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Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig myenteric plexus

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1 The dose-related inhibition of the twitch responses of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine by cannabinoid (CB) agonists, (+)-WIN 55212 and CP 55940 during stimulation at 0.1 Hz with supramaximal voltage was confirmed. These agonists inhibited acetylcholine (ACh) release in the presence of physostigmine (7.7 μM) thus indicating a prejunctional site of action.

2 Inhibition of twitch responses and ACh release by CB agonists was reversed by the CB_1-selective cannabinoid receptor antagonist, SR141716A. Dose-response curves to (+)-WIN 55212 and CP 55940 were shifted to the right, with no reduction of maximal response, by pretreatment with SR141716A (31.6–1000 nM), but not its vehicle, Tween 80 (1 μM). However, at very high concentrations (25–400 μM), Tween 80 itself caused a dose-related inhibition of the twitch response which was significantly reduced in the presence of SR141716A (1 μM). The opioid receptor antagonist, naloxone (1 μM) had no significant effect on the inhibition by CP 55940 of the twitch response.

3 (+)-WIN 55212, CP 55940 and Tween 80 (50 μM) had no effect on responses to exogenous ACh, confirming that their actions were prejunctional. SR141716A (1 μM) did not increase the sensitivity of the longitudinal muscle to either ACh or histamine, but inhibited the responses to high doses of ACh.

4 The (−)-enantiomer of WIN 55212, was approximately 300 times less active than the (+) enantiomer in inhibiting the twitch response, had no CB_1 antagonist activity against the active isomer and did not inhibit the release of ACh in the presence of physostigmine.

5 The dissociation constant (K_D) values for SR 141716A against the inhibitory effect of (+)-WIN 55212 and CP 55940 on the twitch response were 12.07 nM (95% confidence intervals 8.55 and 20.83) and 6.44 nM (95% confidence intervals 4.70 and 10.24), respectively. In experiments in which the release of ACh was inhibited by (+)-WIN 55212, the K_D values were 9.21 nM and 10.53 nM at SR141716A concentrations of 31.6 nM and 100 nM, respectively. The K_D values for the antagonism by naloxone of the inhibition of the twitch responses and the inhibition of ACh release by normorphine in this preparation were found to be 2.38 ± 0.69 nM and 2.00 ± 0.9 nM, respectively.

6 During maximal inhibition of ACh release by (+)-WIN 55212, the addition of normorphine (400 nM) caused a further significant decrease in ACh output.

7 SR141716A alone produced a significant increase in ACh release in both the absence and presence of exogenous cannabinoid drugs, hence we conclude that it has a presynaptic site of action. We also conclude that SR141716A acts either by antagonizing the effect of an endogenous CB_1 receptor agonist or by having an inverse agonist effect at these receptors.

Keywords: Myenteric plexus; guinea-pig small intestine; acetylcholine release; cannabinoid receptor agonists; cannabinoid receptor antagonist; SR141716A; anandamide; inverse agonist

Introduction

Together with the mouse isolated vas deferens, the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine has been used as a functional *in vitro* model in which to study the mode of action of psychotropic cannabinoid agonists (Pertwee *et al*., 1992; 1996b). Such drugs show high potency and stereoselectivity in their inhibition of the evoked contractile responses of these preparations to electrical stimulation, an action which is reversed by SR141716A (Coutts *et al*., 1995; Pertwee *et al*., 1996b), a selective antagonist of the cannabinoid CB_1 receptor (Rinaldi-Carmona *et al*., 1994). The mechanism by which cannabinoids depress the electrically-evoked contractions of the longitudinal muscle of the small intestine is deemed to be presynaptic since concentrations of Δ^9-tetrahydrocannabinol which depress the twitch have no effect on contractile responses to exogenous acetylcholine (ACh) (Gill *et al*., 1970; Layman & Milton, 1971; Roth, 1978), and depress the spontaneous release of ACh from segments of whole ileum (Layman & Milton, 1971) or innervated ileal longitudinal muscle (Paton *et al*., 1972).

The present experiments were undertaken to investigate whether changes in evoked responses of the innervated longitudinal muscle strip due to cannabinoid agonists were accompanied by parallel changes in evoked release of ACh from the myenteric plexus. Similar experiments were performed to determine whether the antagonism of these CB_1 effects by the selective antagonist, SR141716A, was also due to corresponding changes in ACh release. In the absence of exogenous cannabinoid drugs, it was observed (Coutts *et al*., 1995; Pertwee *et al*., 1996b) that this antagonist was able to increase the contractile responses to electrical stimulation. In these experiments we have sought to demonstrate corresponding changes in ACh release. We have also been able to substantiate earlier findings of lack of postjunctional effects of cannabinoid agonists and extend these to include the effects of SR141716A, and of high concentrations of the vehicle, Tween 80, which are also inhibitory in this preparation (Pertwee *et al*., 1996b). Further demonstration of the receptor-mediated mode of action of cannabinoids was sought by comparing the effects of enantiomers of the agonist WIN 55212 on both electrically-evoked twitches and ACh release.

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The presynaptic action of morphine and opioid drugs, acting through μ opioid receptors and clonidine, acting via α_2-adrenoceptors in the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine is well documented. These drugs reduce the twitch responses to low frequency stimulation by reducing the release of ACh (Greenberg *et al*., 1970). It was important, therefore, to differentiate between the site of action of cannabinoid drugs and μ and α_2-adrenoceptors. We have already shown (Pertwee *et al*., 1996b) that dose-response curves for the inhibitory effect of normorphine and clonidine on evoked contractile responses are unaffected by the CB_1 antagonist SR141716A. Further experiments were designed to investigate interactions between the inhibitory effects of opioid and cannabinoid drugs on the evoked contractile responses and ACh release from this preparation. The evoked release of ACh is highly dependent on the frequently of stimulation (Cowie *et al*., 1968). Previous studies have shown that inhibitory drugs which act presynaptically in this tissue are more effective at low than at high frequencies of stimulation (Greenberg *et al*., 1970; Kilbinger, 1977). Therefore in all the experiments described in the present study the frequency of stimulation was 0.1 Hz.

Methods

Tissue experiments

Segments of small intestine were dissected from male albino Dunkin-Hartley guinea-pigs (282–820 g) and set up for field stimulation by the method of Paton & Zar (1968). For the recording of contractile responses to electrical stimulation, tissues were mounted in 4 ml organ baths under an initial tension of 0.5 g with the method described by Pertwee *et al*. (1996b). The baths contained Krebs solution which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (mM): NaCl 118.2, KCl 4.75, KH_2PO_4 1.19, $NaHCO_3$ 25.0, $CaCl_2.6H_2O$ 2.54, $MgSO_4.7H_2O$ 1.19 and glucose 11.0. Isometric contractions were evoked by continuous stimulation at 0.1 Hz with pulses of 110% maximal voltage and 0.5 ms pulse duration from Grass 88 stimulators through platinum electrodes attached to the upper and lower ends of each bath. Contractions were recorded via Dynanometer UF1 transducers (Ether) linked to a pen oscillograph (Grass Polygraph 7D). For cumulative concentration-response curves for the effect of cannabinoid agonists on the twitch response, a 20 min interval was left between consecutive additions of drug.

