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Cannabinoids inhibit pre- and postjunctionally sympathetic neurotransmission in rat mesenteric arteries

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Abstract

The effects of cannabinoids on sympathetic neurotransmission in the rat isolated perfused mesenteric arterial bed, were investigated. Electrically evoked sympathetic neurogenic vasocontraction was inhibited by the cannabinoid receptor agonists 11-hydroxy-dimethylheptyl-Δ^8-tetrahydrocannabinol (HU210), (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)-cyclohexanol (CP55,940) and methanandamide, and by (+)-11-hydroxy-Δ^8-tetrahydrocannabinol (HU211), a (+)-stereoisomer of HU210. The inhibition was unaffected by cannabinoid CB_1 and CB_2 receptor antagonists. Electrically evoked release of endogenous noradrenaline from sympathetic nerves was inhibited by HU210, but not by HU211. Inhibition was blocked by a cannabinoid CB_1, but not a CB_2, receptor antagonist. HU210 attenuated contractions to noradrenaline, and all of the cannabinoids blocked contractions to KCl. Capsaicin pre-treatment had no significant effect on HU210- and CP55,940-mediated inhibition of sympathetic neurogenic contraction, but partly blocked inhibition mediated by methanandamide. These data show that cannabinoids can inhibit, by distinct pre- and postjunctional actions, sympathetic neurotransmission in the rat mesenteric arterial bed. The pre-junctional action is mediated by a cannabinoid CB_1-like receptor, but the postjunctional action does not appear to involve either cannabinoid CB_1 or CB_2 receptors.

Keywords: Cannabinoid; Sympathetic neurotransmission; Sensory nerve; Mesenteric arterial bed, rat; Capsaicin; Methanandamide

1. Introduction

Cannabinoids elicit pronounced effects on the cardiovascular system including bradycardia, hypotension and endothelium-dependent and -independent vasorelaxation (Wagner et al., 1999; Randall et al., 1996; Randall and Kendall, 1998; White and Hiley, 1998; Harris et al., 1999; Járai et al., 1999). Two main types of cannabinoid receptors, both G protein-coupled, have been identified. Cannabinoid CB_1 receptors are found principally in the brain, but also in the peripheral nervous system, whilst cannabinoid CB_2 receptors are found mainly in the periphery, in association with immune tissues (see Randall and Kendall, 1998). The possible involvement of cannabinoid receptor(s) in the regulation of vascular tone, however, is unclear. Indeed, it was recently shown that vanilloid VR1 receptors on perivascular sensory nerves play a crucial role in mediating vascular responses to anandamide and methanandamide, being activated by these cannabinoids to cause a release of calcitonin gene-related peptide and vasorelaxation (Zygmunt et al., 1999; Ralevic et al., 2000). The synthetic cannabinoid receptor agonists 3-(1,1-dimethylheptyl)-*O*-11-hydroxy-Δ^8-tetrahydrocannabinol (HU210) and (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)-cyclohexanol (CP55,940), however, are inactive at vanilloid VR1 receptors (Zygmunt et al., 1999). Furthermore, cannabinoid actions at non-CB_1 non-CB_2 vascular receptors/sites have recently been reported (Járai et al., 1999; Ford et al., 2002).

Cannabinoid receptors have been described on sympathetic nerves in some peripheral tissues, and mediate inhibition of neurotransmitter release. In the pithed rat, stimulated outflow of [^{3}H]noradrenaline is attenuated by cannabinoid receptor agonists, and blockade of this effect by the selective cannabinoid CB_1 receptor antagonist *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (SR141716A) indicates an action at cannabinoid CB_1 receptors located presynaptically on sympathetic nerves (Malinowska et al., 1997). Cannabinoid CB_1 receptors mediate inhibition of the accelerator action of sympathetic nerves in the rabbit heart, also likely via a prejunc-

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tional action (Szabo et al., 2001). Cannabinoid CB_1 receptors also mediate inhibition of [^{3}H]noradrenaline release in rat vas deferens and atria (Ishac et al., 1996), whilst in the mouse vas deferens both cannabinoid CB_1 and CB_2 receptors on sympathetic nerves mediate inhibition of the twitch response (Griffin et al., 1997; Lay et al., 2000). Little is known, however, about cannabinoid modulation of sympathetic neurotransmission in blood vessels. Indeed, there is some evidence which indicates that perivascular sympathetic neurotransmission is *not* modulated by cannabinoids. In the rat tail artery, there was no effect of synthetic cannabinoids on electrically evoked [^{3}H]noradrenaline overflow or on responses to exogenous noradrenaline (Malinowska et al., 1997), and cannabinoids have also been reported not to affect sympathetic neurotransmission in rat isolated small mesenteric arteries (Lay et al., 2000).

In preliminary studies, however, we have shown that cannabinoids can modulate sympathetic neurotransmission in the rat isolated mesenteric arterial bed (Ralevic and Kendall, 2000). Thus, the aim of the present study was to characterise pharmacologically the actions of endocannabinoid-like (methanandamide) and synthetic (HU210 and CP55,940) cannabinoid receptor agonists as modulators of sympathetic neurotransmission in this vascular preparation. Their effects on sympathetic neurogenic contractile responses to electrical field stimulation and to exogenous agonists were investigated. In addition, we measured noradrenaline levels in fractions of effluent, collected from the perfused mesentery during electrical field stimulation, in the absence and presence of HU210 (and its inactive (+)-stereoisomer HU211), with and without antagonists, in order to determine directly whether cannabinoids can act prejunctionally to modulate sympathetic neurotransmitter release. Rat mesenteric arteries are richly innervated by both sympathetic and sensory nerves, and the two nerve populations can be concomittantly activated by electrical field stimulation. As endogenous cannabinoids can activate perivascular sensory nerves (Zygmunt et al., 1999; Ralevic et al., 2000), and sensory nerves can modulate sympathetic neurotransmission (Li and Duckles, 1992), we additionally investigated the possible involvement of sensory nerves in cannabinoid modulation of sympathetic neurotransmission.

2. Materials and methods

2.1. Isolated mesenteric arterial bed preparation

Male Wistar rats (250–300 g) were killed, after exposure to CO_2, by decapitation. Mesenteric beds were isolated and set up for perfusion as described previously (Ralevic et al., 1995). In brief, the abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 ml of Krebs' solution and the gut dissected away carefully from the mesenteric vasculature. The preparation was mounted on a stainless steel grid (7 × 5 cm) in a humid chamber and perfused at a constant flow rate of 5 ml min^{-1} using a peristaltic pump (model 7554-30, Cole-Parmer Instrument, Chicago, IL). The perfusate was Krebs' solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2–5% CO_2 and maintained at 37 °C. Responses of the mesenteric arterial beds were measured as changes in perfusion pressure (mm Hg) with a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA) on a side arm of the perfusion cannula, and recorded on a polygraph (model 7D, Grass Instrument, Quincy, MA). Preparations were allowed to equilibrate for 30 min prior to experimentation.

