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Evidence for presynaptic cannabinoid CB_1 receptor-mediated inhibition of noradrenaline release in the guinea pig lung

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Abstract

Using neurochemical method, evidence was obtained that cannabinoid CB_1 receptors are localized on noradrenergic terminals and their stimulation by WIN-55,212-2 reduces the release of [^{3}H]noradrenaline evoked by axonal activity in a frequency-dependent manner. At stimulation rates of 1 and 3 Hz, there was significant inhibition of noradrenaline release, with IC_{50} of WIN-55,212-2 41.5 $\pm$ 2.6 and 320.5 $\pm$ 28.2 nM, for 1 and 3 Hz, respectively. Cannabinoid CB_1 receptor antagonist SR 141716A completely prevented WIN-55,212-2 from reducing the release. The release of noradrenaline is negatively modulated by presynaptic α_2-adrenoceptors. Because BRL-44408, an α_{2B}-adrenoceptor, and prazosin, an α_1- and α_{2B}-adrenoceptor antagonist, both increased the release of [^{3}H]noradrenaline, it seems likely that the α_{2B} subtype is responsible for the negative feedback modulation of noradrenaline release. In the presence of α_2-adrenoceptor antagonism, cannabinoid CB_1 receptor activation by WIN-55,212-2 was much more effective in inhibiting the release of [^{3}H]noradrenaline. Using a specific antibody against the C-terminus of the rat cannabinoid CB_1 receptor and also against neuropeptide Y, ultrastructural evidence was obtained that cannabinoid CB_1 receptors are exclusively localized on neuropeptide Y-positive noradrenergic varicosities.

Since the sympathetic innervation of the human airway smooth muscle is sparse, and mainly the circulating adrenaline relaxes the airways via activation of β_2-adrenoceptor localized on the smooth muscle, it is suggested that inhibition of noradrenaline release by cannabinoids, and the subsequent bronchospasm, may be limited to those cases when noradrenaline released from sympathetic varicosities is involved in airway relaxation.

Keywords: Cannabinoid CB_1 receptor; Lung; Noradrenaline release; Bronchospasm

1. Introduction

Marijuana, the common name of *Cannabis sativa*, and its psychoactive ingredient, (−)-Δ^9-tetrahydrocannabinol, both influence the central (CNS) and peripheral nervous systems. Besides its pharmacological effects in the CNS (anti-emesis, analgesia, anticonvulsive action, etc.), it inhibits the release of some neurotransmitters (Rinaldi-Carmona et al., 1994; Pertwee et al., 1995, 1996; Schlicker et al., 1997; Coutts and Pertwee, 1997; Göbel et al., 2000; Kim and Thayer, 2000; Szabó et al., 2000; Katona et al., 1999, 2000). Recently it was shown (Calignano et al., 2000) that the endocannabinoid anandamide exerts dual effects on bronchial responsiveness in guinea pigs: it inhibits capsaicin-induced bronchospasm and cough, but produces bronchospasms when the effect of vagal tone is removed. Both actions are mediated via cannabinoid CB_1 receptors. Because evidence was obtained that anandamide is locally generated in guinea pig lung, it was suggested that endocannabinoids are involved in the intrinsic control of bronchial responsiveness (Calignano et al., 2000). It is known that sympathetic axon terminals are equipped with inhibitory α_2-adrenoceptors. Therefore, the effects of cannabinoid CB_1 receptor activation on noradrenaline release were studied in the presence and absence of α_2-adrenoceptor-mediated negative feedback control of noradrenaline release.

2. Materials and methods

In the release experiments, the resection specimens (bronchi) were excised from the lung of guinea pigs and

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immediately placed in oxygenated (95% O_2 + 5% CO_2) ice-cold Krebs solution of the following composition (in mM): NaCl 113, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.25, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, ascorbic acid 0.2, pargyline 0.02 (pH 7.4). The animals of both sexes (400–480 g) were killed by cervical dislocation.

