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The actions of the cannabinoid receptor antagonist, SR 141716A, in the rat isolated mesenteric artery

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1 The actions of the cannabinoid receptor antagonist, SR 141716A, were examined in rat isolated mesenteric arteries. At concentrations greater than 3 μM, it caused concentration-dependent, but endothelium-independent, relaxations of both methoxamine- and 60 mM KCl-precontracted vessels.

2 SR 141716A (at 10 μM, but not at 1 μM) inhibited contractions to Ca^{2+} in methoxamine-stimulated mesenteric arteries previously depleted of intracellular Ca^{2+} stores. Neither concentration affected the phasic contractions induced by methoxamine in the absence of extracellular Ca^{2+}.

3 SR 141716A (10 μM) caused a 130 fold rightward shift in the concentration-response curve to levcromakalim, a K^+ channel activator, but had no effect at 1 μM.

4 SR 141716A (10 μM) attenuated relaxations to NS 1619 (which activates large conductance, Ca^{2+}-activated K^+ channels; BK_{Ca}). The inhibitory effect of SR 141716A on NS 1619 was not significantly different from, and was not additive with, that caused by a selective BK_{Ca} inhibitor, iberiotoxin (100 nM). SR 141716A (1 μM) did not effect NS 1619 relaxation.

5 SR 141716A (10 μM) had no effect on relaxations to the nitric oxide donor S-nitroso-N-acetylpenicillamine, or relaxations to carbachol in the presence of 25 mM KCl.

6 The results show that, at concentrations of 10 μM and above, SR 141716A causes endothelium-independent vasorelaxation by inhibition of Ca^{2+} entry. It also inhibits relaxations mediated by K^+ channel activation. This suggests that such concentrations of SR 141716A are not appropriate for investigation of cannabinoid receptor-dependent processes.

Keywords: SR 141716A; rat mesenteric artery; levcromakalim; K^+ channels; NS 1619; cannabinoid receptor antagonist; Ca^{2+} entry; EDHF; nitric oxide; endothelium

Introduction

The cannabinoid receptor antagonist, SR 141716A (Rinaldi-Carmona *et al*., 1994) has been widely used as a pharmacological tool for the identification of cannabinoid receptors in a variety of tissues (Ishac *et al*., 1996; Coutts & Pertwee, 1997; Randall & Kendall, 1998). Although relatively little is known about the cardiovascular actions of cannabinoids, Randall *et al*. (1996) showed that SR 141716A attenuated endothelium-dependent relaxations attributed to endothelium-derived hyperpolarizing factor (EDHF) in the rat perfused mesenteric bed, and postulated that an endogenous cannabinoid such as arachidonylethanolamide (anandamide) might represent an EDHF. However, more recent studies have shown that relaxations caused by anandamide occur through different mechanisms to EDHF-mediated relaxations (Plane *et al*., 1997; White & Hiley, 1997a; Zygmunt *et al*., 1997), and have therefore cast doubt as to the selectivity of SR 141716A.

Knowledge of cannabinoid receptor pharmacology has hitherto been limited by the lack of selective ligands. Indeed, despite the recent characterization of SR 144528 (Rinaldi-Carmona *et al*., 1998) and LY 320135 (Felder *et al*., 1998), SR 141716A remains the only commercially available cannabinoid receptor antagonist. SR 141716A has been shown to bind with high affinity to CB_1 receptors (11.8 nM; Felder *et al*., 1995) and CB_{1A} receptors (43.3 nM; Rinaldi-Carmona *et al*., 1996), although it also binds with rather lower affinity to CB_2 receptors (702 nM; Showalter *et al*., 1996). However, recent work has suggested that the actions of SR 141716A are more complex than was first thought. Evidence from both functional (Coutts & Pertwee, 1997) and binding studies (Bouaboula *et al*., 1997) indicates that SR 141716A may be an inverse agonist rather than a competitive antagonist. Moreover, Pratt *et al*. (1998) provided evidence that SR 141716A may inhibit mobilization of arachidonic acid by calcium ionophore A23187 in bovine coronary artery endothelial cells, although they did not examine whether this was a cannabinoid receptor-dependent effect.

Here, we have examined the selectivity of SR 141716A in the rat isolated mesenteric artery, as SR 141716A may have actions that cannot be explained by competitive antagonism of cannabinoid receptors, and which may be important when this agent is used at high concentrations such as those necessary to antagonize CB_2 receptor-mediated responses (Griffin *et al*., 1997), and also to inhibit the actions of EDHF (White & Hiley, 1997a, b). In particular, we have examined the actions of SR 141716A alone, and its effects on both intracellular Ca^{2+} release and Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCC). We have also used the K^+ channel activating agents, levcromakalim and NS 1619, to investigate whether SR 141716A inhibits relaxation mediated through K^+ channel activation.

A preliminary account of some of these results was presented at the Harrogate meeting of the British Pharmacological Society in December 1997 (White & Hiley, 1998a).

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Methods

Male Wistar rats (250–350 g; Tucks, Rayleigh, Essex) were killed with an overdose of sodium pentobarbital (120 mg kg^{-1}, i.p., Sagatal, Rhone Merieux, Harlow, Essex). The mesentery was then removed and placed in ice-cold, gassed (95% O_2/5% CO_2), Krebs-Henseleit solution of the following composition (mM): NaCl, 118; KCl 4.7; $MgSO_2$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; D-glucose, 5.5. Segments (2 mm in length) of third order branches of the superior mesenteric artery were removed and mounted in a Mulvany-Halpern myograph (Model 500A, JP Trading, Denmark). Vessels were allowed to equilibrate under zero tension for 60 min at 37°C in Krebs-Henseleit solution, containing indomethacin (10 μM) and bubbled with 95% O_2/5% CO_2. After equilibration, vessels were set to a normalized tension, as described in White & Hiley (1997a). The mean vessel diameter under these conditions was 350 ± 4 μm ($n = 126$). The vessels were left for another 30 min before experiments commenced. In experiments for which the endothelium was not required, it was removed by rubbing the intimal surface with a human hair (Andriantsitohaina *et al.*, 1991).