2.2. Experimental protocol

Electrical field stimulation (10–64 Hz, 90 V, 1 ms, for 5 s, at 2-min intervals) was applied in order to generate frequency-dependent contractile responses. Responses were blocked by guanethidine (5 μM) indicating that they were mediated by sympathetic nerves, and by prazosin (1 μM), indicating that they are mediated primarily by the release of noradrenaline acting at α_1-adrenoceptors (data not shown). Three responses at each frequency were obtained and the response calculated as the mean of the second and third contractions. Three frequency response curves were constructed in each preparation. In another group of preparations, exogenous noradrenaline (0.5–150 nmol) was applied by bolus injections (50 μl) into norprene rubber tubing proximal to the preparations and three dose–response curves were constructed in each preparation. Preliminary studies had showed that, in a series of three consecutive response curves to electrical field stimulation or exogenous noradrenaline, reproducibility was obtained between the second and third response curves. Responses curves for both electrical field stimulation and exogenous noradrenaline were constructed in about 30 min, and an interval of 20 min was allowed between response curves. Cannabinoid receptor agonists were added to the perfusate after the second frequency– or dose–response curve and, after 15 min, their effects on the third response curve to electrical field stimulation or noradrenaline were tested. Cannabinoid receptor antagonists were added either after the second frequency response curve, in order to evaluate (after 15 min exposure) their effects alone on electrical field stimulation (third frequency response curve), or after the first frequency–response curve, in order that responses to electrical field stimulation in the presence of antagonist (second frequency response curve) could be compared to responses to electrical field stimulation in the presence of antagonist plus agonist (third frequency response curve). Thus, antagonists were in contact with the tissue for about 1 h prior to challenge with agonists.

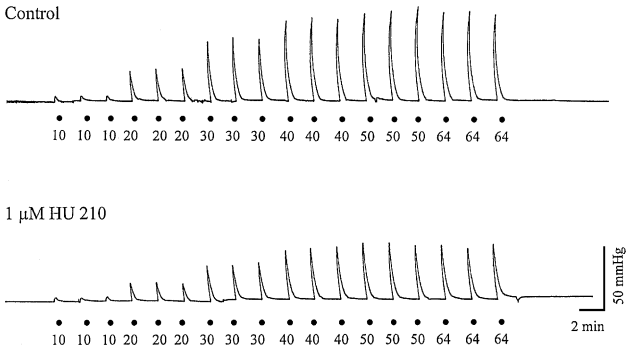

Fig. 1. Representative trace showing the effect of cannabinoid HU210 on contractile responses to electrical field stimulation (10–64 Hz) in a single rat isolated perfused mesenteric arterial bed. Contractions are shown in the absence (control) and in the presence of 1 μM HU210. Responses were attenuated by HU210.

In another group of preparations contractile responses to doses (50 μl) of methoxamine (0.5 nmol–5 μmol), α,β-methylene ATP (α,β-meATP; 0.05–5 nmol), KCl (5–150 μmol) and vasopressin (0.005–1.5 nmol) were investigated under control conditions (absence of agents or in the presence of dimethylsulphoxide) or in the presence of methanandamide, CP55,940, HU210 and HU211 (all at 1 μM; added 15 min prior to the onset of experimentation). In another group of preparations, a control response curve to electrical field stimulation was constructed and then preparations were pre-treated with capsaicin at 10 μM for 1 h, followed by 30 min washout, in order to cause selective desensitization and/or sensory neurotransmitter depletion (Ralevic et al., 2000). Two further frequency response curves to electrical field stimulation were generated after capsaicin pre-treatment, and in time-matched control experiments these were shown to be reproducible. The effects of cannabinoids were tested on the third frequency–response curve and compared to the second frequency response curve. Only a single agonist, or agonist plus antagonist was tested per preparation. In control experiments neither ethanol (0.03%) nor dimethylsulphoxide (0.03%) affected sympathetic or sensory neurogenic responses to electrical field stimulation. Dimethylsulphoxide (0.01%) had no effect on responses to exogenously applied vasoconstrictors.

2.3. Measurement of endogenous noradrenaline release

Fractions of the effluent of a group of mesenteric beds were collected on dry ice and stored at −20 °C for assay of levels of endogenous noradrenaline. After equilibration of the preparations for 1 h, two fractions were collected, one before and one during electrical field stimulation (nine stimulations at 64 Hz, 1 ms, 90 V applied for 5 s, every 30 s) in the absence of drugs. HU210 (3 μM), or HU211 (3 μM), or the equivalent concentration of the vehicle dimethylsulphoxide, was added and, after a further 1 h equilibration, two further fractions were collected, one before and one during electrical field stimulation in the presence of drug or dimethylsulphoxide. In a separate series of experiments 1 μM SR144528 or 1 μM LY321035, added at the onset of the first period of equilibration, were present throughout the experiment with HU210. Noradrenaline was assayed by high performance liquid chromatography with electrochemical detection as described by Forster and Macdonald (1999) and levels are expressed as nmol l^{-1}.

2.4. Data analysis

Contractile responses of the mesenteric arterial beds were measured as increases in perfusion pressure (mm Hg) above baseline. F_{50} is the frequency of stimulation (Hz) required to elicit a half maximal response. Assessment within preparations of the effect of cannabinoids on electrical field stimulation were made using paired Student's *t*-test. Assessment of the effect of treatments on values between more than two groups was by analysis of variance (ANOVA) with Tukey's post-hoc test. A value of $P<0.05$ was taken to indicate a statistically significant difference.

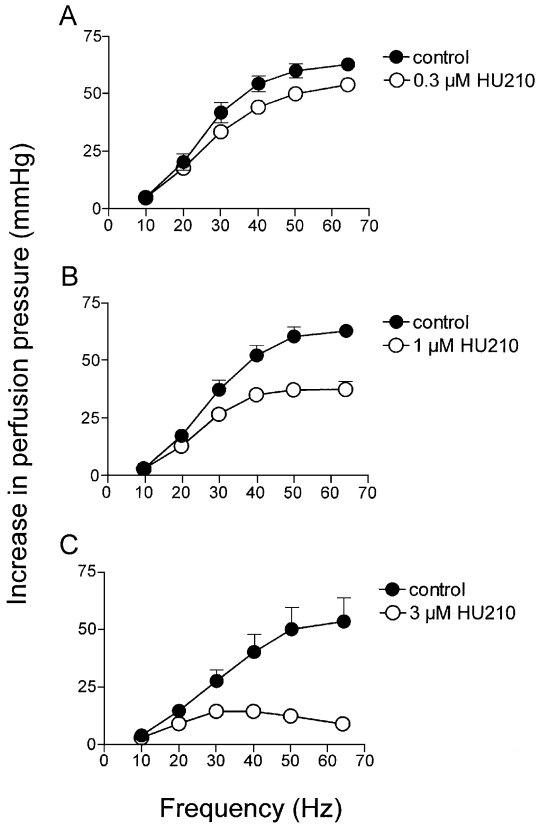

Fig. 2. Effect of HU210 on frequency-dependent contractions to electrical field stimulation (Hz) of rat mesenteric arterial beds. (A) 0.3 μM HU210 ($n=4$); (B) 1 μM HU210 ($n=4$); (C) 3 μM HU210 ($n=4$). Data are given as means and bars indicate S.E.M. Maximal contraction was significantly reduced at all concentrations of HU210 ($P<0.05$), but only at 3 μM HU210 was there a significant effect (decrease) in the frequency required to produce a half maximal response.