2.1. [^{3}H]noradrenaline release

[^{3}H]noradrenaline release experiments were performed as described previously (Sumiya et al., 2001; Taoda et al., 2001). Tissues were cut into small pieces, washed with 5 ml of Krebs, and loaded for 45 min with [^{3}H]noradrenaline (L-7.8-[^{3}H]noradrenaline, 37 MBq, 40 Ci/mmol^{-1}, Amersham) at a concentration of 10 μCi ml^{-1} of Krebs solution. During the entire experiment, the medium was bubbled with a mixture of 95% oxygen–5% carbon dioxide. After incubation, the slices were washed with 5 ml of Krebs solution and transferred into a four-channel microvolume perfusion system (Vizi et al., 1985). Three pieces were placed into each chamber and the preparation was superfused with Krebs solution at 37 °C at a rate of 0.6 ml min^{-1} for 60 min (pre-perfusion period), and the effluent was discarded. Subsequently, 3-min fractions were collected. During the sample collection period, electrical field stimulation was applied twice, 30 min apart (S_1, S_2), using a Grass 88 stimulator. The parameters of electrical field stimulations applied during the 3rd (S_1) and 13th (S_2) fractions were 25 V; 3 ms; 1, 3 and 10 Hz; for 180, 60, and 18 s (180 pulses). Preliminary experiments confirmed that the stimulation-evoked release of [^{3}H]noradrenaline is sensitive to the sodium channel inhibitor tetrodotoxin (1 μM) using this stimulation paradigm. The effects of drugs on the basal efflux and stimulation-evoked release of [^{3}H]noradrenaline were evaluated by comparing the basal or stimulation-evoked release (S_2) obtained in the presence of drugs to the basal efflux or stimulation-evoked overflow (S_1) assayed in the absence of drugs. Data were expressed as absolute amount of radioactivity in Bq/g (disintegration per second per gram of tissue) or as fractional release (FR). Fractional release (FR) is expressed as a percentage of the total stored radioactivity in the tissue. The effects of drugs were expressed as FRS_2/FRS_1 ratios, measured in the absence and presence of the drug. The release in response to stimulation was estimated by the area-under-the-curve method using a computer program. Drugs were added from the eighth fraction, 15 min before the second stimulation, until the end of the experiment. At the end of the experiment, the pieces were removed from the chamber and homogenized in 0.5 ml of 10% trichloroacetic acid. A 0.5-ml aliquot of the superfusate and 0.1 ml of the tissue supernatant were added to 2 ml of scintillation cocktail (Ultima GOLD Packard). Tritium was measured with a Packard 1900 TR liquid scintillation counter using an internal standard.

All results were expressed as means ± S.E.M. The potency of the cannabinoid CB_1 receptor agonist (WIN-55,212-2) was expressed as a pEC_{50} value relative to the individual maxima. In some of the experiments, the apparent pK_B values for antagonists were estimated from the equation:

$$\text{Apparent } pK_B = \log(CR - 1) - \log(B)$$

where CR is the concentration ratio derived from the agonist EC_{50} values in the presence and absence of a concentration of antagonist (B), resulting in rightward shift of the concentration–response curve, with a minimum reduction of the E_{max}. The analysis assumes that at this concentration of antagonist, there is a competitive interaction with a slope of 1.

2.2. Immunocytochemistry

Three guinea pigs were perfused through the right ventricle with a phosphate-buffered (0.1 M) fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.1% glutaraldehyde. The lungs were cut into blocks and were further postfixed for 12 h. Eighty-micrometer-thick lung sections were cut on a vibratome. Immunostainings were carried out in a manner as described previously for brain sections (Katona et al., 1999). Briefly, the lung sections were washed extensively and then incubated for 48 h in a rabbit primary antibody generated against the C-terminus of the rat cannabinoid CB_1 receptor, at a dilution of 1:5000. The staining pattern and intensity was similar between the rat and guinea pig hippocampus, indicating that this antibody recognizes the CB_1 receptor protein in the guinea pig tissue with the same efficacy as in rats. This was expected in light of the very high homology in the amino acid sequence of CB_1 receptor among several mammalian species. The specificity of the antibody has been confirmed by the lack of staining in cannabinoid CB_1 receptor knockout mice (Hájos et al., 2000). After incubation, the sections were washed several times, then incubated again for 6 h in a secondary antibody conjugated to 1-nm-thick gold particles. The size of the gold particles had been increased further by silver intensification. For the second immunostaining, the sections were incubated in a primary antibody against neuropeptide Y (1:20,000, Csiffáry et al., 1990) and then developed by using the conventional ABC method.

For electron microscopy, bronchi and bronchioli containing several neuropeptide Y-positive axons were selected using the light microscope and re-embedded into Durcupan. Ultrathin sections were cut by a Reichert ultramicrotome. In the electron microscope, neuropeptide Y-immunoreactive axons were followed through serial sections and the individual silver–gold particles attached to the inner surface of the plasma membrane were counted to determine the cannabinoid CB_1 receptor-positivity of a given fiber.

2.3. Drugs

The following drugs were used: [^{3}H]noradrenaline (Amersham, Little Chalfont, UK, spec. activity 86 Ci/mmol, 4 μCi/ml), WIN-55,212-2 (RBI, Natick, MA), SR 141716A (NIDA, USA), tetrodotoxin (Sigma, St. Louis, MO), prazosin (Pfizer), CH-38083 (7,8-(methylenedioxy)-14-α-hydoxyalloberbane HCl), Vizi et al., 1986), BRL-44408 [(±)-2-(4,5-dihydro-1H-imidazol-2-yl)methyl)-2,3-dihydro-1-methyl-1H-isoindole] and ARC-239 [2-(2,4-(O-piperazine-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H) isoquinoline-dione chloride]. Fine chemicals were purchased from Sigma. All solutions were freshly prepared on the day of use.

