

Pharmacological Effects of Cannabinoids on the Caco-2 Cell Culture Model of Intestinal Permeability

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ABSTRACT

Activation of cannabinoid receptors decreases emesis, inflammation, gastric acid secretion, and intestinal motility. However, the effects of cannabinoids on intestinal permeability have not yet been established. The aim of the present study is to examine the effects of cannabinoids on intestinal permeability in an in vitro model. Caco-2 cells were grown until fully confluent on inserts in 12-well plates. Transepithelial electrical resistance (TEER) measurements were made as a measure of permeability. EDTA (50 μ M) was applied to reversibly increase permeability (reduce TEER). The effects of cannabinoids on permeability in combination with EDTA, or alone, were assessed. Potential target sites of action were investigated using antagonists of the cannabinoid (CB)₁ receptor, CB₂ receptor, transient receptor potential vanilloid subtype 1 (TRPV1), peroxisome proliferator-activated receptor (PPAR) γ , PPAR α , and a proposed cannabinoid receptor. When applied to the apical or

basolateral membrane of Caco-2 cells, Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) enhanced the speed of recovery of EDTA-induced increased permeability. This effect was sensitive to cannabinoid CB₁ receptor antagonism only. Apical application of endocannabinoids caused increased permeability, sensitive to cannabinoid CB₁ receptor antagonism. By contrast, when endocannabinoids were applied basolaterally, they enhanced the recovery of EDTA-induced increased permeability, and this involved additional activation of TRPV1. All cannabinoids tested increased the mRNA of the tight junction protein zona occludens-1, but only endocannabinoids also decreased the mRNA of claudin-1. These findings suggest that endocannabinoids may play a role in modulating intestinal permeability and that plant-derived cannabinoids, such as THC and CBD, may have therapeutic potential in conditions associated with abnormally permeable intestinal epithelium.

Introduction

Cannabinoids are compounds derived from *Cannabis sativa* (phytocannabinoids) or produced endogenously in the body (endocannabinoids). The most prevalent phytocannabinoids include Δ^9 -tetrahydrocannabinol (THC), the psychotropic constituent of *Cannabis*, and cannabidiol (CBD), which lacks psychoactivity (for a recent of phytocannabinoid pharmacology, see Pertwee, 2008). Cannabinoids act through at least two cloned G protein-coupled receptors: the CB₁ recep-

tor (Matsuda et al., 1990) and the CB₂ receptor (Munro et al., 1993). The discovery of endogenous ligands, such as anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), indicate the presence of a functional "endocannabinoid system" (for a recent review of endocannabinoid pharmacology, see Reggio, 2010). Cannabinoids also activate transient receptor potential vanilloid subtype 1 (TRPV1; Zygmunt et al., 1999), peroxisome proliferator-activated receptors (PPARs) (O'Sullivan, 2007), the orphan receptor GPR55 (Baker et al., 2006), and as yet uncloned cannabinoid receptors (Breivogel et al., 2001; Begg et al., 2005).

The presence of a functional endocannabinoid system has been identified in the gut. In 1995, 2-AG was isolated in the canine intestine (Mechoulam et al., 1995), and in 2001, AEA

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ABBREVIATIONS: THC, Δ^9 -tetrahydrocannabinol; CBD, cannabidiol; CB, cannabinoid; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; TRPV1, transient receptor potential vanilloid subtype 1; PPAR, peroxisome proliferator-activated receptor; TJ, tight junction; TEER, transepithelial electrical resistance; PBS, phosphate-buffered saline; FD, fluorescent dextran; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; GW9662, 2-chloro-5-nitro-*N*-phenylbenzamide; GW6471, [(2*S*)-2-[[[1*Z*]-1-methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]propyl]-carbamic acid ethyl ester; O-1918, 1,3-dimethoxy-5-methyl-2-[(1*R*,6*R*)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene; ZO-1, zona occludens-1; ANOVA, analysis of variance; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl [(4-methoxyphenyl)methanone].