Acetylcholine release in the presence of physostigmine (7.7 μM) was measured by bioassay on a myenteric plexus-longitudinal muscle preparation with a method modified from Waterfield (1973). Stimulation period was 4 min, with 10–20 min between consecutive stimulation periods, depending on whether the experimental conditions had been altered e.g. by increasing the drug concentration or by introducing a new drug. In release experiments, each preparation was subjected to all the experimental conditions described for that experiment, except for experiments for the determination of K_D values in which different preparations were used for each concentration of SR141716A. Therefore control release and that in the presence of drugs were determined in the same tissue. The effects of drugs on resting release of ACh were not determined. The values of ACh release in each experiment was the mean of triplicate samples which were collected under the same experimental conditions. Experiments showed that none of the drugs tested for an effect on output in the course of these experiments had any effect on the sensitivity of these preparations to ACh (see Results section), therefore these drugs were omitted from standard solutions of ACh. When the effects of drugs on ACh release were being measured, after determination of control output, preparations were exposed to the new drug environment for 20 min before the start of collection of samples. The responses to both unknown samples and standard solutions of ACh were abolished in the presence of atropine (100 nM). The output of ACh was calculated in pmol g^{-1} per pulse.

Dose-response curves for ACh and histamine were performed with a 2 min dose cycle, the bath fluid being renewed by overflow after each contraction had reached maximum. After control dose-response curves had been determined, preparations were preincubated with cannabinoid drugs for 20 min before the ACh or histamine dose-response curves were constructed. Cannabinoid drugs were replaced in the organ bath after each washout.

Drugs

Acetylcholine chloride was obtained from BDH and choline chloride, clonidine HCl, physostigmine sulphate, histamine acid phosphate, naloxone HCl and Tween 80 from Sigma. (+)-WIN 55212 (mesylate (**R**)-(+)-[2,3-dihydro-5-methyl-3-[4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl] (1-naphthyl)methanone) was supplied by Sterling Winthrop and (−)-WIN 55212 by Research Biochemicals International. CP 55940 ((−)-3-[2-hydroxy-4-(1,1-dimethyl heptyl)phenyl]-4-(3-hydroxy propyl)cyclohexan-1-ol) was obtained from Pfizer and normorphine from Burroughs Wellcome. SR 141716A (N-(piperidin-1-yl)-5-(-4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride) was a gift from Sanofi. The WIN 55212 isomers, CP 55940 and SR 141716A were each mixed with 2 parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) before addition to the organ bath. Other drugs were dissolved in saline. It was not possible to reverse the inhibitory effect of cannabinoids on either the twitch response or on ACh release by washing them out of the organ bath, therefore cumulative log dose-response curves were constructed. In control experiments, Tween 80 was added instead of cannabinoid receptor agonists or SR 141716A in concentrations corresponding to those required to disperse these agents. Since Tween 80 is a mixture of organic acids and its concentration cannot be expressed in molar terms, in this paper the 'molarity' given for Tween 80 refers to the molarity of the agent for which it was the vehicle control.

Analysis of data

Values are expressed as means and limits of error as s.e.mean (mean ± s.e.mean). Inhibition of the electrically-evoked twitch response is expressed in percentage terms and has been calculated by comparing the amplitude of the twitch response after each addition of a twitch inhibitor with its amplitude immediately before the first addition of the inhibitor. Levels of significance were calculated between two sets of results by paired or unpaired Student's t test as appropriate. Comparison of more than two sets of values was carried out by analysis of variance followed by Scheffé's test (Super ANOVA, Abacus Concepts Inc., Berkeley). A P value <0.05 was considered to be significant.

Dose-ratios have been determined by symmetrical (2 + 2) dose parallel line assays (Colquhoun, 1971) with pairs of agonist concentrations located on the steepest part of each log concentration-response curve. The limits of error are given as 95% confidence intervals.

The method of determination of dissociation constant (K_D) has been described previously (Pertwee *et al*., 1996b). K_D values were calculated from the slope ($1/K_D$) of the best fit straight line of the plot of (x − 1) against B, constrained to pass through the origin (Tallarida *et al*., 1979) and calculated by linear regression analysis with GraphPad InPlot (GraphPad Software, San Diego). The equation for this graph is $(x-1) = B/K_D$, where x (dose-ratio) is the ratio of concentrations of agonist which produce the same degree of inhibition in the presence to that in the absence of a given concentration, B, of antagonist. The value of the Schild slope for the interaction between SR141716A and WIN 55212 or CP 55940 was ob-

tained from the best fit straight line of a plot of log (x−1) against −log B. The equation for this graph, log (x−1)=(log B)−log K_D, predicts a slope of 1 for all receptor-mediated interactions between agonists and antagonists that are competitive and reversible. Values for the Schild slope have been calculated by linear regression analysis by use of GraphPad InPlot.

Results

Effects of cannabinoid agonists on the twitch response

In the present experiments the cannabinoid agonists (+)-WIN 55212 and CP 55940 produced dose-related inhibition of evoked twitch responses. Dose-response curves for these drugs were unaffected by the opioid receptor antagonist, naloxone (1 μM) as illustrated for CP 55940 in Figure 1a. The results for the effect of naloxone on the inhibition by (+)-WIN 55212 are not shown. The vehicle for SR141716A and for cannabinoid agonists, Tween 80, in a concentration equivalent to that used to disperse 1000 nM cannabinoid drug, had no significant effect on dose-response curves to cannabinoid agonists as shown for (+)-WIN 55212 (Figure 1b). However, these curves were shifted in parallel to the right when the CB_1-selective cannabinoid receptor antagonist, SR141716A (100 nM), was present in the organ bath ($n=8$) (Figure 2) producing a dose-ratio of 30.54. In the pooled results from 27 experiments, (+)-WIN 55212 (135 nM) caused 67.33±2.19% inhibition of the twitch which was reversed in a dose-related fashion by the addition of increasing concentrations of SR141716A (Figure 3a).

At high concentrations (25–400 μM), which are well in excess of those required to disperse cannabinoid drugs, Tween 80 caused a dose-dependent inhibition of the twitch response which was significantly reduced in the presence of the cannabinoid antagonist SR141716A (1 μM) (Figure 3b).

The effects of cannabinoid drugs on the sensitivity to ACh and histamine

The responses to ACh added to the organ bath were unaffected by the presence of (+)-WIN 55212 (200 nM) (Figure 4a), a concentration which causes near maximal inhibition of the evoked contractile response to electrical stimulation. A similar lack of effect was found with CP 55940 (200 nM) in another 6 preparations (not shown). Tween 80 (50 μM) also had no effect on the dose-response curves to ACh (Figure 4b).

In the presence of SR 141716A (1 μM), the responses to relatively low doses (<1.4 μM) of ACh were not significantly affected compared with controls (Figure 4c). However, the responses to high doses of ACh (1.4 and 4.14 μM) were significantly reduced by 20.5% and 26.2%, respectively. The concentration-response curve to histamine was unaffected by SR141716A (1 μM) (Figure 4d).