2.4. Statistical analysis

All data are expressed as means (S.E.M.). Statistical significance was determined using analysis of variance (ANOVA) followed by Dunn's test; $P < 0.05$ was considered significant.

3. Results

After loading of the tissue with [^{3}H]noradrenaline, the total tissue [^{3}H] content was 740 157 ± 49 562 Bq/g (n = 24). Using HPLC combined with a radioactivity assay, of the total radioactivity, 94.5 ± 5.7% (n = 4) was found to be [^{3}H]noradrenaline. This indicates that [^{3}H]noradrenaline taken up by the tissue may be stored in such a way that it is protected from degradation, since only a small amount (5.5%) can be accounted for by metabolites. The resting release of radioactivity was 3162 ± 174 Bq/g in 3 min (n = 48), representing 0.472 ± 0.072 % of the radioactivity present in the tissue.

3.1. Effect of cannabinoid CB_1 receptor activation on [^{3}H]noradrenaline release from the bronchi

When the bronchi were stimulated by the same number of pulses (180) but at different frequencies, there were significant increases in the release of radioactivity. At 1, 3 and 10 Hz stimulation rates the release was 4790 ± 240, 5252 ± 1587 and 8950 ± 740 Bq/g, respectively (n = 6–6). Tetrodotoxin at 0.5 μM completely inhibited the release at all frequencies applied (data not shown).

The cannabinoid receptor agonist WIN-55,212-2 (Coutts and Pertwee, 1997) produced a concentration-dependent inhibition of noradrenaline release (Fig. 1), but its potency depended on the frequency of stimulation applied. The IC_{50} of WIN-55,212-2 was 41.5 ± 2.6 and 320.5 ± 28.2 nM and the E_{max} 61.5 ± 4.3% and 31.7 ± 2.2% inhibition (n = 5–5) at 1 and 3 Hz, respectively. The cannabinoid CB_1 receptor antagonist SR 141716A (Rinaldi-Carmona et al., 1994) was studied with WIN-55,212-2 at concentrations of 0.081 and 0.01 μM. It shifted the concentration–inhibition curves to the right in a parallel way. The pK_B value was 9.2 at 1 Hz, and 9.3 at 3 Hz. The shift was compatible with competitive interaction.

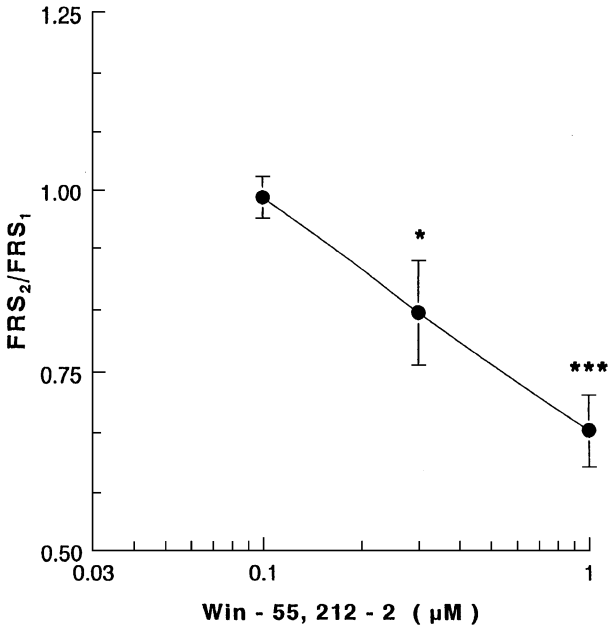

Fig. 1. Effect of WIN-55,212-2 on the release of [^{3}H]noradrenaline. Field stimulation: 3 Hz, 180 shocks and 3 ms impulse duration. WIN-55,212-2 was added to the perfusion Krebs' solution 15 min before the second stimulation (S_2) and kept in the solution throughout the experiments. Note that the cannabinoid CB_1 receptor agonist inhibits [^{3}H]noradrenaline release in a concentration-dependent manner. In control experiments, the FRS_2/FRS_1 ratios were 0.97 ± 0.06 (n = 8). $^*P < 0.05$; $^{**}P < 0.01$. For further information, see Materials and methods.

SR 141716A administered alone at a concentration of 1 μM failed to increase the release (Table 1) evoked by 1 Hz stimulation, but completely prevented WIN-55,212-2 from reducing the release of [^{3}H]noradrenaline (Table 1).

3.2. Effect of cannabinoid CB_1 receptor activation in the presence and absence of α_2-adrenoceptor-mediated negative feedback modulation

It is generally accepted that noradrenergic transmission is subject to α_2-adrenoceptor-mediated negative feedback modulation (cf. Starke, 1977). This was the case with the release of noradrenaline in the bronchi. When CH-38083, a selective α_2-adrenoceptor antagonist (Vizi et al., 1986), was used to exclude α_2-adrenoceptor-mediated negative feedback modulation of noradrenaline release, the release in response to field stimulation (3 Hz, 180 shocks) was significantly higher (Fig. 2A), it amounted to 16 616 ± 3811 Bq/g vs. 510 ± 1573 Bq/g measured in control experiments. Indeed, α_2-adrenoceptor antagonism significantly enhanced the stimulation-evoked release (Fig. 2A and B, Table 1).

