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Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB_1 receptors on peripheral sympathetic nerves

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1 Activation of CB_1 receptors by plant cannabinoids or the endogenous ligand, anandamide, causes hypotension via a sympathoinhibitory action in anaesthetized rats. In mouse isolated vas deferens, activation of CB_1 receptors inhibits the electrically evoked twitch response. To determine if these effects are related to presynaptic inhibition of noradrenaline (NA) release, we examined the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), anandamide and the CB_1 antagonist, SR141716A, on exocytotic NA release in rat isolated atria and vasa deferentia.

2 In isolated atria and vasa deferentia preloaded with [^{3}H]-NA, electrical field stimulation caused [^{3}H]-NA release, which was abolished by tetrodotoxin 0.5 μM and concentration-dependently inhibited by Δ^9-THC or anandamide, 0.3–10 μM. The inhibitory effect of Δ^9-THC and anandamide was competitively antagonized by SR141716A, 1–10 μM.

3 Tyramine, 1 μM, also induced [^{3}H]-NA release, which was unaffected by tetrodotoxin, Δ^9-THC or anandamide in either atria or vasa deferentia.

4 CB_1 receptor mRNA is present in the superior cervical ganglion, as well as in whole brain, cerebellum, hypothalamus, spleen, and vas deferens and absent in medulla oblongata and atria, as demonstrated by reverse transcription-polymerase chain reaction. There was no evidence of the presence of CB_{1A} receptor mRNA in ganglia, brain, or cerebellum. These results suggest that activation of presynaptic CB_1 receptors located on peripheral sympathetic nerve terminals mediate sympathoinhibitory effects *in vitro* and *in vivo*.

Keywords: Cannabinoids; CB_1 receptors; SR141716A; anandamide; Δ^9-tetrahydrocannabinol; presynaptic receptors; noradrenaline release

Introduction

The neurobehavioural effects of marijuana and related cannabinoids are thought to be mediated by a specific cannabinoid receptor, the CB_1 receptor subtype, originally identified and widely expressed in the brain (Herkenham *et al.*, 1990; Matsuda *et al.*, 1990; Howlett *et al.*, 1992). These receptors recognise not only plant-derived cannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC) but also some endogenous ligands, such as anandamide (Devane *et al.*, 1992), and are selectively inhibited by the antagonist, SR141716A (Rinaldi-Carmona *et al.*, 1994). More recently, a peripheral cannabinoid receptor, CB_2, has been cloned from lymphoid tissue (Munro *et al.*, 1993). While CB_1 receptors have also been identified in peripheral organs such as the spleen (Kaminski *et al.*, 1992) and testis (Gerard *et al.*, 1991), the physiological functions of CB_1 receptors outside the central nervous system have not been clearly defined. Anandamide as well as plant-derived cannabinoids inhibit the electrically evoked twitch response of the mouse isolated vas deferens without affecting the contractile response to exogenous noradrenaline (NA) (Pertwee *et al.*, 1992), and this effect is competitively antagonized by SR141716A (Rinaldi-Carmona *et al.*, 1994). It is plausible that this effect involves activation of presynaptic CB_1 receptors on sympathetic nerve terminals, stimulation of which would inhibit NA release, although direct evidence for this has not yet been presented.

The existence of such presynaptic CB_1 receptors is also suggested by *in vivo* studies of the cardiovascular effects of cannabinoids. In anaesthetized rats, the CB_1 receptor-mediated hypotensive response to Δ^9-THC or anandamide is abolished by α-adrenoceptor blockade or cervical spinal cord transection, suggesting sympathetic nervous system involvement (Varga *et al.*, 1995). Furthermore, anandamide was found to inhibit the pressor response to electrical stimulation of sympathetic premotor neurones in the rostral medulla of barodenervated rats, without reducing postganglionic sympathetic nerve activity or the pressor response to phenylephrine (Varga *et al.*, 1996). Presynaptic inhibition of NA release by anandamide may account for these effects.

In the present experiments we demonstrate that both Δ^9-THC and anandamide inhibit the exocytotic release of NA from sympathetic neurones in rat atria and vas deferens via an interaction with CB_1 receptors. Furthermore, CB_1 receptor mRNA is detected in sympathetic ganglia, suggesting that CB_1 receptors localized presynaptically on postganglionic sympathetic nerve terminals can mediate sympathoinhibitory effects *in vivo*.

