



**Figure 3** Concentration-response curves for the inhibition of the electrically-evoked contraction of the rat vas deferens smooth muscle preparation by WIN 55,212-2 in the absence or presence of the indicated concentrations of the cannabinoid antagonists, SR141716A (A) or LY320135 (B). Data shown are the mean±s.e.mean of 4–6 experiments per curve.

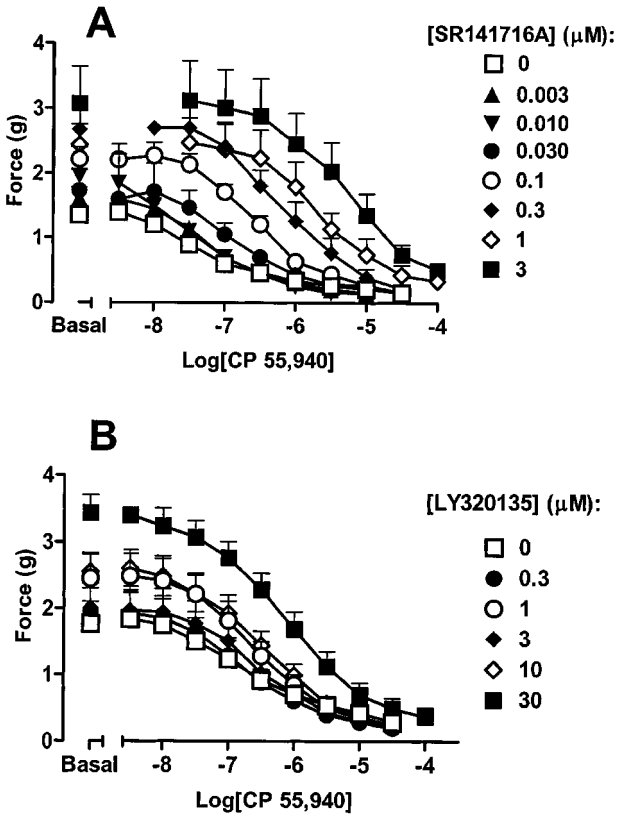



**Figure 4** Concentration-response curves for the inhibition of the electrically-evoked contractions of the rat vas deferens smooth muscle preparation by CP 55,940 in the absence or presence of the indicated concentrations of the cannabinoid antagonists, SR141716A (A) or LY320135 (B). Data shown are the mean±s.e.mean of 4–6 experiments per curve.

**Table 1** Cannabinoid antagonist potency estimates for the inhibition of agonist-mediated responses in the rat electrically-stimulated vas deferens

| | | $pEC_{50}$* | | $pEC_{0.4/0.5\ g}$** | | |
|---|---|---|---|---|---|---|
| *Agonist* | *Antagonist* | $pK_B$† | *Slope*‡ | $pK_B$† | *Slope*‡ | *d.f.*# |
| WIN 55,212-2 | SR141716A | 7.51±0.27 | 1.12±0.30 | 7.91±0.22 | 0.85±0.14 | 24 |
| CP 55,940 | | 7.49±0.25 | 0.85±0.17 | 7.90±0.21 | 1.03±0.14 | 24 |
| WIN 55,212-2 | LY320135 | 5.27±0.42 | 0.78±0.19 | 6.09±0.19 | 0.84±0.18 | 34 |
| CP 55,940 | | 5.10±0.27 | 0.71±0.29 | 6.04±0.26 | 0.92±0.26 | 30 |

*Antagonist parameter estimates were derived by nonlinear regression analysis of agonist $pEC_{50}$ values, obtained in the presence or absence of increasing concentrations of antagonist according to equation (2) of the Methods. **Antagonist parameter estimates were derived by nonlinear regression analysis of equieffective agonist concentrations, corresponding to 0.4 g (WIN 55,212-2 experiments) or 0.5 g (CP 55,940 experiments) absolute force, obtained in the presence or absence of increasing concentrations of antagonist, according to equation (2) of the Methods. †Negative logarithm of the antagonist potency, derived using equation (2) of the Methods. ‡Antagonist slope factor estimated from equation (2) of the Methods. All values were not significantly different ($P>0.05$) from unity. #Degrees of freedom.

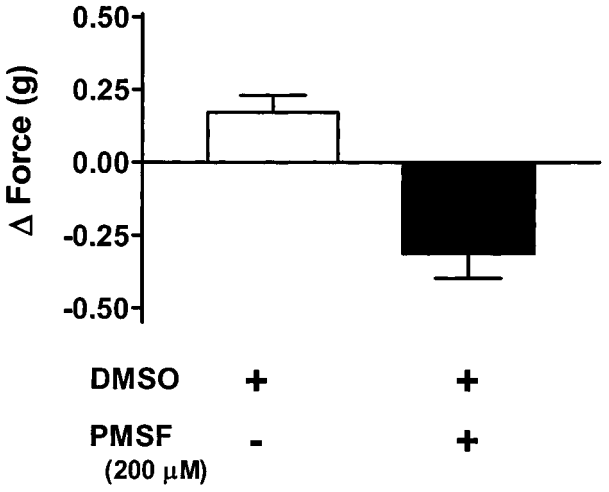



**Figure 5** Effect of DMSO vehicle or PMSF (200 $\mu$M) on the basal electrically-evoked contractile response of the rat vas deferens smooth muscle. Tissues were exposed to either agent for 60 min prior to stimulation. Bars represent the mean ± s.e.mean of six experiments.