was identified in the rat small bowel (Izzo et al., 2001). CB₁ receptors have been shown to be located in the gastrointestinal tract of many species, including rats, guinea pigs, and humans (Crocì et al., 1998; Kulkarni-Narla and Brown, 2000; Coutts et al., 2002; Casu et al., 2003). Immunohistochemical studies indicate that the enteric nervous system appears to be the main site of action for cannabinoids in the gastrointestinal tract (Coutts et al., 2002). In human colonic tissue, CB₁ receptors have been shown in normal colonic epithelium, smooth muscle, and the submucosal myenteric plexus (Wright et al., 2005). Prejunctional CB₁ receptors in the human ileum also have been detected using electrophysiological studies (Crocì et al., 1998). The CB₂ receptor has been detected in rat peritoneal mast cells (Facci et al., 1995) and enteric neurons (Duncan et al., 2008). In human colonic tissue, CB₂ has been shown to be expressed in plasma cells and the lamina propria (Wright et al., 2005). CB₂ immunoreactivity also has been observed in the epithelium of colonic tissue characteristic of inflammatory bowel disease (Wright et al., 2005; Izzo, 2007).

Several studies have shown that cannabinoid receptors are up-regulated in inflammatory conditions; increased CB₁ receptor expression is observed in croton oil-induced inflammation of the small bowel in mice (Izzo et al., 2001), and CB₂ is expressed in colonic tissue in patients with inflammatory bowel disease (Wright et al., 2005). Cannabinoids also have been shown to exert anti-inflammatory actions in the gut through activation of CB₁ receptors using genetically modified mice and CB₁ antagonism (Massa et al., 2004). CB₂ activation also reduces gastrointestinal contractility in a lipopolysaccharide model of inflammation (Duncan et al., 2008), and CB₂ activation is now suggested to be a key process in inflammation of the gut (for review, see Wright et al., 2008). CBD in particular has been shown in several studies to exert numerous beneficial effects in the inflamed gut (Capasso et al., 2008; Borrelli et al., 2009).

The term “intestinal permeability” refers to the barrier properties of the intestinal mucosa that prevent harmful substances from penetrating the mucosa. Gut-derived Gram-negative bacteria and their endotoxins are causal or complicating factors in many life-threatening diseases, such as septicemia and septic shock. Multiple organ dysfunction syndrome develops in approximately 15% of patients admitted to intensive care unit and remains the major cause of death in critically ill patients. The translocation hypothesis postulates that in a wide variety of acute surgical and medical illnesses, gut bacteria and toxins leak via an abnormally permeable intestinal epithelial barrier. Therefore, modulation of intestinal permeability has become an increasingly important therapeutic target.

Cannabinoids have been shown to be beneficial in the treatment of several gastrointestinal conditions, but to date, no studies have examined the effects of cannabinoids on intestinal permeability. Therefore, the aim of the present study was to investigate the effects of various cannabinoids on intestinal permeability using Caco-2 cells as a model of intestinal permeability. Confluent Caco-2 cells express several morphological and biological characteristics of small intestinal enterocytes, including brush-border microvilli, tight junctions (TJs), and dome formation, making them an attractive model for studying intestinal permeability (Pinto et al., 1983; van Breemen and Li, 2005).

Materials and Methods

Cell Culture. Caco-2 cells (European Collection of Cell Cultures, Salisbury, UK; passages 48–56) were cultured in Eagle’s minimal essential medium supplemented with L-glutamine, 10% fetal bovine serum, and 1% penicillin and streptomycin mixture. Cells were kept at 37°C in 5% CO₂ and 95% humidity. Cells were grown in 12-well plates and seeded at ~50,000 cells per insert onto 12-mm-diameter, 0.4-μm pore polycarbonate membrane inserts (Scientific Laboratory Supplies Ltd., Nottingham, UK). Cells were used for experimentation between days 14 and 21. The medium was changed on alternate days.