The inhibitory effects of cannabinoid agonists on the evoked release of ACh from the myenteric plexus and reversal with SR141716A

CP 55940 and (+)-WIN 55212 inhibited the release of ACh from this preparation in a concentration-dependent manner. In a concentration of 200 nM, which caused near maximal inhibition of the twitch response in the absence of physostigmine (Figure 1a), CP 55940 reduced the ACh release from this preparation by 34.7% from 144.1±26.2 to 90.0±15.9 pmol g^{-1} per pulse ($P<0.005$; paired t test, $n=14$). Tween 80 (1 μM) had no significant effect on ACh release (see below). The inhibition due to CP 55940 was reversed by the additional presence of SR141716A (1 μM) (Figure 5a). That these changes were not artifacts due to time was demonstrated by the failure of the output to alter significantly in the presence of Tween 80 (equivalent to 1 μM SR141716A) (Figure 5b).

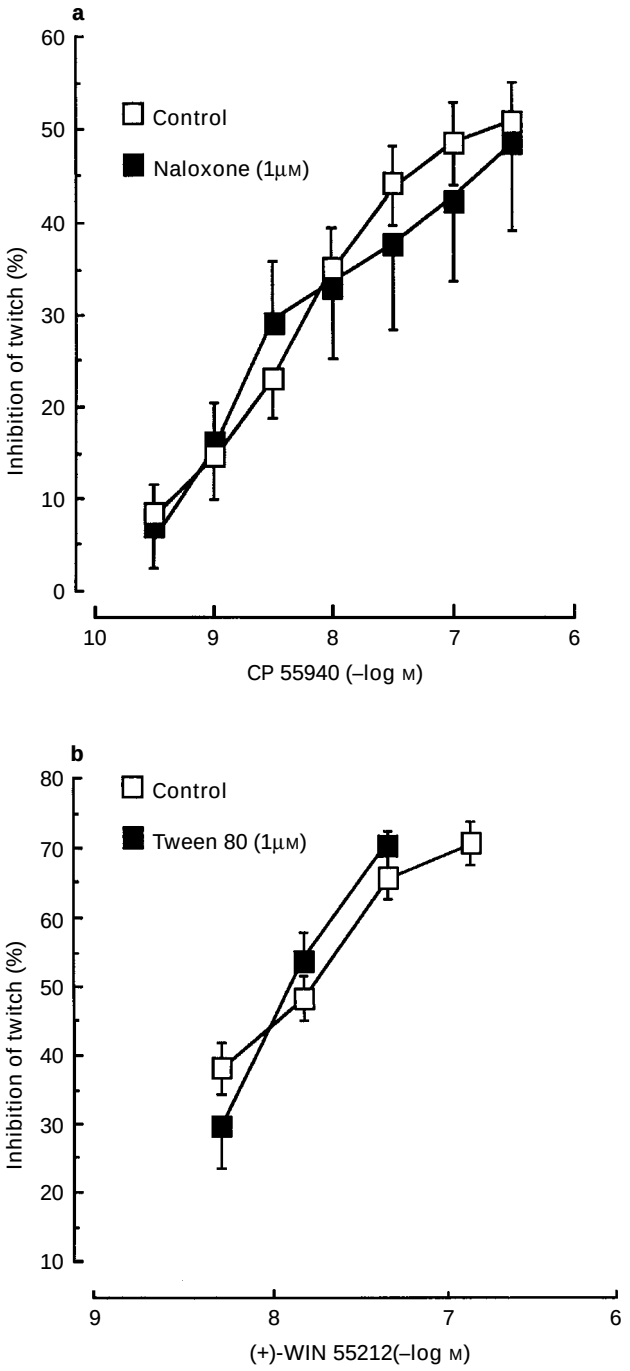

Figure 1 (a) Mean concentration-response curves for CP 55940 in the absence and presence of naloxone (1 μM, $n=9$). (b) Mean concentration-response curves for (+)-WIN 55212 in the absence ($n=4$) and presence ($n=15$) of Tween 80 (1 μM). Each symbol represents the mean value, and vertical lines show s.e.mean, of inhibition of electrically-evoked contractions of the guinea-pig isolated myenteric plexus-longitudinal muscle, expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of the cannabinoid agonist to the organ bath.

(+)-WIN 55212 was also shown to inhibit the release of ACh during stimulation at 0.1 Hz (Figure 2c). This effect was dose-related, was maximal at a concentration of 200 nM and was shifted in parallel to the right in the presence of the CB_1 antagonist SR141716A (Figure 2b). In 4 experiments, (+)-WIN 55212 (200 nM) inhibited the ACh output by 35.1±4.19% from 105.5±22.7 to 65.7±10.6 pmol g^{-1} per pulse ($P=0.01$; paired t test). This inhibitory response was not increased further when the concentration of (+)-WIN 55212 was doubled, but was significantly reversed ($P<0.05$; paired t test) in the