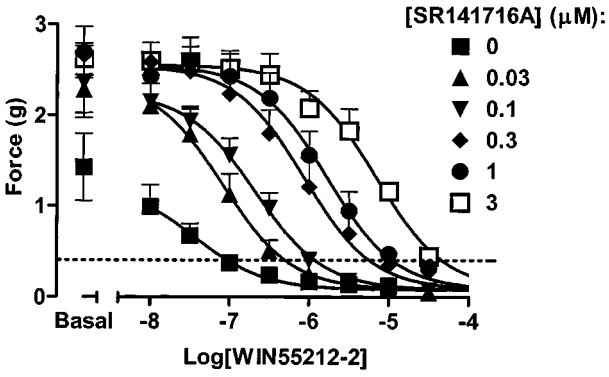



**Figure 6** Nonlinear curve-fitting of the WIN 55,212-2 versus SR141716A data from Figure 3A showing the model fit based on hyperbolic curves with a common maximal response. Also indicated on the Figure is the 0.4 g response level (dashed line; representing approximately 80% of the maximal agonist response) that was used for the derivation of equieffective agonist concentrations for subsequent determination of the antagonist p$K_B$ from equation (2) of the Methods. All other details as for Figure 3A.

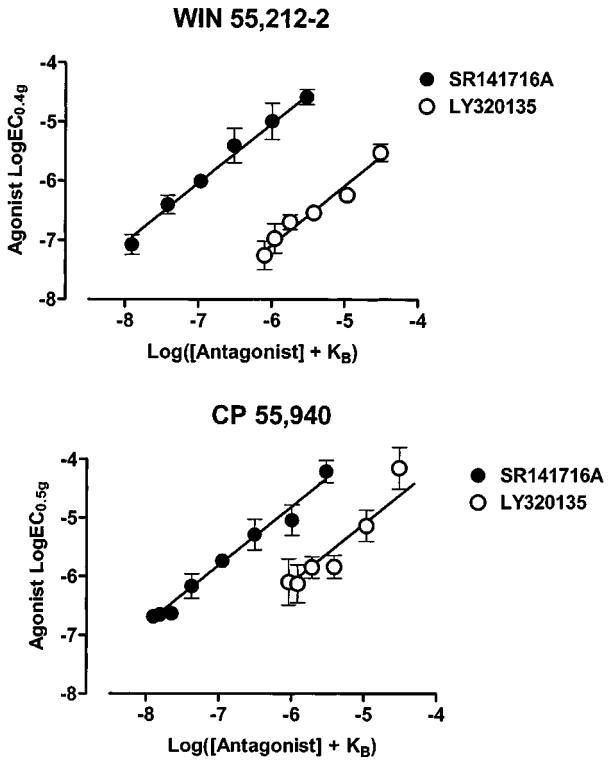



**Figure 7** Clark plots of the interaction between increasing concentrations of either antagonist, SR141716A or LY320135, with either agonist, WIN 55,212-2 (top panel) or CP 55,940 (bottom panel). Antagonist p$K_B$ estimates were first derived by nonlinear regression analysis of equieffective agonist concentrations according to equation (2) in the Methods, and were subsequently used in the construction of the Clork plot. Ordinate, logarithm of the equieffective agonist concentrations obtained in the absence or presence of antagonist. Response level (in grams) is also shown. Abscissa, logarithm of the sum of the antagonist concentration causing the observed curve shifts and the derived $K_B$ value from the nonlinear regression analysis. Error bars represent the mean ± s.e.mean of 4–6 determinations per point.

release of endocannabinoids, such as anandamide, and we have developed an optimized analytical procedure for characterizing antagonist potency under such conditions. Application of this approach to our data yielded p$K_B$ estimates for the antagonists SR141716A and LY320135 consistent with their activities at cannabinoid $CB_1$ receptors.

Because the vas deferens twitch response is known to be composed of an initial ATP-mediated component followed by a noradrenaline-mediated contraction, we chose our assay conditions to minimize the potential for confounding effects of one component on the other. Specifically, we utilized single pulse stimuli in the presence of nifedipine at a concentration that effectively inhibits the ATP component *via* blockade of post-junctional $P_{2X}$ cation channels, thus allowing a relatively unambiguous measurement of a monophasic twitch mediated by noradrenaline (see Figure 1). This differs from our previous study (Lay *et al*., 2000a), where the twitch response was measured under stimulation parameters that favoured the ATP-mediated phase. These differences in conditions may explain why that study found the rat vas deferens to be relatively insensitive to the cannabinoid agonists, $\Delta^9$-tetrahydrocannabinol and anandamide. In contrast, our present experiments with the agonists WIN 55,212-2 and CP 55,940 revealed that both agents elicited a marked and potent inhibition of the rat vas deferens contractile response under the current conditions. Overall, however, the absolute potencies of both agonists tended to be lower than those commonly reported using the mouse vas deferens bioassay (Lay *et al*., 2000a; Pertwee *et al*., 1995c), but this may be ascribed to either a reduced ligand penetration into the thicker rat tissue relative to the mouse, differences in ligand affinity between species, or due to reduced stimulus-response coupling mechanisms in the rat. Indeed, the G protein coupling efficiency of cannabinoid $CB_1$ receptors has been shown to display regional differences in the brain (Sim *et al*., 1995; 1996), and striking differences in stimulus-response coupling mechanisms have also been noted in peripheral