Experimental protocol

After the equilibration period, the integrity of the endothelium was assessed by precontracting the vessels with methoxamine (10 μM) and then adding carbachol (10 μM). The mean tension generated by vessels in response to methoxamine was 12.2 ± 0.3 mN ($n = 126$). Tissues which relaxed to carbachol by greater than 90% were designated as endothelium-intact, and those in which carbachol caused less than 10% relaxation were designated as endothelium-denuded.

Determination of concentration-relaxation relations

After the test for endothelial integrity, vessels were left for 30 min before being precontracted with 10 μM methoxamine in order to obtain cumulative concentration-relaxation data to the appropriate vasorelaxant. When used as an antagonist, SR 141716A (1 or 10 μM) was added to the bath 30 min before construction of the concentration-response curve to the vasorelaxant under study, and was then present throughout the remainder of the experiment. In order to examine its possible effects on relaxations mediated by K^+ channel activation, SR 141716A was used as an antagonist against the K^+ channel opening agents, levcromakalim and NS 1619. The effect of SR 141716A on relaxations induced by nitric oxide was also examined by using SR 141716A as an antagonist against relaxations induced by carbachol, and the nitric oxide donor agent SNAP.

In experiments investigating the relaxant response to SR 141716A, vessels were left for 30 min after the test for endothelial integrity, then precontracted with 10 μM methoxamine, after which SR 141716A was added cumulatively to the bath. Relaxations to SR 141716A were examined in both the presence and absence of endothelium. In some experiments, vessels were precontracted with high K^+ (25 or 60 mM) Krebs-Henseleit solution instead of methoxamine. This was prepared by equimolar substitution of NaCl for KCl in the standard Krebs-Henseleit buffer described above. The mean tension generated by vessels in response to 60 mM KCl was 13.0 ± 1.9 mN ($n = 6$), which was not significantly different from the response to 10 μM methoxamine. As treating vessels with 25 mM KCl alone produced only small contractions, methoxamine (1–5 μM) was added to give a similar level of tone to that induced by 10 μM methoxamine in the test of endothelial integrity.

Effect of SR 141716A on intracellular Ca^{2+} release

In order to investigate possible effects of SR 141716A on agonist-induced release of intracellular Ca^{2+}, vessels were treated according to the methods used by Julou-Schaeffer & Freslon (1988). Briefly, intracellular stores were replenished by incubating vessels for 45 min in normal Krebs-Henseleit solution, with the bath solution being changed four times during this period. Extracellular Ca^{2+} was then removed by washing vessels three times with Ca^{2+}-free Krebs-Henseleit solution (composition the same as normal Krebs-Henseleit buffer but with $CaCl_2$ omitted), and then incubating them for a further 45 min in Ca^{2+}-free solution, with the bath solution being changed a further four times during this period. Release of intracellular stores was then determined by eliciting phasic contractions to methoxamine (10 μM), after which the washing process was repeated. Contractions to methoxamine were found to be reproducible in any given vessel, hence after eliciting a control response, a single test response was determined after incubating vessels with SR 141716A (1 or 10 μM) for 30 min prior to the addition of methoxamine. After determination of a control and a test response, vessels were discarded.

Effect of SR 141716A on extracellular Ca^{2+} entry

The effect of SR 141716A on influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels was examined in vessels depleted of intracellular Ca^{2+} stores according to the method of Julou-Schaeffer & Freslon (1988). Briefly, vessels were stimulated with 10 μM methoxamine in Ca^{2+}-free Krebs-Henseleit solution containing 1 mM EGTA, followed by washing with solution of the same composition, until no contractile response to methoxamine was observed, at which point intracellular stores were considered to have been depleted. Vessels were then washed with Ca^{2+}-free Krebs-Henseleit solution without EGTA, and 10 μM methoxamine was added. Methoxamine alone had no effect under these conditions. Cumulative contractions to $CaCl_2$ (10 μM to 10 mM) were then elicited, and the washing process was then repeated. Concentration-response curves to $CaCl_2$ were found to be reproducible in any given vessel, hence after first establishing a control curve and washing the vessels, a second test curve was carried out after incubating vessels for 30 min with SR 141716A (1 or 10 μM). Contractions were normalized to the maximum contraction of the vessel to 10 μM methoxamine. The L-type Ca^{2+} channel inhibitor, verapamil (10 μM for 30 min), was used to investigate the role of these channels in the agonist-stimulated influx of extracellular Ca^{2+}.

Data and statistical analysis

All relaxation responses are expressed as the percentage relaxation of the tone induced by methoxamine or 60 mM K^+. Data are given as the means ± s.e.mean. Where appropriate, EC_{50} values for vasorelaxant responses were obtained from individual concentration-response curves by fitting the data to the logistic equation:

$$R = \frac{R_{max} \cdot A^{n_H}}{EC_{50} \cdot A^{n_H} + A^{n_H}}$$

where R is reduction in tone, A the concentration of the agonist, R_{max} the maximum reduction of established tone, n_H

the slope function and EC_{50} the concentration of relaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph software (Version 3.0.2, Synergy Software, Reading, PA, U.S.A.) running on a Macintosh computer. Statistical analysis of the variables was carried out by two-way analysis of variance and an *F*-test.