TEER Measurement. Transepithelial electrical resistance (TEER) measurement is used as an index of monolayer confluence and integrity in cell culture experiments (Huynh-Delerme et al., 2005). TEER also has been used to measure paracellular permeability of cell monolayers (Madara et al., 1988). The TEER of the monolayer in the present study was determined using an EVOM volt-ohm meter (World Precision Instruments, Inc., Sarasota, FL) according to the methods of Wells et al. (1998). Cells were used for experimentation between days 14 and 21, each insert with a TEER value greater than 1000 Ω · cm².

Transport Studies. Caco-2 cells were seeded on a BD BioCoat Transwell system with 6.5-mm diameter, 0.4-μm polycarbonate pore size inserts (BD Biosciences, Oxford, UK) at a density of 20,000 cells/insert and incubated in 24-well culture plates with a medium change every other day. Confluent monolayers were washed three times with prewarmed phosphate-buffered saline (PBS), left for 30 min at 37°C to equilibrate, and then baseline TEER was measured. EDTA was used as a pharmacological tool to increase permeability in the Caco-2 cells (Tomita et al., 1994; Quan et al., 1998; Ward et al., 2000). Increasing concentrations of EDTA (0, 20, 50, and 100 μM) were added to the apical site of the inserts (*n* = 4) for 5 min. PBS (310 μl) containing increasing molecular weight fluorescent dextrans (FDs; 25 mg · ml⁻¹) were then added to the apical side: 4 (FD4), 10 (FD10), and 20 kDa (FD20). After 1 h at 37°C, 100-μl aliquot samples were withdrawn from the basolateral sites. The amounts of different fluorescence in the samples were determined using a Fluoroskan Ascent FL2.5 fluorometer (Thermo Fisher Scientific, Waltham, MA), with excitation and emission wavelengths of 485 and 520 nm, respectively. The apparent permeability coefficients (*P*_{app}) of different fluorescent agents used were measured using the following equation:

$$P_{app} = \frac{dq}{dt} \times \frac{1}{A \times C^o}$$

where *dq* is the amount of fluorescence in the basolateral side (milligrams per milliliter), *dt* is a function of time per second, *A* is the surface area of the inserts (0.64 cm²), and *C*^o is the initial concentration of fluorescent applied in apical compartment (milligrams per milliliter).

Effects of Cannabinoids on Caco-2 Cell Monolayer Integrity (Apical Application). To induce an increase in permeability, that is, a decrease in TEER, cells were treated with the permeability enhancer EDTA applied to the apical site of the inserts. We choose a concentration of 50 μM, which was found to cause the transport of FD4 and FD10 but not FD20 (see Fig. 1).

Five minutes after EDTA application, fresh medium with or without cannabinoids (10 μM) was applied to the apical side of the insert. Vehicle controls (ethanol or dimethyl sulfoxide) were used as appropriate. TEER values were then obtained every 30 min until recovery.

Our initial experiments showed that a single concentration of THC (10 μM) or CBD (10 μM) reversed the increase in permeability, i.e., the fall in TEER over time, caused by EDTA. We therefore attained concentration-response curves to THC and CBD by adding increasing concentrations of THC or CBD (100 nM–30 μM) to inserts after EDTA administration. TEER values were monitored every 30 min until they returned to basal values.

Because our initial experiments showed that both AEA and 2-AG (10 μM) caused a further increase in permeability (reduction in TEER) in addition to the effects of EDTA, we established the effects of AEA and 2-AG alone (without EDTA) on intestinal permeability to assess whether AEA and 2-AG cause a fall in TEER by themselves. Increasing concentrations (100 nM–30 μM) of AEA or 2-AG were added to inserts, and TEER was monitored until it returned to baseline.

Target Sites of Action of Cannabinoids in Caco-2 Cells. To establish the receptor sites of action for cannabinoids, the following antagonists were coapplied with the cannabinoids (all cannabinoid agonists in these experiments were used at 10 μM): AM251 (CB₁ receptor antagonist), AM630 (CB₂ receptor antagonist), capsazepine (TRPV1 antagonist), GW9662 (PPAR γ antagonist), GW6471 (PPAR α antagonist), and O-1918 (proposed cannabinoid receptor antagonist). All antagonists were used at 1 μM , and appropriate vehicles were applied to control inserts.