tissue bioassays of cannabinoid activity. For instance, the cannabinoid ligand, AM630, behaves as a competitive antagonist in the mouse vas deferens assay (Pertwee *et al*., 1995a) and an agonist in the guinea-pig myenteric plexus longitudinal muscle (Pertwee *et al*., 1996), but appears to possess no detectable cannabinoid activity in the mouse urinary bladder (Pertwee & Fernando, 1996). In CHO cell lines transfected with the human $CB_1$ receptor, AM630 has even been shown to possess inverse agonist properties (Landsman *et al*., 1998). In an electrophysiological study on guinea-pig myenteric neurones, López-Redondo *et al*. (1997) have also described agonist-like effects of SR141716A, a compound that is almost invariably classed as either a cannabinoid antagonist or inverse agonist (see below). These findings highlight the need for a variety of both cell-based and tissue-based bioassays in order to properly define the efficacy spectrum of cannabinoid ligands.

Previous studies by Pertwee *et al*. (1993) and Ishac *et al*. (1996) have provided molecular biological and some pharmacological evidence for the existence of cannabinoid $CB_1$ receptors in the rat vas deferens, thus suggesting the utility of this tissue as a cannabinoid receptor bioassay. However, a complete analysis with a range of concentrations of selective receptor antagonists has not been reported until the present study, where increasing concentrations of either SR141716A or LY320135, both $CB_1$-selective antagonists (Pertwee, 1997; 1999), caused progressive dextral displacements of both the WIN 55,212-2 concentration-response curve (Figure 3) and the CP 55,940 concentration-response curve (Figure 4). In each instance, the shifts were parallel and surmountable, two important criteria consistent with simple competition (Kenakin, 1997), and on the surface may appear amenable to standard analyses of agonist-antagonist interaction. However both antagonists increased the basal tissue response in their own right, a finding that is inconsistent with simple competition.

In the case of SR141716A, these findings parallel those already reported in the literature by other researchers using isolated tissue preparations including the mouse urinary bladder (Pertwee & Fernando, 1996), the guinea-pig myenteric plexus longitudinal muscle (Pertwee *et al*., 1996; Coutts & Pertwee, 1997; Coutts *et al*., 2000) and the guinea-pig ileal circular smooth muscle (Izzo *et al*., 1998). Furthermore, *in vivo* administration of SR141716A has been shown to produce hyperalgesic effects, in direct contrast to analgesia associated with the administration of cannabinoid agonists (Richardson *et al*., 1997; Lay *et al*., 2000b). These findings have been interpreted in terms of either a true inverse agonist effect of SR141716A in the presence of agonist-independent, constitutive receptor activity, or an antagonist-mediated inhibition of endogenous agonist-mediated receptor activation. To date, however, SR141716A has only been conclusively shown to behave as an inverse agonist in a number of recombinant cell-based assays of cyclic AMP accumulation or [$^{35}$S]-GTP$\gamma$S incorporation (Bouaboula *et al*., 1997; Landsman *et al*., 1997; MacLennan *et al*., 1998). In the case of LY320135, even fewer studies have examined its functional pharmacology. Felder *et al*. (1998) described a stimulatory effect of LY320135 on cyclic AMP accumulation in a CHO cell line, in contrast to the inhibitory effects of anandamide, but the cannabinoid agonist was subsequently found to mimic the stimulation caused by LY320135 once the $G_{i/o}$ proteins in the CHO cells had been inactivated by pertussis toxin. Thus, it is likely that this particular stimulatory effect on cyclic AMP accumulation represents a distinct signalling pathway common to a number of ligands acting *via* the $CB_1$ receptor, and not an example of inverse agonism by LY320135. Furthermore, in a series of experiments monitoring cannabinoid-mediated [$^3$H]-noradrenaline release from human right atrial appendages, Molderings *et al*. (1999) failed to find any evidence of tonic receptor activation, as neither LY320135 nor SR141716A caused any effect on basal [$^3$H]-noradrenaline release in the absence of cannabinoid agonist. Since no detailed studies of LY320135 in constitutively active receptor systems have yet been reported, any possible inverse agonist effects of this compound remain undetermined.