2.5. Drugs

N-([1s)-Endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) was obtained from Sanofi; (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)-cyclohexanol (CP55,940) was from Pfizer; [6-methoxy-2-(4-methoxyphenyl)benzo[*b*]thien-3-yl][4-cyanophenyl]methanone (LY320135) was from Pfizer. Methanandamide was from RBI. 3-(1,1-Dimethylheptyl)-*O*-11-hydroxy-Δ^8-tetrahydrocannabinol (HU210) was from Tocris. (+)-11-Hydroxy-Δ^8-tetrahydrocannabinol (HU211) was a gift from R. Mechoulam, Hebrew University, Jerusalem, Israel. α,β-Methylene ATP, 8-methyl-*N*-vanillyl-6-nonenamide (capsaicin), methoxamine (hydrochloride), noradrenaline (bitartrate), prazosin and vasopressin were from Sigma (Poole, UK). CP55,940, LY320135 and SR144528 were dissolved in ethanol. HU210, HU211 and capsaicin were dissolved in dimethylsulphoxide. All other drugs were dissolved in distilled water.

3. Results

3.1. Effects of HU210, HU211, CP55,940 and methanandamide on sympathetic neurogenic contractions to electrical field stimulation

The cannabinoid receptor agonist HU210 concentration-dependently attenuated contractile responses to electrical field stimulation (Figs. 1 and 2). At 0.3 μM HU210, R_{max} was reduced by 9 ± 1.9 mm Hg (from 63 ± 2.8 to 54 ± 2.6 mm Hg, $P<0.05$). At 1 μM HU210, R_{max} was reduced by 27 ± 1.5 mm Hg (from 63 ± 1.9 to 37 ± 3.4 mm Hg, $P<0.001$), and at 3 μM HU210, R_{max} was reduced by 54 ± 5.6 mm Hg (from 63 ± 2.8 to 9 ± 3.4 mm Hg ($P<0.05$) (Fig. 2). Neither 0.3 or 1 μM HU210 had a significant effect on F_{50}. At 3 μM HU210, however, F_{50} was reduced (from 29.7 ± 1.6 to 16.2 ± 1.4 Hz, $P<0.05$). The effect of HU210 (1 μM) was not reversed after 1 h washout and responses to electrical field stimulation at this stage were virtually abolished. HU211 (1 μM), a stereoisomer of HU210, also inhibited R_{max}, by 33 ± 9% ($n=4$), which was not significantly different to inhibition of R_{max} by 1 μM HU210 (41 ± 4%).

In the presence of 1 μM CP55,940, R_{max} was reduced by 25 ± 5.4 mm Hg (from 57 ± 4.4 to 33 ± 4.7 mm Hg, $P<0.01$) (Fig. 3). CP55,940 (1 μM) had no significant effect on F_{50}. Methanandamide was slightly more potent as an inhibitor of sympathetic neurotransmission than HU210 and CP55,940 (all at 1 μM) (ANOVA, $P<0.05$), attenuating R_{max}, by 39 ± 3.2 mm Hg (from 69 ± 9.1 to 30 ± 6.7 mm Hg) (Fig. 3). The F_{50} was also reduced (from 26.3 ± 1.3 to 21.8 ± 1.3 Hz) ($P<0.001$).

3.2. Effects of LY320135 and SR144528 on inhibition by cannabinoids of contractions to electrical field stimulation

LY320135 (1 μM; $n=4$), a cannabinoid CB_1 receptor antagonist, and SR144528 (1 μM; $n=8$), a cannabinoid CB_2 receptor antagonist, alone had no significant effect on contractions to electrical field stimulation. LY320135 (1 μM) and SR144528 (1 μM) had no significant effect on inhibition by HU210 (1 μM) (Fig. 4), CP55,940 (1 μM) or methanandamide (1 μM) of contractions to electrical field stimulation.

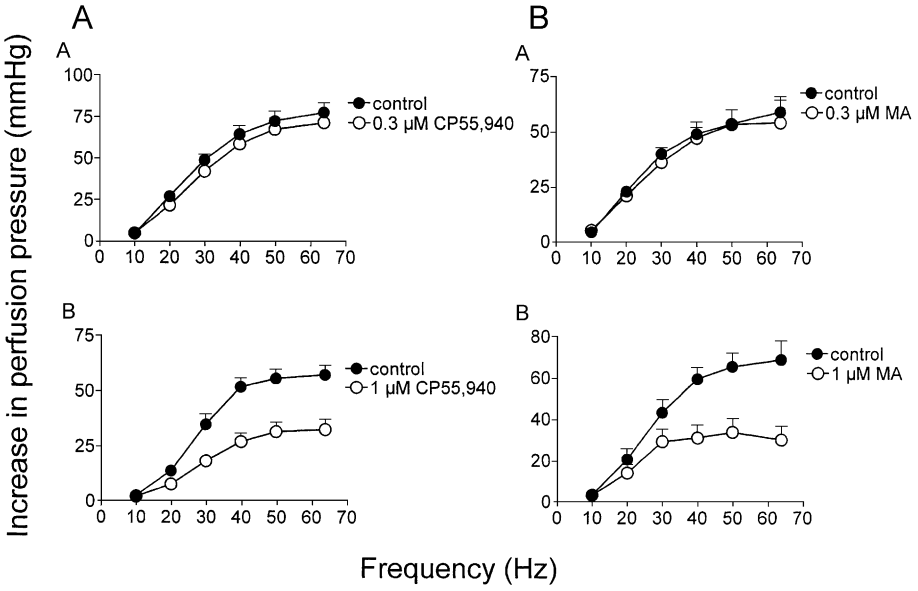

Fig. 3. Effect of cannabinoid agonists CP55,940 and methanandamide (MA) on frequency-dependent contractions to electrical field stimulation (Hz) of the rat isolated mesenteric arterial bed. (A) a: 0.3 μM CP55,940 ($n=4$); b: 1 μM CP55,940 ($n=7$). (B) a: 0.3 μM methanandamide ($n=4$); b: 1 μM methanandamide ($n=4$). Data are given as means and bars indicate S.E.M. Maximal contraction was significantly reduced at 1 μM CP55,940 ($P<0.01$) and methanandamide ($P<0.001$).