In Fig. 4C, AM251 and AEA appeared to cause an increase in TEER (decreased permeability). We therefore assessed whether AM251 caused any effects on TEER either on its own or after the application of EDTA. We found that at the higher concentrations of 1 and 10 μM , AM251 increased TEER or reversed the effects of EDTA but had no effect on TEER at 100 nM. Therefore, in subsequent experiments, AM251 was used at 100 nM. We also repeated the experiment in Fig. 4C and confirmed that 100 nM AM251 was capable of inhibiting the effects of AEA.

Effects of Phytocannabinoids on the Effects of Endocannabinoid in Caco-2 Cells. In some experiments, the effects of THC or CBD (10 μM) on the fall in TEER caused by AEA or 2-AG (10 μM) were examined.

Effects of Cannabinoids on Caco-2 Cell Monolayer Integrity (Basolateral Application). All of the above-mentioned protocols were repeated with cannabinoid compounds applied to the basolateral membrane.

Effects of Cannabinoids on Expression of mRNA of Tight Junction Proteins. 5XT75 flasks were grown until fully confluent. Flasks were treated with vehicle THC, CBD, AEA, or 2-AG (10 μM) for 3 days. Total RNA was extracted with TRI reagent (Invitrogen, Paisley, United Kingdom) following the manufacturer's protocol. Reverse transcription of total RNA to cDNA suitable for quantitative polymerase chain reaction was performed using the High-Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA).

mRNA levels for each of the three tight junction proteins [zona occludens-1 (ZO-1), claudin-1, and occludin] were determined using TaqMan gene expression assays (Applied Biosystems). Reactions were run on a Chromo 4 Real-Time PCR Detector (Bio-Rad Laboratories Inc, Hercules, CA), and the data were collected during the exponential phase of the reactions using the opticon monitor software (Bio-Rad Laboratories, Hemel, Hempstead, UK). All samples were assayed in quadruplicate and replicated three times. Standard curves were produced using a standard source of cDNA. In our case, this was identified by using vehicle cDNA for a specific tight junction gene and housekeeping gene (B2M) at five known dilutions. Loga-

arithmic standard curves were generated for both the mRNA of interest and B2M, and slope and intercept of these curves used to generate the quantity of mRNA and B2M. The quantity of a specific mRNA was then normalized to B2M. The average relative quantity of mRNA and the S.E.M. were calculated.

Chemicals. All chemicals were purchased from Sigma Chemical (Poole, Dorset, UK) unless otherwise mentioned. EDTA was purchased from BDH (Poole, Dorset, UK). All cannabinoids and antagonists were purchased from Tocris Bioscience (Bristol, UK) except THC and capsazepine, which were obtained from Sigma Chemical. CBD, THC, capsazepine, AEA, and 2-AG were dissolved in ethanol to a stock concentration of 10 mM, with further dilutions made in PBS. GW9662, GW6471, AM251, and AM630 were dissolved in dimethyl sulfoxide to 10 mM, with further dilutions made in PBS.

Statistical Analysis. In each protocol, values are expressed as mean \pm S.E.M. of $n = 3$ inserts. Data were compared, as appropriate, by Student's *t* test or by analysis of variance (ANOVA), with statistical significance between manipulations and controls determined by Dunnett's post hoc test.

Results

Effects of EDTA on Transport of Fluorescent Markers across Caco-2 Cell Monolayers. Figure 1 shows the effects of various EDTA concentrations (0, 20, 50, and 100 μM) on the permeability to increasing sizes of fluorescent markers FD4, FD10, and FD20. EDTA (20 μM) did not alter the transport of any of the fluorescent markers. EDTA (50 μM) caused significant transport of FD4 and FD10 ($P < 0.001$) but not FD20 (Fig. 1). EDTA (100 μM) caused significant transport of all markers compared with control ($P < 0.001$).

In all subsequent experiments, 50 μM EDTA was used as a permeability enhancer and caused a fall in TEER of approximately 20% in all experiments (Figs. 2, 3, and 6–8). Full recovery of TEER values was usually observed within 4 h in control groups.

