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SPECIAL REPORT

In vitro functional evidence of neuronal cannabinoid CB_1 receptors in human ileum

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We investigated the effect of the cannabinoid agonist (+)WIN-55212-2 on human ileum longitudinal smooth muscle preparations, either electrically stimulated or contracted by carbachol. Electrical field stimulation mostly activated cholinergic neurons, since atropine and tetrodotoxin (TTX), alone or co-incubated, reduced twitch responses to a similar degree (85%). (+)WIN-55212-2 concentration-dependently inhibited twitch responses (IC_{50} 73 nM), but had no additive effect with atropine or TTX. The cannabinoid CB_1 receptor antagonist SR 141716 (pA_2 8.2), but not the CB_2 receptor antagonist, SR 144528, competitively antagonized twitch inhibition by (+)WIN-55212-2. Atropine but not (+)WIN-55212-2 or TTX prevented carbachol-induced tonic contraction.

These results provide functional evidence of the existence of prejunctional cannabinoid CB_1-receptors in the human ileum longitudinal smooth muscle. Agonist activation of these receptors prevents responses to electrical field stimulation, presumably by inhibiting acetylcholine release. SR 141716 is a potent and competitive antagonist of cannabinoid CB_1 receptors naturally expressed in the human gut.

Keywords: Cannabinoids; CB_1 receptors; WIN-55212-2; SR 141716; SR 144528; human ileum

Introduction Cannabinoid agonists are powerful psychoactive drugs that activate two receptor subtypes, CB_1 and CB_2. The cannabinoid CB_1 receptor was found in the brain, where it is believed to be responsible for psychoactive effects of cannabinoids, and in peripheral tissues, where agonists inhibit gut motility *in vitro* and *in vivo* in different animal species (Rinaldi-Carmona *et al*., 1998; Santucci *et al*., 1996; Calignano *et al*., 1997; Coutts & Pertwee, 1997; Colombo *et al*., 1998). The cannabinoid agonist (+)WIN-55212-2 was reported to inhibit electrically evoked contractions in rat ileum (Coutts & Pertwee, 1997; Izzo *et al*., 1998) and mouse *vas deferens* (Pertwee *et al*., 1995), through CB_1 receptor activation.

The cannabinoid CB_2 receptor has been found mainly in tissues responsible for the cannabinoids' effect on immune functions (Herkenham, 1995; Felder & Glass, 1998).

Whether cannabinoid receptors have any physiological or pharmacological role in the human gut remains to be established. Thus, we used human ileum longitudinal smooth muscle preparations contracted by electrical field stimulation or by carbachol, to study *in vitro* the pharmacological properties of the cannabinoid receptor agonist (+)WIN-55212-2 and its susceptibility to specific antagonists.

Methods *Tissue preparation* Specimens of human proximal or distal ileum were obtained from macroscopically normal regions of patients (12 males and 2 females, aged 42–83) undergoing surgery for duodenal or colonic cancer at the San Raffaele Hospital, Milan. Patients did not receive radiotherapy and had not been treated chronically with steroids, opioids or chemotherapy. Specimens were available at the operating theatre, each consisting of a whole ileum segment; they were washed in saline and immediately placed in a cold (4°C) pre-aerated (95% O_2, 5% CO_2) Krebs' solution (composition, mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7) and transported to Sanofi laboratories within about 30 min.

Mucosa and sub-mucosa were gently removed and longitudinal muscular regions were cut into strips approximately 3 mm wide (total length of each preparation 2.5 cm). Smooth muscle strips were used immediately or stored overnight (16–18 h) in cold (4°C) pre-aerated Krebs' solution; in the latter experimental condition, the strips maintained full sensitivity to (+)WIN 55212-2.

Experimental conditions Sixteen strips were dissected from each specimen, allowing a direct comparison of the (+)WIN-55212-2 response in the presence or absence of the different concentrations of antagonists.

Ileum strips were mounted in a 20-ml organ bath containing warm (37°C) aerated (95% O_2, 5% CO_2) Krebs' solution and stretched with 2 g; they were washed and isotonic contractions were evoked by an electrical field stimulation. Two platinum wire electrodes were placed on the top and bottom of the organ bath and electric field stimulation was elicited by a stimulator (Hugo Sachs Electronik, Friburg, Germany) coupled to a multiplexing pulse booster (Basile, Varese, Italy). Supramaximal stimulation (20 Hz; pulse width 2 ms; trains of 10 s every 2 min; 120–150 mA) was set up to elicit maximal strip contractility, then mA were reduced to obtain submaximal stimulation (20% reduction of maximal twitch response). After stabilization, about 2 h later, a cumulative WIN-55212-2 concentration response curve was plotted (contact time about 30 min). The response to atropine (1 μM) was determined at the end of the experiment for each strip and was used as reference for calculating the agonists' responses. At least one preparation was used as control for each specimen. Some strips were also contracted with a submaximal dose of carbachol

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(0.5 μM); these preparations developed a stable tonic contraction.