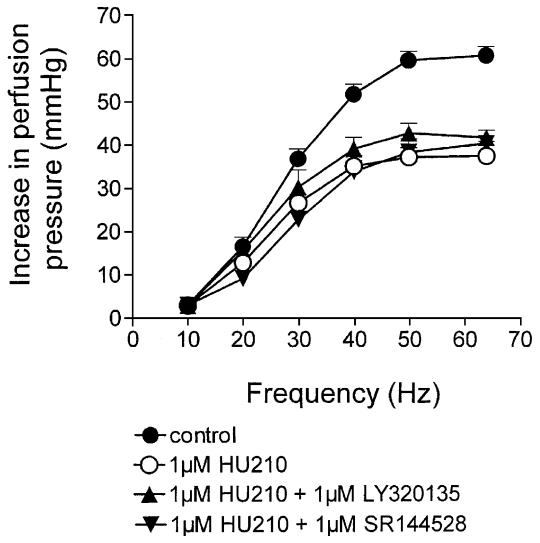

Fig. 4. Effect of the cannabinoid receptor antagonists LY320135 and SR144528 on inhibition by HU210 of contractions to electrical field stimulation (Hz) of the rat mesenteric arterial bed. Control = absence of agents ($n = 12$); 1 μM HU210 alone ($n = 4$), 1 μM HU210 + 1 μM LY320135 ($n = 4$); 1 μM HU210 + 1 μM SR144528 ($n = 4$). Data are given as means and bars indicate S.E.M. The inhibitory action of HU210 was not significantly different in absence and presence of the antagonists.

CP55,940 inhibited R_{max} by 43 ± 6% ($n = 3$) and 54 ± 2% ($n = 3$) in the presence of LY320135 and SR144528, respectively, which was not significantly different to inhibition of R_{max} by CP55,940 in the absence of antagonists (42 ± 7%, $n = 7$). Methanandamide inhibited R_{max} by 47 ± 5% ($n = 8$) and 46 ± 7% ($n = 6$) in the presence of LY321035 and SR144528, respectively, which was not significantly different to inhibition of R_{max} by methanandamide in the absence of antagonists (58 ± 6%, $n = 4$).

3.3. Effect of HU210 on electrically evoked noradrenaline release

Endogenous noradrenaline released during electrical field stimulation was detected in the perfusate of the mesenteric

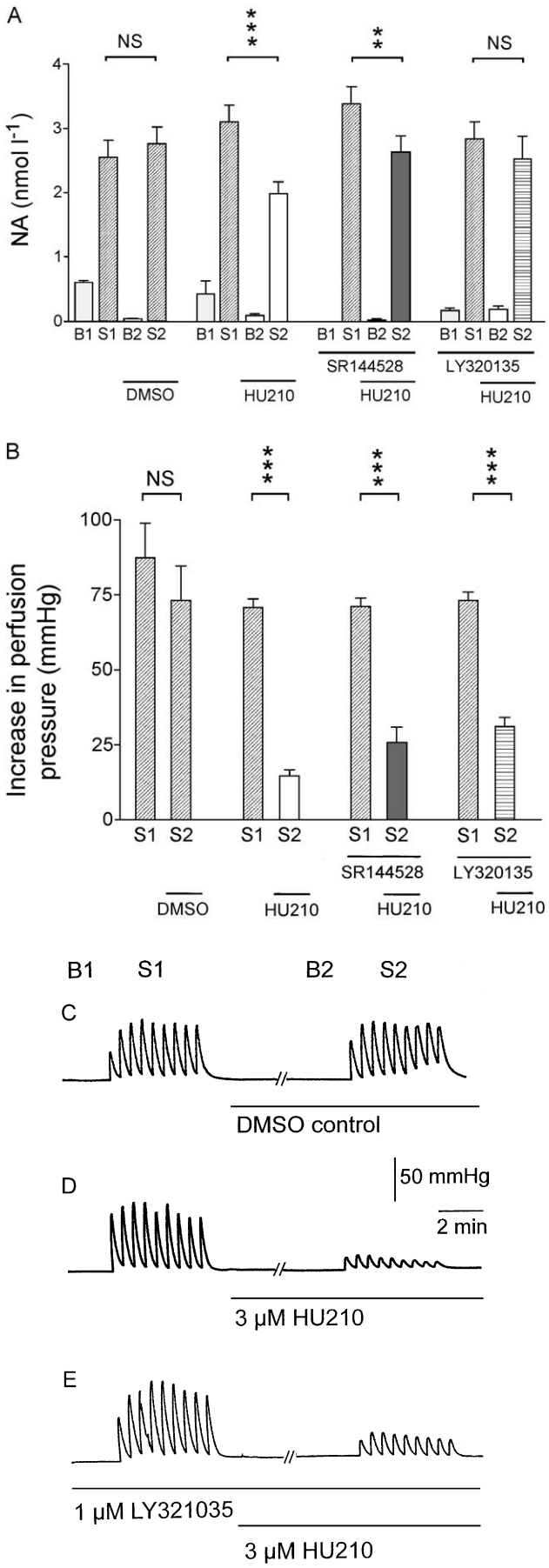

Fig. 5. The effect of HU210 on electrically evoked (A) noradrenaline (NA) release and (B) contractile activity, of the rat isolated mesenteric arterial bed. (A) Electrical field stimulation (nine stimulations at 64 Hz, 1 ms, 90 V, applied for 5 s every 30 s) evoked a significant release of NA into the perfusate on each of two occasions (S1 and S2), where S1 was carried out in the absence of drugs, and S2 was carried out in the presence of vehicle (dimethylsuphoxide, DMSO) ($n = 8$). In another group of mesenteric arterial beds, 3 μM HU210 inhibited NA release during S2 compared to release during S1 ($P < 0.001$; $n = 6$). LY320135 (1 μM; $n = 5$), but not SR144528 (1 μM; $n = 5$), antagonised HU210-mediated inhibition of NA release. B1, B2 = pre-stimulation fractions; S1, S2 = fractions collected during electrical field stimulation. (B) The functional correlate to the release experiment in (A) shows that inhibition by HU210 (3 μM) of neurogenic contractions is not blocked by SR144528 and LY321035. Data are given as means and vertical bars indicate S.E.M. (C–E) Representative traces of experiments used in (A,B). These show that the vehicle control, DMSO, had no effect on the contractile response (C), but contractions were inhibited by HU210 (D), and the inhibition was not reversed by LY320135 (E). ** $P < 0.01$; *** $P < 0.001$.

arterial beds (Fig. 5A). The noradrenaline level was not significantly different between fractions collected during the first (S1) and second periods of electrical field stimulation (S2) in the presence of vehicle (dimethylsulphoxide) alone. HU210 (3 μM) caused a significant inhibition of noradrenaline release (from 3.18 ± 0.19 to 1.99 ± 0.19 nmol noradrenaline l^{-1}; $P < 0.001$), equivalent to a $37 \pm 7\%$ decrease in neurotransmitter release (Fig. 5A).