Methods

Isolated tissue preparations

Male Sprague-Dawley rats weighing 250–300 g were killed by decapitation and the heart and vasa deferentia were removed. The left and right atria were isolated, the vasa deferentia were cleared of connective tissue, and the preparations were mounted between field stimulating platinum electrodes in individual organ baths containing oxygenated (95% O_2/5% CO_2) Krebs buffer at 37°C.

[^{3}H]-noradrenaline release

The tissues were preincubated with 3 μCi of (-)-[7-^{3}H]-noradrenaline (^{3}H-NA, 10.5 Ci mmol^{-1}, 0.1 μM) for 30 min, then

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washed with Krebs' buffer for 60 min, during which there was a priming stimulation (4 Hz, 45 V, 1 ms, 60 s train) to remove loosely bound radioactivity. Subsequent stimulations using the same parameters were carried out in 30 min test cycles, during which 3 samples of the medium were collected for measurement of the released radioactivity: 3 min basal, 3 min test period, and 3 min washout, followed by 21 min of recovery. The tissues were electrically stimulated using the above parameters during the first 1 min of the 3 min test period. Stimulation-induced release was calculated as radioactivity collected during the test period minus the mean of the radioactivity collected in the basal and washout periods. Yohimbine, 1 μM, and cocaine, 1 μM, were present in the tissue bath throughout the experiment to block presynaptic α_2-adrenoceptors and neuronal uptake of noradrenaline, respectively. When the effect of an agonist on [^{3}H]-NA release was tested, the drug was added to the medium 3 min prior to the first 3 min collection period and was removed at the end of the test period. When used, the antagonist SR141716A was added 15 min prior to the basal collection period and remained in the medium until the end of the washout period. At the end of the experiments the tissues were removed, solubilized in Soluene-350 (Packard), and the total radioactivity remaining in the tissue was measured and used to calculate tissue content for each test period (final tissue content + amounts released after each subsequent test period). Fractional release is calculated for each test period as amount of ^{3}H released/total tissue content at the time of stimulation. The neural origin of the [^{3}H]-NA released was verified by the ability of 0.5 μM tetrodotoxin to abolish release completely. To examine drug effects on non-exocytotic NA release, tissues were challenged with (1 μM) tyramine.

Detection of CB_1 receptor mRNA by reverse transcription–polymerase chain reaction (RT-PCR)

To detect the presence of CB_1 receptor mRNA in various tissues, total RNA was extracted from the superior cervical ganglia, atria, spleen, vasa deferentia, whole brain (brain minus cerebellum), cerebellum, hypothalamus and medulla oblongata of rats, using the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987). Poly(A)$^+$ RNA was isolated by standard oligo(dT) cellulose chromatography. First strand cDNA was obtained using 2 μg poly(A)$^+$ as template, oligo(dT) as primer and 200 units of SuperScript II (GIBCO/BRL). After reverse transcription for 1 h at 42°C, 5 μl of the reaction mixture was brought to 100 μl PCR reaction mixture by adding 100 ng each of sense and antisense primers corresponding to appropriate segments of the rat CB_1 receptor gene coding region (see legends for Figures 5 and 6), 200 μM of each dNTP, Taq DNA polymerase buffer and 2.5 units of Taq DNA polymerase. Amplification was done in 35 cycles, and the PCR products were sized on a 2.0% agarose gel and visualised by ethidium bromide staining.

Southern analysis and nucleotide sequencing

The identity of the amplicons was verified not only by their size, but also by Southern blotting and by DNA sequencing. For Southern analysis, the gels were then transblotted onto nitrocellulose and hybridized with a ^{32}P-labelled CB_1 receptor cDNA probe (McLaughlin & Abood, 1993), following a protocol described earlier (Gao & Kunos, 1994). For nucleotide sequencing, the amplicons were excised and electroeluted from the gels and purified with the Geneclean kit (BIO101). The fragments were then subcloned into pCR II vector (Invitrogen) for dideoxy chain termination sequencing.

Drugs

Anandamide (arachidonylethanolamide) and SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl] were synthesized and provided for us by Dr Raj Razdan. Δ^9-Tetrahydrocannabinol (Δ^9-THC) was provided by Dr Billy Martin. Other drugs and reagents were from usual commercial sources.

