Behavioral/Systems/Cognitive

CB$_1$ Receptors Mediate the Analgesic Effects of Cannabinoids on Colorectal Distension-Induced Visceral Pain in Rodents

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Activation of cannabinoid receptors (CB$_1$, CB$_2$ and GPR$_{55}$) produces analgesic effects in several experimental pain models, including visceral pain arising from the gastrointestinal tract. We assessed the role of CB$_1$, CB$_2$, and GPR$_{55}$ receptors and the endogenous cannabinoid system on basal pain responses and acute mechanical hyperalgesia during colorectal distension (CRD) in rodents. The effects of cannabinoid receptor agonists and antagonists on pain-related responses to CRD were assessed in rats and in wild-type and CB$_1$ receptor knock-out mice. The dual CB$_{1/2}$ agonist, WIN55,212-2, and the peripherally acting CB$_1$-selective agonist, SAB-378, inhibited pain-related responses to repetitive noxious CRD (80 mmHg) in a dose-related manner in rats. The analgesic effects of WIN55,212-2 and SAB-378 were blocked by the selective CB$_1$ antagonist SR141716, but were not affected by the selective CB$_2$ antagonist SR144528. SR141716, per se, increased the responses to repetitive noxious CRD, indicative of hyperalgesia, and induced pain-related responses during non-noxious CRD (20 mmHg), indicative of allodynia. The cannabinoid receptor agonists anandamide, virodhamine and O-1602 had no effect. At analgesic doses, WIN55,212-2 did not affect colonic compliance. In accordance to the rat data, WIN55,212-2 produced analgesia, whereas SR141716 induced hyperalgesia, during noxious CRD (55 mmHg) in wild-type but not in CB$_1$-knock-out mice. These data indicate that peripheral CB$_1$ receptors mediate the analgesic effects of cannabinoids on visceral pain from the gastrointestinal tract. The allodynic and hyperalgesic responses induced by SR141716 suggest the existence of an endogenous cannabinoid tone and the activation of CB$_1$ receptors during noxious CRD.

Key words: cannabinoid receptors; endocannabinoids; hyperalgesia; SR141716; visceral pain; WIN55,212-2

Introduction

Biological effects of cannabinoids are mediated primarily through specific G-protein-coupled cannabinoid receptors. Two cannabinoid receptor subtypes (CB$_1$ and CB$_2$) and a putative third cannabinoid receptor (referred to as GPR$_{55}$) have been identified (Howlett et al., 2002; Brown, 2007; Ryberg et al., 2007). All three cannabinoid receptors are activated by endogenous lipid ligands known as endocannabinoids, with anandamide and 2-arachinodonyl glycerol being the most abundant endocannabinoids (for review, see Bisogno, 2008; Di Marzo, 2008). CB$_1$ receptors (CB$_1$Rs) are mostly expressed in the nervous system at both central and peripheral sites, including the enteric nervous system (Matsuda et al., 1990, 1992; Ahluwalia et al., 2000; Coutts et al., 2002; Bridges et al., 2003; MacNaughton et al., 2004). In addition, CB$_1$R expression has also been localized on normal colonic epithelium and smooth muscle (Casu et al., 2003; Ligresti et al., 2003). However, CB$_2$ receptors (CB$_2$Rs) are predominantly present in immune cells (Munro et al., 1993), whereas GPR$_{55}$ has a more promiscuous distribution with high expression levels in the adrenal glands, small intestine and CNS (Ryberg et al., 2007).

Among the roles of the endocannabinoid system, analgesia at central and peripheral levels, particularly associated to somatic pain, is largely documented in both clinical and preclinical studies (Ashton and Milligan, 2008; Hosking and Zajicek, 2008). With respect to visceral pain, the information related to the effects of cannabinoids is still limited and somewhat controversial. For instance, the endocannabinoid system has been involved in both proalgesic (Dinis et al., 2004) and analgesic responses in the urinary tract (Farquhar-Smith et al., 2002). A limited number of studies have implicated both CB$_1$Rs and CB$_2$Rs in the modulation of gastrointestinal functions, including sensitivity (for review, see Hornby and Prouty, 2004; Di Marzo and Izzo, 2006; Storr and Sharkey, 2007; Storr et al., 2008; Wright et al., 2008). So far, only two independent studies have evaluated the role of CB$_1$Rs and CB$_2$Rs in viscerosensitivity during colorectal distension in rats (Sanson et al., 2006; Kikuchi et al., 2008). According to these studies, both CB$_1$Rs and CB$_2$Rs elicited analgesic responses under basal conditions (Sanson et al., 2006) and during states of inflammation-induced hyperalgesia (Sanson et al., 2006; Kikuchi et al., 2008). Interestingly, the only data available in humans showed that the CB$_1$/CB$_2$ nonselective agonist, dronabinol, relaxed the colon but increased sensation ratings to distension in healthy volunteers (Esfandyari et al., 2007). This was interpreted as an interaction between the colonic motor effects and the central action of cannabinoids modulating perception (increasing awareness).

The aim of the present study was to further assess the role of cannabinoid receptors and the endogenous cannabinoid system on basal pain responses and acute mechanical hyperalgesia during colorectal distension (CRD)-elicited visceral pain in rodents. For this, the effects of different cannabinoids (agonists and antagonists) with various selectivity for CB$_1$Rs and CB$_2$Rs were assessed on visceral pain-related responses during CRD in rats and in wild-type and CB$_1$-knock-out mice.

Materials and Methods

Animals
Adult Sprague Dawley female rats (250–350 g; Harlan Netherlands) were used. In addition, adult female mice with a C57BL/6JOlaHsd background, normal (CB$_1$$^{+/+}$, wild-type mice) or deficient for the gene encoding for the CB$_1$R [CB$_1$$^{-/-}$, knock-out mice; originally created by Zimmer et al. (1999)] were used in some of the experiments. Mice were bred in house (Astra-Zeneca R&D Mölndal) and genotyped by PCR on DNA isolated from tail biopsy samples. Homozygous mutant mice grew and reproduced normally, with no obvious phenotypical differences when compared with age- and gender-matched wild-type control mice. Animals were housed in group cages (2–5 animals per cage) in an enriched environment with free access to food (Standard pellets, R3, Lactamin) and tap water on a 12:12 h light-dark cycle and controlled conditions of temperature (21°C) and humidity (50%). Before starting any experimental procedure, animals were allowed to acclimatize to the animal facility for at least 1 week after arrival. The phase of the oestrus cycle was not taken into account. All experiments were approved by the local animal ethics review committee in Göteborg, Sweden.

Colorectal distension
For CRD in rats, a 3 cm polyethylene balloon with a connecting catheter (made in-house) was inserted into the distal colon, 2 cm from the base of the balloon to the anus, during light isoflurane anesthesia (Forene, Abbott Scandinavia). The catheter was fixed to the base of the tail with tape. At the same time, an intravenous catheter (Neoflon, Becton Dickinson) was inserted in a tail vein for compounds administration. Similar procedures were followed in mice. In that case, a 2 cm polyethylene balloon with a connecting catheter (in-house made) was inserted in the distal colon, 0.5 cm proximal to the anus, during light isoflurane anesthesia (Forene), and fixed to the tail with tape to avoid any displacement. For compound administration, when needed, a polyethylene catheter (PE25) was placed subcutaneously in the neck region. Thereafter, animals were placed in Bollmann cages and allowed to recover from sedation for at least 15 min before starting the experiments.

During the CRD procedure, the balloons, through the connecting catheter, were connected to pressure transducers (P-602, CFM-k33, 100 mmHg; Bronkhorst Hi-Tec). A customized barostat (Astra-Zeneca) was used to control the air inflation and intraballoon pressure. A customized computer software (PharmLab on-line 4.0.1) running on a standard PC was used to control the barostat and to perform data collection and storage. The distension paradigms generated by the barostat were achieved by generating pulse patterns on an analog output channel. When assessing pain relates responses in rats, two CRD paradigms were used: (1) repeated noxious phasic distensions, 12 times at 80 mmHg, with a pulse duration of 30 s at 5 min intervals; and (2) repeated non-noxious phasic distensions, 10 times at 20 mmHg, with a pulse duration of 30 s at 5 min intervals. Distensions at 20 mmHg are considered non-noxious because they are below the estimated threshold pressure for pain in this model and do not evoke a significant visceromotor response (Tammpere et al., 2005; Martínez el al., 2007). In mice, the CRD paradigm used consisted on repeated noxious phasic distensions, 12 times at 55 mmHg, with a pulse duration of 10 s at 5 min intervals. Similar CRD paradigms have been used previously to assess pain-related responses in mice and rats (Tammpere et al., 2005; Arvidsson et al., 2006; Martinez and Melgar, 2008).