For concentration-response curves to Ca^{2+}, where concentration-response data could not be fitted to a logistic function, statistical comparison of the data was carried out by Student's unpaired *t*-test at individual concentrations. For comparison of phasic contractions to methoxamine in Ca^{2+}-free conditions, the paired *t*-test was used.

In the case of concentration-response curves to SR 141716A and NS 1619, solubility limitations prevented the definition of a true maximum response. Hence data are expressed as $pEC_{40\%}$ and $pEC_{50\%}$ for SR 141716A and NS 1619 respectively, where $pEC_{40\%}$ and $pEC_{50\%}$ represent the mean of the negative logarithm of the concentration of agent producing 40% or 50% relaxation of tone respectively, and the maximum relaxations given are those obtained at the highest concentration that could be used.

P values less than 0.05 were considered to be statistically significant.

Drugs

All solutions were prepared on the day of the experiment. Methoxamine, L-NAME and carbachol (Sigma Chemical Company, Poole, Dorset, U.K.) were dissolved in distilled water. EGTA (Sigma) was dissolved in Krebs-Henseleit solution. Iberiotoxin (Research Biochemicals International, Natick, MA, U.S.A.) was reconstituted in distilled water. NS 1619 (RBI), verapamil (Sigma) and SNAP (Calbiochem, Nottingham, U.K.) were dissolved in 100% ethanol. SR 141716A was provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health (Contract N01MH30003), and was dissolved in 100% ethanol. Levcromakalim (SmithKline Beecham, Betchworth, Surrey, U.K.) and pinacidil (RBI) were dissolved as 10 mM stock solutions in 70% (v/v) ethanol. Indomethacin (Sigma) was dissolved in 5% (w/v) $NaHCO_3$ solution. Dilutions were made in distilled water.

Results

Relaxations to SR 141716A in the presence and absence of endothelium

Figure 1a shows that SR 141716A caused concentration-dependent relaxations of methoxamine-induced tone in vessels with intact endothelium ($pEC_{40\%} = 4.64 \pm 0.08$; maximum relaxation at 100 μM = $78.5 \pm 2.4\%$; $n = 8$). Removal of the endothelium from vessels did not significantly affect the relaxation response to SR 141716A (in the absence of endothelium, $pEC_{40\%} = 4.51 \pm 0.06$; maximum relaxation at 100 μM = $68.8 \pm 4.3\%$; $n = 8$).

Relaxations to SR 141716A in endothelium-denuded vessels precontracted with 60 mM KCl

SR 141716A induced relaxation of methoxamine-precontracted vessels without endothelium with a $pEC_{40\%} = 4.51 \pm 0.09$, and a maximum relaxation at 100 μM = $72.8 \pm 6.2\%$ ($n = 4$). Precontracting vessels with 60 mM KCl Krebs-Henseleit solution did not produce a significantly different curve for SR 141716A-induced relaxation ($pEC_{40\%} = 4.51 \pm 0.06$; maximum relaxation at 100 μM = $58.5 \pm 7.6\%$; $n = 6$; Figure 1b).

Effect of SR 141716A on contractions to $CaCl_2$ in endothelium-denuded vessels depleted of intracellular Ca^{2+}

In vessels depleted of intracellular Ca^{2+} stores, and stimulated with methoxamine (10 μM) as described in Methods, $CaCl_2$ (0.01 – 1 mM) produced concentration-