3.4. Effects of SR144528 and LY320135 on HU210-mediated inhibition of electrically evoked noradrenaline release

Neither LY320135 (1 μM) nor SR144528 (1 μM) alone significantly affected electrically evoked noradrenaline release (Fig. 5A). In the presence of LY321035, but not SR144528, the inhibitory effect of HU210 on evoked noradrenaline release was blocked (Fig. 5A). Fig. 5B is the functional correlate for the noradrenaline release experiment in Fig. 5A, and shows that neither SR144528 nor LY320135 significantly blocked inhibition by HU210 of the contractile response to electrical field stimulation, consistent with the pharmacological studies reported in Section 3.2. Fig. 5C–E are representative traces of experiments presented in Fig. 5A and B. An inhibitory action of HU210 on the sympathetic contractile response to electrical field stimulation of the mesenteric beds is clearly seen, whilst the vehicle, dimethylsulphoxide, had no effect.

3.5. Effect of HU211 on electrically evoked noradrenaline release

HU211 (3 μM) inhibited the contractile response to electrical field stimulation of the mesenteric arterial beds (Fig. 6B,D) ($P < 0.001$; $n = 7$), as reported in Section 3.1. However, HU211 had no significant effect on evoked noradrenaline release from the mesenteric bed (Fig. 6C) ($n = 7$), indicating that its action was purely postjunctional.

3.6. Effects of HU210, CP55,940 and methanandamide on contraction to noradrenaline, methoxamine, α,β-meATP, vasopressin and KCl

HU210, 1 μM, had no significant effect on dose-dependent contraction mediated by exogenous noradrenaline (0.5–150 nmol) (Fig. 7B). At 3 μM HU210, responses to noradrenaline were reduced ($P < 0.001$) (Fig. 7D). Methanandamide (1 μM) and CP55,940 (1 μM) had no significant effect on dose-dependent contractions to noradrenaline (Fig. 7A,C).

Dose–response curves to methoxamine, α,β-methylene ATP and vasopressin were significantly different when compared in the absence and presence of the cannabinoids, and there was a trend for responses to be reduced in the presence of the cannabinoids (Fig. 8A,B,D). When responses at individual doses were evaluated, however, this revealed a significant difference only for the effect of methanandamide on responses to methoxamine, and methanandamide and HU211 on responses to vasopressin, at the doses indicated in the figures. In contrast, contractions to KCl, particularly those at low doses, were almost abolished by all of the cannabinoids (Fig. 8C).

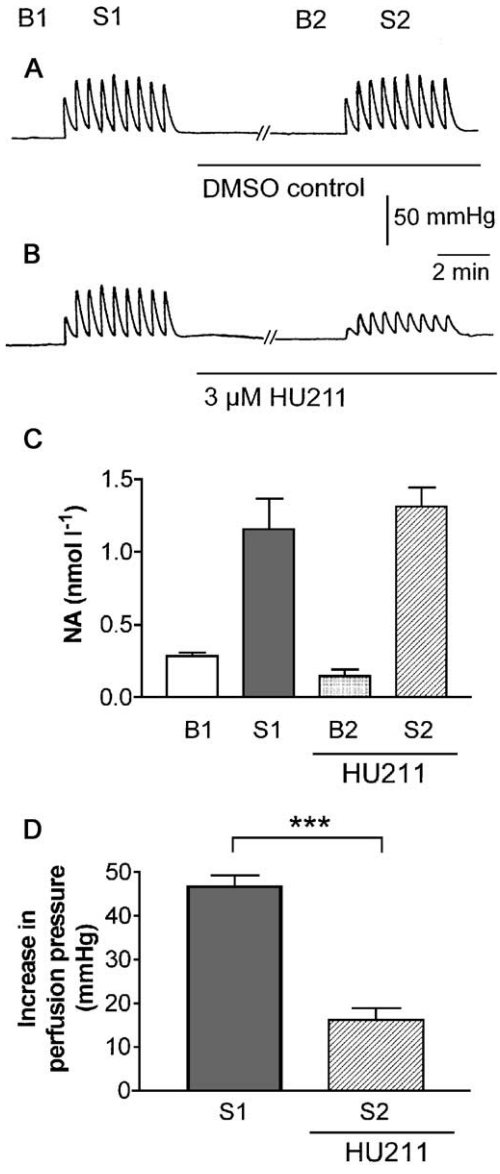

Fig. 6. Effect of HU211 on electrically evoked noradrenaline (NA) release and contractile activity of the rat isolated mesenteric arterial bed. (A,B) Representative traces showing effect of the vehicle (dimethylsuphoxide, DMSO) and HU211 (3 μM) on the contractile response to electrical field stimulation (nine stimulations at 64 Hz, 1 ms, 90 V, applied for 5 s every 30 s). HU211 had no significant effect on the evoked release of NA into the perfusate (C), but blocked the contractile response (D) ($n = 7$), indicating that its action was post-, and not pre-, junctional. B1, B2 = pre-stimulation fractions; S1, S2 = fractions collected during electrical field stimulation. ***$P < 0.001$.

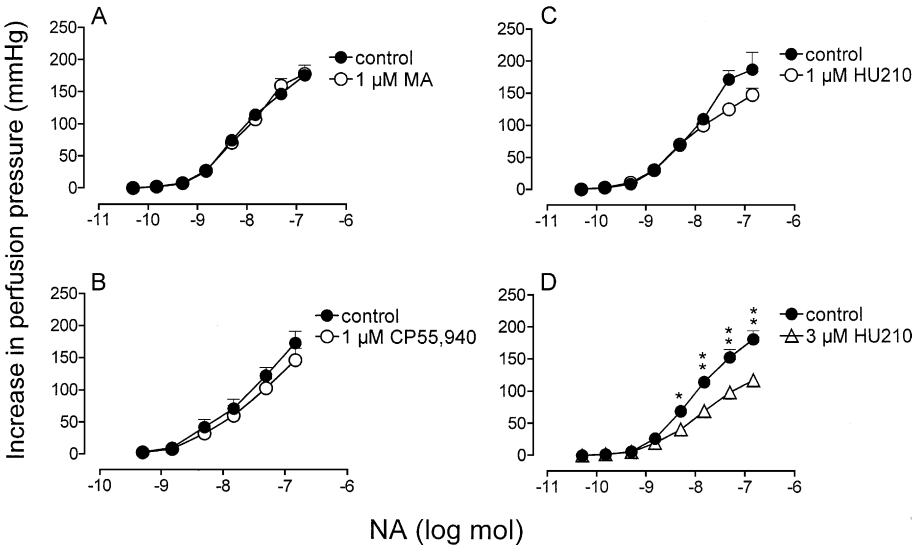

Fig. 7. Effect of HU210 (1 and 3 μM), methanandamide (MA; 1 μM) and CP55,940 (1 μM) on contractile responses to exogenous noradrenaline (NA; 0.5–150 nmol) in the rat isolated mesenteric arterial bed. (A) 1 μM methanandamide ($n=8$); (B) 1 μM CP55,940 ($n=7$); (C) 1 μM HU 210 ($n=6$); (D) 3 μM HU210 ($n=6$). Only in the presence of 3 μM HU210 was there a significant difference between the dose–response curves. Data are given as means and bars indicate S.E.M. * $P<0.05$; ** $P<0.01$.

