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Effects of cannabinoid receptor ligands on electrophysiological properties of myenteric neurones of the guinea-pig ileum

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1 The effect of cannabinoid receptor agonists was studied in guinea-pig myenteric neurones *in vitro* by use of conventional intracellular recording techniques.

2 Exposure of myenteric neurones of the S-cell type to the cannabinoid receptor agonists WIN 55,212-2 (100 nM) and CP 55,940 (100 nM) reversibly and significantly depressed the amplitude of fast excitatory synaptic potentials (fast e.p.s.ps) by 46% and 37%, respectively.

3 The depressant effect of WIN 55,212-2 and CP 55,940 on fast e.p.s.p. amplitude (expressed as the area above the amplitude-time curve (mVs)) was significantly greater than that of the vehicle, Tween 80, which had no detectable effect.

4 The inhibitory effect of WIN 55,212-2 appeared to be concentration-dependent over the range 1–100 nM. WIN 55,212-3, its (−)-enantiomer (100 nM), was inactive.

5 The cannabinoid CB_1 receptor antagonist, SR141716A (1 μM), reversed the inhibitory effects of WIN 55,212-2 on fast e.p.s.ps in 38% of neurones tested (3/8) and acetylcholine (ACh)-induced depolarizations in 42% of neurones tested (5/12).

6 When tested on its own, SR141716A (1 μM) caused a 40–50% reduction in the amplitude of fast e.p.s.ps ($n=9$).

7 WIN 55,212-2 reversibly depressed the amplitude of the slow e.p.s.p. and, in 2 out of 7 neurones, this effect was reversed by SR141716A (1 μM).

8 It is concluded that cannabinoid-induced inhibition of fast cholinergic synaptic transmission occurred by reversible activation of both presynaptic and postsynaptic CB_1 receptors and that slow excitatory synaptic transmission can also be reversibly depressed by cannabinoids. Furthermore, it would seem that subpopulations of myenteric S-neurones and their synapsing cholinergic and non-cholinergic, non-adrenergic terminals are not endowed with cannabinoid receptors.

Keywords: Cannabinoid CB_1 receptors; cholinergic neurotransmission; NANC neurotransmission; fast e.p.s.p.; slow e.p.s.p.; guinea-pig myenteric neurones

Introduction

The endogenous fatty acid amide, arachidonoylethanolamide (anandamide), activates cannabinoid CB_1 receptors, members of the G protein-coupled superfamily (Devane *et al*., 1992; Pertwee, 1997). It has recently been established that cannabinoids inhibit electrically-induced contractions of the guinea-pig myenteric plexus-longitudinal muscle preparation by activation of cannabinoid CB_1 receptors (Pertwee *et al*., 1996), for which SR141716A is known to be a highly potent and selective antagonist (Rinaldi-Carmona *et al*., 1994). A likely mechanism is closure of N-type and P/Q-type (ω-conotoxin (MCVIII)-sensitive) calcium channels responsible for acetylcholine release from the prejunctional cholinergic terminal (Caulfield & Brown, 1992; Mackie & Hille, 1992; Mackie *et al*., 1993; 1995; Pan *et al*., 1996; Hong *et al*., 1996; Pertwee, 1997).

Since there is little direct evidence concerning the actions of cannabinoids on peripheral neurones, it seemed imperative to investigate the effects of cannabinoids on myenteric neurones with excitatory synaptic potentials (e.p.s.ps), a subset of which are the final motor neurones to the longitudinal muscle (Brookes *et al*., 1992).

A preliminary account of the results described in this paper was presented at a recent meeting of the British Pharmacological Society (López-Redondo *et al*., 1996)

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Methods

Tissue dissection

Myenteric plexus-longitudinal muscle preparations were made from a segment of ileum (3–4 cm) isolated from guinea-pigs (320–550 g; Dunkin Hartley) killed by cervical dislocation and exsanguination. Briefly, a segment of small intestine was slipped onto a glass rod and the longitudinal muscle, with the adherent myenteric plexus, was separated from the circular muscle layer by means of tangential strokes with fine cotton wool dampened with Krebs solution. The tissue was pinned to the Sylgard base of a small recording chamber placed on the stage of a Vickers Instruments M15 microscope (×400). The preparation was superfused with warm (36–37°C) Krebs solution of the following composition (mM): NaCl 118.6, KCl 4.69 $CaCl_2$ 2.54, KH_2PO_4 1.35, $MgSO_4$ 1.18, $NaHCO_3$ 25 and D-glucose 11.1; the pH was 7.4 when gassed with 95% O_2/5% CO_2. This solution also contained nicardipine (0.5 μM) and hyoscine (1 μM) to minimise movements of the longitudinal muscle during intracellular recording and to block muscarinic cholinoceptors.