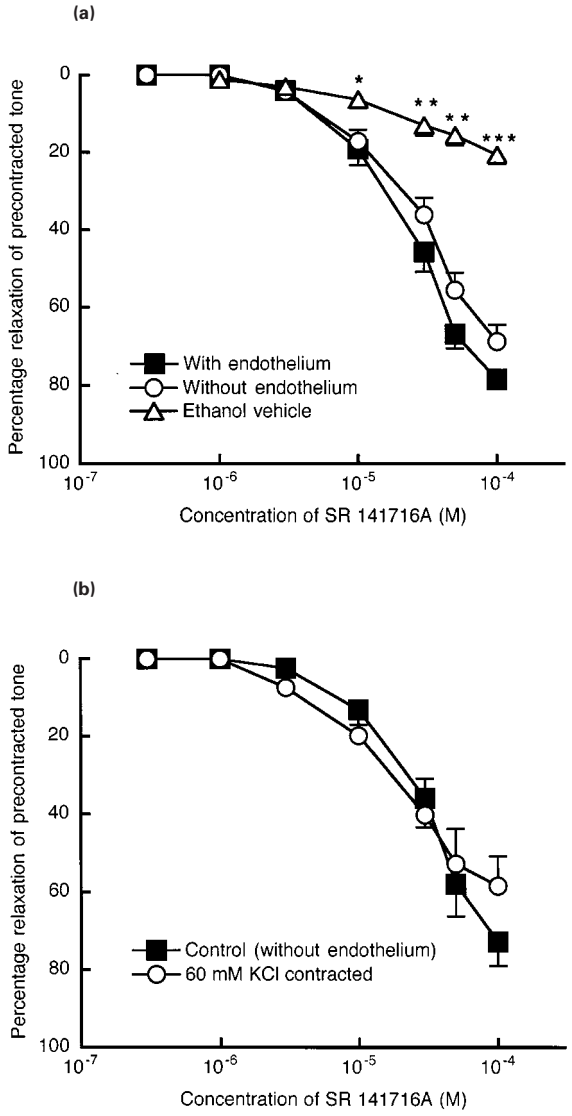

Figure 1 Concentration-response curves for relaxation of methoxamine- or KCl-induced tone in the rat isolated mesenteric artery. Relaxation was induced by SR 141716A and was determined (a) in the presence ($n = 8$) or absence ($n = 8$) of a functional endothelium, and compared with the effects of the appropriate concentration of vehicle, 100% ethanol ($n = 4$). In (b), relaxations to SR 141716A were determined in vessels precontracted with either methoxamine ($n = 4$) or 60 mM KCl ($n = 6$). Values are shown as means and vertical lines indicate s.e.mean. Concentration-response data for SR 141716A were compared by comparison of $pEC_{40\%}$ and maximum relaxation values as described in Methods, and the parameters are given in the text. Comparison of relaxations to SR 141716A with those of its vehicle were made by unpaired *t*-test at individual concentrations. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ indicate significant differences of vehicle-induced relaxations from those in the presence of SR 141716A.

dependent contractions up to 1 mM, but caused relaxation at higher concentrations ($n=8$). Figure 2 shows that the response to $CaCl_2$ was not affected by prior incubation of vessels for 30 min with 1 μM SR 141716A ($n=4$). However, 10 μM SR 141716A caused a significant rightward shift in the Ca^{2+} concentration-contraction curve ($n=4$). Figure 2 also shows that the L-type Ca^{2+} channel inhibitor, verapamil (10 μM), greatly reduced contractions to $CaCl_2$ ($n=4$).

Effect of SR 141716A on phasic contractions to methoxamine in endothelium-denuded vessels in the absence of extracellular Ca^{2+}

Methoxamine (10 μM) caused phasic contractions in vessels in the absence of extracellular Ca^{2+}. Figure 3 shows that prior incubation of preparations for 30 min with either 1 μM ($n=3$) or 10 μM SR 141716A ($n=6$) had no effect on the magnitude of the response to methoxamine.

Effect of SR 141716A on relaxations to levcromakalim in vessels without endothelium

Figure 4 shows that levcromakalim caused concentration-dependent relaxations of methoxamine-induced tone in endothelium-denuded vessels ($EC_{50}=0.64\pm0.08$ μM; $R_{max}=81.6\pm3.5\%$; $n=10$). The potency of levcromakalim was not affected by 1 μM SR 141716A ($EC_{50}=0.70\pm0.03$ μM; $n=10$), but was significantly ($P<0.001$) reduced by approximately 130 fold in the presence of 10 μM SR 141716A ($EC_{50}=82.7\pm20.7$ μM; $n=4$). Neither concentration of SR 141716A altered the maximum response to levcromakalim (1 μM SR 141716A, $R_{max}=76.6\pm1.4\%$; 10 μM SR 141716A, $R_{max}=79.9\pm6.7\%$; Figure 4a). Similar results were obtained using pinacidil, another K_{ATP} activator (data not shown).

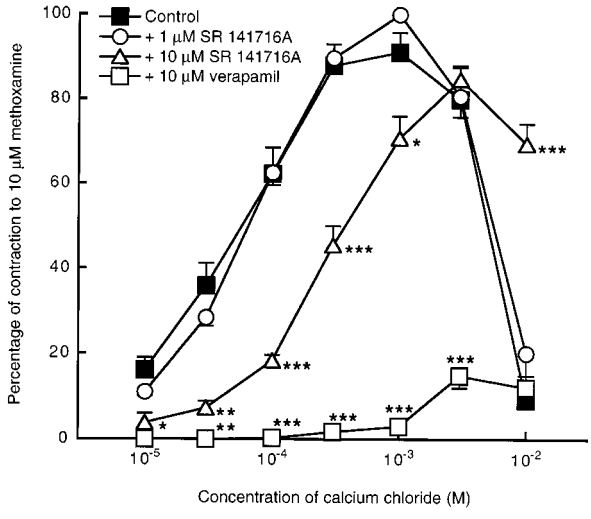

Figure 2 Concentration-response curves for $CaCl_2$-induced contractions of methoxamine-stimulated (10 μM) rat isolated mesenteric arteries depleted of intracellular Ca^{2+} stores with EGTA as described in Methods. All vessels were denuded of endothelium. Contractions were determined in the absence ($n=8$) or presence of 1 μM SR 141716A ($n=4$) or 10 μM SR 141716A ($n=4$), or in the presence of 10 μM verapamil ($n=4$). Values are shown as means and vertical lines indicate s.e.mean. Statistical comparisons were made by unpaired t-test at individual concentrations. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ indicate significant differences from control values.

Effect of SR 141716A on relaxations to NS 1619 in endothelium-denuded vessels

NS 1619 caused concentration-dependent relaxation of methoxamine-induced tone in endothelium-denuded vessels (Figure 5; Table 1). The results show that although 1 μM SR 141716A had no significant effect on relaxations to NS 1619, 10 μM SR 141716A significantly attenuated relaxations to the K^+ channel activator. The BK_{Ca} channel blocker, iberiotoxin (100 nM) also reduced the responses to NS 1619, and the effect of iberiotoxin was not significantly different from that of 10 μM SR 141716A. Figure 5 and Table 1 also show that the inhibitory effect of a combination of 10 μM SR 141716A and