Responses to CRD were assessed by recording and quantitation of phasic changes in intraballoon pressure during the distending pulses, as previously described (Tammpere et al., 2005; Arvidsson et al., 2006). Pressure oscillations during the isobaric inflation of the intracolonic balloon reflect abdominal muscle contractions associated to the distension procedure and, therefore, are considered a valid assessment of the visceromotor response associated to the presence of pain of visceral origin (Tammpere et al., 2005; Arvidsson et al., 2006).

For compliance measurements, ascending phasic isobaric distensions from 2 to 20 mmHg, with a pulse duration of 1 min at 5 min intervals, were used. In this case, pressure dependent changes in volume during isobaric CRD were taken as a measure of colorectal compliance (Käll et al., 2007; Martínez et al., 2007). When assessing compliance, low distension pressures (within a range considered non-noxious) were chosen to avoid pain-related visceromotor responses that might interfere with the measurements of the intraballoon volume. In this case, the barostat system was used to inflate the balloon to a predetermined pressure and to simultaneously measure pressure and volume changes.

Data collection and analysis
The analog input channels were sampled with individual sampling rates and digital filtering was performed on the signals. The balloons were connected to pressure transducers (P-602, CFM-k33, 100 mmHg, Bronkhorst Hi-Tec) and the pressure signals sampled at 50 Hz. A highpass filter at 1 Hz was used to separate the contraction-induced pressure changes from the slow varying pressure generated by the barostat. A resistance in the airflow between the pressure generator and the pressure transducer further enhanced the pressure variations induced by the abdominal contractions of the animal. In addition, a band-stop filtered at 49–51 Hz was used to remove line frequency interference. A customized computer software (PharmLab off-line 4.0.1) was used to quantify the phasic changes of the balloon pressure signals. Changes in the highpass filtered balloon pressure signals were determined for the 30 s (rat) or 5 s (mouse) period before each pulse (baseline) and for each pulse. When assessing CRD in mice, and to minimize variability due to movement-related artifacts, responses to CRD were determined for each 3 consecutive pulses as a group, as previously described (Arvidsson et al., 2006; Martinez and Melgar, 2008).

Drugs
WIN55,212-2 [[(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenyl-methanone], mesylate form; Tocris Bioscience] (D'Ambra et al., 1992) was dissolved in 5% ethanol:5% Solutol HS 15:90% saline (v:v:v). Anandamide (arachidonoylethanolamide, N-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; Cayman Chemical) was dissolved in 5% (v:v) N,N-dimethylacetamide (DMA) in water. SAB-378 [naphthalen-1-yl-(4-pentyloxynaphthalen-1-yl)methanone (Astra-Zeneca R&D Mölndal] was administered as a nanosuspension in 5% DMA. O-1602 (5-methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol, Tocris Cookson) was administered as a nanosuspension in 10% DMA. SR141716 [5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; rimonabant; Astra-Zeneca R&D, Mölndal] (Rinaldi-Carmona et al., 1995) and SR144528 [N-[(1S)-endo-1,3,3-trimethyl bicycle [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; Astra-Zeneca R&D Mölndal) (Rinaldi-Carmona et al., 1998) were administered as nanosuspensions in 3% DMA. The corresponding vehicles were used as controls. Doses were selected based on preliminary experiments and published data showing efficacy in different pain models (Dziadulewicz et al., 2007). See Table 1 for biological activity and relative receptor affinity of compounds used in this study.

Experimental protocols
Before starting the experiments, to minimize the effects of restraint and to avoid movement-related artifacts, animals were habituated to Bollmann cages [Plexi-glass tubes; 18 cm length × 6 cm internal diameter (rat) or 10 cm length × 2.6 cm internal diameter (mouse); Astra-Zeneca Mölndal] 30 min per day, for at least 3 d. Animals were used in multiple occasions, with at least a 5 d interval between consecutive experiments.

Effects of cannabinoid receptor agonists on pain-related responses to CRD in rats. During the noxious, repetitive CRD paradigm, after the third distension, either anandamide (0.1 or 3 μmol/kg; corresponding to ~0.3 and 9 mg/kg, respectively), WIN55,212-2 (30, 50 or 100 nmol/kg; corre-

sponding to ~16, 26 and 52 μg/kg, respectively), O-1602 (20 μmol/kg, corresponding to ~5 mg/kg), SAB-378 (0.27, 1.4, 2.7 or 5.4 μmol/kg; equivalent to ~0.1, 0.5, 1 and 2 mg/kg, respectively) or their respective vehicles (1 ml/kg) was administered as a slow intravenous bolus injections over a 1 min period. Effects on the pain-related responses to CRD were assessed during distensions 4–12. In a separate experiment, the effects of the repeated administration of WIN55,212-2 (100 nmol/kg), given intravenously (1 ml/kg) after pulses 3 and 7 (total dose of 200 nmol/kg), were also tested. Virodhamine (3 μmol/kg; equivalent to ~1 mg/kg) or its vehicle (1 ml/kg) was administered intraperitoneally 10 min before starting the noxious, repetitive CRD protocol.

In a separate experiment after 3 phasic distensions at 80 mmHg, SR141716 (300 nmol/kg, corresponding to ~0.14 mg/kg), SR144528 (300 nmol/kg, corresponding to ~0.14 mg/kg) or their vehicle (1 ml/kg) was administered as an slow intravenous bolus injection, over a 1 min period, immediately before the administration of WIN55,212-2 (100 nmol/kg, i.v.); thereafter the responses to CRD were assessed during the remaining 9 phasic distensions.

Based on the former experiments, the effects of SR141716 on SAB-378 effects on CRD-induced pain-related responses were also investigated. For these experiments, SR141716 (300 nmol/kg) or vehicle (1 ml/kg) was administered intravenously after the third distension during the repetitive noxious CRD paradigm. Thereafter, after the seventh distension of the CRD protocol, either SAB-378 (2.7 μmol/kg) or its vehicle (1 ml/kg) were administered intravenously and the response to CRD during distensions 7–12 assessed.

Effects of the cannabinoid receptor antagonist SR141716 on pain-related responses to CRD in rats. During the noxious CRD paradigm, after three phasic distensions at 80 mmHg, SR141716 (300 or 1000 nmol/kg) or vehicle (1 ml/kg) was administered as a slow intravenous bolus injection over a 1 min period and the responses to CRD assessed for nine additional pulses at 80 mmHg.

In a separate experiment, after five consecutive non-noxious distensions (20 mmHg), SR141716 (300 nmol/kg) or its vehicle (1 ml/kg) was administered intravenously and the responses to five additional distensions at 20 mmHg assessed.

Effects of WIN55,212-2 on colonic compliance in rats. When assessing compliance, vehicle (1 ml/kg) or WIN55,212-2 (100 nmol/kg) was administered intravenously at the beginning of the ascending phasic CRD (2–20 mmHg) paradigm. In a set of experiments, treatments were repeated after the CRD at 10 mmHg (total dose of WIN55,212-2 administered: 200 nmol/kg). The maximal intracolonic volume achieved during each distension was determined and pressure-volume curves were constructed as a measure of colonic compliance (Käll et al., 2007; Martínez et al., 2007; Ravnefjord et al., 2008).

Pain-related responses to CRD in wild-type and CB$_1$-knock-out mice and effects of WIN55,212-2 and SR141716. Normal pain responses to mechanical stimulation of the colon were assessed in wild-type and CB$_1$-knock-out mice during the repetitive phasic (12 pulses × 55 mmHg) CRD protocol.