Intracellular recordings and ionophoresis

Intracellular recordings were made with glass microelectrodes (74–130 MΩ) filled with 2 M KCl, by use of a NL102G DC pre-amplifier (Neurolog, Digitimer), and displayed on an oscilloscope (Tektronix 5112). The recordings were simultaneously monitored on a pressure ink chart recorder (Gould 2600S) and digitized at 40 kHz with a MacLab/4 data acqui-

sition system (AD Instruments) for later analysis with Scope v3.4.1 software on a Macintosh LC computer. Experiments commenced not less than 45 min after impalement. Synaptic responses were evoked by single cathodal current pulses (0.12–0.3 ms duration, 0.7–3 mA passed through glass microelectrodes (tip dia. 10 μm). These were filled with a modified Krebs solution of the following composition (mM): NaCl 143, KCl 4.75 and $CaCl_2$ 2.54, and placed on the surface of the ganglia or interconnecting strands.

For ionophoresis of acetylcholine (ACh), sharp glass microelectrodes were filled with a solution of ACh (1 M) connected to a micro-ionophoresis programmer (WPI, M-160); the tip of each microelectrode was adjusted until the rate of rise of the depolarization (ACh-potential), as well as its peak amplitude, matched that of the fast e.p.s.p. it was intended to mimic as closely as possible, without the neurone generating an action potential.

Drugs

Drugs and test solutions were applied by changing the superfusing solution by means of a three-way valve. The drugs used were stored in ethanol and protected from the light. Before use, they were mixed with two parts of Tween 80 (Sigma) by weight. The ethanol was then eliminated by evaporation under vacuum and the drugs dispersed in 0.9% w/v NaCl solution (saline) as described previously for delta-9-tetrahydrocannabinol (Pertwee *et al.*, 1992).

The compounds used in this study included: WIN 55,212-2 ((**R**)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone; RBI), its inactive (−)-enantiomer, WIN 55,212-3, CP 55,940 ((−)-3-[2-hydroxy 4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; from Dr L.S. Melvin (Pfizer)), SR141716A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4,-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; Sanofi Recherche). Other chemicals were purchased from BDH Laboratory Supplies, Merck Ltd.

Analysis of data

Values are expressed as means and limits of error as s.e.mean. The degree of inhibition of fast e.p.s.p. amplitude is sometimes expressed in percentage terms. This was calculated by comparing fast e.p.s.p. amplitude immediately before drug application with the amplitude after drug administration. Data concerning inhibition of fast synaptic transmission have also been presented as the area above the amplitude-*versus*-time curve of fast e.p.s.ps, elicited at 20 s intervals, during 5 min of drug superfusion. The area was calculated as the sum of partial areas determined by triangulation of the curve.

Statistical comparisons were made by use of either Student's paired t test or one-way analysis of variance (ANOVA) followed by Newman-Keuls test. A P value <0.05 was considered to be significant.

Results

Stable intracellular recordings were made from a total of 132 neurones in 117 preparations of myenteric plexus. Intracellular recordings were considered to be satisfactory if (i) the resting membrane potential was stable and more negative than −45 mV, (ii) the input membrane resistance was greater than 80 MΩ, and (iii) action potentials induced by direct somal depolarizations or focal stimulation of preganglionic fibre tracts showed unequivocal overshoot. Unless stated otherwise, the effects of drugs were tested on S-neurones (Bornstein *et al.*, 1994).