As it stands, therefore, the evidence for unambiguous inverse agonist effects of cannabinoid ligands in native tissues under physiological conditions remains elusive. In contrast, although endogenous anandamide release has only been conclusively demonstrated in the brain (Deutsch & Chin, 1993), evidence does exist for anandamide removal mechanisms in some peripheral tissues (Deutsch & Chin, 1993; Pertwee *et al*., 1995b), and support for this mechanism being operative in the rat vas deferens is provided in the present study. Specifically, incubation of the tissue in the presence of the amidase inhibitor, PMSF, resulted in a significant reduction in the basal tissue contractile response (Figure 5). In fact, given that the DMSO vehicle had a slight potentiating effect in its own right, the observed reduction of the twitch response in the presence of PMSF is probably an underestimate of the true effect. Since PMSF is known to inhibit the degradation of the endogenous cannabinoid anandamide, our findings may be taken as presumptive evidence of a basal level of tonic cannabinoid receptor activation due to anandamide in the rat vas deferens. In light of this observation, the concentration-dependent stimulatory effects of both antagonists on the tissue response in the absence of agonist are most readily reconciled with an inhibition of this endogenous basal agonist release.

Unfortunately, there are no readily-available pharmacological agents that can be used to effectively cancel the impact of endogenous agonist tone on the determination of exogenous cannabinoid agonist-antagonist interactions, and it is thus possible that tonic receptor activation may confound the interpretation and/or analysis of agonist-antagonist interactions in a variety of systems. In order to examine the theoretical impact of this effect, we developed a model based on our experimental findings. This model is presented in detail in the Appendix for the case of hyperbolic concentration-response curves, as were observed in this study. From the simulations, it was found that the indiscriminate use of $pEC_{50}$ values for the determination of antagonist potency will underestimate the true $pK_B$ (see Figure 9 in the Appendix). The optimal determination of antagonist potency may be made by using equieffective agonist concentrations as close as possible to the agonist maximal response. In practice, a trade-off will usually have to be made between the maximum response level chosen for the determination of equiactivity and the decreased confidence in the reliability of the datasets as the response approaches its asymptotic maximum. In our case, we have utilized an agonist response level that corresponds to approximately 80% of the maximal

response for both WIN 55,212-2 and CP 55,940, which, at worst, is expected to over estimate the $pK_B$ by less than 0.2 log units, well within experimental error. From Table 1, it can be seen that the use of the $pEC_{50}$ concentrations yielded estimates of the antagonist $pK_B$ values that were consistently lower for each combination of agents than the corresponding $pK_B$ values derived from the comparison of equieffective concentrations. In particular, the $pK_B$ estimates for LY320135 showed a discrepancy of almost an order of magnitude between the two approaches. In contrast, the potency estimates derived for each of the antagonists using the equieffective concentration approach were in excellent agreement with the range of $pK_B$ values reported for these ligands in other functional assays of $CB_1$ receptor activity (Rinaldi-Carmona *et al*., 1994; Pertwee & Fernando, 1996; Coutts & Pertwee, 1997; Felder *et al*., 1998). Furthermore, the standard errors associated with both the $pK_B$ and slope parameter estimates were also reduced when the optimized null-method was used compared to the $pEC_{50}$ approach. This is of particular import for the slope parameters derived from equation (2). As with the classic Schild approach (see Kenakin, 1997), the antagonist slope parameter is used to assess the conformity of the data to a model of simple competition. Large errors in the slope parameter lead to a high degree of uncertainty in the validity of constraining the slope to 1 in order to estimate $pK_B$ values; this would be exacerbated when the agonist concentrations used in the determination of the slope parameter are not equieffective because the error would then not only reflect variance in the data, but also an inappropriate match of the data to the model.

It should be noted that the analytical method described in this study is not without its limitations. In particular, the method is likely to give spurious $pK_B$ estimates in those situations where tonic receptor activation occurs as a consequence of high concentrations of a low efficacy endogenous agonist (i.e., $>2\times K_D$). However, we are unaware of any real-life examples of this particular situation. Obviously, the ideal solution would be to remove the influence of endogenous agonist tone, but if this is not possible, then the current approach represents a preferred alternative to the use of $pEC_{50}$ values. Although not addressed explicitly in this study, it is also worth noting that the method is expected to work under circumstances where tonic receptor activation is in fact due to true constitutive receptor activity. Additional simulations (not shown) revealed that agonist-inverse agonist interactions can yield identical curve patterns to those observed for the interaction between two agonists and a competitive antagonist and thus, in principle at least, the analytical approach described here should be applicable irrespective of which mechanism is operative.

In conclusion, we have provided evidence that inhibitory cannabinoid $CB_1$ receptors located on peripheral nerve endings in the rat vas deferens may be under endogenous cannabinoid tone. This tone can explain the concentration-dependent enhancement of the basal twitch response by the antagonists SR141716A and LY320135 and can lead to a distortion of antagonist potency estimates, especially when limited antagonist concentrations are utilized. In addition, we have developed a theoretical model and an analytical approach that may prove useful in optimizing antagonist potency estimates under conditions of tonic receptor activation. Application of this approach to the interaction between SR141716A or LY320135 with WIN 55,212-2 or CP 55,940 resulted in antagonist $pK_B$ estimates that are in good agreement for a pharmacological profile in this tissue corresponding to the $CB_1$ receptor.