In separate experiments, the effects of WIN55,212-2 and SR141716 on pain responses to CRD were also assessed. In wild-type and CB$_1$-knock-out mice, WIN55,212-2 (200 nmol/kg), SR141716 (2000 nmol/kg), or the appropriate vehicles (5 ml/kg) was administered subcutaneously, as a slow bolus injection, after the third distension at 55 mmHg and the responses to CRD evaluated for the following nine distensions at 55 mmHg.

Statistical analysis
Data are expressed as mean ± SEM. In some cases data represent the cumulative response during the part of the CRD procedure after treatments (area under the curve, AUC). Differences between two groups were determined using paired or unpaired Student's *t* test, as appropri-

Table 1. Biological activity and relative receptor affinity of cannabinoids and cannabinoid-related compounds

Compound	Activity	CB$_1$	CB$_2$	GPR$_{55}$	Reference
Anandamide	Agonist	31 ± 6[a]	27 ± 6[a]	18 ± 3[a]	Ryberg et al., 2007
WIN55,212-2	Agonist	18 ± 3[a]	1 ± 0.2[a]	>30,000[a]	Ryberg et al., 2007
SAB-378	Agonist	15 ± 5[b]	98 ± 7.6[c]		Dziadulewicz et al., 2007
O-1602	Agonist	>30,000[a]	>30,000[a]	13 ± 2[a]	Ryberg et al., 2007
Virodhamine	Agonist	2920 ± 325[a]	381 ± 34[a]	12 ± 3[a]	Ryberg et al., 2007
SR-141716	Antagonist/Inverse agonist	12[d]	790[d]		Silvestri et al., 2008
SR-144528	Antagonist/Inverse agonist	>2820[d]	5.4[d]		Silvestri et al., 2008

[a]EC$_{50}$ (nM) for the stimulation of GTPγS binding on membranes from human embryonic kidney-HEK293s cells transiently transfected with human CB$_1$ or CB$_2$ receptors.

[b]EC$_{50}$ (nM) inhibiting specific binding of [^{3}H]CP55,940 in cell membranes from HEK293s cells transfected with the human CB$_1$ receptor.

[c]EC$_{50}$ (nM) inhibiting specific binding of [^{3}H]CP55,940 in cell membranes from CHO-K1 cells transfected with the human CB$_2$ receptor.

[d]K_i (nM) values for the inhibition of [^{3}H]CP55,940 binding in membranes from HEK cells transfected with human CB$_1$ or CB$_2$ receptors.

ate. Differences between multiple groups were assessed by ANOVA followed, when necessary, by a Student-Newman–Keuls multiple comparisons test. Within-group differences in the responses to repeated distensions were assessed by repeated measures ANOVA followed, when necessary, by a Student-Newman–Keuls multiple comparisons test. ED$_{50}$ values were determined by nonlinear regression to a sigmoidal equation with variable slope (GraphPad Prism, version 4.0.3, GraphPad Software). Data were considered statistically significant when p was ≤0.05.

Results
Effects of cannabinoid receptor agonists on pain-related responses to CRD in rats
Under basal conditions, activity of the abdominal musculature was low (mean basal activity: 0.06 ± 0.01) and stable over the experimental time. Phasic CRD at 80 mmHg induced abdominal contractions, as revealed by the oscillations in intraballoon pressure during the distension time (0.19 ± 0.04; $p < 0.05$ vs basal) (Fig. 1*A*). In addition, during repetitive distensions at 80 mmHg in vehicle-treated rats ($n = 8$), the response to distension increased over time by 113 ± 38% (first distension: 0.16 ± 0.03; 12th distension: 0.30 ± 0.05 mmHg; $p < 0.05$) (Fig. 1*A*).

WIN55,212-2 (100 nmol/kg, $n = 8$), administered intravenously between distensions 3 and 4, inhibited the overall response to CRD (distensions 4–12) by 40 ± 9.5% ($p < 0.05$ vs vehicle) (Fig. 1*A*,*C*). The effects of WIN55,212-2 were transitory and present between distension 4 and 7 (~15 min), reaching values similar to those observed for the basal activity of the abdominal musculature. Thereafter, responses to CRD recovered and reached values similar to those observed in the vehicle-treated group (Fig. 1*A*). Based on these observations, the effects of two consecutive doses of WIN55,212-2 were also determined. Two doses of WIN55,212-2 (100 nmol/kg each, $n = 7$), administered after distensions 3 and 7, respectively, resulted in an enhanced inhibitory effect of CRD responses, with an overall inhibition of 63 ± 14% of the response to CRD (distensions 4–12) (Fig. 1*B*,*C*). Lower doses of WIN55,212-2 (30 or 50 nmol/kg) had no significant effects on the responses to CRD (Fig. 1*C*).