Statistical analyses

For comparing the effect of a single concentration of an agonist in the absence and presence of the antagonist in the same preparation, the paired *t* test was used. The concentration-dependence of the effects of agonists was determined by a two-factor analysis of variance (ANOVA) with repeated measures. A one-factor ANOVA with repeated measures followed by Dunnett's test was used to determine if the response to a given concentration of a drug or drugs was significantly different from the drug-free control response. Use of these parametric tests was justified by the normal distribution of the data. Probability levels of <0.05 were considered statistically significant.

Results

[^{3}H]-NA release in isolated atria

Electrical field stimulation of isolated atria preloaded with [^{3}H]-NA resulted in a significant increase in the release of radioactivity into the medium as compared to unstimulated basal release. Stimulation-induced fractional release in the pesence of yohimbine and cocaine represented about 3% of the total tissue content and it remained constant when stimulation was repeated up to 5 times in the same preparation. This stimulation-induced release was completely abolished in the presence of tetrodotoxin, 0.5 μM (Figure 1a), whereas the release of radioactivity triggered by tyramine, 1 μM was unaffected by tetrodotoxin (Figure 1c), which confirms the exocytotic nature of the release triggered by electrical field stimulation. There was no difference between left and right atria regarding fractional [^{3}H]-NA release in response to electrical stimulation or the modification of this release by drugs. Therefore, the results obtained were pooled.

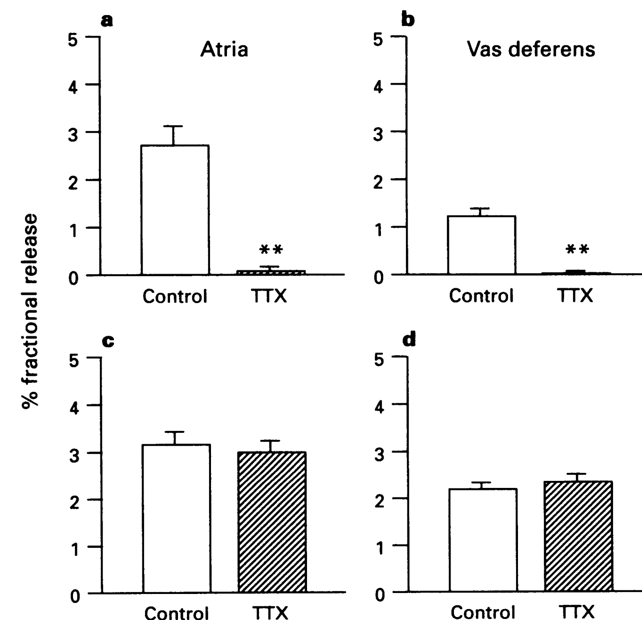

Figure 1 Tetrodotoxin inhibits [^{3}H]-NA release induced by electrical field stimulation but not by tyramine. Atria (a,c) and vasa deferentia (b,d) were electrically stimulated (a,b) or exposed to 1 μM tyramine (c,d) in the absence (Control) or presence of 0.5 μM tetrodotoxin (TTX), as described under Methods. Columns are means $\pm$ s.e. of fractional release, as measured in 4–6 preparations. Significant difference from release during control stimulation: $^{**}P<0.005$).

When the atria were field stimulated in the presence of Δ⁹-THC (10 μM) or anandamide (10 μM), [³H]-NA release was significantly suppressed, as compared to control release (Figure 2a, b and c). Exposure of atria to SR141716A (10 μM) did not affect stimulation-induced [³H]-NA release (Figure 2d), but completely blocked the effect to both Δ⁹-THC and anandamide (Figure 2e and f). In separate experiments, [³H]-NA release induced by tyramine (1 μM) (3.3±0.1%, $n=9$) was similar in magnitude to that caused by electrical stimulation, but tyramine-induced release was unaffected by either Δ⁹-THC (3.1±0.3%) or anandamide (3.4±0.5%).