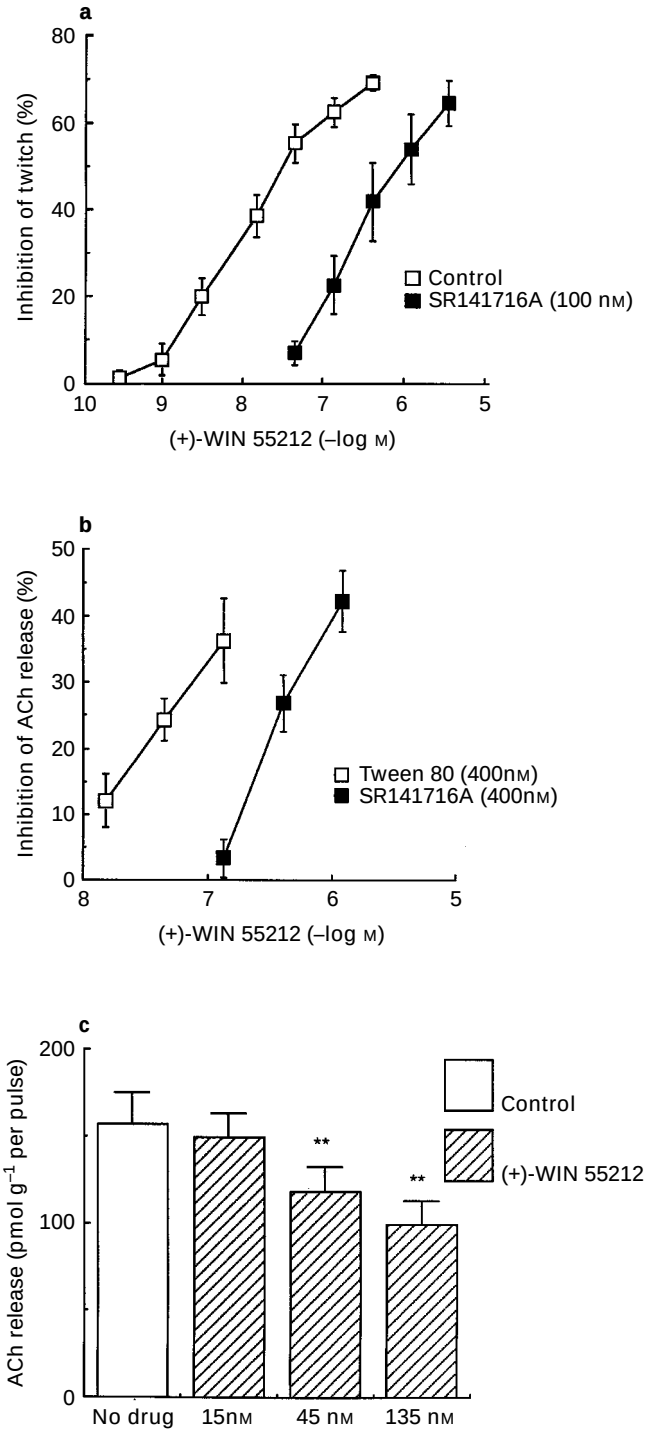

Figure 2 The shift by SR141716A of concentration-response curves to (+)-WIN 55212. Mean concentration-response curves for (a) the inhibition of electrically-evoked contractions by (+)-WIN 55212 in the presence of Tween 80 or SR141716A (100 nM) ($n=8$). SR141716A caused a significant rightward shift (symmetrical (2+2) dose parallel line assay, $P<0.05$) giving a dose-ratio of 30.54 (95% confidence intervals were 5.89 and 183.87). (b) The inhibition of the evoked release of ACh by (+)-WIN 55212 in the presence of Tween 80 or SR141716A (400 nM) ($n=5$). SR141716A caused a significant rightward shift (symmetrical (2+2) dose parallel line assay, $P<0.02$) giving a dose-ratio of 9.87 (95% confidence intervals were 5.01 and 19.81). Each symbol represents the mean values and vertical lines show s.e.mean. (c) The effect of increasing concentrations of (+)-WIN 55212 on ACh release. The mean control output (No drug) and that in the presence of 15 nM, 45 nM and 135 nM (+)-WIN 55212 are shown in pmol g^{-1} per pulse (mean of 12 donor preparations). Vertical lines indicate s.e.mean. The double asterisks denote a significant difference ($P<0.0001$) between the ACh output compared with both the control output and that in the presence of 15 nM (+)-WIN 55212. The difference between the ACh output in the presence of 45 nM and 135 nM WIN 55212 was also significant ($P<0.005$) (paired t test).

presence of SR141716A (1 μM) when the output increased to 94.7 ± 17.5 pmol g^{-1} per pulse.

Dissociation constant of SR141716A at CB_1 receptors as measured in the myenteric plexus

The dissociation constant was determined for the inhibition of the twitch response by CP55940 and its antagonism by SR141716A. Dose-response curves to CP55940 were constructed in the absence of any antagonist and after pretreatment of preparations with 31.6, 100, 316 or 1000 nM

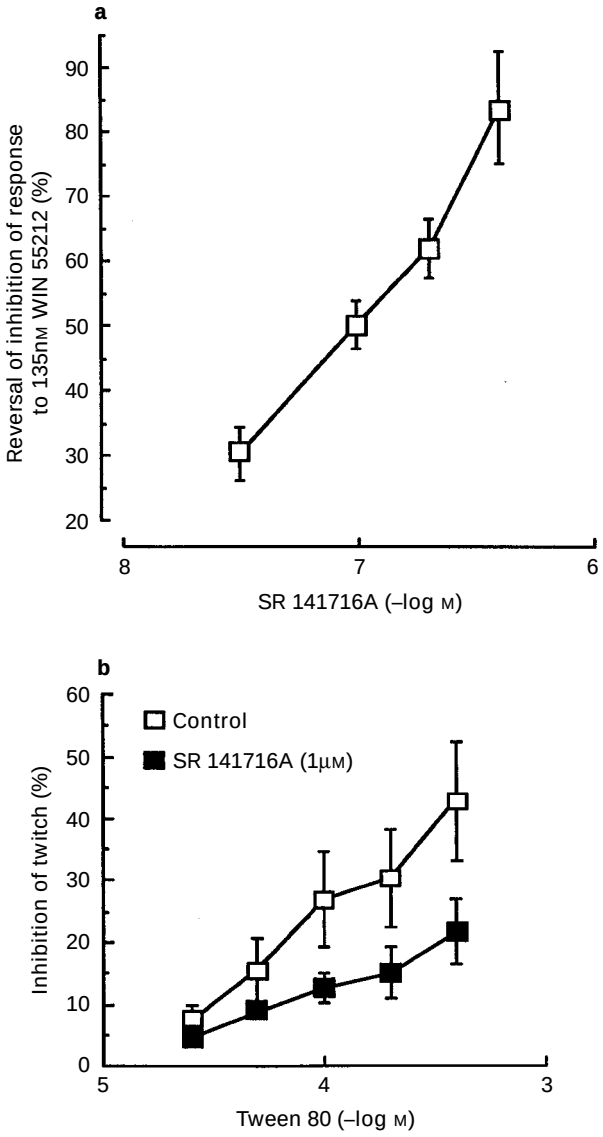

Figure 3 (a) The concentration-effect curve for the reversal by SR141716A of the inhibition of the twitch response of the myenteric plexus-longitudinal muscle by WIN 55212 (135 nM, $n=5-8$). (b) Effect of SR141716A on dose-response curves to Tween 80. Mean concentration-response curves for the inhibitory effect of Tween 80 on electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation in the absence ($n=9$) and presence ($n=9$) of SR141716A (1 μM). Statistical comparison by symmetrical (2+2) dose parallel line assay of the responses to doses of 100 μM and 400 μM Tween 80 showed a significant reduction in response in the presence of SR141716A ($P<0.001$). In (a) and (b), each symbol represents the mean and vertical lines show s.e.mean.