The authors are grateful to Sanofi-Recherche and Eli Lilly for their generous gifts of SR141716A and LY320135, respectively, and to Dr Tracie L. Pierce for critical review of the manuscript. The authors also gratefully acknowledge the financial support of the AMRAD Drug Discovery Laboratory in the Department of Pharmacology, University of Melbourne, by AMRAD Corporation Ltd, Melbourne, Australia. Arthur Christopoulos is a C.R. Roper Research Fellow of the Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne.

## References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.,* **14,** 48–57.

BLACK, J.W. & LEFF, P. (1983). Operational models of pharmacological agonism. *Proc. Roy. Soc. (Lond.) B.,* **220,** 141–162.

BOUABOULA, M., PERRACHON, S., MILLIGAN, L., CANATT, X., RINALDI-CARMONA, M., PORTIER, M., BARTH, F., CALANDRA, B., PECCEU, F., LUPKER, J., MAFFRAND, J.-P., LE FUR, G. & CASELLAS, P. (1997). A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J. Biol. Chem.,* **272,** 22330–22339.

CHAKRABARTI, A., ONAIVI, E.S. & CHAUDHURI, G. (1995). Cloning and sequencing of a cDNA encoding the mouse brain-type cannabinoid receptor protein. *DNA Seq.,* **5,** 385–388.

CHRISTOPOULOS, A. & LEW, M.J. (2000). Optimising Schild analysis: What to do when the baseline moves. *Proc. ASCEPT,* **7,** 46.

COUTTS, A.A., BREWSTER, N., INGRAM, T., RAZDAN, R.K. & PERTWEE, R.G. (2000). Comparison of novel cannabinoid partial agonists and SR141716A in the guinea-pig small intestine. *Br. J. Pharmacol.,* **129,** 645–652.

COUTTS, A.A. & PERTWEE, R.G. (1997). Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig myenteric plexus. *Br. J. Pharmacol.,* **121,** 1557–1566.

DEUTSCH, D.G. & CHIN, S.A. (1993). Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem. Pharmacol.,* **46,** 791–796.

DEVANE, W.A., DYSARZ, F.A.D., JOHNSON, M.R., MELVIN, L.S. & HOWLETT, A.C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.,* **34,** 605–613.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science,* **258,** 1946–1969.

DEWEY, W.L. (1986). Cannabinoid pharmacology. *Pharmacol. Rev.,* **38,** 151–178.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., GLASS, M., MACKIE, K.P., FAHEY, K.J., CULLINAN, G.J., HUNDEN, D.C., JOHNSON, D.W., CHANEY, M.O., KOPPEL, G.A. & BROWNSTEIN, M. (1998). LY320135, a novel cannabinoid $CB_1$ receptor antagonist, unmasks coupling of the $CB_1$ receptor to stimulation of cAMP accumulation. *J. Pharmacol. Exp. Ther.,* **284,** 291–297.

ISHAC, E.J., JIANG, L., LAKE, K.D., VARGA, K., ABOOD, M.E. & KUNOS, G. (1996). Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid $CB_1$ receptors on peripheral sympathetic nerves. *Br. J. Pharmacol.,* **118,** 2023–2028.

IZZO, A.A., MASCOLO, N., BORRELLI, F. & CAPASSO, F. (1998). Excitatory transmission to the circular muscle of the guinea-pig ileum: evidence for the involvement of cannabinoid $CB_1$ receptors. *Br. J. Pharmacol.,* **124,** 1363–1368.

KENAKIN, T.P. (1997). *Pharmacologic Analysis of Drug-Receptor Interaction*. Philadelphia, PA: Lippincott-Raven.

LANDSMAN, R.S., BURKEY, T.H., CONSROE, P., ROESKE, W.R. & YAMAMURA, H.I. (1997). SR141716A is an inverse agonist at the human cannabinoid $CB_1$ receptor. *Eur. J. Pharmacol.,* **334,** R1–R2.

LANDSMAN, R.S., MAKRIYANNIS, A., DENG, H., CONSROE, P., ROESKE, W.R. & YAMAMURA, H.I. (1998). AM630 is an inverse agonist at the human cannabinoid $CB_1$ receptor. *Life Sci.,* **62,** 109–113.

LAY, L., ANGUS, J.A. & WRIGHT, C.E. (2000a). Pharmacological characterisation of cannabinoid $CB_1$ receptors in the rat and mouse. *Eur. J. Pharmacol.,* **391,** 151–161.

LAY, L., ANGUS, J.A. & WRIGHT, C.E. (2000b). Antinociceptive effects of intrathecal cannabinoid $CB_1$ and $CB_2$ ligands in a rat acute pain model. *Proc. ASCEPT,* **7,** 34.

LAY L., COLES, P., ANGUS, J.A. & CHRISTOPOULOS, A. (1999). Masking of cannabinoid $CB_1$ receptor activity by endogenous cannabinoid-like tone in the rat vas deferens. *Proc. ASCEPT,* **6,** 60.

LEFF, P., DOUGALL, I.G. & HARPER, D. (1993). Estimation of partial agonist affinity by interaction with a full agonist: a direct operational model-fitting approach. *Br. J. Pharmacol.,* **110,** 239–244.