SAB-378 inhibited the responses to repetitive noxious CRD in a dose-related manner. At doses of 1.4 ($n = 4$), 2.7 ($n = 8$) and 5.4 μmol/kg ($n = 4$), SAB-378 inhibited the response to CRD (distension 4–12) by 82 ± 3%, 98 ± 6% and 90 ± 2%, respectively (all $p < 0.05$ vs vehicle) (Fig. 2). A lower dose, 0.27 μmol/kg ($n = 4$) inhibited the responses to CRD by 28 ± 15%, without reaching statistical significance. According to these data, the calculated ED$_{50}$ was 0.48 μmol/kg (95% confidence interval: 0.33–0.67 μmol/kg; $r^2 = 0.9085$). At effective doses, the effects of SAB-378 were long-lasting (distensions 4–12) and lead to a complete inhibition of the response to distension with values comparable to the basal activity of the abdominal musculature and no signs of
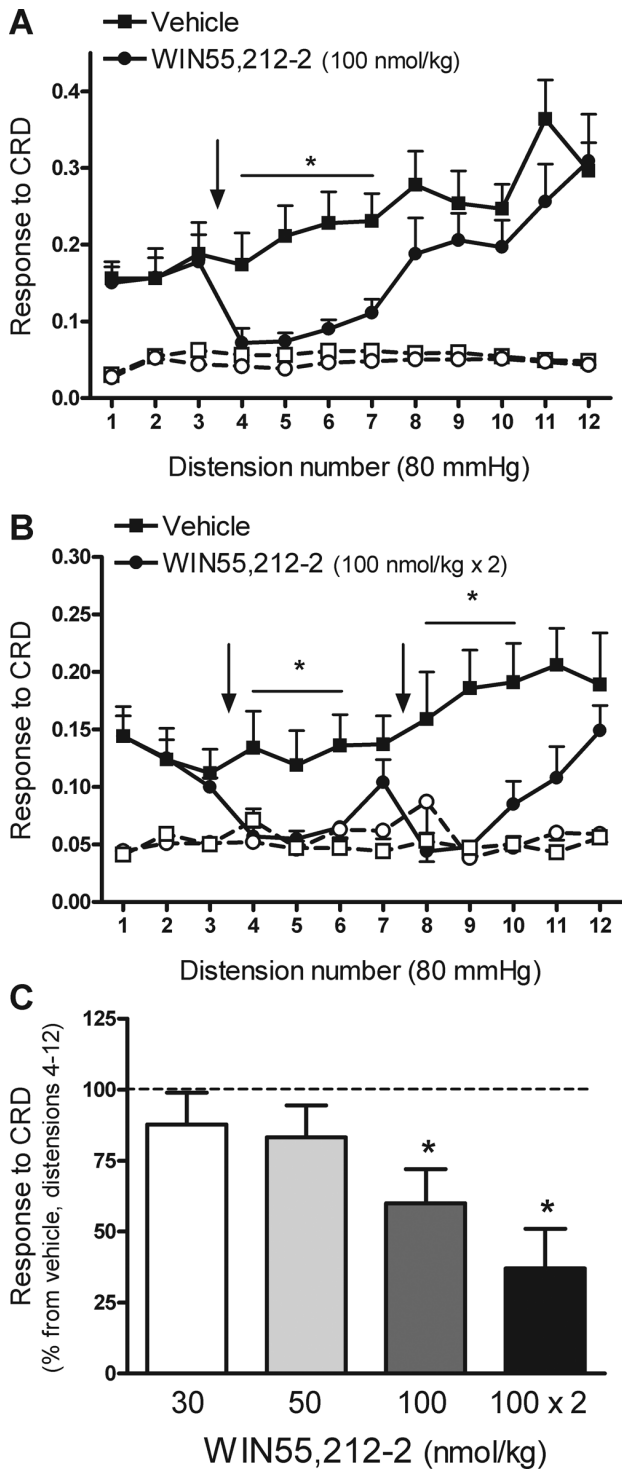
Figure 1. Effects of WIN55,212-2 on repetitive noxious CRD-evoked pain related responses in rats. **A**, Effects of WIN55,212-2 (100 nmol/kg) or vehicle (1 ml/kg), administered intravenously between distensions 3 and 4, on CRD-evoked pain related responses. Graphs with open symbols and broken lines correspond to the basal activity of the abdominal musculature between distensions. The arrow indicates the point of WIN55,212-2 or vehicle administration. Data are mean ± SEM of eight animals per group. *$p < 0.05$ versus corresponding response in the vehicle-treated group. **B**, Effects on pain-related responses to CRD of repeated administration of WIN55,212-2 (100 nmol/kg) between distensions 3 and 4 and 7 and 8 (as indicated by the arrows). Graphs with open symbols and broken lines correspond to the basal activity of the abdominal musculature between distensions. Data are mean ± SEM of seven animals per group. *$p < 0.05$ versus corresponding response in the vehicle-treated group. **C**, Overall effects of WIN55,212-2 (% inhibition vs the response in the vehicle group, set up at 100%; broken line) on pain-related responses during distensions 4–12 of the noxious CRD protocol. Data are mean ± SEM of 4–8 animals per group. *$p < 0.05$ versus vehicle.
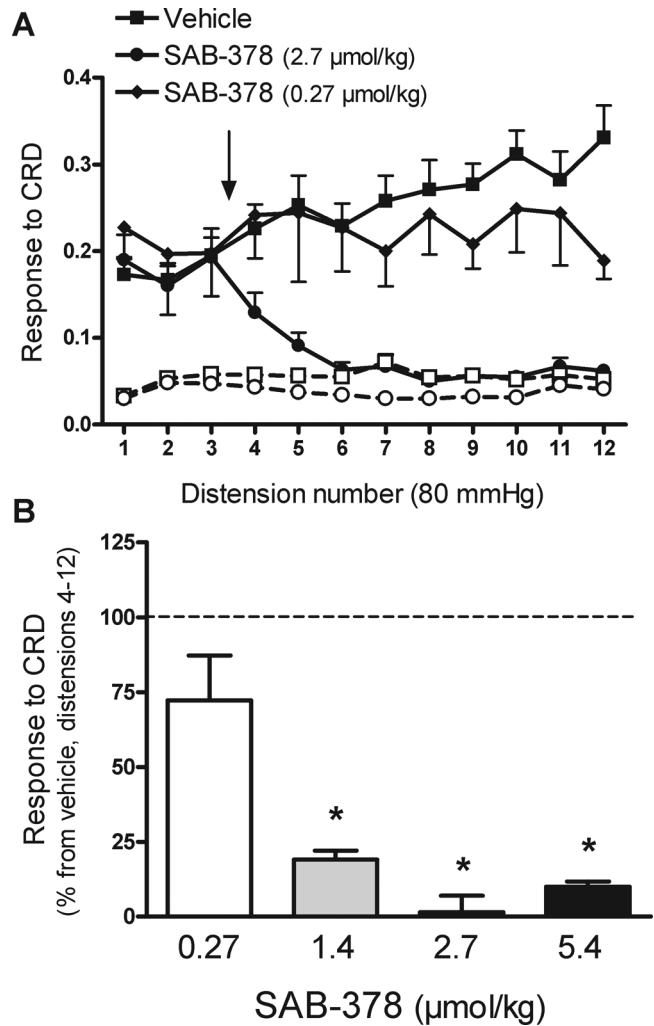

Figure 2. Effects of SAB-378 on repetitive noxious CRD-evoked pain related responses in rats. **A**, Effects of SAB-378 (0.27 or 2.7 μmol/kg) or vehicle (1 ml/kg), administered intravenously between distensions 3 and 4, on CRD-evoked pain-related responses. Graphs with open symbols and broken lines correspond to the basal activity of the abdominal musculature between distensions. The arrow indicates the point of SAB-378 or vehicle administration. Data are mean ± SEM of 4–8 animals per group. **B**, Overall effects of SAB-378 (% inhibition vs the response in the vehicle group, set up at 100%; broken line) on pain-related responses during distensions 4–12 of the noxious CRD protocol. Data are mean ± SEM of 4–8 animals per group. *$p < 0.05$ versus vehicle.

recovery at the end of the CRD protocol (~45 min posttreatment) (Fig. 2).

Neither anandamide (0.1 or 3 μmol/kg; $n = 3$ and 8, respectively) nor virodhamine (3 μmol/kg, $n = 4$) nor O-l602 (20 μmol/kg, $n = 4$) affected the response to CRD (Fig. 3).

At the doses tested, no gross side-effects were observed for none of the compounds used.

Effects of the cannabinoid receptor antagonists SR141716 and SR144528 on pain-related responses to CRD and on WIN55,212-2 and SAB-378 effects in rats

In vehicle + WIN55,212-2 (100 nmol/kg, i.v.)-treated rats ($n = 6$), the response to CRD was inhibited between distensions 4 and 8. Responses to distension during this period were similar in magnitude to the basal activity of the abdominal musculature (Fig. 4A), consistent with the experiments described above. SR141716 (300 nmol/kg, i.v.), administered immediately before
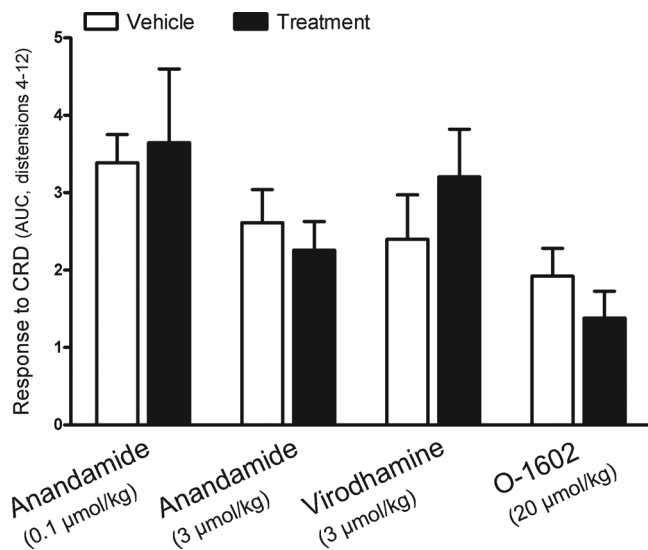
Figure 3. Effects of the cannabinoid agonists anandamide, virodhamine, and O-1602 on repetitive noxious CRD-evoked pain-related responses in rats. Compounds, or their respective vehicles, were administered intravenously between distensions 3 and 4 and the data represent the overall response (AUC) for distensions 4–12. In the case of virodhamine, treatments were applied before starting the CDR protocol (see Materials and Methods for details) and data represent the overall response (AUC) for the complete protocol (distensions 1–12). Data are mean ± SEM of 3–8 animals per group.

WIN55,212-2 (100 nmol/kg, i.v.) ($n = 6$), completely prevented the inhibitory effects of WIN55,212-2 on the pain responses to CRD (Fig. 4A). In contrast, pretreatment with SR144528 (0.3 μmol/kg, $n = 8$) did not affect the inhibitory effects of WIN55,212-2 on CRD responses (Fig. 4B).

Similarly, pretreatment with SR141716 (0.3 μmol/kg, i.v.) completely prevented SAB-378 (2.7 μmol/kg, i.v.)-induced inhibition of pain responses during noxious CRD (Fig. 5).

Based on these observations, the effects of SR141716, per se, on visceromotor responses to noxious and non-noxious CRD were assessed in separate experiments. SR141716 (0.3 or 1 μmol/kg, i.v.) administered between distensions 3 and 4 of the repeated noxious CRD protocol resulted in an increase in the response to CRD compared with vehicle-treated rats. Overall, SR141716 increased the response to CRD by 47 ± 27% and 57 ± 29% at 0.3 and 1 μmol/kg, respectively ($n = 8$ for each, both $p < 0.05$ vs vehicle) (Fig. 6).