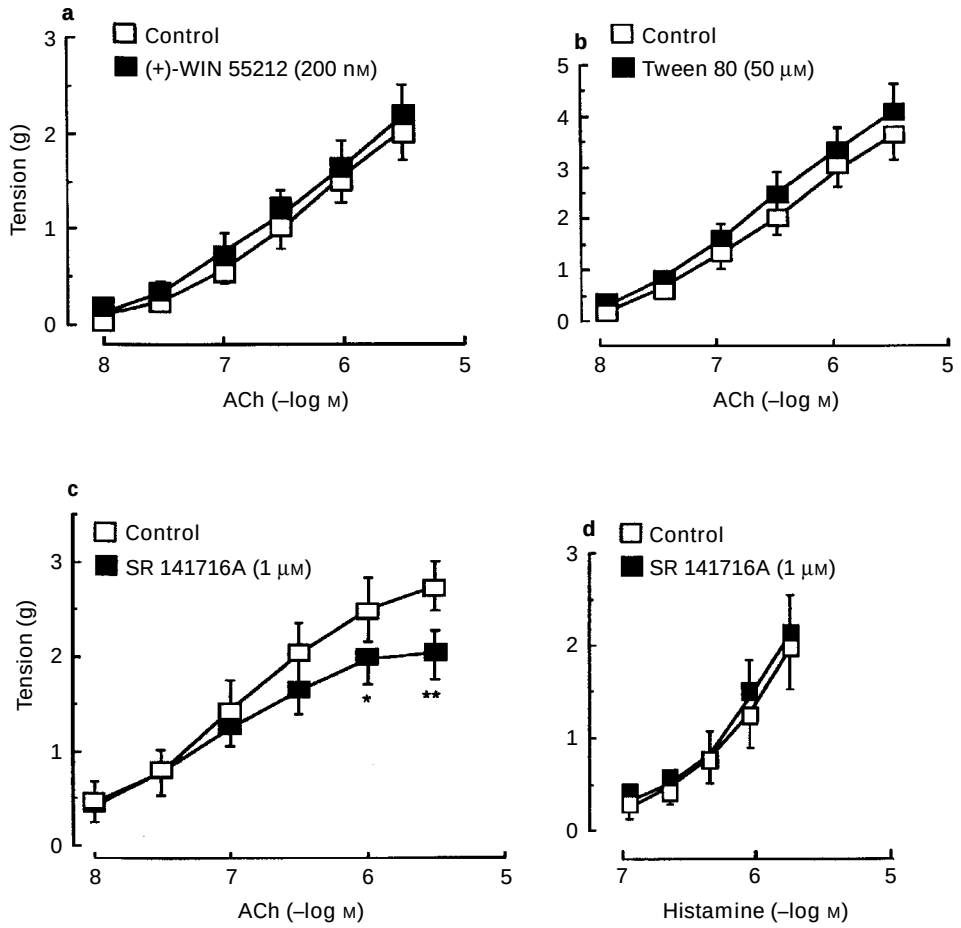

Figure 4 The effects of cannabinoid drugs on the sensitivity of the myenteric plexus-longitudinal muscle preparation to exogenous ACh and histamine. Ordinate scales, tension (g) and abscissa scales, final bath concentration of ACh or histamine (−log M). (a) Responses to ACh in the absence and presence of (+)-WIN 55212 (200 nM), (b) responses to ACh in the absence and presence of Tween 80 (equivalent to that used to disperse 50 μM (+)-WIN 55212), (c) responses to ACh in the absence and presence of SR141716A (1 μM), $^*P<0.01$, $^{**}P<0.0001$, paired t test. (d) Responses to histamine in the absence and presence of SR141716A (1 μM); $n=6$ for all experiments. Each symbol represents the mean value and vertical lines show s.e.mean.

SR141716A. These concentrations of antagonist produced dose-ratios of 5.17, 27.81, 33.15 and 80.04, respectively. Experiments carried out with (+)-WIN 55212 as agonist on the twitch response and SR141716A concentrations of 10, 31.6 and 100 nM produced dose-ratios of 1.93, 4.12 and 17.14, respectively. K_D values of 12.07 nM and 6.45 nM were obtained for the antagonism of CP 55940 and (+)-WIN 55212, respectively (Table 1). A similar series of experiments was carried out for the inhibition of ACh release in the presence of physostigmine by (+)-WIN 55212 and its antagonism by SR141716A at concentrations of 31.6 nM, 100 nM and 400 nM. The resulting dose-ratios were 4.43 ± 1.94 ($n=3$), 10.5 ± 4.00 ($n=4$) and 9.55 ± 3.52 ($n=5$), respectively. As the Schild plot (Figure 6) generated from the release data gave a slope significantly less than 1, it would appear that at high concentrations SR141716A may not be acting in a simple competitive manner on the release of ACh. Therefore the K_D values given in Table 1 were calculated from the dose-ratios obtained with 31.6 nM and 100 nM only.

Dissociation constant of opioid receptors for naloxone

The dissociation constant for the effect of naxolone in antagonizing the inhibitory effects of normorphine on the twitch response was compared with that for its antagonism of the inhibition due to normorphine of the release of ACh. In this series of experiments, cumulative dose-response curves for the inhibitory effect of normorphine (50–3200 nM) on the twitch response were constructed before and after the addition of naloxone (final bath concentration 10 nM) to the organ bath. The maximum inhibition of the twitch was $70.15\pm9.58\%$ and $68.7\pm6.04\%$ ($n=5$) in the absence and presence of naloxone and the corresponding IC_{50} values were 89.0 nM and 652.3 nM, respectively. In these experiments, naloxone produced a mean dose-ratio of 6.91 ± 1.61. The mean value for the dissociation constant determined from these data was 2.38 ± 0.69 nM. The dissociation constant for the antagonism by naloxone (10 nM) of the inhibition of ACh release from the myenteric plexus by normorphine (50–4050 nM) was found to be 2.00 ± 0.90 nM (mean ± s.e.mean for 3 experiments). The maximum inhibition due to normorphine on ACh release was $53.1\pm4.7\%$ in the absence and $53.9\pm3.0\%$ in the presence of naloxone, and the mean dose-ratio was 7.93 ± 2.16. When the maximum inhibition of output in each experiment was taken as 100% maximum inhibition, the mean IC_{50} values for the inhibitory effect of normorphine on ACh release in the absence and presence of naloxone were 158.3 nM and 1021 nM, respectively.

Comparison of the responses to (+)- and (−)-isomers of the cannabinoid agonist WIN 55212

Dose-response curves for the (−)-isomer of WIN 55212 in inhibiting the twitch response were compared with those for the (+)-isomer. The responses to Tween 80, in concentrations equivalent to those used to disperse (−)-WIN 55212 are also shown (Figure 7a). (−)-WIN 55212 was approximately 300 times less potent than (+)-WIN 55212 on the

twitch response, though slightly more active than the vehicle alone. In 4 experiments, dose-response curves to (+)-WIN 55212 were constructed in the presence of (−)-WIN 55212 (1 μM) and were not different from control curves (Figure 7b). These results indicate that (−)-WIN 55212 is also devoid of cannabinoid receptor antagonist activity.

When the effects of (−)-WIN 55212 were measured on the release of ACh from the myenteric plexus, in sequential treatments of the same preparations, the output of ACh was not changed in the presence of (−)-WIN 55212 (1 μM) or its vehicle, Tween 80, but was significantly reduced by subsequent exposure of the preparation to the active isomer, (+)-WIN 55212 (200 nM) (Figure 7c).