In order to analyse concentration-response relationships, in a separate set of experiments concentration-response curves for Δ⁹-THC and anandamide were established in the absence or presence of SR141716A. In these experiments, one atrium was sequentially exposed to 0.3, 1, 3 and 10 μM of a given agonist (one concentration per 30 min test cycle), and the other atrium from the same animal was exposed to the same agonist concentrations in the presence of SR141716A, 1 μM. According to preliminary experiments, 10 μM was a maximally effective concentration for both agonists. This approach is justified by the lack of significant change in fractional release in response to control test stimulations repeated five times in the same preparation. As shown in Figure 3, both agonists caused concentration-dependent inhibition of [³H]-NA release, and the curve for both agonists was shifted to the right by approximately one order of magnitude in the presence of the antagonist.

[³H]-NA release in vasa deferentia

Electrical field stimulation also triggered [³H]-NA release in vasa deferentia, although fractional release was less than in atria and it amounted to 1.2±0.1% of tissue content. Exposure of vasa deferentia to tyramine, 1 μM caused a greater fractional [³H]-NA release (2.8±0.2%, $n=9$) but, as in atria, the field stimulation-induced release was completely blocked by tetrodotoxin (0.5 μM), whereas the tyramine-induced release was unaffected (Figure 1b and d).

Similar to atria, at a concentration of 10 μM both Δ⁹-THC and anandamide significantly suppressed electrical stimulation-induced [³H]-NA release, and these effects were similarly blocked in the presence of SR141716A (10 μM) (Figure 4). Concentration-response relationships were not established in

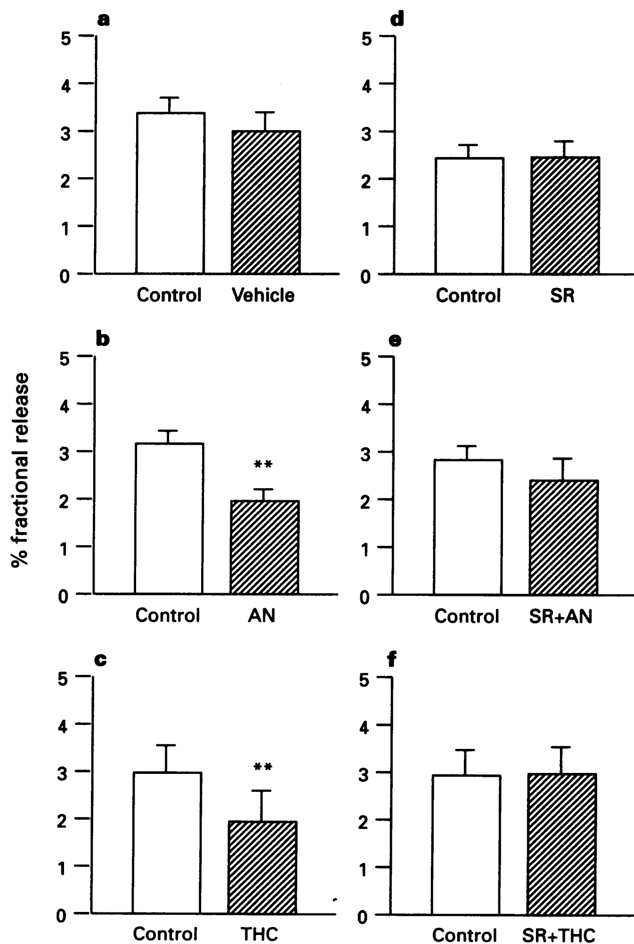

Figure 2 Effects of Δ⁹-THC (THC), anandamide (AN), and SR141716A (SR) on electrical stimulation-induced [³H]-NA release in isolated atria. Isolated left and right atria preloaded with [³H]-NA were subjected to two cycles of field stimulation, one in the absence of drugs (Control) and one in the presence of vehicle or drug(s) as indicated. The concentration of all three drugs used was 10 μM. Means±s.e. from 6 experiments are shown. Significant difference from corresponding control value: $^{**}P<0.005$).