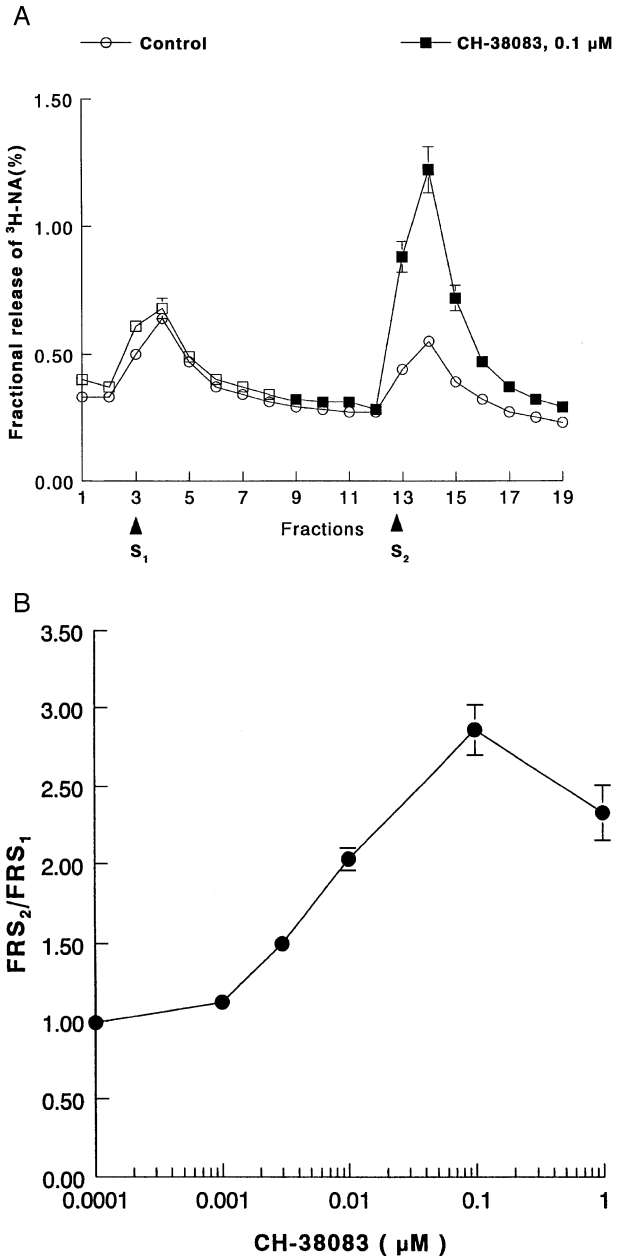

Fig. 2. Effect of α_2-adrenoceptor blockade on the release of [^{3}H]noradrenaline. Stimulations: 3 Hz (180 shocks) at 3rd (S_1) and 13th (S_2) collection periods. CH-38083, a selective α_2-adrenoceptor antagonist at a concentration of 0.1 μM was added to the perfusion fluid from the 8th collection period and continued throughout the experiments (A). Effects of CH-38083 on FRS_2/FRS_1 ratios at different concentrations (B). The fact that CH-38083 increased the release, indicated by the increase of the FRS_2/FRS_1 value, shows that there is a strong tonic control of noradrenaline release via α_2-adrenoceptors.

In using α_{2A}- or α_{2B}-adrenoceptor-selective antagonists, it turned out that at a concentration of 0.1 μM, the α_{2B} subtype-selective α_2-adrenoceptor antagonist BRL-44408 significantly enhanced the release (Table 1), and the α_{2A} subtype-selective ARC-239 had no effect at all. At higher concentrations, both drugs were effective. Prazosin, an α_1- and α_{2B}-adrenoceptor antagonist, also enhanced the release (Table 1), confirming that α_{2B}-subtypes of α_2-adrenoceptor are involved in the negative feedback modulation of noradrenaline release in the lung.