LEW, M.J. & ANGUS, J.A. (1995). Analysis of competitive agonist-antagonist interactions by nonlinear regression. *Trends Pharmacol. Sci.,* **16,** 328–337.

LÓPEZ-REDONDO, F., LEES, G.M. & PERTWEE, R.G. (1997). Effects of cannabinoid receptor ligands on electrophysiological properties of myenteric neurones of the guinea-pig ileum. *Br. J. Pharmacol.,* **122,** 330–334.

MACLENNAN, S.J., REYNEN, P.H., KWAN, J. & BONHAUS, D.W. (1998). Evidence for inverse agonism of SR141716A at human recombinant cannabinoid $CB_1$ and $CB_2$ receptors. *Br. J. Pharmacol.,* **124,** 619–622.

MARTIN, B.R. (1986). Cellular effects of cannabinoids. *Pharmacol. Rev.,* **38,** 45–74.

MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, M.J., YOUNG, A.C. & BONNER, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature,* **346,** 561–564.

MOLDERINGS, G.J., LIKUNGU, J. & GOTHERT, M. (1999). Presynaptic cannabinoid and imidazoline receptors in the human heart and their potential relationship. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **360,** 157–164.

MUNRO, S., THOMAS, K.L. & ABU-SHAAR, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* **365,** 61–65.

PERTWEE, R.G. (1997). Pharmacology of cannabinoid $CB_1$ and $CB_2$ receptors. *Pharmacol. Ther.,* **74,** 129–180.

PERTWEE, R.G. (1999). Pharmacology of cannabinoid receptor ligands. *Curr. Med. Chem.,* **6,** 635–664.

PERTWEE, R.G. & FERNANDO, S.R. (1996). Evidence for the presence of cannabinoid $CB_1$ receptors in mouse urinary bladder. *Br. J. Pharmacol.,* **118,** 2053–2058.

PERTWEE, R.G., FERNANDO, S.R., GRIFFIN, G., ABADJI, V. & MAKRIYANNIS, A. (1995b). Effect of phenylmethylsulphonyl fluoride on the potency of anandamide as an inhibitor of electrically evoked contractions in two isolated tissue preparations. *Eur. J. Pharmacol.,* **272,** 73–78.

PERTWEE, R.G., FERNANDO, S.R., NASH, J.E. & COUTTS, A.A. (1996). Further evidence for the presence of cannabinoid $CB_1$ receptors in guinea-pig small intestine. *Br. J. Pharmacol.,* **118,** 2199–2205.

PERTWEE, R., GRIFFIN, G., FERNANDO, S., LI, X., HILL, A. & MAKRIYANNIS, A. (1995a). AM630, a competitive cannabinoid receptor antagonist. *Life Sci.,* **56,** 1949–1955.

PERTWEE, R.G., GRIFFIN, G., LAINTON, J.A. & HUFFMAN, J.W. (1995c). Pharmacological characterization of three novel cannabinoid receptor agonists in the mouse isolated vas deferens. *Eur. J. Pharmacol.,* **284,** 241–247.

PERTWEE, R.G., STEVENSON, L.A., ELRICK, D.B., MECHOULAM, R. & CORBETT, A.D. (1992). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br. J. Pharmacol.,* **105,** 980–984.

PERTWEE, R.G., STEVENSON, L.A., FERNANDO, S.R. & CORBETT, A.D. (1993). *In vitro* effects of the cannabinoid, CP 55,940 and of its (+)-enantiomer, CP 56,667. In *Problems in Drug Dependence, 1992: Proceeding of the 54th Annual Scientific Meeting of the College on Problems of Drug Dependence, Inc.* ed. Harris, L. pp. 374. Rockville, USA: National Institute of Drug Abuse.

RICHARDSON, J.D., AANONSEN, L. & HARGREAVES, K.M. (1997). SR 141716A, a cannabinoid receptor antagonist, produces hyperalgesia in untreated mice. *Eur. J. Pharmacol.,* **319,** R3–R4.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERARRA, P., SOUBRIE, P. BRELIERE, J.C. & LE FUR, G. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.,* **350,** 240–244.

SHIRE, D., CALANDRA, B., DELPECH, M., DUMONT, X., KAGHAD, M., LE FUR, G., CAPUT, D. & FERRARA, P. (1996). Structural features of the central cannabinoid CB1 receptor involved in the binding of the specific CB1 antagonist SR 141716A. *J. Biol. Chem.,* **271,** 6941–6946.

SIM, L.J., SELLEY, D.E. & CHILDERS, S.R. (1995). In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[$\gamma$-[$^{35}$S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7242–7246.

SIM, L.J., SELLEY, D.E., XIAO, R. & CHILDERS, S.R. (1996). Differences in G-protein activation by $\mu$ and $\delta$-opioid, and cannabinoid, receptors in rat striatum. *Eur. J. Pharmacol.,* **307,** 97–105.

STONE, M. & ANGUS, J.A. (1978). Development of computer-based estimation of $pA_2$ values and associated analysis. *J. Pharmacol. Exp. Ther.,* **207,** 705–718.