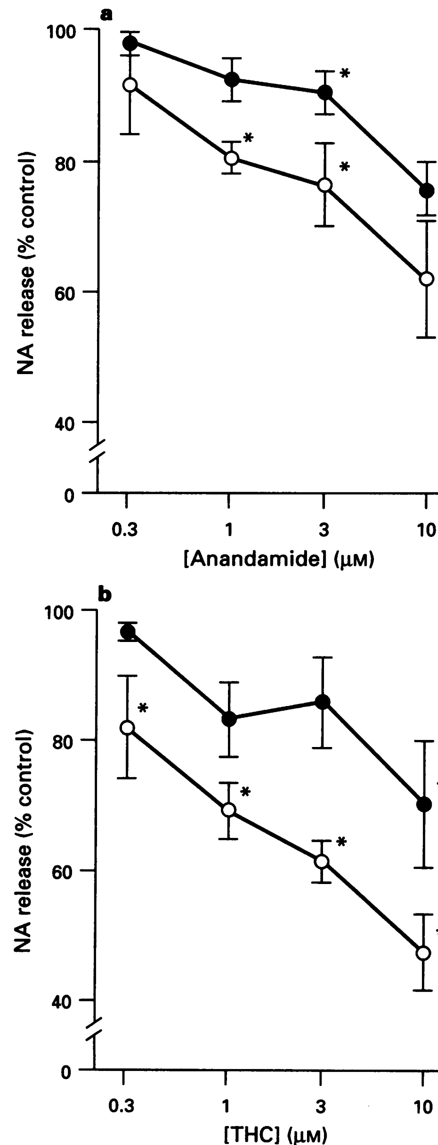

Figure 3 Concentration-dependent inhibition of [³H]-NA release in isolated atria by anandamide (a) and Δ⁹-THC (b) in the absence of antagonist (○) or in the presence of 1 μM SR141716A (●). Each concentration-response curve was established in 3 experiments; values are mean±s.e.mean. Significant difference $^{*}P<0.05$, from stimulated NA release in the absence of drugs.

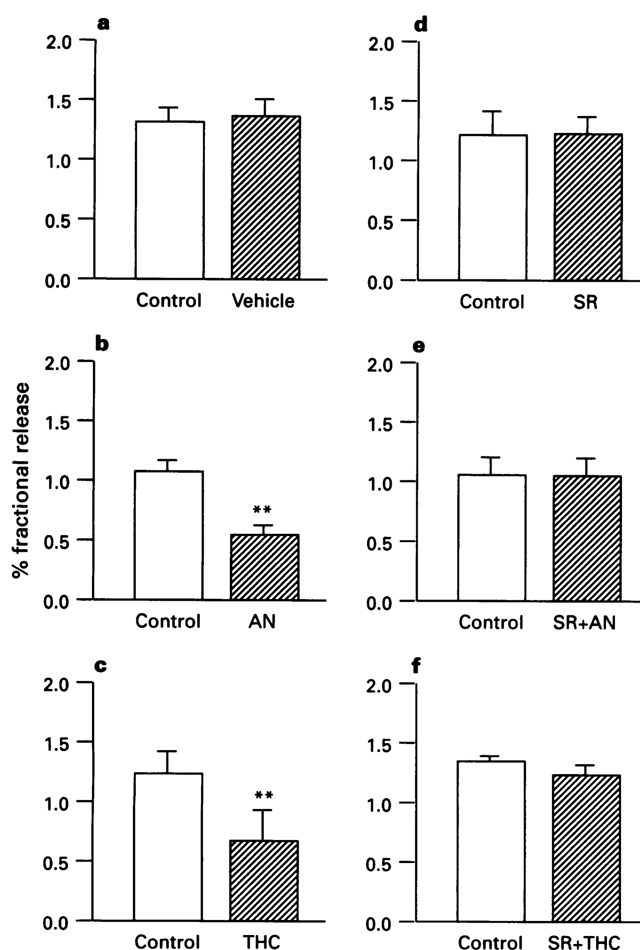

Figure 4 Effects of Δ^9-THC, anandamide, and SR141716A on electrical stimulation-induced [^{3}H]-NA release in isolated vasa deferentia. Conditions as described in the legend for Figure 2.

the vas deferens. As in atria, tyramine-induced release of [^{3}H]-NA remained unaffected by either Δ^9-THC ($2.6 \pm 0.4\%$) or anandamide ($3.1 \pm 0.6\%$).

Presence of CB_1 receptor mRNA in various tissues

The results of RT-PCR analysis indicate that, in addition to brain tissue, CB_1 receptor mRNA is also present in sympathetic ganglia as well as in some other peripheral organs, as indicated by the appearance of an amplicon of the expected size (Figure 5a) that hybridizes with the CB_1 receptor mRNA (Figure 5b). The absence of a band in lane 9, which contains the PCR products of non-reverse transcribed poly(A)$^+$ RNA from whole brain, confirms the absence of genomic DNA contamination of the mRNA. The specificity of the amplicons was also verified by DNA sequencing, which indicated 100% homology with the relevant segment of the rat CB_1 receptor gene coding region (Matsuda *et al.*, 1990; McLaughlin & Abood, 1993).