3.7. Effect of capsaicin pre-treatment on inhibition by cannabinoids of the response to electrical field stimulation

Capsaicin pre-treatment (10 μM, 1 h), augmented the contractile response to electrical field stimulation; there was an increase in R_{max}, from 60 ± 4.5 to 85 ± 3.8 mm Hg ($P<0.001$) and decrease in F_{50}, from 23.4 ± 1.1 to 19.2 ± 0.5 Hz ($P<0.01$) (Fig. 9). In time-control experiments, this augmented response was shown to be reproducible between the two frequency response curves that were generated after capsaicin treatment (R_{max} 82 ± 12.6 mm Hg, F_{50} 20.4 ± 1.8 Hz, and R_{max} 81 ± 7.8 mm Hg, F_{50} 21 ± 1.0 Hz, for the

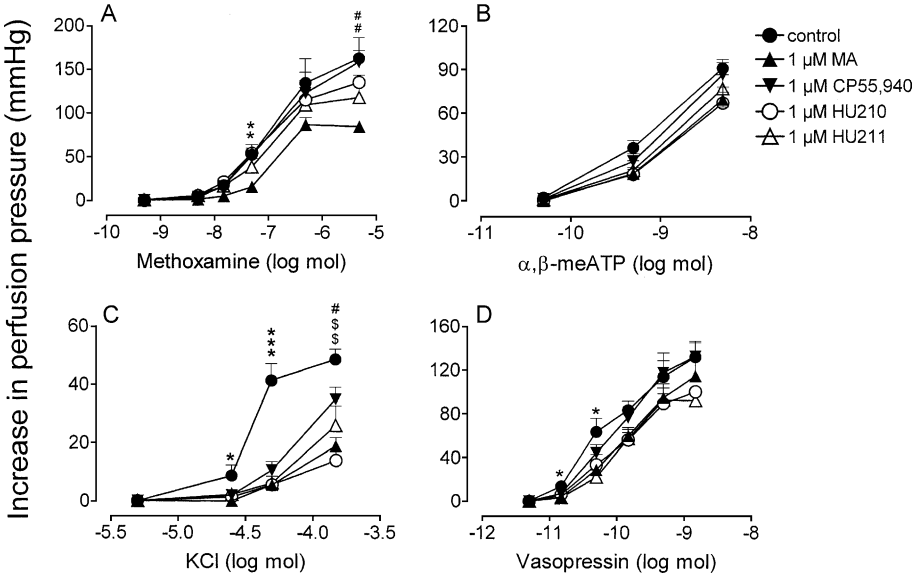

Fig. 8. Contractile responses to methoxamine, α,β-methylene ATP (α,β-meATP), KCl and vasopressin in the rat isolated mesenteric arterial bed in the absence of drugs or presence of dimethylsulphoxide (0.0001%; $n=5$) or in the presence of methanandamide (MA; 1 μM, $n=6$), CP 55,940 (1 μM, $n=4$), HU 210 (1 μM, $n=6$) and HU211 (1 μM, $n=4$). (A) ** denotes significant differences between methanandamide vs. control, CP55,940 and HU210 ($P<0.01$); ## denotes significant differences between methanandamide vs. control and CP55,940 ($P<0.01$). (B) Although there was a highly significant difference between the curves, no differences at individual doses were detected. (C) *($P<0.05$) and ***($P<0.001$) denote significant differences between control vs. all other groups; $$ denotes significant difference between control vs. methanandamide, HU210 and HU211 ($P<0.01$); # denotes significant difference between CP55,940 vs. methanandamide and HU210 ($P<0.05$). (D) * denotes significant difference between control vs. methanandamide and HU211 ($P<0.05$).

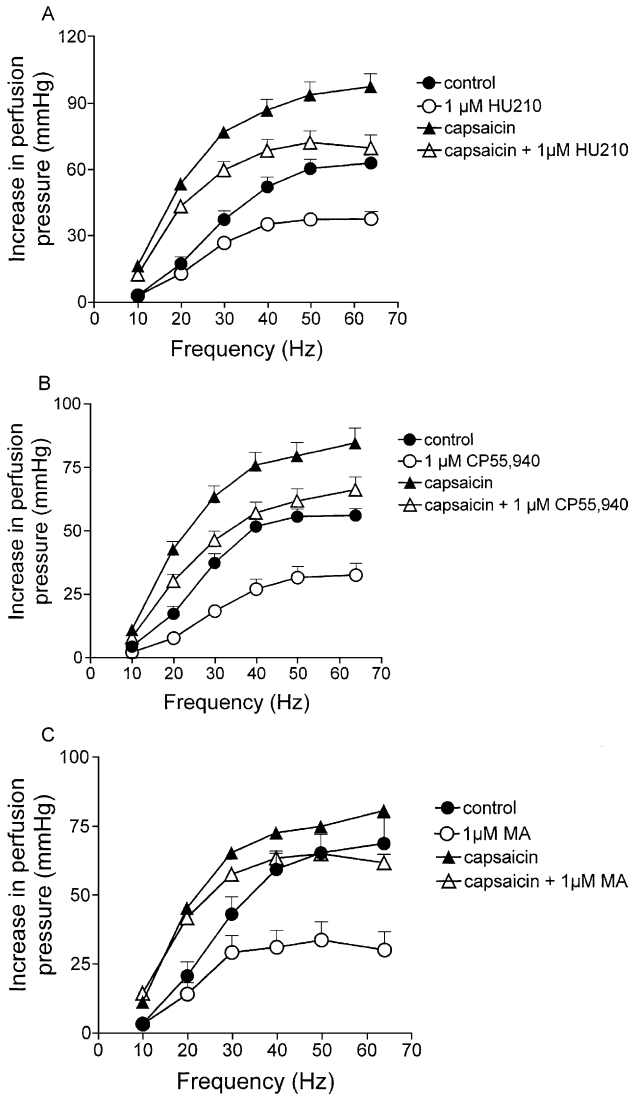

Fig. 9. Effect of capsaicin pre-treatment on inhibition by HU210, CP55,940 and methanandamide of contractions to electrical field stimulation (Hz) of the rat isolated mesenteric arterial bed. (A) Control and 1 μM HU210 ($n=4$), and capsaicin pre-treatment without and with 1 μM HU210 ($n=4$). (B) control and 1 μM CP55,940 ($n=7$), and capsaicin pre-treatment without and with 1 μM CP55,940 ($n=8$). (C) control and 1 μM methanandamide ($n=4$), and capsaicin pre-treatment without and with 1 μM methanandamide ($n=4$). Data are given as means and bars indicate S.E.M. HU210 and CP55,940 elicited a similar decrease in the maximal response without and with capsaicin pre-treatment. In contrast, the inhibitory effect of methanandamide was significantly smaller in capsaicin pre-treated preparations ($P<0.001$).

first and second frequency response curves, respectively, $n=4$).