Inhibition of fast synaptic transmission

When administered alone, Tween 80 had a slight depressant effect on the amplitude of fast e.p.s.ps (10 ± 0.2 mV, control vs 9 ± 0, Tween-treated; $n=6$; $P<0.01$, unpaired t test) but had no depressant effect on depolarizations induced by ionophoresis of acetylcholine (ACh potentials; control, 9 ± 1 mV cf Tween-treated 8 ± 1 mV, $n=7$). In contrast, in 21/30 neurones studied, the cannabinoid receptor agonist, WIN 55,212-2 (100 nM), significantly ($P<0.05$) depressed the amplitude of fast e.p.s.ps from their control (i.e. neither drug- nor vehicle-treated) values (Figure 1). The inhibitory effect of WIN 55,212-2, which was reversible on discontinuing exposure to the drug, was apparently concentration-dependent in the range 1–100 nM; at 1 nM ($n=6$), the amplitude of the fast e.p.s.p. was depressed by $27\pm5\%$ ($P<0.01$, ANOVA, followed by Newman-Keuls), whilst at 100 nM ($n=6$), the reduction was $63\pm4\%$ ($P<0.01$, ANOVA, followed by Newman-Keuls). At a concentration of 1 μM, WIN 55,212-2-induced inhibition ($57\pm6\%$) was not significantly different from that produced by 100 nM. The degree of maximum inhibition ranged from 33 to 100% but was rarely greater than 60%. The depressant effects of this agent (1, 100, 1000 nM) on fast e.p.s.p. amplitude was also significantly greater than that of the vehicle, Tween, at each concentration tested ($P<0.01$; $n=4$, 5 and 4, respectively). For the purposes of this comparison, the inhibitory effect was expressed as the area above the amplitude-time curve (mVs); the values were $5{,}420\pm1{,}650$ ($n=5$), $14{,}069\pm4{,}774$ ($n=5$) and $14{,}145\pm5{,}828$ ($n=4$), respectively, and were all significantly different from the Tween-treated value ($1{,}047\pm2423$, $n=8$; $P<0.01$, ANOVA, followed by Newman-Keuls).

Another cannabinoid receptor agonist, CP 55,940 (1, 100, 1000 nM) also produced an inhibitory effect on fast e.p.s.p. amplitude in 16 out of 25 neurones tested. The depression was $13\pm4\%$ ($n=6$) at 1 nM, $44\pm2\%$ ($n=6$) at 100 nM and $40\pm2\%$ ($n=6$) at 1 μM; the values at 100 nM and 1 μM were both significantly different from the Tween-treated and 1 nM drug-treated values given above (ANOVA, followed by Newman-Keuls), but not different from each other.

At a concentration of 100 nM, WIN 55,212-3, the (−)-enantiomer, had no detectable inhibitory effect on fast e.p.s.p. amplitude (11 ± 1 mV), whereas a subsequent application to the same neurones of WIN 55,212-2, produced a significant decrease (8 ± 2 mV) compared with the control response (12 ± 2 mV) (Figure 2).

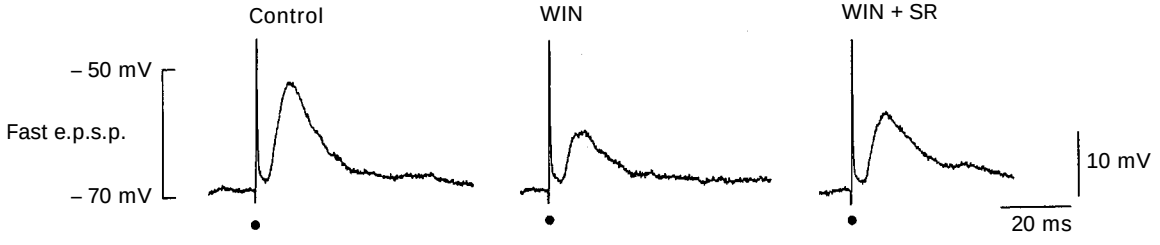

Figure 1 Intracellular electrophysiological recordings showing inhibitory effect of WIN 55,212-2 (WIN, 100 nM) and its reversal by the selective cannabinoid CB_1 receptor antagonist, SR141716A (SR, 1 μM), in a single myenteric neurone. Dots indicate the stimulus artifacts of focal electrical stimulation.

Mechanisms of presynaptic and postsynaptic inhibition of fast e.p.s.ps

In concentrations of up to 10 μM, WIN 55,212-2 and CP 55,940 had no statistically significant effect on the resting membrane potential ($n=119$ neurones; 74 S- and 26 AH-neurones), on the input resistance ($n=27$ S- and 5 AH-neurones), membrane time constant (8 S-neurones) or on the threshold, amplitude or duration of action potentials of either S- ($n=6$) or AH-neurones ($n=13$) (data not shown). Thus, changes in active and passive properties of myenteric neurones were excluded as possible explanations for the depressant effect of cannabinoids on ganglionic transmission.