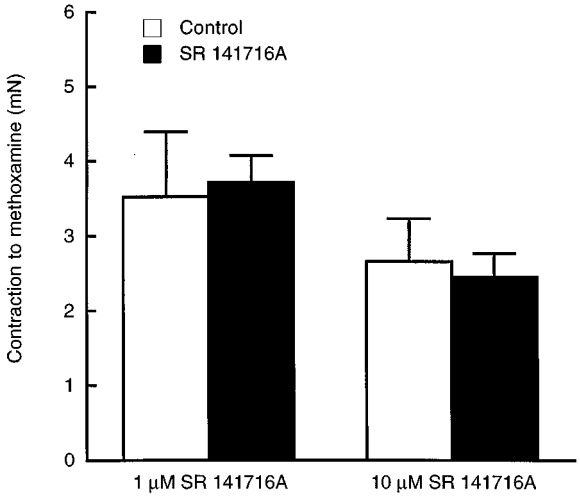

Figure 3 Contractions of rat isolated mesenteric artery induced in the absence of extracellular Ca^{2+} by methoxamine (10 μM) either in the absence or presence of 1 μM ($n=3$) or 10 μM SR 141716A ($n=6$). All vessels were denuded of endothelium. Values are shown as means and vertical lines indicate s.e.mean. Control and test data were compared by paired t-test as indicated; no significant differences were found between control contractions and those determined in the presence of SR 141716A.

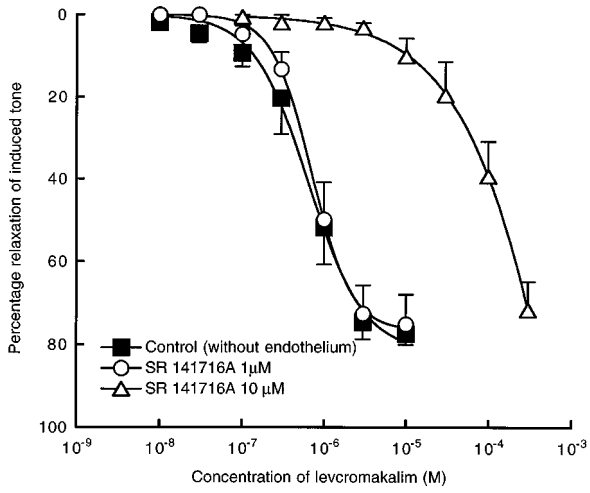

Figure 4 Concentration-response curves for relaxation of methoxamine-induced tone in the endothelium-denuded rat isolated mesenteric artery. Relaxations were elicited by levcromakalim alone ($n=10$), and in the presence of 1 μM ($n=10$) or 10 μM SR 141716A ($n=4$). Values are shown as means and vertical lines indicate s.e.mean. Data were fitted to a logistic function, and the parameters for the curve fitting procedure are given in the text.

100 nM iberiotoxin was not significantly greater than the inhibition caused by either agent alone.

Effect of SR 141716A on relaxations to SNAP and carbachol

Figure 6a shows that carbachol induced endothelium-dependent relaxation in the presence of 300 μM L-NAME ($EC_{50}=0.65\pm0.02$ μM; $R_{max}=93.2\pm0.4\%$; $n=6$). SR 141716A (1 μM) caused a 3.4 fold rightward shift in the concentration-response curve ($P<0.01$) but did not alter the maximum response (in the presence of 1 μM SR 141716A, $EC_{50}=2.22\pm0.07$ μM; $R_{max}=95.5\pm0.9\%$; $n=6$). In the presence of 300 μM L-NAME, SR 141716A (10 μM) reduced the maximum response to carbachol to $10.8\pm3.2\%$ ($n=4$).

The nitric oxide donor, SNAP, induced relaxation of endothelium-denuded vessels ($EC_{50}=0.16\pm0.02$ μM; $R_{max}=102.1\pm2.8\%$; $n=4$) which was not affected by 10 μM SR 141716A (SNAP: $EC_{50}=0.19\pm0.03$ μM; $R_{max}=104.4\pm2.5\%$; n=4; Figure 6b).

Carbachol also induced endothelium-dependent relaxation of methoxamine-induced tone in the absence of L-NAME (control $EC_{50}=0.66\pm0.05$ μM; $R_{max}=96.4\pm1.9\%$; $n=7$). The potency of carbachol, but not the maximum relaxation, was reduced in the presence of 25 mM KCl ($EC_{50}=2.08\pm0.59$ μM; $R_{max}=88.6\pm7.1\%$; $n=4$). Neither the potency nor maximum relaxation of carbachol in the presence of 25 mM KCl were affected by 10 μM SR 141716A ($EC_{50}=3.92\pm0.97$ μM; $R_{max}=94.0\pm6.4\%$; $n=4$; Figure 6c).

1 μM SR 141716A was found to have no effect on either SNAP-induced relaxations or those to carbachol in the absence of L-NAME (data not shown).

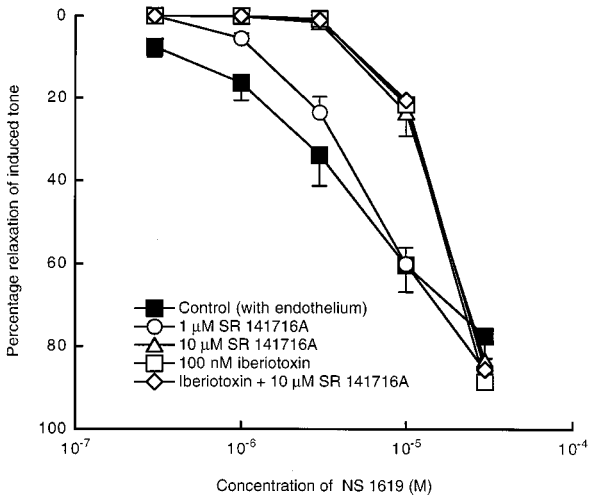

Figure 5 Concentration-response curves for relaxation of methoxamine-induced tone by NS 1619 in the endothelium-denuded rat isolated mesenteric artery. Relaxations were elicited by NS 1619 alone ($n=5$), or in the presence of 1 μM ($n=6$) or 10 μM SR 141716A ($n=4$), or in the presence of 100 nM iberiotoxin ($n=3$) or a combination of 10 μM SR 141716A with 100 nM iberiotoxin ($n=3$). Values are shown as means and vertical lines indicate s.e.mean. Concentration-response data for NS 1619 were compared by comparison of $pEC_{50\%}$ and maximum relaxation values as described in Methods, and the parameters are given in Table 1.