Since evidence was obtained (Vasquez and Lewis, 1999) that the activation of cannabinoid CB_1 receptors causes

Table 1
Effects of different cannabinoid CB_1 receptor agonists (WIN-55,212-2), antagonist (SR 14716A) and α_2-adrenoceptor antagonists on the release of [^{3}H]noradrenaline from guinea pig bronchus strip

	Drugs (concentration)	*n*	FRS_2/FRS_1[a]	*P*[b]
1	control	9	0.99 ± 0.03	
2	CH-38083, 0.1 μM	5	2.86 ± 0.16	< 0.0001 (2:1)
3	WIN-55,212-2, 0.3 μM	4	0.57 ± 0.05	< 0.0004 (3:1)
4	CH-38083, 0.1 μM + WIN-55,212-2, 0.3 μM	5	1.98 ± 0.18	< 0.0065 (4:2) < 0.0004 (4:3)
5	(CH-38083, 0.1 μM, SR 14716A, 1 μM) + WIN-55,212-2, 0.3 μM	4	3.24 ± 0.11	< 0.0025 (5:4) > 0.05 (5:2)
6	CH-38083, 0.1 μM + SR 14716A, 1.0 μM	3	3.06 ± 0.27	> 0.52 (6:2)
7	SR 141716A, 1.0 μM	3	0.98 ± 0.14	> 0.92 (7:1)
8	SR 141716A, 1.0 μM + WIN-55,212-2, 0.3 μM	6	0.96 ± 0.05	> 0.95 (8:7)
9	ARC 239, 0.1 μM	6	0.96 ± 0.06	< 0.63 (9:1)
10	ARC 239, 10 μM	4	2.28 ± 0.21	< 0.0001 (10:1)
11	BRL-44408, 0.1 μM	6	1.66 ± 0.13	< 0.0003 (11:1)
12	BRL-44408, 1.0 μM	4	2.52 ± 0.18	< 0.0001 (12:1)
13	Prazosin, 1.0 μM	4	1.50 ± 0.10	< 0.0004 (13:1)

Electrical field stimulation: 3 Hz, 3 ms, 180 shocks. For further details see Materials and methods.

[a] FRS_2/FRS_1 is the ratio between the fractional release of [^{3}H]noradrenaline by the second (S_2) and first (S_1) stimulation. Drugs were added between first and second stimulation and kept in the perfusion fluid throughout the experiment. If there is no significant change in the ratio (compared to control) it means that the drug has no effect on the release. If the ratio is higher, the release is higher, if less than the control, it indicates that the release is reduced.

[b] Different experimental groups (1–13) were statistically compared as indicated.

Table 2
Effects of WIN-55,212-2 on [^{3}H]noradrenaline release in the absence and presence of α_2-adrenoceptor-mediated negative feedback modulation

	n	Δ Reduction of [^{3}H]noradrenaline release (Bq/g) by CB_1 activation
Control	5	1227 ± 103
Without α_{2B}-adrenoceptor-mediated negative feedback	5	4278 ± 260[a]

WIN-55,212-2 was administered at a concentration of 1 μM 12 min before S_2 and kept in the perfusion fluid throughout the experiment. In order to inhibit α_2-adrenoceptor-mediated negative feedback modulation, CH-38083 was added to the Krebs solution at a concentration of 1 μM 15 min prior to S_2 and kept in solution. Note that the effect of cannabinoid CB_1 receptor activation is greater in the absence than in the presence of negative feedback modulation.

[a] Difference from control, $P < 0.01$. Stimulation: 3 Hz, 180 shocks, 3 ms.

sequestration of $G_{i/o}$-proteins from a common pool and prevents other $G_{i/o}$-coupled receptors sensitive to noradrenaline and somatostatin, the effect of WIN-55,212-2 on noradrenaline release was studied under conditions in which the tonic inhibitory influence of endogenous noradrenaline was excluded by α_2-adrenoceptor antagonists. Under conditions in which the α_2-adrenoceptor-mediated negative feedback modulation was prevented by CH-38083, WIN-55,212-2 was more potent (Table 2): the amount (Δ 4278 ± 260 Bq/g) of noradrenaline prevented to be released was significantly higher. In control experiments, however, in which the α_2-adrenoceptor-mediated negative feedback modulation was also in operation (see Fig. 2B) the activation of cannabinoid CB_1 receptors results in less inhibition of [^{3}H]noradrenaline release (Δ 1227 ± 103 Bq/g).