In order to determine whether WIN 55,212-2 (100 nM) inhibited fast e.p.s.ps in myenteric neurones by acting presynaptically or postsynaptically, the depression of electrically-evoked cholinergic fast e.p.s.ps in a group of 11 S-neurones was compared directly with its effect on depolarizations induced by the ionophoretic application of ACh, the putative mediator of fast e.p.s.ps. In 5 of the neurones (46%), WIN 55,212-2 reduced the amplitudes of both fast e.p.s.ps and ACh-potentials to $54\pm11\%$ and $63\pm10\%$ of the pre-drug values, respectively (Figures 3 and 4); these values were significantly different from the relevant control values. In another 3 neurones tested (27%), WIN 55,212-2 reduced the amplitude of only fast e.p.s.ps to $53\pm15\%$ and in the remaining 3 neurones (27%), it had no depressant effects (Figure 3).

Inhibition of slow e.p.s.ps

Repetitive focal stimulation of interganglionic nerve fibres is known to result in the occurrence of slow (non-adrenergic, non-cholinergic; NANC) excitatory synaptic potentials in about 50% of myenteric S-neurones of guinea-pig ileum (Lees *et al*., 1989). It was found that WIN 55,212-2 reversibly depressed the amplitude of such slow e.p.s.ps in 5 out of 7 S-neurones (Figures 5 and 6a). Tests for differential effects on the release and postsynaptic actions of the putative mediators of the NANC slow e.p.s.p. were not performed.

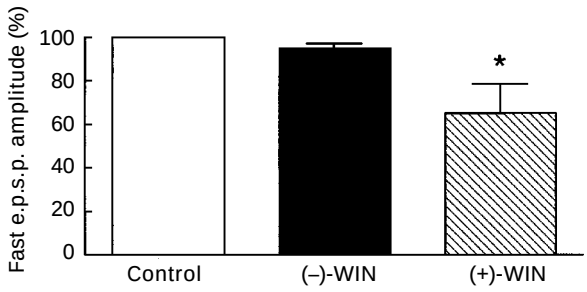

Figure 2 Stereoselectivity of the cannabinoid receptor agonist WIN 55,212-2. Lack of effect of the inactive enantiomer WIN 55,212-3 ((−)-WIN; 100 nM) and subsequent effect of the active enantiomer, WIN 55,212-2 ((+)-WIN; 100 nM), on fast e.p.s.p. peak amplitude applied to the same neurones. Each column represents the mean$\pm$ s.e.mean of the percentage of fast e.p.s.p. amplitude of 3 neurones. *$P<0.05$, ANOVA, followed by Newman-Keuls. Control - before treatment.

Effects of SR141716A

The cannabinoid CB_1 receptor antagonist, SR141716A (1 μM), reversed the inhibitory effects of WIN 55,212-2 on fast e.p.s.ps in 38% of neurones tested (3/8 neurones) and ACh-potentials in 42% of neurones tested (5/12) (Figures 1 and 4). Likewise, the depressant effects of WIN 55,212-2 on slow e.p.s.ps were antagonized by SR141716A (1 μM), in 2 out of 5 S-neurones (Figures 5 and 6a). A satisfactory explanation for the failure of the antagonist to reverse the depression in some neurones was not found.

However, when tested on its own, SR141716A (1 μM) caused a significant reduction of $44\pm7\%$ in the amplitude of fast e.p.s.ps ($P<0.01$, Student's t test; $n=9$). The mean areas above the amplitude-time curves (mVs) for 1 and 10 μM SR141716A were significantly different from each other and from the Tween 80 treated group (Figure 7). In 5 of 10 neurones tested, SR141716A (1 μM) inhibited ACh-potentials by $44\pm7\%$ ($P<0.01$, Student's t test), a finding which suggests a postsynaptic depression in sensitivity to ACh may have been responsible for the inhibition of fast e.p.s.p. amplitude. The effect of SR141716A by itself on slow e.p.s.ps was not tested.