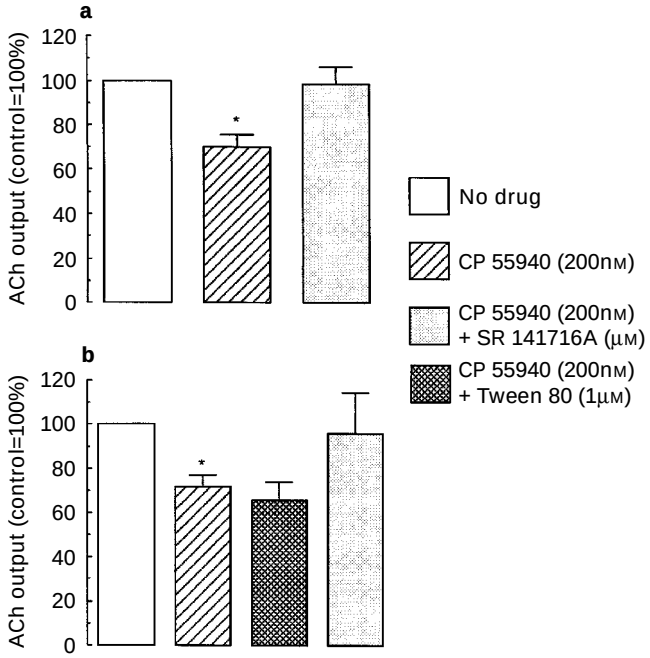

Figure 5 Release of acetylcholine from electrically stimulated strips of myenteric plexus-longitudinal muscle before administration (No drug), in the presence of 200 nM CP 55940 alone and in the presence of 200 nM CP 55940 plus either Tween 80 or 1 μM SR141716A. ACh output is expressed as a percentage of the control output; vertical lines represent s.e.mean. (a) The control output of 65.9 ± 13.0 pmol g^{-1} per pulse (mean ± s.e.mean of 5 experiments) is shown as 100%. ACh output in the presence of CP 55940 alone (asterisk) was significantly less ($P<0.05$, ANOVA Scheffé's test) than both control output and that in the presence of CP 55940 and SR141716A. (b) The control output of 59.35 ± 8.75 pmol g^{-1} per pulse (mean ± s.e.mean of 4 experiments) is shown as 100%. The ACh output in the presence of CP 55940 alone (asterisk) was significantly different ($P<0.05$, ANOVA Scheffé's test) from the control output but was not different from that in the presence of CP 55940 and Tween 80.

Effects of SR141716A on the evoked release of ACh in the absence of exogenous cannabinoids

In 9 experiments, the output of ACh in the presence of Tween 80 (1 μM) was not significantly different from the control output. However, subsequent addition of SR141716A, giving a final bath concentration of 1 μM, resulted in the ACh output being increased by 25.34 ± 9.59% (Figure 8a) compared with the control output ($P<0.05$).

A rise in ACh release due to SR141716A could also be demonstrated in the presence of concentrations of normorphine (100 nM) which produced significant inhibition of ACh release (data not shown; $n=5$).

The effect of normorphine on the evoked release of ACh in the presence of maximal activation of cannabinoid receptors

In 3 experiments, the output of ACh was determined before and after treatment of preparations with a supramaximal dose of (+)-WIN 55212 (1215 nM) and again after subsequent exposure of the preparations to normorphine (400 nM) in the presence of the cannabinoid. (+)-WIN 55212 alone significantly inhibited the output of ACh by 35.6 ± 0.94%. The output was further inhibited to produce a final inhibition of 65.58 ± 0.59% on exposure to normorphine (Figure 8b, $P<0.005$; paired t test).

Discussion

That cannabinoid agonists act presynaptically by reducing ACh release in the myenteric plexus-longitudinal muscle preparation is now quite clear. The present experiments show

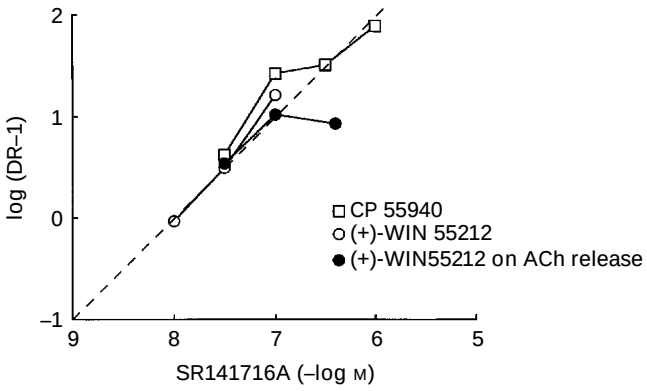

Figure 6 Schild plots for the antagonism by SR141716A of the inhibitory effects of CP 55940 and (+)-WIN 55212 on the twitch response and of (+)-WIN 55212 on the release of acetylcholine from the myenteric plexus. The slopes of the Schild plots are given in Table 1. The dashed line indicates a slope of unity.

Table 1 Dissociation constants (K_D) of SR141716A determined in the presence of CP 55940 AND (+)-WIN 55212 on both the contractile response and the release of ACh from the myenteric plexus-longitudinal muscle preparation

Agonist	K_D (nM)	*95% confidence intervals*	r^2	*Slope*	P (*2-tailed*)
CP 55940*	12.07	8.55 and 20.83	0.9066	0.782 ± 0.198	0.058
(+)-WIN 55212*	6.44	4.70 and 10.24	0.9038	1.241 ± 0.108	0.055
(+)-WIN 55212**	9.21, 10.53τ	–	–	–	–

K_D values were determined by the Tallarida method (mean values and 95% confidence intervals) with at least three of the following concentrations of SR141716A: 10, 31.6, 100, 316.2 and 1000 nM. Slope is that of a Schild plot. P values indicate the levels of significant difference from a slope of unity. *Determined on contractile response. ($n=5-8$ at each concentration of SR141716A). **Determined on release of ACh ($n=3$ or 4 at each concentration of SR141716A). τ values calculated from mean (DR−1) at SR141716A concentrations of 31.6 nM and 100 nM, respectively.

that, in doses which inhibit the evoked contractile responses at low frequency, these agonists produce a corresponding reduction in ACh release. These results support earlier findings for the effects of Δ^9-tetrahydrocannabinol on resting ileal preparations (Layman & Milton, 1971; Paton *et al*., 1972). In all experiments in which the release of ACh was determined, the rate of release was not significantly changed by time or by incubation with the vehicle, Tween 80 alone. Similar presynaptic CB_1-receptor activation has been shown to inhibit the release of [^{14}C]-ACh from hippocampal slices (Gifford &