Repetitive distensions at 20 mmHg did not elicit any increase in the activity of the abdominal musculature over basal values (mean basal: 0.05 ± 0.01; mean response to five repeated distensions at 20 mmHg: 0.06 ± 0.01; pooled data from all animals corresponding to the five consecutive distensions before treatments, $n = 24$). After five consecutive distensions at 20 mmHg, administration of SR141716 (0.3 μmol/kg, i.v., $n = 12$) resulted in an increase in the response to subsequent distensions (Fig. 7A). Peak response to distension reached values (0.13 ± 0.02) similar to those observed during distensions at 80 mmHg in previous control experiments (0.19 ± 0.04). Overall, during the experimental time after SR141716 administration (distension 6–10) the response to CRD increased by 100 ± 33% and 110 ± 49% over the basal response before treatment or the response in vehicle-treated animals, respectively (both $p < 0.05$) (Fig. 7B).
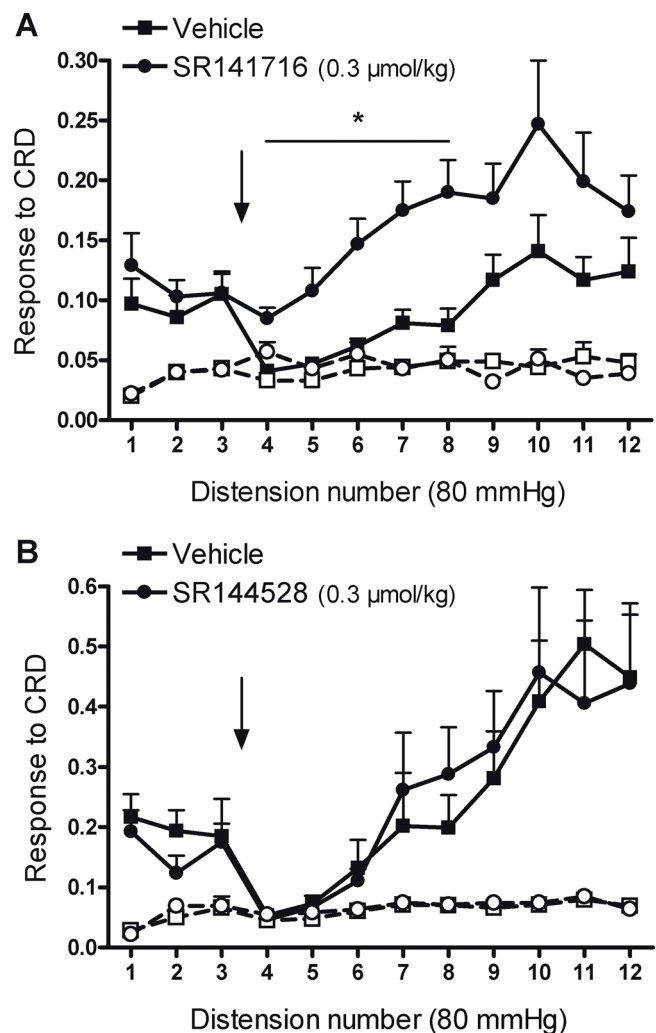

Figure 4. A, B, Effects of the selective CB$_1$ antagonist, SR141716 (A), and the selective C$_{B2}$ antagonist, SR141528 (B), on WIN55,212-2-induced analgesic responses during repetitive noxious CRD ni rats. SR141716 (0.3 μmol/kg), SR144528 (0.3 μmol/kg), or vehicle (1 ml/kg) was administered intravenously between distensions 3 and 4 and immediately before the intravenous administration of WIN55,212-2 (100 nmol/kg) (indicated by the arrow). Graphs with open symbols and broken lines correspond to the basal activity of the abdominal musculature between distensions. Data are mean ± SEM of 6–8 animals per group. *$p < 0.05$ versus corresponding response in the vehicle-treated group.

Effects of WIN55,212-2 on pressure-volume responses during CRD in rats

In vehicle-treated animals ($n = 8$), during phasic CRD at 2–20 mmHg, intracolonic volume increased in a pressure-related manner (Fig. 8). WIN55,212-2 (100 nmol/kg or 100 nmol/kg × 2; $n = 8$ for each) did not affect the volume-pressure relationship during CRD (Fig. 8).

Pain-related response to CRD and effects of WIN55,212-2 and SR141716 in wild-type and CB$_1$-knock-out mice

Basal activity of the abdominal musculature was similar in wild-type (0.020 ± 0.002, $n = 8$) and CB$_1$-knock-out mice (0.016 ± 0.001, $n = 5$; $p > 0.05$ vs wild type). CRD at 55 mmHg elicited a distension-related increase in the activity of the abdominal musculature, with similar magnitude in both groups of animals (overall response to distension, wild type: 0.26 ± 0.02; CB$_1$-knock-out: 0.31 ± 0.08; $p > 0.05$).
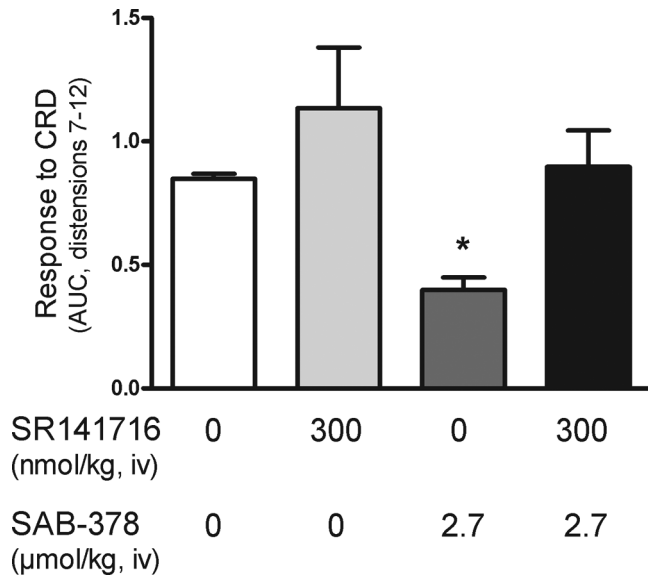
In wild-type mice, WIN55,212-2 (200 nmol/kg, $n = 7$), ad-

Figure 5. Blocking effects of the selective CB$_1$ antagonist, SR141716, on SAB-378-indcued analgesia during noxious CRD in rats. Data represent the overall response to CRD (AUC of distensions 7–12) in animals pretreated with either vehicle or SR141716 (300 nmol/kg) and receiving thereafter either vehicle or SAB-378 (2.7 μmol/kg). Data are mean $\pm$ SEM of 4–5 animals per group. *$p < 0.05$ vs other experimental groups.

Figure 6. Effects of the selective CB$_1$ antagonist, SR141716 on visceral pain-related responses during repetitive noxious CRD ni rats. **A**, SR141716 (0.3 or 1 μmol/kg) or vehicle (1 ml/kg) was administered intravenously between distensions 3 and 4 of the noxious CRD protocol (arrow). The broken line represents the mean basal activity of the abdominal musculature between distensions. **B**, Overall response to CRD (area under the curve, AUC, for distensions 4–12) for the different experimental groups. Data are mean $\pm$ SEM of 8 animals per group. *$p < 0.05$ versus the vehicle group.

ministered subcutaneously after the third distension, reduced the responses to CRD compared with vehicle-treated animals. Inhibition of CRD responses was observed between distensions 4 and 9; thereafter the response to CRD was similar to that observed in vehicle-treated wild-type mice (Fig. 9*A*). Overall, WIN55,212-2 inhibited the response to CRD by 30 $\pm$ 7% for distensions 4–12 ($p < 0.05$ vs vehicle) (Fig. 8*C*). Under the same experimental conditions, WIN55,212-2 (200 nmol/kg, $n = 7$) did not affect the responses to CRD in CB$_1$-knock-out mice (Fig. 9*B*,*C*).

In wild-type mice, SR141716 (2000 nmol/kg, $n = 7$), administered subcutaneously after the third distension, increased the overall response to CRD by 93 $\pm$ 24% over the response to distension observed in vehicle-treated wild-type animals (Fig. 10*A*,*C*). In CB$_1$-knock-out mice, administration of SR141716 (2000 nmol/kg, $n = 7$), did not affect the responses to CRD when compared with the response in vehicle-treated animals (Fig. 10*B*,*C*).