Discussion

This study provides evidence that the cannabinoid receptor antagonist, SR 141716A, has vasorelaxant effects in vascular smooth muscle that appear to be independent of cannabinoid receptor blockade. It seems likely that SR 141716A causes its endothelium-independent vasorelaxation through inhibition of extracellular Ca^{2+} entry through L-type Ca^{2+} channels, and it also inhibits relaxation induced by K^+ channel activating agents.

SR 141716A caused concentration-dependent relaxations of methoxamine-induced tone that were independent of the presence of endothelium. The threshold concentration, that is the highest concentration at which relaxations to SR 141716A were not significantly greater than relaxations to its vehicle, ethanol, was 3 μM. Hence experiments examining the actions of SR 141716A as an antagonist were carried out using both sub-threshold (1 μM) and supra-threshold (10 μM) concentrations.

We have shown previously that SR 141716A (1 μM) inhibits endothelium-dependent relaxations in the rat isolated mesenteric artery which are attributed to EDHF (White & Hiley, 1997a,b); this was confirmed in the present study which found that, in the presence of the nitric oxide synthase inhibitor L-NAME, 1 μM SR 141716A caused a 3.4 fold shift in the concentration-response curve to carbachol. Pratt *et al*. (1998) found that, in the bovine coronary artery, SR 141716A inhibited the release of arachidonic acid by A23187, and argued that this might represent the mechanism by which this cannabinoid antagonist attenuates EDHF-mediated relaxation. It seems unlikely, however, that such a mechanism underlies the relaxant effects of higher concentrations of SR 141716A in the rat mesenteric artery, as arachidonic acid has very little relaxant effect in endothelium-intact rat mesenteric arteries ($7.8\pm2.6\%$ relaxation of methoxamine-induced tone by 10 μM arachidonic acid; $n=4$; White & Hiley, 1998b) indicating that it is probably not metabolized to vasoactive metabolites under the conditions of this study.

In the present study, SR 141716A caused relaxation of tone induced by depolarizing K^+ solution (60 mM). This

Table 1 Effects of SR 141716A and iberiotoxin on relaxation to NS 1619 in rat isolated mesenteric artery without endothelium

	$pEC_{50\%}$	*Maximum relaxation (%)*	n
Control	5.23 ± 0.14	77.6 ± 5.2	6
+10 μM SR 141716A	4.79 ± 0.04***	83.4 ± 2.2	4
+100 nM iberiotoxin	4.81 ± 0.03***	88.6 ± 1.2	3
+10 μM SR 141716A and 100 nM iberiotoxin	4.78 ± 0.01***	85.6 ± 1.8	3

Data are expressed as means$\pm$s.e.mean. $pEC_{50\%}$ values indicate the mean of the negative logarithm of the concentrations of NS 1619 causing 50% relaxation of methoxamine-induced tone. Maximum relaxations refer to the relaxation caused by the highest concentration of NS 1619 used (30 μM). *n* values indicate the number of animals used. *** indicates significantly different from control values with $P<0.001$. $pEC_{50\%}$ values for NS 1619 in the presence of both iberiotoxin and SR 141716A were not significantly different from values obtained in the presence of either iberiotoxin or SR 141716A alone.