Discussion

Four important points emerge from this investigation, which we believe to be the first study of the effects of cannabinoid

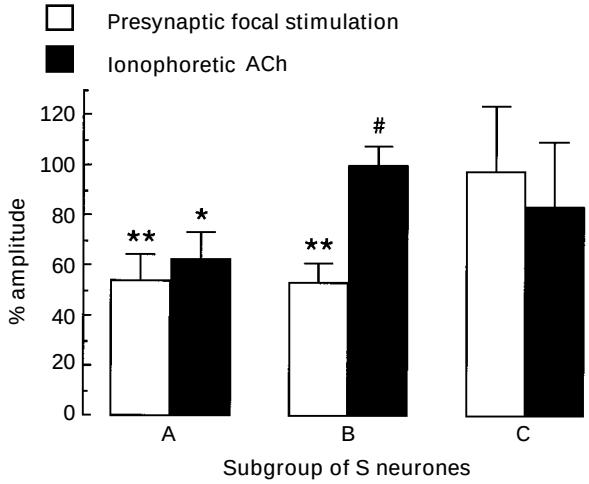

Figure 3 Direct comparison of the effects of WIN 55,212-2 on responses to presynaptic focal stimulation and acetylcholine (ACh) ionophoresis application in 11 myenteric S-neurones. Ordinate scale, amplitude of the response, expressed as a percentage of its relevant control. In group A ($n=5/11$; 46%), WIN 55,212-2 reduced the amplitudes of both fast e.p.s.ps and ACh-potentials. These values were significantly different from controls (*$P<0.05$, **$P<0.01$, paired t test) but not from each other. In group B ($n=3/11$; 27%), WIN 55,212-2 induced a significant reduction only in the amplitude of fast e.p.s.ps (**$P<0.01$, paired t test; #$P<0.05$ vs ACh-potentials). In group C ($n=3/11$; 27%), WIN 55,212-2 did not produce any depressant effects.

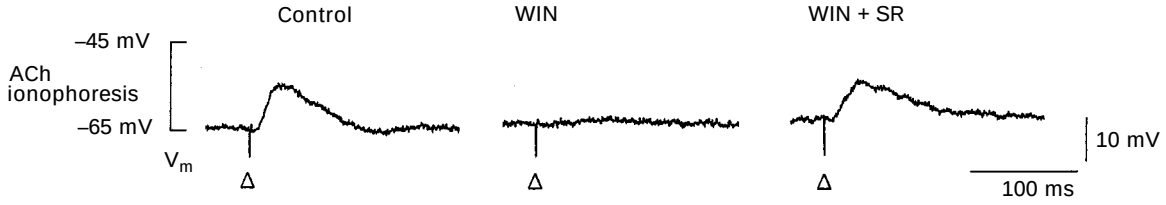

Figure 4 Intracellular electrophysiological traces of ACh-potentials in a single myenteric S-neurone of guinea-pig ileum showing the effect of application of WIN 55,212-2 (WIN; 100 nM) for 15 min, and its reversal at 5 min by the selective cannabinoid CB_1 receptor antagonist, SR141716A (SR; 1 μM). Triangles indicate stimulus artifacts for ionophoretic application of ACh.

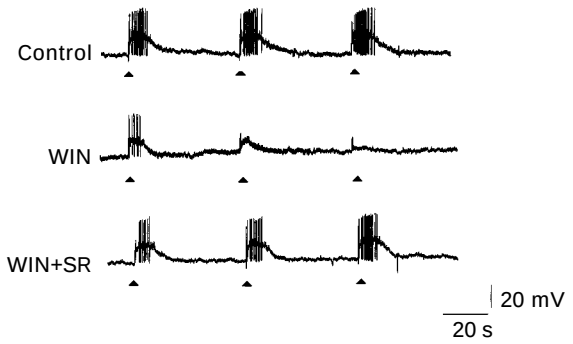

Figure 5 Inhibitory effect of WIN 55,212-2 (WIN; 100 nM) on non-adrenergic, non-cholinergic (NANC) slow e.p.s.ps in a single myenteric S-neurone of guinea-pig ileum and its reversal by the cannabinoid CB_1 receptor antagonist, SR141716A (SR; 1 μM). The resting membrane potential was -57 mV, throughout. Solid triangles indicate the stimulus artifact of repetitive focal electrical stimulation (10 pulses at 20 Hz) to activate the synaptic input.