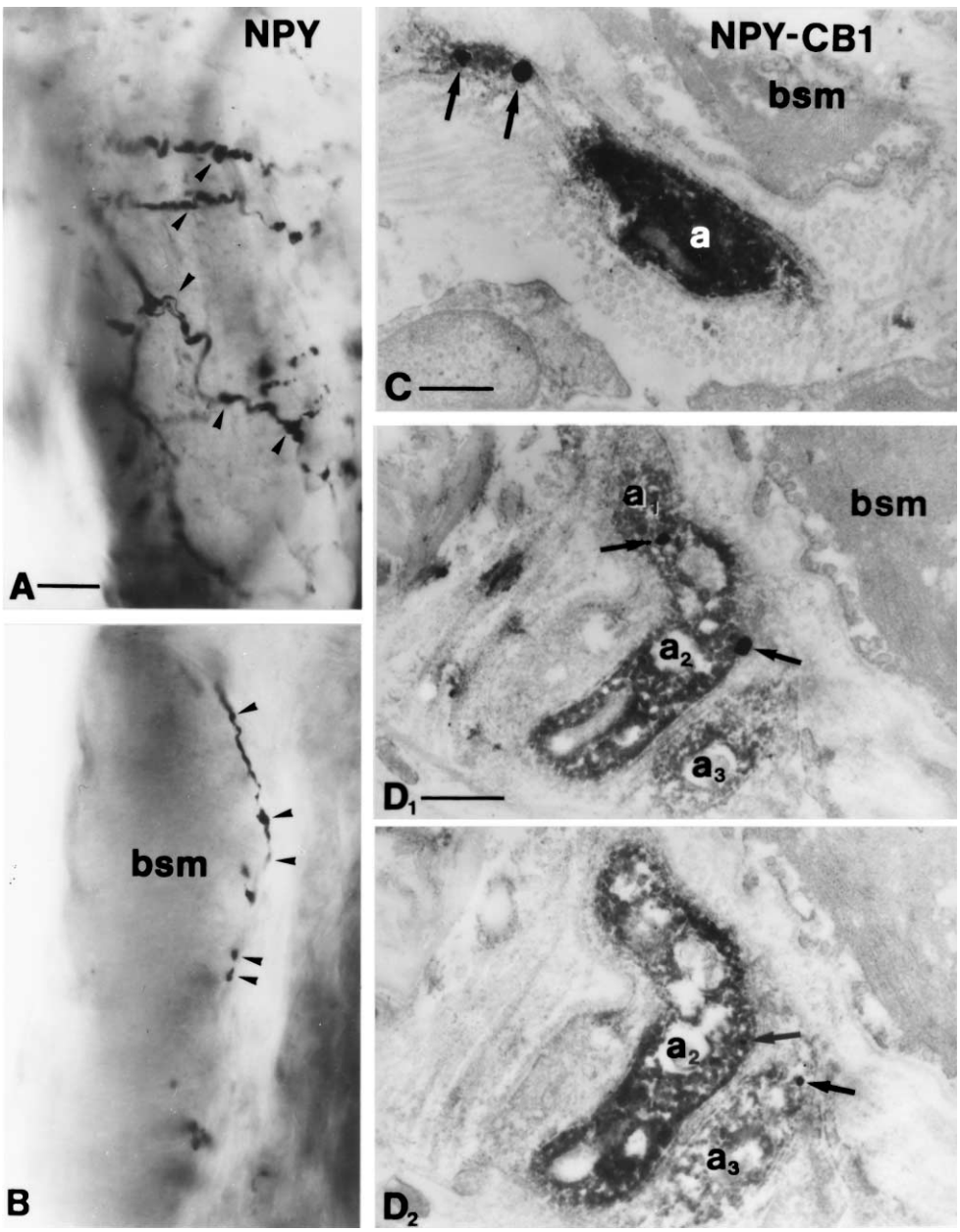

Fig. 3. (A, B) Light micrographs of lung sections immunostained for neuropeptide Y. Highly varicose (arrowheads) neuropeptide Y-positive (presumed sympathetic) fibers are running through the bronchi, in the immediate vicinity of smooth muscle cells (bsm). (C, D_1, D_2) Electron micrographs of lung tissue double-immunostained for neuropeptide Y (DAB, diffuse electron-dense end product) and cannabinoid CB_1 receptor (arrows, silver-enhanced colloidal gold). Double-labelled axons ("a" in (C), and serial sections of "a_{1-3}" in (D_1 and D_2)) are running in clusters adjacent to the bronchial smooth muscle (bsm) cells. Note that the cannabinoid CB_1 receptor antibody recognizes the intracellular C-terminus epitope of the receptor, therefore the gold particles are attached to the inner surface of the plasma membrane. Interestingly, cannabinoid CB_1 receptor labeling was found more often on pre-terminal axons than on varicosities (e.g. as in (C)). Scales: (A, B) 20 μm; (C, D_1, D_2) 0.5 μm.

3.3. Localization of CB_1 cannabinoid receptor on sympathetic nerve terminals in the guinea pig lung

To verify the localization of cannabinoid CB_1 receptors on sympathetic nerve terminals, we combined immunogold and immunoperoxidase staining for the cannabinoid CB_1 receptor and neuropeptide Y, respectively. Neuropeptide Y was used as a marker for sympathetic nerve fibers (Barnes, 1992). Since the intensity of immunogold staining for cannabinoid CB_1 receptor was insufficient to reveal the nerve fibers under the light microscope, neuropeptide Y immunostaining, providing more detailed fiber staining (see Fig. 3A and B), was used to select axons for further electron microscopic investigation.

In the electron microscope, most of the neuropeptide Y-positive axonal fibers were shown to express cannabinoid CB_1 receptor immunoreactivity. In the detailed analysis, five axon bundles scattered among the bronchial smooth muscle cells, containing 21 individual axons, were followed through approximately 25–30 serial ultrathin sections. From these, 12 were neuropeptide Y-positive. None of the neuropeptide Y-negative axons, but 9 out of the 12 neuropeptide Y-positive fibers, were positive for the cannabinoid CB_1 receptor (Fig. 3C and D1–2). The number of gold particles representing the localization of the receptor varied considerably; we found axons with 19 gold particles, but two contained only one in the analyzed 2-μm-thick slabs of tissue.