Capsaicin pre-treatment had no effect on attenuation by HU210 (1 μM) of responses to electrical field stimulation; R_{max} was reduced by 29 ± 2.2 mm Hg (from 98 ± 5.8 to 70 ± 6.0 mm Hg; $P<0.001$) (Fig. 9A). F_{50} values were not significantly different with and without HU210. Capsaicin pre-treatment also had no effect on inhibition of sympathetic neurotransmission by CP55,940: R_{max} was reduced by 18 ± 1.7 mm Hg (from 85 ± 5.6 to 66 ± 5.1 mm Hg, $P<0.05$) (Fig. 9B). F_{50} values were not different. In contrast, capsaicin pre-treatment attenuated inhibition of responses to electrical field stimulation by methanandamide (Fig. 9C): R_{max} was reduced by 19 ± 3.7 mm Hg (from 81 ± 2.3 to 62 ± 3.1 mm Hg), which was significantly less than the attenuation observed without capsaicin pretreatment (39 ± 3.2 mm Hg) ($P<0.01$). The F_{50} value was reduced by methanandamide (from 18.6 ± 0.1 to 16.6 ± 0.3 Hz; $P<0.01$).

Neither LY320135 (1 μM; $n=4$), nor SR144528 (1 μM; $n=4$), blocked the inhibitory action of CP55,940 after capsaicin pre-treatment; CP55,940 elicited a decrease in R_{max} of 30 ± 6.4 mm Hg in the presence of LY320135 and 24 ± 1.8 mm Hg in the presence of SR144528.

3.8. Effect of capsazepine on methanandamide-induced inhibition of contraction to electrical field stimulation

Capsazepine (3 μM) alone inhibited contraction mediated by electrical field stimulation (reduction in R_{max} of $32 \pm 5\%$, $n=4$). In the presence of methanandamide (1 μM) there was a further reduction in R_{max} by $44 \pm 2\%$ ($n=4$). There was a trend towards inhibition by methanandamide in the presence of capsazepine being smaller than that evoked by methanandamide alone ($58 \pm 6\%$, $n=4$), although this did not reach statistical significance.

4. Discussion

The present study demonstrates clearly, and for the first time, that cannabinoids can inhibit, pre- and postjunctionally, sympathetic neurotransmission in the rat isolated mesenteric arterial bed. The prejunctional action involves a cannabinoid CB_1-like receptor, whilst the postjunctional action does not appear to involve either cannabinoid CB_1 or CB_2 receptors.

HU210 and the other cannabinoids were active at micromolar concentrations in inhibiting sympathetic neurotransmission in the rat mesenteric arterial bed. Micromolar concentrations of cannabinoids were also needed to inhibit electrically evoked release of [^{3}H]noradrenaline from sympathetic neurons in rat atria, and to evoke relaxation in a number of different blood vessels (Zygmunt et al., 1997; White and Hiley, 1998; Harris et al., 1999). However, the concentration of HU210 used in these and the present study is greater than that which caused inhibition of electrically evoked contractions of the vas deferens and myenteric plexus (EC_{50} 0.15 and 1.4 nM, respectively) (Pertwee et al., 1992). The 10-fold difference in potency of HU210 between the latter preparations (Pertwee et al., 1992) indicates that sequestration of drug in different tissues may be a crucial factor in affecting potency, which may be significant with respect to cannabinoid actions in the fatty mesenteric arterial bed. The potency of cannabinoids may also be influenced by their route of administration. In the present

study they were perfused luminally through the mesenteric vasculature, but sympathetic neurotransmission is most significant in the outermost layers of the blood vessel, in the adventitia and media.

Two previous studies which investigated cannabinoid modulation of sympathetic perivascular neurotransmission, reported no effect of micromolar concentrations of cannabinoids on sympathetic neurotransmission in the rat tail artery (Malinowska et al., 1997) and in rat isolated small mesenteric arteries (Lay et al., 2000). The reason for the difference between these and the present study is unclear, especially as drug concentrations and equilibration times were similar. However, our study is in line with those showing cannabinoid-mediated inhibition of sympathetic neurotransmission in the rat and mouse vas deferens (Ishac et al., 1996; Griffin et al., 1997; Lay et al., 2000), rat atria (Ishac et al., 1996) and rabbit heart (Szabo et al., 2001). In addition, the present study shows clearly that cannabinoid-mediated inhibition of sympathetic neurotransmission in blood vessels can occur via both pre- and postjunctional actions.

4.1. Prejunctional inhibition of sympathetic neurotransmission by cannabinoids

Direct evidence for a prejunctional action of cannabinoids on sympathetic perivascular neurotransmission was provided with the demonstration that HU210 attenuated the level of endogenous noradrenaline released during electrical field stimulation. Furthermore, HU211, a (+)-stereoisomer of HU210 with >500 times lower potency at cannabinoid receptors in the guinea-pig vas deferens and myenteric plexus (Pertwee et al., 1992), had no effect on evoked endogenous noradrenaline release from the mesenteric arterial bed. The inhibitory action of HU210 was antagonised by LY320135, but not by SR144528, indicating the likely involvement of a cannabinoid CB_1 or CB_1-like receptor. This is consistent with evidence for the expression of cannabinoid CB_1 receptors on sympathetic nerves in other peripheral tissues (Ishac et al., 1996; Griffin et al., 1997; Malinowska et al., 1997; Lay et al., 2000).

Noradrenaline and ATP are released as cotransmitters from sympathetic nerves in many blood vessels, including rat mesenteric arteries (Burnstock, 1990; Sjöblom-Widfeldt et al., 1990), although in the rat whole mesenteric arterial bed preparation the adrenoceptor antagonist prazosin abolishes the sympathetic neurogenic contractile response. Whether the release of noradrenaline and ATP from sympathetic nerves can be differentially modulated by cannabinoids remains to be investigated.