Antagonist incubation times were 1 h for naloxone, SR 141716 and SR 144528, 30 min for atropine and 15 min for TTX. (+)WIN 55212-2, SR 141716 and SR 144528 were dissolved in dimethylsulphoxide and other drugs in distilled water. Control tissues were incubated only with the vehicles.

Calculations and statistical analysis The agonist concentration producing 50% maximal effect (IC_{50}) was calculated using a four-parameter logistic model according to Ratkovsky & Reedy (1986), with adjustment by non-linear regression using the Levenberg-Marquard algorithm in RS/1 software.

The pA_2 for antagonists, as defined by Arunlakshana & Schild (1959), was obtained from linear regression of mean values of the log (Dr-1) vs the negative log of the antagonist concentration. When the Schild plot slope was not significantly different from 1, it was constrained to unity. Computer analysis was done as described by Tallarida & Murray (1987).

Chemicals SR 141716 (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride) and SR 144528 (N-(1S)-endo-1,3,3-trimethyl bicyclo (2.2.1)-heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylphenyl)-pyrazole-3-carboxamide) were synthesized at Sanofi Recherche, Montpellier, France. The following chemicals were purchased from the commercial sources indicated: Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.) TTX, carbachol, atropine sulphate, naloxone, (+)WIN 55212-2 and papaverine.

Results Electrical field stimulation of human ileum longitudinal smooth muscle preparations evoked regular twitch responses that were reduced by 1 μM atropine and 1 μM TTX: % inhibition (means$\pm$s.e.mean) 85$\pm$5 and 86$\pm$6, n=5.

As shown in Figures 1a and 2a, (+)WIN-55212-2 (10 nM–10 μM) dose-dependently inhibited the electrical twitch responses: its IC_{50} was 73 nM (40–120, n=7) (in parentheses 95% confidence limits) and its maximal effects vs 1 μM atropine 89$\pm$4% (means$\pm$s.e.mean). The inhibitory action of (+)WIN-55212-2 was competitively antagonized by the selective cannabinoid CB_1 antagonist SR 141716 (Figures 1 and 2) as shown by Schild regression analysis that gave a pA_2 of 7.93$\pm$0.04, n=5 and a slope of 1.26$\pm$0.05 (means $\pm$s.e.mean), not significantly different from unity; after constraining the slope to unity, pA_2 value was 8.2$\pm$0.07. SR 141716, up to the highest concentration tested, had no effect on electrical twitch responses. Naloxone (1 μM) had no effect either on the ability of WIN-55212-2 (10 nM–10 μM) to inhibit electrical twitch responses: IC_{50} 97 nM (60–124, n=4), or on the antagonist potency of SR 141716 (pA_2 7.9$\pm$0.04, n=4, slope 1).

The concentrations of WIN-55212-2 inhibiting electrical twitch responses did not change –IC_{50} 80 nM (52–106, n=4)- in the presence of the cannabinoid CB_2 receptor antagonist SR 144528 (0.1 μM). Neither (+)WIN-55212-2, nor SR 141716, TTX, or naxolone inhibited carbachol (0.5 μM) induced tonic contractions (data not shown); only atropine (1 μM) completely prevented the carbachol response, 98$\pm$2%, n=3 vs 0.1 mM papaverine.

Discussion The present results provide functional evidence of the presence in the human ileum longitudinal smooth muscle of prejunctional cannabinoid CB_1 receptors through which cannabinoid agonists inhibit electrically-evoked contractile responses, presumably by reducing acetylcholine release. Thus, (+)WIN-55212-2 concentration-dependently inhibited the electrical twitch response and this effect was competitively antagonized by the cannabinoid CB_1 receptor antagonist SR 141716 (Rinaldi-Carmona *et al.*, 1994) but not by the CB_2

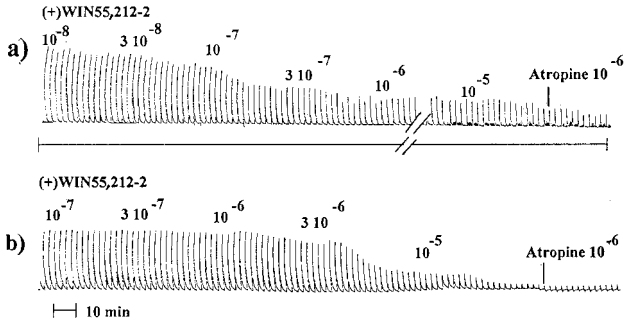

Figure 1 Representative tracings of the contractile response of human ileum longitudinal smooth muscle to electrical field stimulation and its inhibition by (+)WIN-55212-2 (10 nM–10 μM) in the absence (a) or presence (b) of the cannabinoid CB_1 antagonist, SR 141716 (0.1 μM).