4. Discussion

Two cannabinoid receptors have been identified to date; cannabinoid CB_1 receptor is localized mainly in the central nervous system, whereas cannabinoid CB_2 receptor is located predominantly in immune cells (cf. Pertwee, 1997; Lay et al., 2000). Rather strong neurochemical (Katona et al., 1999, 2000), immunohistochemical (Katona et al., 1999, 2000), and electrophysiological (Ameri et al., 1999; Misner and Sullivan, 1999; Hájos et al., 2000; Al-Hayani and Davies, 2000; Hoffman and Lupica, 2000) evidence is available that the cannabinoid CB_1 receptor subtype of cannabinoid receptors is located presynaptically.

Recently it was shown that there are release-modulating cannabinoid receptors localized on the axon terminals of GABA(ergic) interneurons in rat (Katona et al., 1999) and human (Katona et al., 2000) hippocampus. In these studies, neurochemical and immunohistochemical evidence was obtained that the activation of cannabinoid CB_1 receptors results in inhibition of GABA release in response to axonal stimulation, and that this subset of GABA(ergic) axons is immunoreactive for cannabinoid CB_1 receptor. In pharmacological experiments, presynaptic inhibitory cannabinoid CB_1 receptors were shown on the cholinergic terminals in the hippocampus (Gifford and Ashby, 1996). As far as the effect of cannabinoids on transmitter release in the periphery is concerned, it was shown that tetrahydrocannabinol or WIN-55,212-2 and the endogenous ligand anandamide inhibit the stimulation-evoked release of endogenous acetylcholine from guinea pig myenteric plexus (Coutts and Pertwee, 1997) and [^{3}H]noradrenaline from vas deferens (Ishac et al., 1996; Trendelenburg et al., 2000) and from the atria via an interaction with cannabinoid CB_1 receptors (Ishac et al., 1996; Niederhoffer and Szabo, 1999). A cannabinoid CB_2 receptor-mediated inhibition was shown on acetylcholine release (Spicuzza et al., 2000) with CP 55,940. A cannabinoid CB_1 receptor-mediated increase in hippocampal acetylcholine release was shown (Acquas et al., 2001) in response to cannabinoid agonists assayed in vivo in microdialysis study. Regional differences in the cannabinoid receptor mRNA levels in response to WIN-55,212-2 suggest different weights of cannabinoid influence among areas in the brain (Romero et al., 1999).

In our experiments, stimulation of cannabinoid CB_1 receptors inhibited the release of labelled noradrenaline from isolated bronchi in a concentration-dependent manner. At a lower stimulation rate (1 Hz), the EC_{50} value for WIN-55,212-2-mediated inhibition of noradrenaline release (41.5 nM) is in reasonable agreement with the values obtained by others for inhibition of [^{3}H]acetylcholine release (Gifford and Ashby, 1996) and [^{3}H]GABA release (Katona et al., 1999) in hippocampal slices. At a higher stimulation rate, the EC_{50} value for WIN-55,212-2 is 320 nM. The inhibitory effect of WIN-55,212-2 is mediated by cannabinoid CB_1 receptors: SR 141716A, a cannabinoid CB_1 receptor-selective antagonist, fully antagonized the effect of WIN-55,212-2 and shifted the dose–response curves to the right. SR 141716A by itself failed to increase the release of noradrenaline, indicating that there was no ongoing release of endocannabinoids under our experimental conditions.

It is generally accepted that chemical signal transmission is subject to presynaptic modulation via auto- (for review see Starke, 1977) and heteroreceptors (for review see Vizi, 1985; Fuder and Muscholl, 1995; Schlicker and Gothert, 1998). In human and animal tissues, it has been shown that these receptors located on the axon terminals are targets of endogenous ligands and different drugs (cf. Vizi, 2000). Indeed, the noradrenergic terminals in the bronchi are also equipped with α_2-adrenoceptors, whose activation results in inhibition of noradrenaline release. Using α_{2A}-(ARC-239) and α_{2B}-(BRL-44408)-selective antagonists, it was shown that BRL-44408 and prazosin increased release indicating that α_{2B}-subtypes of α_2-adrenoceptors are present on the varicosities involved in negative feedback modulation (Table 1).