Discussion

The present data indicates that CB$_1$Rs, but not CB$_2$Rs or the putative cannabinoid receptor, GPR$_{55}$, are involved in the modulation of basal visceral sensitivity in a model of visceral pain in rodents. Enhancement of visceral pain-related responses, resulting in a hyperalgesic state, after treatment with the CB$_1$ antagonist/inverse agonist, SR141716, suggests the endogenous release of endocannabinoids during the stimulation of pain-related pathways. Moreover, the presence of allodynic-like responses after treatment with SR141716 suggests the existence of an endogenous cannabinoid tone, likely modulating (containing) pain-related responses.

Cannabinoids are well known analgesics, particularly as it relates to somatic pain (Rice at el., 2002; Fox and Bevan, 2005). Recent studies in several species, including humans, support a role for endocannabinoids modulating visceral pain (Jaggar et al., 1998; Farquhar-Smith et al., 2002; Sanson et al., 2006; Esfandyari et al., 2007; Rousseaux et al., 2007; Kikuchi et al., 2008). Sanson et al. (2006) implicated CB$_{1/2}$Rs in the modulation of basal viscer-
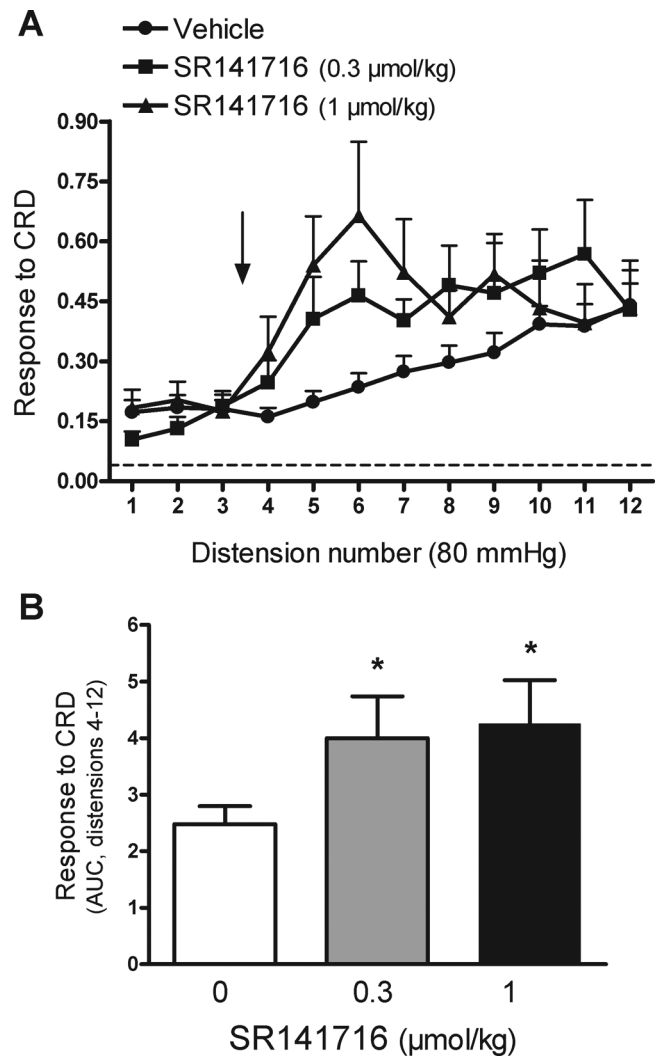
osensitivity in rats. However, according to the present results, CB$_1$Rs account for the full analgesic effects of cannabinoids on basal CRD-associated pain responses. Several observations support a predominant role of CB$_1$Rs mediating the visceral analgesic effects of cannabinoids. First, the distribution of CB$_1$Rs is consistent with a role in mediating pain responses. CB$_1$Rs are present in enteric primary sensory neurons, considered the primary nociceptive pathway within the gut (Ahluwalia et al., 2000). Moreover, CB$_1$Rs are present in the dorsal horn of the spinal cord and in brain nuclei involved in the integration of pain-related responses (Farquhar-Smith et al., 2000; Salio et al., 2002a,b; Cristino et al., 2006). Pharmacological data also support a role for CB$_1$Rs because the effects of WIN55,212-2 were blocked by SR141716, but not by the CB$_2$ antagonist/inverse agonist, SR144528. Moreover, the CB$_1$-preferring agonist, SAB-378, also inhibited the responses to CRD in a SR141716-sensitive manner. These observations in rats might be considered biased from having chosen a noninactive per se dose of SR141716. However, a
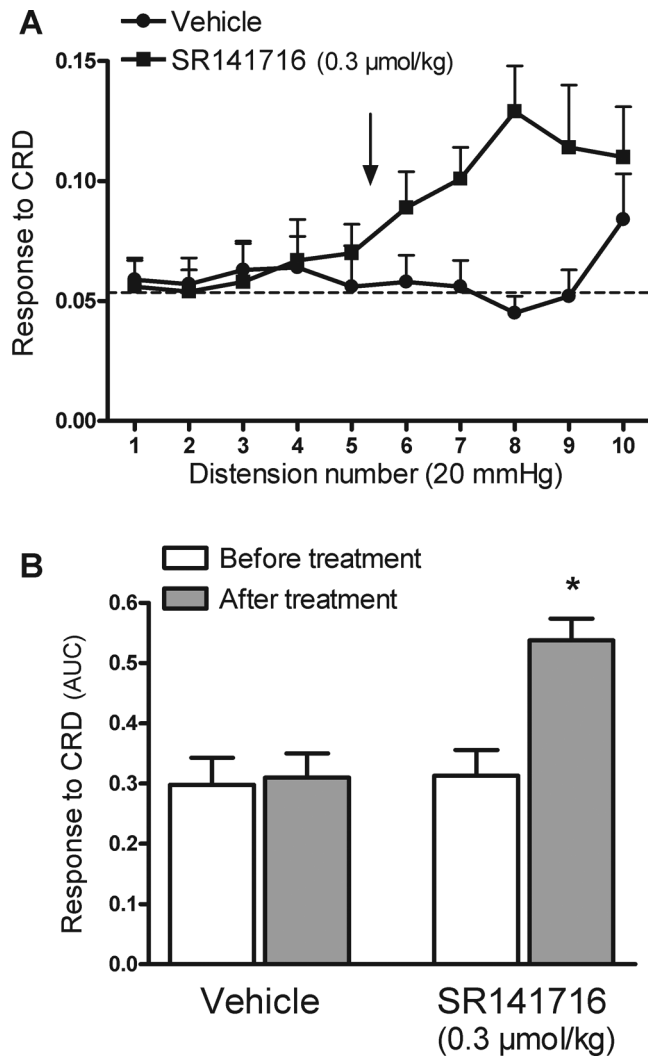
A

B

Figure 7. Effects of the selective CB_1 antagonist, SR141716 on visceral pain-related responses during repetitive non-noxious CRD ni rats (10 distensions at 20 mmHg). **A**, SR141716 (0.3 μmol/kg) or vehicle (1 ml/kg) was administered intravenously between distensions 5 and 6 of the non-noxious CRD protocol (arrow). The broken line represents the mean basal activity of the abdominal musculature between distensions. **B**, Overall response to CRD (area under the curve, AUC, before and after treatments, distensions 1–5 and 6 –10, respectively) for the different experimental groups. Data are mean $\pm$ SEM of 12 animals per group. $*p < 0.05$ versus the response to CRD before treatment.