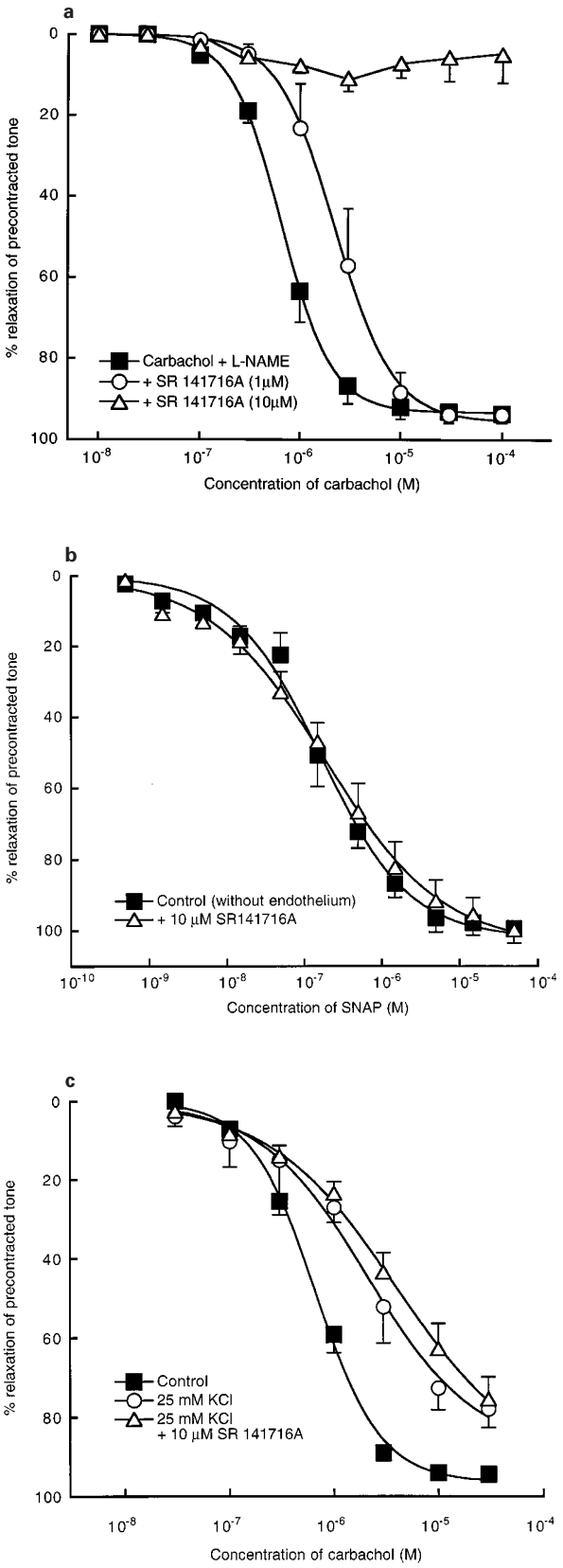

Figure 6 Concentration-response data for relaxation of methoxamine-precontracted rat isolated mesenteric arteries by (a) carbachol in the presence of L-NAME, (b) SNAP and (c) carbachol in the absence of L-NAME. (a) Relaxations to carbachol were determined in the presence of the endothelium and 300 μM L-NAME ($n=6$) and with either 1 μM ($n=6$) or 10 μM ($n=4$) SR 141716A. (b) Relaxations to SNAP were determined in the absence ($n=4$) or presence of 10 μM SR 141716A ($n=4$). All vessels were denuded of endothelium. (c) Relaxations to carbachol were determined in vessels with endothelium in the absence ($n=7$) or presence of 25 mM KCl ($n=4$), or in the presence of 25 mM KCl and 10 μM SR 141716A ($n=4$). Values are shown as means and vertical lines indicate s.e.mean. Data were fitted to a logistic function, and the parameters for the curve fitting procedure are given in the text.

shows that SR 141716A does not cause relaxation through activation of K^+ channels, as the electrochemical gradient for K^+ ion efflux is abolished in high K^+ solution. We have also carried out electrophysiological studies (White & Hiley, unpublished observations) in endothelium-denuded rat mesenteric arteries; intracellular recordings using glass microelectrodes filled with 1 M KCl (resistance 50–80 MΩ) under current-clamp conditions showed that 1 μM and 10 μM SR 141716A caused no significant change in membrane potential (0.0 ± 0.0 mV, $n=4$, and 0.8 ± 2.2 mV, $n=6$ respectively). These results confirm the finding of Chataigneau *et al.* (1998) that SR 141716A does not cause hyperpolarization of the rat mesenteric artery.

Tone induced by depolarizing K^+ solution is mediated almost entirely through the opening of voltage-operated Ca^{2+} channels (VOCC; Karaki *et al.*, 1997). Hence the ability of SR 141716A to induce relaxation under these conditions suggests that this agent may cause inhibition of VOCC opening. In order to provide further evidence for such a mechanism, we examined the effect of SR 141716A on contractions to cumulative addition of Ca^{2+} to vessels that had previously undergone depletion of intracellular stores (Julou-Schaeffer & Freslon, 1988). Under the protocol employed, the contractile effect of Ca^{2+} added to methoxamine-stimulated vessels in Ca^{2+}-free solution occurs almost entirely through influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels. Our observation that the L-type Ca^{2+} inhibitor, verapamil caused almost complete inhibition of Ca^{2+}-induced contractions confirms that Ca^{2+} influx under these conditions occurs mainly through VOCC. SR 141716A, at a concentration of 10 μM, caused a significant rightward shift of approximately 10 fold in the concentration-response curve to Ca^{2+} whereas, at 1 μM, it had no effect. These results suggest that the major relaxant effect of SR 141716A involves inhibition of Ca^{2+} influx through VOCC. It is notable that high concentrations of Ca^{2+} (>1 mM) induced vasorelaxation, confirming previous findings in the rat mesenteric artery (Julou-Schaeffer & Freslon, 1986). The relaxant effects of high concentrations of Ca^{2+} have been attributed to its 'membrane stabilizing' effects (Rothstein, 1968). The solubility limitations of $CaCl_2$ and the shift in the concentration-response curve with 10 μM SR 141716A meant that it was not possible to determine if this Ca^{2+}-induced relaxation could still occur in the presence of SR 141716A.

SR 141716A (1 or 10 μM) was found to have no significant effect on the phasic contractions to methoxamine elicited in the absence of extracellular Ca^{2+}. This shows that it has no effect on (a) the binding of methoxamine to adrenoceptors; (b) adrenoceptor-G-protein coupling; (c) IP_3-mediated release of intracellular Ca^{2+} stores, or (d) the sensitivity of contractile proteins to Ca^{2+}.

We have also examined the nature of the inhibition of EDHF by SR 141716A. We previously showed that 10 μM SR 141716A almost completely abolished EDHF-mediated relaxations to carbachol and calcium ionophore A23187 in the rat mesenteric artery (White & Hiley, 1997a); this was confirmed in the present investigation. As EDHF acts through the opening of K^+ channels (Cohen & Vanhoutte, 1995), we

postulated that SR 141716A might inhibit EDHF through inhibition of K^+ channel opening. The K^+ channel activating agents, levcromakalim (an opener of ATP-sensitive K^+ channels or K_{ATP}) and NS 1619 (an opener of large conductance Ca^{2+}-activated K^+ channels or BK_{Ca}; Holland *et al*., 1996) were used to test this hypothesis.