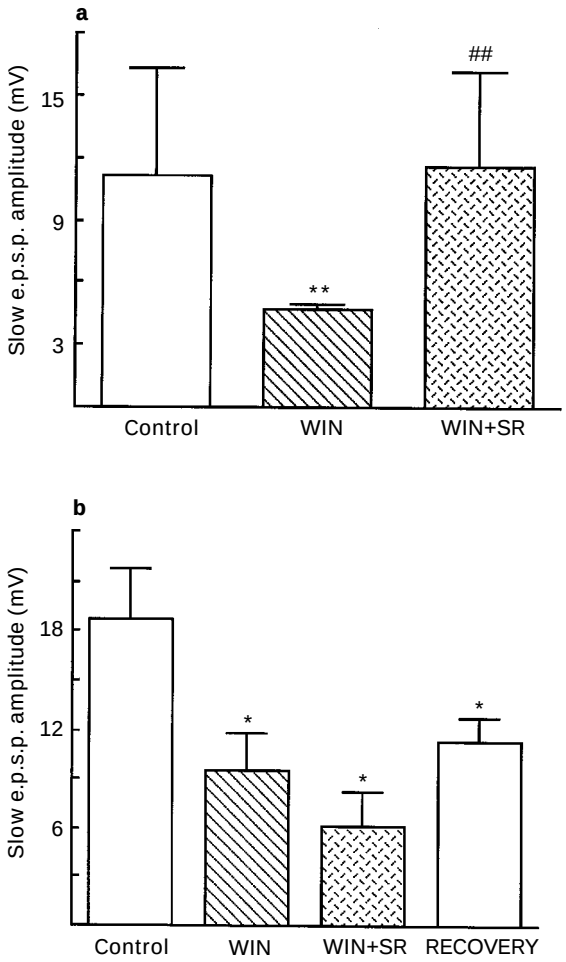

Figure 6 Attempted reversal of the inhibitory effect of WIN 55,212-2 on slow excitatory synaptic potentials by SR141716A. (a) Inhibition of NANC slow e.p.s.p. amplitude by WIN 55,212-2 (WIN; 100 nM; $**P<0.01$) and the reversal in 2 out of 7 neurones by SR141716A (SR; 1 μM) of the inhibitory effect of WIN 55,212-2 ($\#\#P<0.01$, ANOVA, followed by Newman-Keuls). (b) Lack of reversal by SR141716A (1 μM) of inhibition due to WIN 55,212-2 (100 nM) in 5 out of 7 neurones in which WIN 55,212-2 produced a significant decrease in the amplitude of the NANC slow e.p.s.p. ($*P<0.05$, ANOVA, followed by Newman-Keuls); 20 min after exposure to the two agents had been discontinued, the amplitude of the slow e.p.s.p. was still significantly depressed and no significant recovery had occurred.

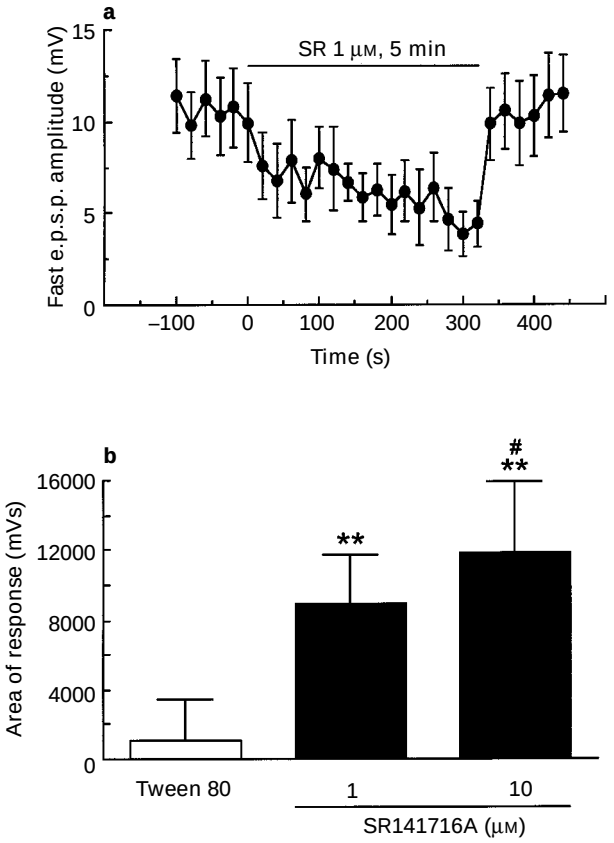

Figure 7 Depressant effect of SR141716A on fast e.p.s.p. amplitude in guinea-pig myenteric S-neurones. (a) Time-course of inhibition of transmission by SR141716A (SR; 1 μM) during 5 min application ($n=6$). (b) Effects of SR141716A (1 and 10 μM) presented as the area above the amplitude-versus-time curve. $**P<0.01$, ANOVA, followed by Newman-Keuls (significant differences vs Tween 80); $\#P<0.05$, ANOVA, followed by Newman-Keuls (significant differences vs effect of 1 μM SR). Data are mean$\pm$s.e.mean of 5–9 cells.