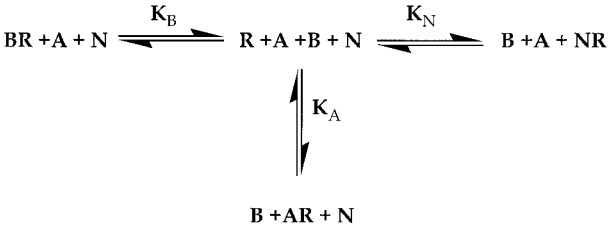
VENTURA, S. (1998). Autoinhibition, sympathetic cotransmission and biphasic contractile responses to trains of nerve stimulation in the rodent vas deferens. *Clin. Exp. Pharmacol. Physiol.,* **25,** 965–973.

*(Received July 26, 2000*
*Revised November 15, 2000*
*Accepted January 5, 2001)*

# Appendix

## *Determination of antagonist potency in the presence of an endogenous agonist*

A scheme for the interaction between an exogenous agonist, A, an endogenous agonist, N, and a competitive antagonist, B, at the same receptor type, R, is given below:




**Scheme 1**

In the above model, $K_A$, $K_N$ and $K_B$ denote the equilibrium dissociation constants of ligands A, N and B, respectively.

The equilibrium occupancy of the receptor by either agonist in the concomitant presence of each other and the competitive antagonist is given below in the following relationships:

$$[AR] = \frac{[R_T][A]}{[A] + K_A\left(1 + {}^{[N]}/_{K_N} + {}^{[B]}/_{K_B}\right)} \quad (A1)$$

$$[NR] = \frac{[R_T][N]}{[N] + K_N\left(1 + {}^{[A]}/_{K_A} + {}^{[B]}/_{K_B}\right)} \quad (A2)$$

The agonist-receptor complexes, AR and NR, are able to impart stimuli, $S_A$ and $S_N$, respectively, to the system. The stimuli are assumed to be proportional to the intrinsic efficacies, $\varepsilon_A$ and $\varepsilon_N$, of agonist A and N, respectively. The observed effect (E) is a function of the total stimulus ($S_T$), which may be defined as:

$$S_T = S_A + S_B = \epsilon_A[AR] + \epsilon_N\,[NR] \quad (A3)$$

Substituting equations A1 and A2 into equation A3 yields:

$$S_T = \frac{\epsilon_A[R_T][A]K_N + \epsilon_N[R_T][N]K_A}{[A]K_N + [N]K_A + K_AK_N + K_AK_N{}^{[B]}/_{K_B}} \quad (A4)$$

The relationship relating total stimulus ($S_T$) to observed response (E) is assumed to be adequately described by a logistic function, specifically:

$$E = \frac{E_m S_T{}^n}{1 + S_T{}^n} \quad (A5)$$

where $E_m$ denotes the maximum possible effect of the system and n is a slope factor describing the shape of the transducer function linking occupancy to response (Black & Leff, 1983). Values of n different from 1 describe non-hyperbolic concentration-response curves.

Substituting equation A4 into equation A5 and simplifying yields the relationship between agonist occupancy and final response:

$$E = \frac{E_m(\epsilon_A[A][R_T]K_N + \epsilon_N[N][R_T]K_A)^n}{\left([A]K_N + [N]K_A + K_AK_N + K_AK_N{}^{[B]}/_{K_B}\right)^n + (\epsilon_A[A][R_T]K_N + \epsilon_N[N][R_T]K_A)^n} \quad (A6)$$

Adopting the terminology of the operational model of Black & Leff (1983), the products $\varepsilon_A[R_T]$ and $\varepsilon_N[R_T]$ can be redefined as $\tau_A$ and $\tau_N$ respectively, giving the following equation:

$$E = \frac{E_m(\tau_A[A]K_N + \tau_N[N]K_A)^n}{\left([A]K_N + [N]K_A + K_AK_N + K_AK_N{}^{[B]}/_{K_B}\right)^n + (\tau_A[A]K_N + \tau_N[N]K_A)^n} \quad (A7)$$

It should be noted that this equation is an extension of one previously derived by Leff *et al*. (1993) to describe the operational modelling of the interaction between a full and partial agonist.

Based on our experimental observations, we utilized equation A7 to simulate a series of hyperbolic agonist concentration-response curves that mimicked the pattern observed with the cannabinoid agonists in the presence of increasing concentrations of antagonist in the rat vas deferens. The resulting curves are shown in Figure 8 and the parameters indicated in the legend to the Figure. For simplicity, it was assumed that the agonist and endogenous agonist had the same affinity and intrinsic efficacy for the receptor. In this instance, the parameters yield a basal level of approximately 0.5 that of the maximum response. Also shown in the Figure are the midpoint location parameters for each curve (corresponding to the $EC_{50}$ values), as well as the response level corresponding to 55% and 95% of the tissue maximum (dashed lines).