It has been shown (Vasquez and Lewis, 1999; Calandra et al., 1999) that cannabinoid CB_1 receptor activation can sequester $G_{i/o}$-proteins making them unavailable to couple to receptors, i.e. α_2-adrenoceptors for noradrenaline and somatostatin (Vasquez and Lewis, 1999). Therefore, the

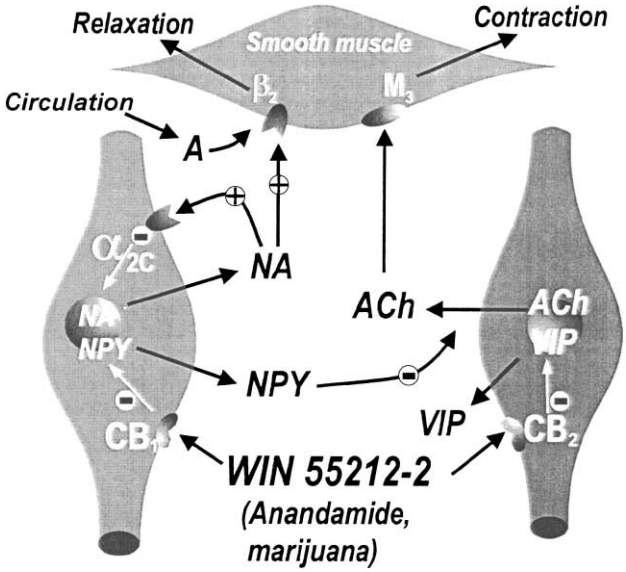

Fig. 4. Functional interaction between cholinergic and noradrenergic innervation of guinea pig airways: possible effects of cannabinoid CB_1 receptor activation. Stimulation of parasympathetic pathways results in acetylcholine and VIP release and causes bronchoconstriction, mucus secretion and bronchovasodilatation. Acetylcholine acts on the M_3 subtype of muscarinic receptors located on the smooth muscle. Sympathetic control of airways is mainly mediated via indirect action resulting in bronchodilatation. There is a functional interaction between noradrenergic and cholinergic neurons which are located nearby (Jones et al., 1991): noradrenaline released from the sympathetic nerve inhibits the release of acetylcholine via activation of α_2-adrenoceptors (Paton and Vizi, 1969; Grundström et al., 1981). Neuropeptide Y has a similar action. In addition, the circulating adrenaline (A), especially during stress may inhibit the release of acetylcholine and may have effect on β_2-adrenoceptors located on the smooth muscle cells resulting in bronchodilatation. Note that cannabinoid CB_1 receptors are located on the noradrenergic varicosities. Activation of these receptors by WIN 55,212-2 results in a decrease in the release of noradrenaline and neuropeptide Y. The endogenous anandamide might have additional effects on vanilloid receptors. Consequently, the release of acetylcholine increases and the activation of β_2-adrenoceptors located on the smooth muscle diminishes, resulting in a M_3-receptor-mediated bronchospasm. It has been shown (Spicuzza et al., 2000) that cannabinoid agonist CP 55,940 (CB_1/CB_2) and anandamide inhibit acetylcholine release via activation of CB_2 receptors, but this inhibitory action does not result in changes in functional responses, a fact that may be due to several factors able to influence responsiveness of the airways (Folkerts et al., 2001).

role of the cannabinoid CB_1 receptors in heteroreceptor-mediated inhibition of noradrenaline release was also studied under conditions in which the negative feedback modulation of noradrenaline release via α_2-adrenoceptor was absent. In the absence of α_2-adrenoceptor-mediated tonic control, the release of [^{3}H]noradrenaline was much higher and cannabinoid CB_1 receptor activation was more effective: the amount of [^{3}H]noradrenaline (expressed in Bq/g) prevented from release was greater in the absence of negative feedback modulation (Table 2). These facts indicate that noradrenaline release is able to escape from presynaptic tonic control by the endogenous ligand anandamide released locally in the lung in response to receptor activation (cf. Piomelli et al., 2000), under conditions in which the α_2-adrenoceptor-mediated tonic control is dominant.

At the ultrastructural level, the cannabinoid CB_1 receptor-immunoreactivity was exclusively confined to neuropeptide Y-positive axons. Since neuropeptide Y is a co-transmitter in noradrenergic neurons (cf. Barnes, 1992), the present finding indicates that in the lung, the noradrenergic axon terminals are equipped with cannabinoid CB_1 receptors. This is in line with the recent observations of Calignano et al. (2000). In contrast, the present results also suggest that neuropeptide Y-negative, probably parasympathetic fibers in the lung do not express cannabinoid CB_1 receptors, which is supported by recent pharmacological evidence for the lack of cannabinoid CB_1 receptor-mediated effect on acetylcholine release from guinea pig trachea (Spicuzza et al., 2000). The difference between the present study and Calignano et al. (2000) may derive from either a species difference or may be due to the inconsistent expression level of neuropeptide Y in sympathetic neurons. The active constituent of marijuana or the endogenous cannabinoid anandamide may produce bronchospasms by reducing the release of noradrenaline (Fig. 4), provided the bronchi are in relaxation in response to the tonic influence of catecholamines via β_2-adrenoceptors (cf. Hauck et al., 1997) in which noradrenaline released from the sympathetic innervation is involved.

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