We also examined the possibility that the hybridizing species may represent mRNA of CB_{1A} receptors, a recently identified splice variant of the CB_1 receptor (Shire *et al.*, 1995), in which a 167 bp segment encoding part of the NH_2 terminal tail of the CB_1 receptor is spliced out. In Figure 6, reverse transcribed mRNA from ganglia (lanes 3 and 5), brain (lanes 1 and 4) and cerebellum (lane 2) was PCR amplified using two sets of primers. The first pair of primers flank the splice sites, whereas the sense primer in the second set is within the spliced region. Using the first set of primers, CB_1 receptor mRNA is expected to yield an amplicon of 307 bp, whereas CB_{1A} mRNA would yield a shorter amplicon of 140 bp. Instead, only the

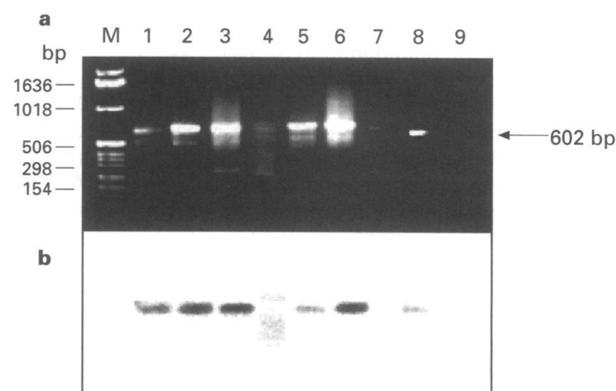

Figure 5 Tissue distribution of CB_1 receptor mRNA determined by RT-PCR and Southern blotting. RT-PCR was performed as described under the Methods section, using 200 ng poly(A)$^+$ RNA from superior cervical ganglia (lane 1), whole brain (lane 2), cerebellum (lane 3), atria (lane 4), vas deferens (lane 5), spleen (lane 6), medulla oblongata (lane 7), and hypothalamus (lane 8). In lane 9, 200 ng of non reverse transcribed poly(A)$^+$ RNA from brain was subjected to PCR to control for genomic DNA contamination. The degenerate primers used were TCC TTC (A,G)C (A,C) GC(T,C) TC(T,A) GTG (sense, corresponding to nt + 598 to + 615, with + 1 being the A in the translation start codon) and AGA G(C,T)(A,G) TA(G,A) ATG A(T,C)(G,A) GG(G,A) TT(C.G) A (antisense, nt + 1199 to + 1178). M: 1 kb DNA ladder. (a) Sizing of amplicons in a 2% agarose gel stained with ethidium bromide; (b) Southern analysis of amplicons transblotted from the same gel, using a ^{32}P-labelled rat CB_1 receptor cDNA. For further explanation, see text.