The determination of antagonist potency from agonist concentration-response data is almost invariably based on the method of Schild (Arunlakshana & Schild, 1959) or its variants. Importantly, the method is based on the comparison of *equieffective* agonist concentrations determined in the absence and presence of antagonist, thus ensuring its null methodology. When the agonist curves obtained in the presence of antagonist show parallelism with no change in upper and lower curve asymptotes, then the $EC_{50}$ concentration of each curve is commonly utilized as the reference point of equiactivity in the construction of the Schild plot. However, in circumstances such as those outlined in Figure 8, it is obvious that the $EC_{50}$ concentrations do *not* result in equivalent levels of response. The use of these agonist concentrations in the determination of antagonist potency violates the assumptions of Schild analysis, yet it is quite common to see this approach utilized in the semi-quantitative determination of antagonist $pK_B$ values.

The lack of validity of this approach can be seen by the estimation of the antagonist $pK_B$ using the $EC_{50}$ values from

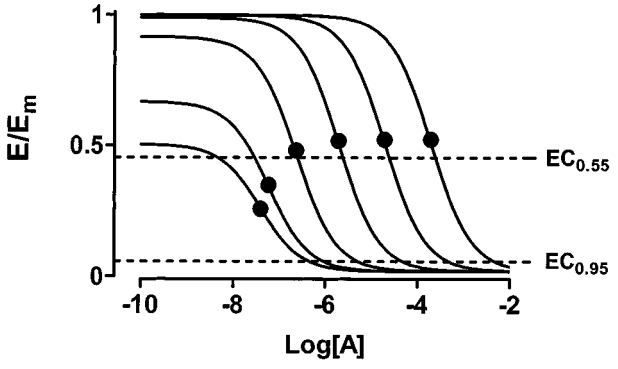



**Figure 8** Simulated concentration-response curves for the interaction between an agonist, A, and a competitive antagonist B, in the presence of a fixed concentration of endogenous agonist, N, according to the model shown in Scheme 1 of the Appendix. Simulations were based on the following parameters. Log $K_A$ = Log $K_N$ = −6, Log $K_B$ = −9, $\tau_A = \tau_N = 50$, Log[N] = −7.7, n = 1. Concentrations of [B] were (from left to right) 0, 1 nM, 10 nM, 100 nM, 0.1 $\mu$M, 1 $\mu$M and 10 $\mu$M, where the parameters are as defined in the Appendix. Closed circles represent the response level corresponding to the $EC_{50}$ for each curve, whereas the dashed lines indicate equieffective response levels corresponding to 55% ($EC_{0.55}$) and 95% ($EC_{0.95}$) of the maximal agonist response.

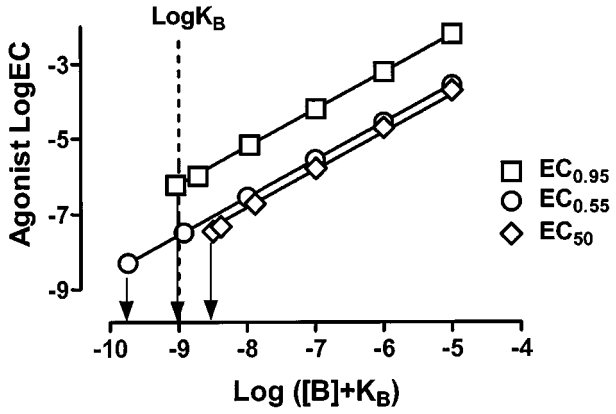



**Figure 9** Clark plots of the agonist-antagonist interaction based on the simulated curves shown in Figure 8. Arrows denote the location on the abscissa that corresponds to the estimate of the antagonist Log $K_B$ value obtained in each instance.

Figure 8. For comparison, the null-method was also utilized, whereby equieffective agonist concentrations corresponding to the 55% ($EC_{0.55}$) and 95% ($EC_{0.95}$) were determined in parallel and used to estimate antagonist p$K_B$ values. Data were analysed by nonlinear regression analysis, as described in the main text, and the resulting analysis is presented in Figure 9 as a group of Clark plots.

Nonlinear regression analysis yielded p$K_B$ estimates of 8.55 for the $EC_{50}$ approach and 9.74 and 9.02 for the $EC_{0.55}$ and $EC_{0.95}$ approaches, respectively. Thus, using the $EC_{50}$ will underestimate the true p$K_B$ whereas using the null method will tend to overestimate it. Unfortunately, in the presence of endogenous tone, even equieffective agonist concentrations do not truly satisfy the null-method criterion of classic Schild-type analysis because the contribution of the endogenous agonist to the overall stimulus in the system invalidates the traditional assumption that equal stimuli in the absence and presence of antagonist imply equal occupancy by the exogenous agonist (Kenakin, 1997). Nevertheless, it can be seen that using equieffective response levels that approach the agonist maximum will result in the closest estimate of the true p$K_B$ being obtained under the present conditions. Practically, the maximum response level that can be utilized in such an analysis will be predominantly determined by the 'noise' in the data and the accuracy of the curve-fit to the data. Based on an additional series of simulations (not shown), equieffective response levels between 75 and 95% should yield more reasonable estimates of the p$K_B$ (9.18 – 9.02 in this instance) than those obtained using the $EC_{50}$.