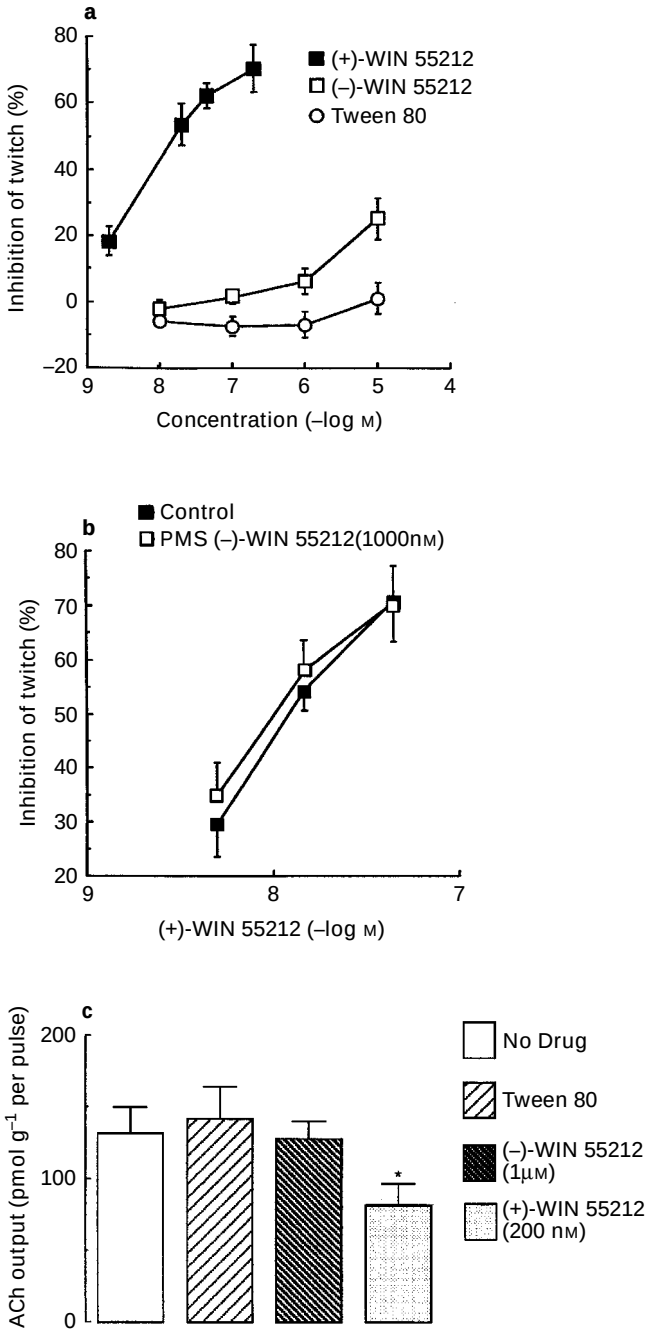

Figure 7 Comparison of (−)- and (+)-enantiomers of WIN 55212. (a) Mean concentration-response curves for the inhibitory effect of (−)-WIN 55212 ($n=6$), (+)-WIN 55212 ($n=5$) and Tween 80 ($n=6$) on electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation. (−)-WIN 55212 was much less potent than (+)-WIN 55212 but was significantly greater than Tween 80 alone ($P<0.05$, unpaired t test). Each symbol represents the mean value. (b) The presence of (−)-WIN 55212 (1000 nM) had no significant effect on the concentration-response curves to (+)-WIN 55212 in the presence of Tween 80 ($n=4$). (c) The control output of ACh from the myenteric plexus (No drug) and in the presence of 1 μM Tween 80, 1 μM (−)-WIN 55212 and 200 nM (+)-WIN 55212. Each column is calculated from the mean of triplicate samples from 4 different donor preparations. ACh release is expressed as pmol g^{-1} per pulse. In (a) and (b) and (c) vertical lines indicate s.e.mean. The asterisk denotes a significant difference between release in the presence of (+)-WIN 55212 and control release ($P<0.05$, paired t test).

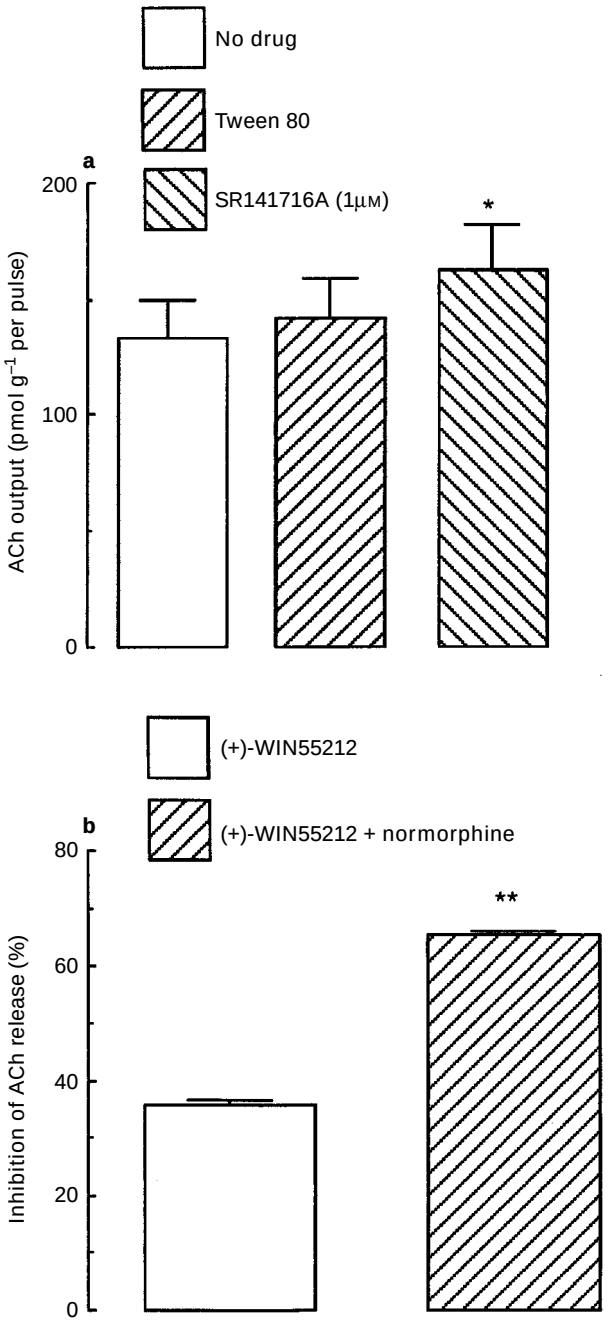

Figure 8 (a) The release of acetylcholine from electrically stimulated strips of myenteric plexus-longitudinal muscle before drug administration (No drug), in the presence of 1 μM Tween 80 alone and in the presence of Tween 80 and 1 μM SR141716A. Each column represents the mean ACh release ($n=9$ different donor preparations); vertical lines indicate s.e.mean. The asterisk denotes a significant difference between release in the presence of SR141716A and control output or the output in the presence of an equivalent concentration of Tween 80 ($P<0.05$, ANOVA Scheffé's test). (b) The effect of normorphine on the inhibition of ACh release from the myenteric plexus in the presence of (+)-WIN 55212 (1215 nM). The ordinate scale represents the inhibition of ACh output (%; mean±s.e.mean) by (+)-WIN 55212 (1215 nM) alone and in the presence of normorphine (400 nM) in 3 experiments. The asterisks denote a significant difference between the release in the presence and absence of normorphine ($P<0.005$, paired t test).

Ashby, 1996), [^{3}H]-noradrenaline from peripheral sympathetic nerves (Ishac *et al*., 1996) and evoked [^{3}H]-noradrenaline and [^{3}H]-dopamine from guinea-pig superfused retinal discs (Schlicker *et al*., 1996).

Antagonism of the effects of cannabinoid agonists on the contractile response or on the evoked release of ACh is demonstrated by the parallel, rightward shift of the agonist dose-response curves by pretreatment of the preparation with SR141716A, the CB_1-selective antagonist. The shifts of the dose-response curves to (+)-WIN 55212 and CP 55940 on twitch responses are in line with those previously published (Pertwee *et al*., 1996b) as are the resulting values for dissociation constants. The exception to this is the failure of the highest dose of SR141716A to cause a further parallel rightward shift of the inhibitory dose-response curve for the effect of (+)-WIN 55212 on the release of acetylcholine. Consequently, the slope for the Schild plot appears to be less than unity. This suggests that the effects of (+)-WIN 55212 in this preparation may not be totally explained in terms of a straightforward competitive surmountable interaction with CB_1 receptors and care must be taken in the interpretation of these findings. It is possible that part of the response to higher concentrations of (+)-WIN 55212, which are required to produce inhibition in the presence of 400 nM SR141716A, may be mediated via a different population of receptors which are poorly antagonized by SR141716A e.g. CB_2 receptors, as (+)-WIN 55212 has been shown to bind preferentially to CB_2 rather than CB_1 receptors (Showalter *et al*., 1996).