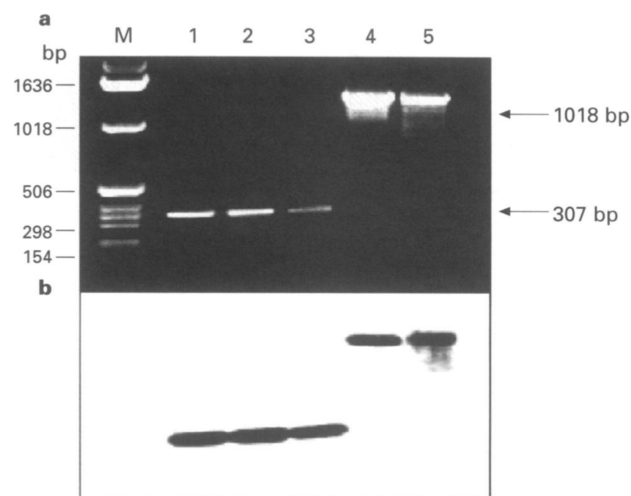

Figure 6 RT-PCR analysis of CB_1 and CB_{1A} receptor transcripts. RT-PCR was performed using 200 ng of poly(A)$^+$ RNA from brain (lanes 1 and 4), cerebellum (lane 2), and superior cervical ganglia (lanes 3 and 5). Lanes 1-3: the primers used were ATG AAG TCG ATC CTA GAT GGC C (sense, corresponding to nt + 1 to + 22 in rat CB1 receptor gene, + 1 being the A in the translation start codon) and AGT TCT CCC CAC ACT GGA TGT T (antisense, nt + 307 to + 286). Lanes 4 and 5: sense primer: AGA TGA CCG CAG GAG ACA AAC (nt + 182 to + 202); antisense: AGA GCA TAG ATG ATG GGG TTC (+ 1199 to + 1179). (a) Sizing of amplicons on a 2% agarose gel stained with ethidium bromide; (b) Southern analysis using a rat CB_1 receptor cDNA as in Figure 5. Note the absence of a second band of 140 bp length expected if CB_{1A} mRNA were present in the tissues.

longer and not the shorter amplicon is evident in all three tissues examined (see lanes 1-3, Figure 6), either by ethidium bromide staining (a) or Southern blotting (b). Using the second set of primers, the 1018 bp-long amplicon obtained in both brain (lane 4) and ganglia (lane 5) could only have originated

from CB_1 receptor mRNA. Thus, if present, CB_{1A} in the tissues examined could have represented but a negligible fraction of CB_1 mRNA.

Discussion

The results presented clearly indicate that activation of CB_1 receptors in two sympathetically innervated peripheral organs, the atria and the vas deferens, inhibit the release of NA from sympathetic nerves. This inhibition can be elicited either by Δ^9-THC or by the endogenous ligand, anandamide. Both Δ^9-THC and anandamide inhibit [^{3}H]-NA release in response to electrical stimulation but not to tyramine, which indicates that exocytotic release is selectively affected. Tetrodotoxin suppresses neuronal depolarization by inhibiting Na^+ channels, which decreases propagated action potentials and the subsequent exocytotic release of NA (Kao, 1966). In contrast, tyramine induces non-exocytotic, calcium-independent release of NA (Smith & Winkler, 1972). The effect of Δ^9-THC on the vas deferens confirms an earlier report that documented the ability of Δ^9-THC to inhibit electrical stimulation-induced [^{3}H]-NA release in rat vas deferens, although without evidence for receptor involvement (Graham *et al.*, 1974). As tested in atria, the inhibition of NA release was concentration-dependent for both Δ^9-THC and anandamide, which were roughly equipotent. This is similar to the situation in the mouse vas deferens, where the two agents are also equipotent in inhibiting the twitch response (see Pertwee *et al.*, 1992; Rinaldi-Carmona *et al.*, 1994), although with absolute potencies somewhat higher than those established here for inhibition of [^{3}H]-NA release in rat atria.

Blockade of the effects of both Δ^9-THC and anandamide by SR141716A strongly suggests that the inhibition of [^{3}H]-NA release is mediated by a CB_1-like receptor. The novel antagonist, SR141716A, is a potent and selective inhibitor of CB_1 receptors with more than a 1,000 fold lower affinity for CB_2 receptors (Rinaldi-Carmona *et al.*, 1994). In an earlier study, SR141716A was found to have a pA_2 value of 8.17 for antagonizing the inhibition of the electrically evoked twitch response of the mouse vas deferens by anandamide (Rinaldi-Carmona *et al.*, 1994). In the present experiments, at a concentration of 10 μM, SR141716A completely blocked the response to a maximally effective concentration of both agonists, whereas at the lower concentration of 1 μM it caused an approximately 10 fold inhibition, as tested in atria. From this latter finding a K_i of 0.1 μM can be estimated for SR141716A, which is compatible with the involvement of CB_1 receptors in the inhibition of [^{3}H]-NA release. Species differences and differences in the type of response measured may account for the somewhat higher antagonist potency observed in the mouse vas deferens.