receptor agonists on enteric neurones. First, evidence that WIN 55,212-2 may have been acting at CB_1 receptors to inhibit fast and slow synaptic transmission was adduced by demonstrating that these inhibitory effects, in at least some enteric neurones, could be attenuated by SR141716A, a selective cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona *et al.*, 1994). Furthermore, this inhibitory effect, which was reversible, was achieved at concentrations as low as 1 nM. Although another agonist, CP 55,940, produced similar inhibitory effects, this compound was less potent than WIN 55,212-2, a finding seemingly contrary to one made previously, in experiments in which the measured response was inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation (Pertwee *et al.*, 1996).

Second, WIN 55,212-2, acted stereoselectively in inducing a reversible depression of fast cholinergic synaptic transmission and the receptor-mediated nature of the inhibition is suggested by the lack of activity of (−)-WIN 55,212-3, its enantiomer.

Third, the differences in the pre- and postsynaptic sensitivity of the S-neurones to the agonists suggest that there may be different subpopulations of S-neurones; some showed evidence for both presynaptic and postsynaptic receptors, whereas others seemed to have only presynaptic receptors. No detectable effects were seen in about one third of S-neurones and were not recorded in AH-neurones. It is tempting to conclude, therefore, that a significant proportion of myenteric neurones may be devoid of cannabinoid receptors.

The finding of inhibitory effects in only about half of the total population of myenteric neurones, together with findings of stereoselectivity of action and the consistent lack of any

alteration in active and passive electrophysiological properties of the recorded neurones, adds weight to our conclusion that the effect of the cannabinoids was not due to a non-specific membrane depressant action. Although there is no *a priori* reason why only certain myenteric neurones and their synaptic inputs should be affected by WIN 55,212-2, further experiments would be required to establish why the depressant effects of WIN 55,212-2 were not observed on all myenteric S-neurones and whether the presence of cannabinoids receptors is confined to one functional class of S-neurone. Certainly, no effects of WIN 55,212-2 were recorded in AH-neurones ($n=26$), which are morphologically, electrophysiologically, immunohistochemically and functionally distinct from S-neurones (Brookes *et al*., 1992: Lees *et al*., 1992; Kunze *et al*., 1993; Bornstein *et al*., 1994; for a cautionary note see Wood, 1994).

In rat nodose ganglion neurones, it was suggested that the 5-HT_3 receptor ion-channel is a site acted upon by cannabinoid agonists (Fan, 1995). Since 5-HT has been proposed as a mediator of NANC slow e.p.s.p in guinea-pig myenteric neurones (Wood & Mayer, 1979), it would seem worthwhile to investigate the possibility that cannabinoid agonists could modulate 5-HT receptors to inhibit the slow synaptic inputs.

Fourth, when administered by itself, SR141716A had an unexpected, concentration-dependent depressant effect on fast e.p.s.p. amplitude, leading us to conclude that this agent may have a more complex spectrum of actions than has previously been supposed. Further work is required to elucidate the mechanism involved which was not identified. The depressant effect of SR141716A on cholinergic fast synaptic transmission contrasts with the increase in electrically-evoked release of ACh, that this compound produces in the myenteric plexus-longitudinal muscle preparation when given alone (Coutts & Pertwee, 1996; Pertwee *et al*., 1996). It is possible that a depressant effect of SR141716A has not been detected before, because most previous studies with this compound have involved the measurement of end-organ responses rather than the responses of individual neurones. There was no obvious explanation for the failure of SR141716A to reverse the depressant actions of WIN 55,212-2 in certain neurones; it may have been that the depressant effect of this agent predominated over its antagonist action in these neurones. However, it is also possible that a type or subtype of cannabinoid receptor that is distinct from the CB_1 receptor could be involved.

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