In experiments in which K_D values were determined on the contractile response, the responses in the presence of cannabinoid agonists were compared with those before the addition of the agonist but after equilibration with SR141716A. These responses were not the same as those of the naive preparation and this poses some difficulty. However, when determined on the twitch responses, the K_D values (see Table 1) generated from this analysis gave apparent pA_2 values of 8.19 for (+)-WIN 55212-2 and 7.94 for CP 55940 which compare favourably with the pA_2 values found for the antagonism of WIN 55212-2 by SR141716A in the mouse isolated vas deferens (Rinaldi-Carmona *et al*., 1994) and cannabinoid-mediated inhibition of dopamine release in the retina (Schlicker *et al*., 1996). The K_D value of 2.38 nM (pA_2 of 8.62) found for the antagonism of normorphine by naloxone agrees well with that previously published in the literature (Lord *et al*., 1977).

In addition, none of the cannabinoid drugs tested showed postjunctional activity, apart from SR141716A which reduced the responses to very high doses of ACh. Since this drug increases electrically-evoked contractile responses, any effect which reduces smooth muscle contractions is unlikely to be related to its effects on the contractile responses to electrical stimulation.

Unlike the twitch response, which can be virtually abolished by CB agonists, the inhibition of ACh release reaches a plateau at 35–40% inhibition. There are several possible explanations for this discrepancy. Firstly, the abolition of the twitch response may leave a residual release of ACh from the nerve terminals which is subthreshold for excitation of the smooth muscle in the absence of physostigmine but is still detectable by bioassay when physostigmine is present. Secondly, ACh is released from sites in the myenteric plexus other than from the terminal varicosities of the final motorneurones, for example from the preganglionic terminals in the myenteric ganglia. These sources may provide a significant contribution to the ACh measured in the presence of physostigmine and may be insensitive to CB_1 receptor activation. Thirdly, the inhibitory effects of CB agonists on ACh release may be partially precluded by the autoinhibition of acetylcholine release in the presence of anticholinesterases (Ten Berge *et al*., 1996). Finally, there is electrophysiological evidence (North & Nishi, 1974) that after pretreatment with physostigmine (10 μM), fast excitatory postsynaptic potentials recorded in S neurones of guinea-pig myenteric ganglia are augmented in amplitude and the duration prolonged, resulting in repetitive firing. This may lead to further release of ACh which does not occur in the absence of physostigmine and is not sensitive to inhibition by cannabinoids.

The ability of normophine to reduce significantly the residual release of ACh after maximal activation of cannabinoid receptors confirms the distinction between opioid and cannabinoid receptors. This is supported by the finding that dose-response curves to cannabinoid agonists are unaffected by the presence of naloxone in the organ bath, and that dose-response curves to the inhibitory effects of normorphine and also clonidine are not antagonized by SR141716A (Pertwee *et al*., 1996b).

The difference in potencies of the stereoisomers of WIN 55212 on the contractile responses and the complementary effects on ACh release is consistent with a specific receptor-mediated mechanism of action of cannabinoids.

Occasionally, the control of ACh output from myenteric plexus-longitudinal muscle preparations is found to be considerably lower than normal despite the sex, weight range and experimental conditions being identical to other experiments (see Figure 5). In terms of sensitivity to cannabinoid drugs, these preparations behave in the same way as those showing 'normal' levels of ACh release. The reason for this observed difference in ACh output remains obscure.

SR141716A has previously been shown to produce a slight, though significant and dose-related increase in twitch tension when added to a naive preparation during stimulation at 0.1 Hz compared with responses in the presence of the equivalent concentration of Tween 80 (Coutts *et al*., 1995; Pertwee *et al*., 1996b). The augmenting action of SR141716A on the twitch response correlates well with the significant increase in ACh release when acetylcholinesterase has been completely inhibited, therefore the increased twitch induced by SR141716A in the absence of a cholinesterase inhibitor is unlikely to result from a reduction in ACh metabolism. Pretreatment of the preparation with normorphine (100 nM) did not prevent this augmenting effect which appeared to result in a physiological partial reversal of the inhibitory response to normorphine on both the twitch response and on ACh release.

This reverse-agonist effect of SR141716A alone is not without precedent. It has been shown that treatment with SR141716A can increase forskolin-induced stimulation of cyclic AMP production in cells transfected with cannabinoid receptors (Felder *et al*., 1995), decrease spontaneous firing of rat substantia nigra pars reticulata neurones (Tersigni & Rosenberg, 1996), increase locomotor activity in mice (Compton *et al*., 1996) and produce signs of increased arousal in rats (Santucci *et al*., 1996). In more comparable experimental situations, SR141716A has been found to augment electrically-evoked twitch responses of mouse isolated vas deferens (Pertwee *et al*., 1996a) and urinary bladder (Pertwee & Fernando, 1996) and facilitate the release of radiolabelled neurotransmitter from superfused retinal discs (Schlicker *et al*., 1996) and hippocampal slices (Gifford & Ashby, 1996). It may be postulated that the increase in twitch response and evoked release by ACh by SR141716A is due to antagonism of an endogenously-released CB_1 receptor agonist. Endogenous cannabinoid ligands which have been found include arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol. Such ligands have been detected in brain (Devane *et al*., 1992; Sugiura *et al*., 1995) and the latter has also been found in the canine intestinal tract (Mechoulam *et al*., 1995). However, these ligands have not been identified in the myenteric plexus-longitudinal muscle preparation of the guinea-pig to date.

Alternatively, the augmenting effect of SR141716A in this tissue may be explained in terms of the 2-state model of receptors such as proposed by Leff (1995). This assumes that the receptor can exist in two states, one active, which can couple to the signal transducer mechanism, and one inactive, which cannot. According to this hypothesis (see Pertwee *et al*., 1996b), the receptor states coexist in equilibrium, even in the absence of any agonist. Preferential binding of SR141716A to

the receptor in the inactive state would be expected to produce a shift in equilibrium away from the active state and hence a reduction in the number of precoupled receptors and an attenuation in inhibitory activity. If this hypothesis were true, then SR141716A would act as an inverse agonist rather than a pure antagonist. Gifford & Ashby (1996) arrived at similar conclusions in a recent study in which SR141716A was shown to enhance the electrically-evoked release of [^{14}C]-ACh from hippocampal slices of rat brain.

In conclusion, the findings of the present study, taken together with the evidence of CB receptor localization on nerve terminals (Herkenham, 1995) and the effects of CB_1 receptor activation on transmitter release in the central nervous system (Gifford & Ashby, 1996) and other peripheral neural tissues (Ishac *et al*., 1996; Schlicker *et al*., 1996; Pertwee *et al*., 1996b), reinforce the hypothesis that cannabinoids perform a neuromodulatory role. Since SR1417167A is such a widely used and invaluable tool in the characterization of cannabinoid receptors in both the central and peripheral nervous system and in cultured cells, it is essential that its mechanism of action is clarified in the near future in order to facilitate the interpretation of experimental findings.

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