Figure 8. Effects of WIN55,212-2 on the pressure–volume relationship during CRD in rats. Vehicle (1 ml/kg $\times$ 2) or WIN55,212-2 (100 nmol/kg, a single dose or two doses) was administered intravenously immediately before starting the CRD procedure (single dose) and after the 5fh distension (repeated treatment), as indicated by the arrows. Data are mean $\pm$ SEM of eight animals per group.

clear-cut evidence for the role of CB_1Rs is provided by the use of CB_1-knock-out mice. Indeed, effects of WIN55,212-2 and SR141716 were absent in CB_1-knock-out mice, but present in wild-type animals. For the first time, a potential role for the putative CB_3 receptor, GPR_{55}, in viscerosensitivity was investigated. None of the GPR_{55}-preferring agonists tested, virrodhamine or O-1602 (Table 1), affected CRD-induced pain responses. All together, these observations strongly indicate that analgesic effects of cannabinoids on visceral pain, in basal conditions, are mediated through the activation of CB_1Rs. This contrasts with reports indicating that CB_2Rs might be involved in normal somatic antinociception (Malan et al., 2001; Nackley et al., 2004), suggesting differences between somatic and visceral pain in cannabinoid-dependent analgesia. In addition, direct and/or indirect effects of cannabionids on TRPV1 channels, also implicated in pain responses, cannot be excluded as contributing mechanisms to the effects observed (De Petrocellis et al., 2001; Dinis et al., 2004; Jeske et al., 2006; Patwardhan et al., 2006; Fioravanti et al., 2008).
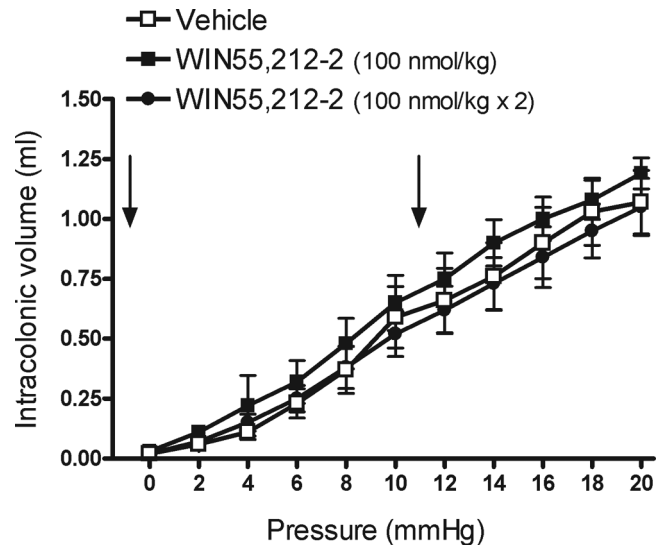
Pharmacokinetic properties of the cannabinoid agonists tested may explain the differences in the effects observed. The relatively short duration of action of WIN55,212-2 (15–20 min in rats and $\sim$30 min in mice) might be explained by its short active half life *in vivo* (Croxford and Miller, 2003). Similarly, low metabolic stability (Khanolkar and Makriyannis, 1999) most likely accounts for the lack of effects of anandamide. Indeed, anandamide inhibited the response to CRD when its half-life was prolonged by inhibiting its endogenous metabolism (data not shown) (Pinto et al., 2002; Capasso et al., 2005; Palmer et al., 2008). Likewise, the long-lasting analgesic effects of SAB-378 probably reflect its long half life *in vivo* after intravenous administration (Dziadulewicz et al., 2007).

CRD-induced pain responses were similar in CB_1-knock-out and wild-type mice. This agrees with data showing that CB_1-knock-out mice are normoalgesic in the tail flick test while being hypoalgesic in the hot plate or the formalin tests (Zimmer et al., 1999), and suggests heterogeneity in the involvement of CB_1Rs in basal pain responses, depending on the nociceptive stimuli and the sensory pathway stimulated. Alternatively, compensatory changes might account for the normalization in visceral pain responses in CB_1-knock-out mice. Nevertheless, the lack of effects of WIN55,212-2 and SR141716 in CB_1-knock-out mice strongly supports a role for CB_1Rs mediating the analgesic effects of cannabinoids on visceral pain. In general, effective doses were higher in mice than in rats, likely due to the different routes of administration used and/or species-related pharmacodynamic differences.

Interestingly, treatment with SR141716 led to the development of both hyperalgesia and allodynia. SR141716 increased pain-related responses to noxious CRD in rats and wild-type mice, but not in CB_1-knock-out mice, indicating the development of visceral hyperalgesia in response to CB_1R-blockade. This suggests that endogenous cannabinoids are released during the CRD procedure, probably due to the activation of pain-related pathways. Indeed, endocannabinoids and other pain-modulatory mediators, such as GABA, seem to be released during
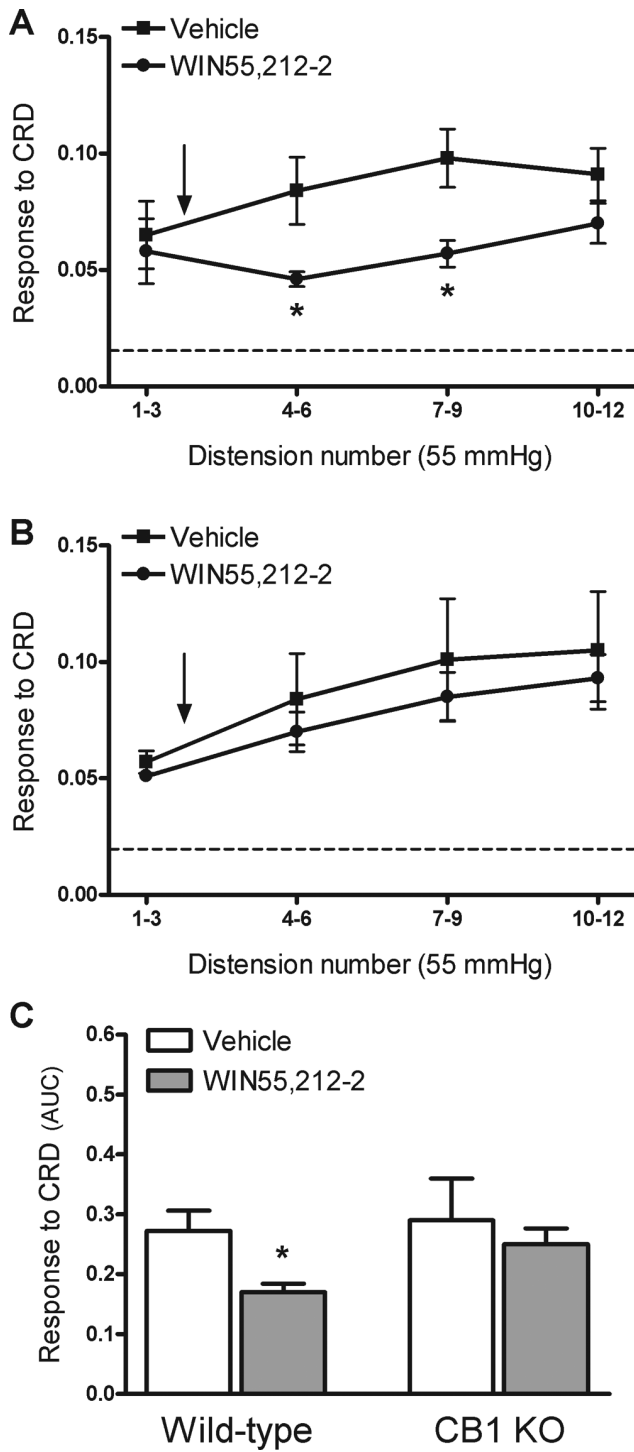
Figure 9. Effects of WIN55,212-2 on visceral pain-related responses to CRD in wild-type and CB$_1$-knock-out (KO) mice. WIN55,212-2 (200 nmol/kg) or vehicle (5 ml/kg) was administered subcutaneously after the third distension at 55 mmHg (arrow). **A**, Responses to CRD in wild-type mice. The broken line represents the mean basal activity of the abdominal musculature between distensions. **B**, Responses to CRD in CB$_1$-knock-out mice. The broken line represents the mean basal activity of the abdominal musculature between distensions. **C**, Overall response to CRD (area under the curve, AUC, for distensions 4–12) in wild-type and CB$_1$-knock-out mice. Data are mean ± SEM of 7–8 animals per group. *$p < 0.05$ versus response in the vehicle-treated group.
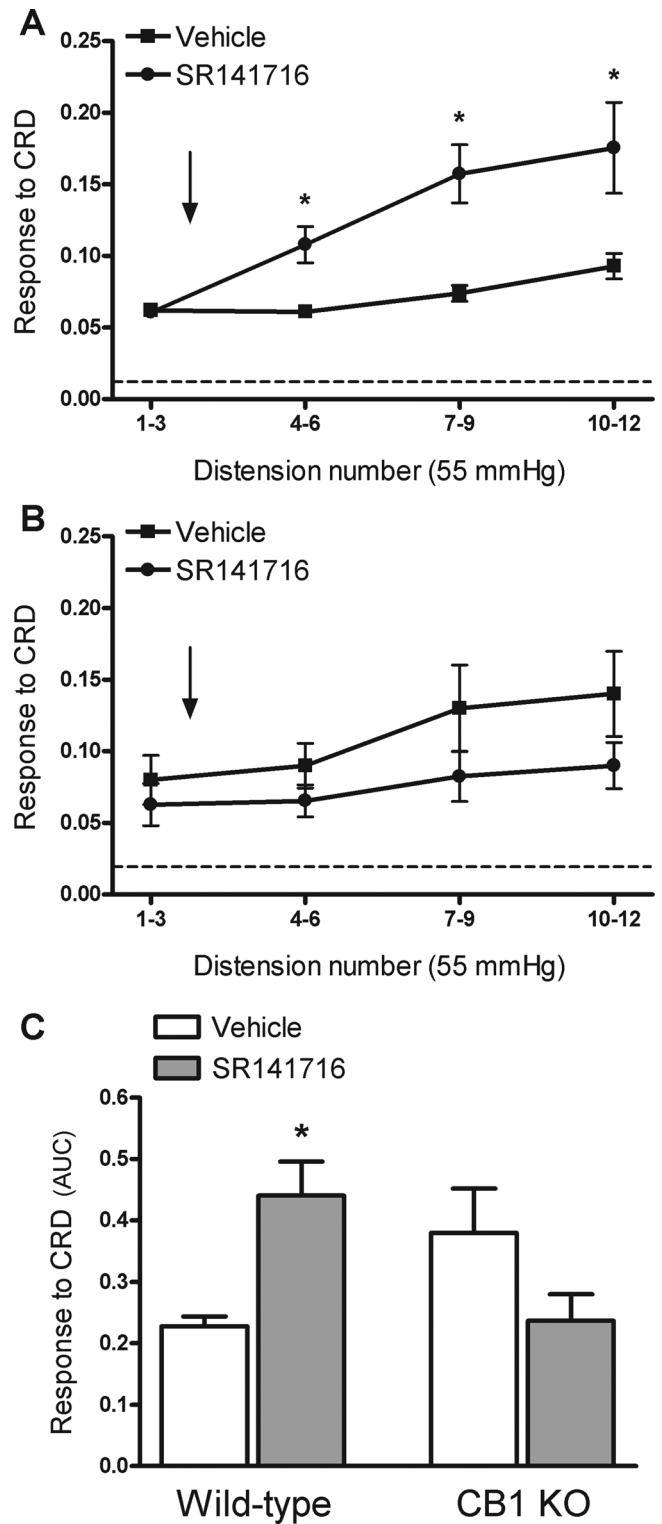
Figure 10. Effects of the selective CB$_1$ antagonist, SR141716 on visceral pain-related responses to CRD in wild-type and CB$_1$-knock-out (KO) mice. SR141716 (2 μmol/kg) or vehicle (5 ml/kg) was administered subcutaneously after the third distension at 55 mmHg (arrow). **A**, Responses to CRD in wild-type mice. The broken line represents the mean basal activity of the abdominal musculature between distensions. **B**, Responses to CRD in CB$_1$-knock-out mice. The broken line represents the mean basal activity of the abdominal musculature between distensions. **C**, Overall response to CRD (area under the curve, AUC, for distensions 4–12) in wild-type and CB$_1$-knock-out mice. Data are mean ± SEM of 4–7 animals per group. *$p < 0.05$ versus response in the vehicle-treated group.