Receptors mediating a decrease in stimulation-induced NA release are probably located presynaptically on sympathetic nerve terminals. The synthesis of such receptors must occur in the cell body of the postganglionic sympathetic neurone, which is also the site where measurable levels of receptor mRNA are localized. We therefore sought to determine whether CB_1 receptor mRNA can be detected in a sympathetic ganglion. Because of the expected low abundance of the message in peripheral tissues, we used the sensitive RT-PCR technique to look for the CB_1 receptor mRNA. The presence of a hybridizing band in lane 1 of Figure 5 is the first direct evidence for the transcription of the CB_1 receptor gene in a sympathetic ganglion. The strong hybridizing bands obtained using mRNA from whole brain or cerebellum (lanes 2 and 3 in Figure 5) are as expected from the known abundance of CB_1 receptors in these tissues (Herkenham *et al.*, 1990). The presence of a weak hybridizing band in the hypothalamus and the absence of a band in the medulla is also in agreement with the sparsity of CB_1 receptors in these latter brain areas (Herkenham *et al.*, 1990). The strong hybridizing band in the spleen confirms the results of an earlier report (Kaminski *et al.*, 1992). It is noteworthy that CB_1 receptor mRNA could be detected in the vas deferens but not in atria, whereas functional receptors are present in both tissues. This may be explained if the expression of CB_1 receptors is limited to sympathetic neurones in both tissues. It is well established that sympathetic innervation of certain organs, such as the vas deferens, is via short postganglionic neurones with cell bodies located in the target organ itself, whereas postganglionic neurones innervating the atria are located outside the heart, in the stellate ganglia (Sjostrand & Swedin, 1968).

Recently, a splice variant of the CB_1 receptor, the CB_{1A} receptor, has been identified and found to be widely expressed, albeit at levels much lower than the CB_1 receptor (Shire *et al.*, 1995). However, we found no evidence for CB_{1A} receptors expressed in the superior cervical ganglion, as PCR amplification using primers that flank the spliced segment yielded a single strong band the size of which matches the corresponding region in the unspliced CB_1 receptor, with no evidence for a shorter product (Figure 6).

The present findings clearly link a peripherally located CB_1 receptor to a well-defined physiological response. The demonstration of the existence of a presynaptic cannabinoid receptor on sympathetic nerve terminals is particularly interesting with regard to the cardiovascular effect of cannabinoids. Previous studies in the rat have established that Δ^9-THC causes a prolonged hypotensive response (Vollmer *et al.*, 1974). More recently, anandamide was found to have a similar effect, and both this and the effect of Δ^9-THC could be inhibited by SR141716A (Varga *et al.*, 1995). α-Adrenoceptor blockade or cervical spinal cord transection were also found to inhibit the hypotensive effect without eliminating the ability of blood vessels to dilate further (Varga *et al.*, 1995). This suggested that activation of CB_1 receptors elicits hypotension by a sympathoinhibitory action that could occur in the CNS, at sympathetic ganglia, or at the level of the sympathetic nerve terminal. More recent, preliminary observations indicate that at doses that produce hypotension, anandamide does not decrease the electrical activity of barosensitive neurones in the rostral ventrolateral medulla (RVLM) or postganglionic splanchnic nerve activity (PSNA). Furthermore, in barodenervated rats, anandamide decreased the pressor response to electrical stimulation of the RVLM, without decreasing PSNA or the pressor response to i.v. phenylephrine (Varga *et al.*, 1996). These observations exclude the CNS or sympathetic ganglia as sites for the hypotensive action of anandamide, and leave presynaptic inhibition of NA release as the only plausible mechanism.

In summary, the present studies provide direct evidence for the existence of presynaptic cannabinoid receptors mediating inhibition of exocytotic NA release in sympathetically innervated peripheral organs. These receptors may be involved in the hypotensive effect of cannabinoids *in vivo*. The susceptibility of this presynaptic effect to inhibition by a selective CB_1 receptor antagonist and the presence of CB_1 receptor mRNA in a sympathetic ganglion suggest that presynaptic cannabinoid receptors are of the CB_1 subtype. However, peripheral tissues such as the spleen may contain more than one type of cannabinoid receptor (Kaminski *et al.*, 1992; Munro *et al.*, 1993). Therefore, we cannot exclude the possibility that presynaptic inhibition of NA release is mediated by an as yet undefined cannabinoid receptor which is CB_1-like in its affinity for SR141716A. Endogenous anandamide has so far been identified only in the brain, and a putative peripheral ligand, 2-arachidonoyl glycerol, has recently been identified (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). It remains to be determined if presynaptic CB_1 receptors are acted upon by an endogenous ligand and, if so, what is the nature of this ligand.

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