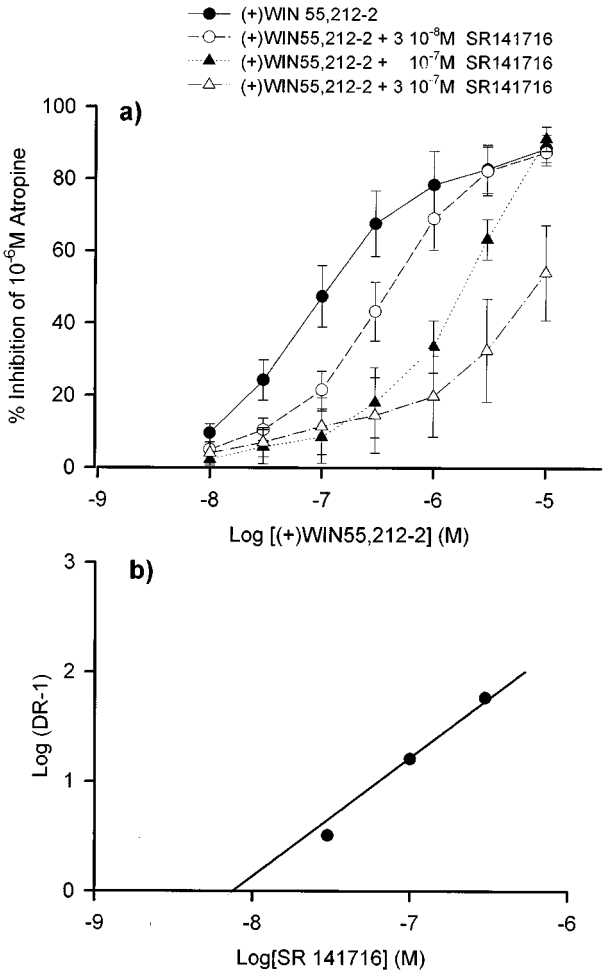

Figure 2 Effect of the cannabinoid agonist WIN-55212-2 on electrical twitch responses in human ileum longitudinal smooth muscle *in vitro*. Concentration response curves in the absence or presence of the cannabinoid CB_1 antagonist SR 141716 (a) and Schild plot (b). Results are means$\pm$s.e.mean of five ileum preparations from different patients.

antagonist SR 144528 (Rinaldi-Carmona *et al*., 1998). The electrical twitch response was almost completely prevented by atropine or TTX, and WIN-55212-2 had no further effect when added to either or both these agents.

The neuronal presynaptic location of cannabinoid CB_1 receptors in the human ileum is further indicated by the fact that (+)WIN-55212-2 did not inhibit the contraction induced by carbachol, which works by activating post-junctional muscarinic receptors on the smooth muscle.

Our results in the human gut are substantially in line with previous animal studies in which the role of neuronal cannabinoid CB_1 receptors modulating the release of acetylcholine was shown in rat hippocampus (Gessa *et al*., 1997) and in preparations of circular (Izzo *et al*., 1998) and longitudinal smooth muscle of guinea-pig ileum (Pertwee *et al*., 1996; Coutts & Pertwee, 1997); in the latter tissues, SR 141716 antagonized cannabinoid-induced inhibition of the twitch response and of acetylcholine release, with affinity comparable to our human preparation.

However, ileum preparations obtained from man and guinea-pigs differ, since in the guinea-pig, but not in human ileum, SR 141716 produced a small but significant increase in the amplitude of electrically-evoked contractions, suggesting a role for an endogenous agonist (Pertwee *et al*., 1996; Izzo *et al*., 1998); consistently, *in vivo* SR 141716 has been shown to increase small intestinal peristalsis of otherwise untreated mice (Colombo *et al*., 1998).

In conclusion, we have obtained functional evidence of the existence in human ileum longitudinal smooth muscle of prejunctional cannabinoid CB_1-receptors, by showing that agonist's responses are susceptible to the potent and selective antagonist SR 141716. SR 141716 thus appears to offer a valid mean of investigating the role of cannabinoid receptors in human gastrointestinal pathophysiology.

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