the activation of pain mechanisms (Petrosino et al., 2007; Brusberg et al., 2009; Hong et al., 2009) or colitis (D'Argenio et al., 2006). This might be interpreted as an endogenous mechanism

containing the activation of pain pathways and thus maintaining the overall response to pain within a physiologically manageable range. Supporting this view, tonic release of endocannabinoids has been described in several systems, including the gastrointestinal tract (Izzo et al., 1999; Pinto et al., 2002; Hentges et al., 2005; Narushima et al., 2007), and has also been postulated from results obtained after the pharmacological blockade (Cippitelli et al., 2008; Moreira et al., 2008) or the genetic deletion of the endocannabinoids catabolic system (Cravatt et al., 2001; Moreira et al., 2008). *In vitro* studies showed that SR141716 behaves as an inverse agonist on the CB$_1$R (Landsman et al., 1997; Shire et al., 1999) and that CB$_1$Rs are constitutively active (Leterrier et al., 2006; Canals and Milligan, 2008). Therefore, although constitutive activity is difficult to demonstrate *in vivo*, the effects observed here could be explained by an inverse agonistic activity of SR141716 on constitutively active CB$_1$Rs. In these conditions, an apparent antagonistic effect will be observed in the absence of any endogenous ligand. Functionally, a tonic constitutive activity of cannabinoid receptors might lead to a sustained tone of analgesia without any tonic endocannabinoid release. Overall, our observations contrast with previous results showing that SR141716 did not affect normal pain responses to CRD in rats (Sanson et al., 2006). All together, these observations warrant further studies assessing the tonic release of endocannabinoids at pain-relevant sites as well as the activity of CB$_1$Rs in normal and pathophysiological conditions (such as during inflammation).

Altered colonic tone/accommodation to distension have been suggested as contributing mechanisms to altered colonic sensitivity in functional gastrointestinal disorders (Delgado-Aros and Camilleri, 2005). Nevertheless, there is not a clear correlation between compliance and pain perception (Käll et al., 2007; Lindström et al., 2008; Ravnefjord et al., 2008). Experimental data in humans showed that the nonselective cannabinoid dronabinol reduced colonic tone and increased compliance while increasing sensation ratings to distension, probably through the central modulation of perception (Esfandyari et al., 2007). In the present experiments, WIN55,212-2, at analgesic doses, did not modify the pressure-volume relationship during distension indicating a lack of effect modulating colonic compliance. These results argue for a lack of direct relationship between analgesia and compliance and suggest differences between humans and rodents in cannabinoid-dependent modulation of colonic tone/compliance.

Analgesic effect of cannabinoids may be exerted at multiple central and peripheral locations. Although the site of action was not directly assessed in the present studies, several observations suggest a peripheral effect. First, as mentioned, CB$_1$Rs are expressed within the gastrointestinal tract and in sensory endings on primary sensory afferents (Kulkarni-Narla and Brown, 2000; Coutts et al., 2002; Bridges et al., 2003; Casu et al., 2003; MacNaughton et al., 2004), allowing a peripheral effect modulating sensory afferent information. Second, SAB-378 is a peripherally acting compound with limited brain access, showing CNS-related effects only at doses ~170-fold greater than that required to produce analgesia (Dziadulewicz et al., 2007). Finally, none of the compounds tested evoked visible CNS-related side effects at the doses used, which would be expected if stimulation of central cannabinoid receptors occurred, although this was not assessed systematically. However, a potential contribution of central CB$_1$Rs in the analgesic responses observed cannot be totally excluded. Nevertheless, the present observations suggest that the peripheral blockade of CB$_1$Rs is sufficient to elicit visceral analgesia. Therefore, highly peripheralized CB$_1$ agonists may be a

valid therapeutic option for the treatment of visceral pain arising from the gut.

In summary, cannabinoids, acting through a CB$_1$-dependent mechanism, have analgesic effects in a rodent model of mechanically induced visceral pain, likely acting at peripheral sites. Observations derived from the use of SR141716 indicate that, through a tonic release of endocannabinoids and/or the constitutive activity of CB$_1$Rs, the endogenous cannabinoid system might be tonically active, likely constraining the activity of pain pathways. These observations suggest that peripherally active CB$_1$ agonists have a potential interest for the treatment of visceral pain-related disorders, such as irritable bowel syndrome, avoiding the undesirable profile associated to the use of centrally acting cannabinoids.

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