At a concentration of 10 μM, SR 141716A caused a rightward shift of 130 fold in the concentration-response curve to levcromakalim; similar results were obtained using another K_{ATP} opener, pinacidil, which suggests that 10 μM SR 141716A is a potent inhibitor of relaxation induced by activation of K_{ATP}. Indeed, Chataigneau *et al*. (1998) recently reported a similar inhibition of K_{ATP}-mediated hyperpolarization. At low concentrations, NS 1619 causes relaxation through activation of BK_{Ca}; however at high concentrations it relaxes vessels through inhibition of VOCC (Holland *et al*., 1996). In the present study, iberiotoxin (100 nM), a potent and selective inhibitor of BK_{Ca} (Galvez *et al*., 1990) significantly attenuated NS 1619-induced relaxation. SR 141716A (10 μM) inhibited relaxation to NS 1619 to a similar degree, and it is notable that the inhibitory effects of SR 141716A and iberiotoxin were not additive. Although the K^+ channels activated by EDHF have not been characterized, our observation that 10 μM SR 141716A causes inhibition of relaxation induced by activation of both K_{ATP} and BK_{Ca} channels, which are structurally distinct, suggests that inhibition of K^+ channels by 10 μM SR 141716A may play some part in its inhibitory effect on EDHF-mediated relaxation.

Although we reported that 1 μM SR 141716A caused a modest inhibition of EDHF-mediated relaxation (White & Hiley, 1997a; this study), this concentration had no effect on relaxation of endothelium-denuded vessels by levcromakalim or NS 1619. However we have recently shown that 1 μM SR 141716A inhibits an endothelium-dependent component of levcromakalim relaxation that may be mediated by EDHF (White & Hiley, 1997b). Hence inhibition of EDHF-mediated relaxation by 1 μM SR 141716A may not involve inhibition of K^+ channels on vascular smooth muscle cells. It is possible that the effect of this lower concentration of SR 141716A is simply due to cannabinoid receptor antagonism, although the effect is much smaller than would be expected for action at CB_1 receptors (White & Hiley, 1997a). It also seems possible, however, that the effect of lower concentrations of SR 141716A on EDHF-mediated relaxation can be explained by the inhibition of arachidonic acid mobilization (Pratt *et al*., 1998), but only if EDHF is a derivative of arachidonic acid, which may not be the case in the rat mesenteric artery (Fukao *et al*., 1997). It seems clear, however, that K^+ channel inhibition accounts for the more pronounced effect of higher concentrations of SR 141716A.

It is notable that the relaxant actions of anandamide were found to be insensitive to SR 141716A in the study of Pratt *et al*. (1998). Hence it seems likely that although 1 μM SR 141716A may inhibit Ca^{2+}-induced mobilization of arachidonic acid in endothelial cells, it does not affect metabolism of anandamide to arachidonic acid and thence to vasoactive derivatives. We have recently shown, however, that relaxation to anandamide in the rat mesenteric artery is sensitive to 1 μM SR 141716A. Furthermore, the synthetic cannabinoid agonists, HU-210 and CP 55,940, cause endothelium-independent relaxation that is antagonized by 1 μM SR 141716A (White & Hiley, 1998b). It is therefore likely that antagonism of anandamide-induced relaxations by SR 141716A at a concentration of 1 μM is a consequence of cannabinoid receptor antagonism.

The effects of SR 141716A on nitric oxide-mediated relaxations were used to investigate whether this agent is a non-specific inhibitor of vasorelaxation. SR 141716A (10 μM) had no effect on relaxations induced by the nitric oxide donor, SNAP, strongly suggesting that it is not a general inhibitor of relaxation. The concentration-response curve for relaxations to carbachol in the absence of nitric oxide synthase inhibitors was shifted approximately 3 fold rightwards by the presence of 25 mM KCl, a treatment which abolishes EDHF-mediated relaxation (Waldron & Garland, 1994). The lack of effect of SR 141716A on SNAP-induced relaxation suggests that this agent does not affect nitric oxide-mediated effects. We therefore postulated that the inhibition of carbachol-induced vasorelaxation caused by 10 μM SR 141716A was due to the abolition of an EDHF-mediated component. Evidence that this is indeed the case was our finding that relaxations induced by carbachol in the presence of 25 mM KCl, which are mediated almost entirely by nitric oxide (Waldron & Garland, 1994), were unaffected by 10 μM SR 141716A.

The precise mechanism by which SR 141716A causes inhibition of K^+ and Ca^{2+} channels is difficult to elucidate, as the second messenger pathways for cannabinoid receptors are poorly understood. Recent evidence suggests that the CB_1 receptor may couple to G-proteins which can both stimulate or inhibit adenylyl cyclase (Felder *et al*., 1998), hence the relative contribution of each may determine the overall functional effect. Moreover, SR 141716A may be an inverse agonist at cannabinoid receptors (Coutts & Pertwee, 1997; Bouaboula *et al*., 1997), hence its effects on second messenger pathways are likely to be very complex. The current lack of availability of other cannabinoid receptor antagonists prevents examination of whether the actions of SR 141716A are dependent or independent of cannabinoid receptors.

In summary, the present study has shown that, at concentrations of 10 μM and above, SR 141716A causes endothelium-independent vascular relaxation through inhibition of VOCC, and also inhibits relaxations mediated by K^+ channel activation. These results suggest that such concentrations of SR 141716A are not appropriate for the investigation of cannabinoid receptor-dependent processes.

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