4.2. Postjunctional inhibition of sympathetic neurotransmission by cannabinoids

There was a trend for some of the cannabinoids, at 1 μM, to inhibit contractile responses to noradrenaline, methoxamine, α,β-methylene ATP, and vasopressin, and at a concentration of 3 μM HU210, inhibition of the response to noradrenaline was observed. Hence, cannabinoids clearly can modulate postjunctionally sympathetic neurotransmission. The experiments with HU211 provided additional evidence for a postjunctional action of cannabinoids on sympathetic neurotransmission, as this compound blocked the neurogenic contractile response, but had no effect on the evoked release of noradrenaline. The fact that LY320135 was able to antagonise HU210-mediated inhibition of evoked noradrenaline release, but not HU210-mediated inhibition of neurogenic contraction, indicates that postjunctional, rather than prejunctional, cannabinoid effects dominate in modulating the sympathetic neurogenic contractile response to electrical field stimulation, under the conditions of the present study. Furthermore, the greater potency of HU210 in causing inhibition of the neurogenic contractile responses, compared to its inhibition of contractions to exogenous agents, suggests that electrically evoked neurogenic contraction may be a more sensitive assay for postjunctional neuromodulation than the contractile response to agents applied exogenously.

Inhibition of the contractile response to electrical field stimulation by HU210, CP55,940 and methanandamide, which as discussed above occurs largely via a postjunctional action, was not blocked by either LY321035 or SR144528. This indicates that the postjunctional sympathoinhibitory effect of these cannabinoids does not involve cannabinoid CB_1 or CB_2 receptors. Moreover, inhibition of contractile responses to electrical field stimulation by HU211, which is weak or inactive at CB_1 and CB_2 receptors, also indicates a postjunctional action in the rat mesenteric arterial bed that must be mediated by a different receptor or different mechanism. Cannabinoid research is a relatively young field and it would not be surprising if novel receptors exist for these compounds that have not yet been identified. Indeed, cannabinoid actions at non-CB_1 non-CB_2 receptors/sites have recently been reported in rat mesenteric arteries (Járai et al., 1999) and hearts (Ford et al., 2002). Although the antagonists employed have nanomolar affinities for cannabinoid CB_1 and CB_2 receptors, respectively (Felder et al., 1998; Rinaldi-Carmona et al., 1998), and thus were used in the present study at relatively high concentrations, this was appropriate as the cannabinoid receptor agonists were also used at high concentrations. Lay et al. used a 10-fold higher concentration of SR144528 (10 μM) against CP55,940-mediated inhibition of contractile responses in the rat vas deferens, but concluded that the small non-parallel shift in the response curve was not due to actions of SR144528 at either a cannabinoid CB_1 or CB_2 receptor (Lay et al., 2000).

The mechanism by which cannabinoids attenuate contractions to the various agonists used is unclear. Receptor-dependent and -independent mechanisms may be involved, including hyperpolarization, inhibition of voltage-operated calcium channels and inhibition of calcium mobilization from intracellular stores (Zygmunt et al., 1997; White and

Hiley, 1998). The pronounced inhibition by all cannabinoids of responses to KCl, indicates that hyperpolarization and/or inhibition of voltage-operated calcium channels may be a principal mechanism whereby cannabinoids block vasoconstriction. Vasorelaxation appears to contribute minimally, if at all, to cannabinoid-mediated inhibition of sympathetic neurotransmission in the mesenteric arterial bed, at least for HU210 and CP55,940 (at 1 μM), as these compounds have little or no effect on tone of the methoxamine-raised tone rat isolated mesenteric arterial bed (unpublished observations), although they are relatively potent vasorelaxants in rat isolated small mesenteric arteries (EC_{50} 1.7 and 3.9 μM, respectively) (White and Hiley, 1998). Capsaicin pre-treatment had no effect on inhibition by HU210 and CP55,940 of contractions to electrical field stimulation, indicating a lack of involvement of sensory nerves.

The actions of methanandamide appear to be more complex as, in addition to possible direct pre- and postjunctional actions, the attenuation of its inhibitory effect on sympathetic neurogenic contraction, by capsaicin pre-treatment, indicates an involvement of sensory nerves. The most likely explanation is that methanandamide is acting at vanilloid VR1 receptors on sensory nerves (Zygmunt et al., 1999; Ralevic et al., 2000), to cause vasorelaxation of the methoxamine-preconstricted rat mesenteric arterial bed (its EC_{50} in this preparation is about 1 μM; Ralevic et al., 2000), and thus functional antagonism of sympathetic vasoconstriction. A prejunctional action of calcitonin gene-related peptide, the principal motor neurotransmitter released from sensory nerves (Kawasaki et al., 1988), is unlikely as this has been shown to inhibit postjunctionally, but not prejunctionally, sympathetic neurotransmission (Maynard and Burnstock, 1994; Al-Kazwini et al., 1986). HU210 and CP55,940 are inactive at VR1 (Zygmunt et al., 1999), thus their inhibitory actions were not blocked by capsaicin pretreatment. As electrical field stimulation can concomittantly activate sympathetic vasocontractile and sensory vasorelaxant nerves, inhibition of sensory nerve activity, by capsaicin pre-treatment, augments sympathetic contractile responses to electrical field stimulation by removing functionally antagonistic sensory vasorelaxation (Kawasaki et al., 1990; Li and Duckles, 1992; present study). An increased amplitude of the sympathetic neurogenic contractile response, however, is unlikely to have been involved in capsaicin-induced block of sympathoinhibition by methanandamide, as capsaicin pre-treatment had no effect on inhibition of the responses by HU210 and CP55,940.

4.3. Effects of cannabinoid receptor antagonists on sympathetic neurotransmission

It has been shown that cloned and native cannabinoid CB_1 receptors in some tissues can exist in a constitutively active conformational state and that SR141716A, a selective cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994), acts as an inverse agonist to relieve inhibition and augment responses (Pertwee and Fernando, 1996; Pan et al., 1998; Coutts et al., 2000). However, we have shown previously that SR141716A *inhibits* sympathetic neurotransmission in the rat mesenteric arterial bed (Ralevic and Kendall, 1999). Furthermore, in the present study, LY320135 and SR144528 alone had no significant effect on sympathetic neurotransmission (functional response and noradrenaline release), which suggests that there is no constitutively active cannabinoid receptor, or evoked release of endogenous cannabinoids, that modulates sympathetic neurotransmission in the rat isolated mesenteric arterial bed. The affinities of LY320135 and SR144528 are 200- and 400-fold lower, respectively, than that of SR141716A for the CB_1 receptor (Felder et al., 1998; Shire et al., 1999), but they were used at a concentration (1 μM) well in excess of their affinities (nanomolar range) for cannabinoid CB_1 and CB_2 receptors.

In conclusion, this study has demonstrated, for the first time, that cannabinoids can inhibit, by distinct pre- and postjunctional actions, sympathetic neurotransmission in the rat mesenteric arterial bed. The pre-junctional action could be mediated by a cannabinoid CB_1 or CB_1-like receptor, but the postjunctional action does not appear to involve either cannabinoid CB_1 or CB_2 receptors.

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