Effects of Cannabinoids on Caco-2 Cell Monolayer Integrity (Apical Application). When Caco-2 monolayers were incubated with cannabinoids (10 μM) after increased permeability (reduced TEER) was stimulated by EDTA, THC and CBD (Fig. 2A) caused a more rapid recovery of TEER than observed in the control group. By contrast, endocannabinoids (AEA and 2-AG) caused a further and sustained increase in permeability (reduced TEER) in addition to the effects of EDTA (Fig. 2B).

Further experiments showed that the ability of THC and CBD to speed the recovery to normal TEER values after EDTA application was concentration-dependent (Fig. 3, A and B; Table 1). To establish the potential receptor sites of

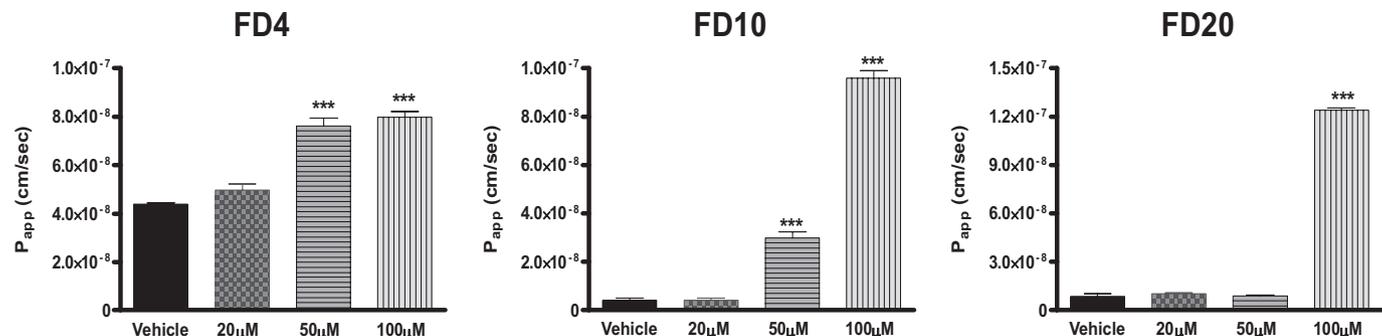


Fig. 1. Effects of increasing concentrations of EDTA on the paracellular transport (permeability coefficients) of fluorescein isothiocyanate-dextran of 4 (FD4), 10 (FD10), and 20 kDa (FD20). Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$, ANOVA).

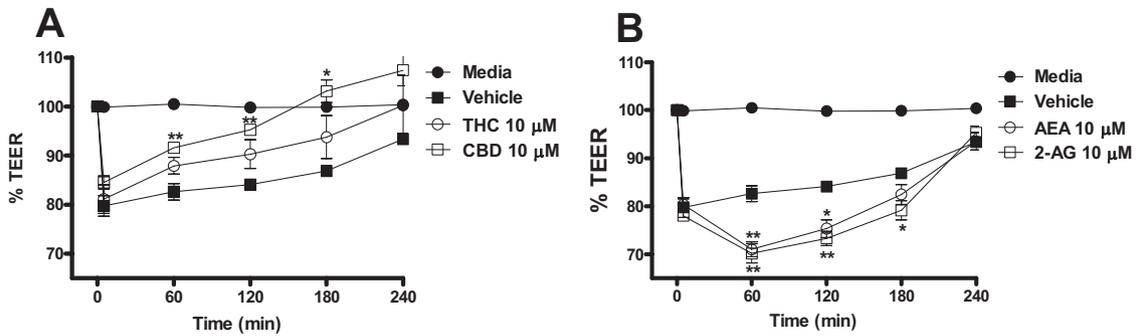


Fig. 2. Effects of THC and CBD (10 μM ; A) and endocannabinoids (AEA and 2-AG, 10 μM ; B) on the fall of TEER caused by apical EDTA (50 μM) application to Caco-2 cells. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$, ANOVA).

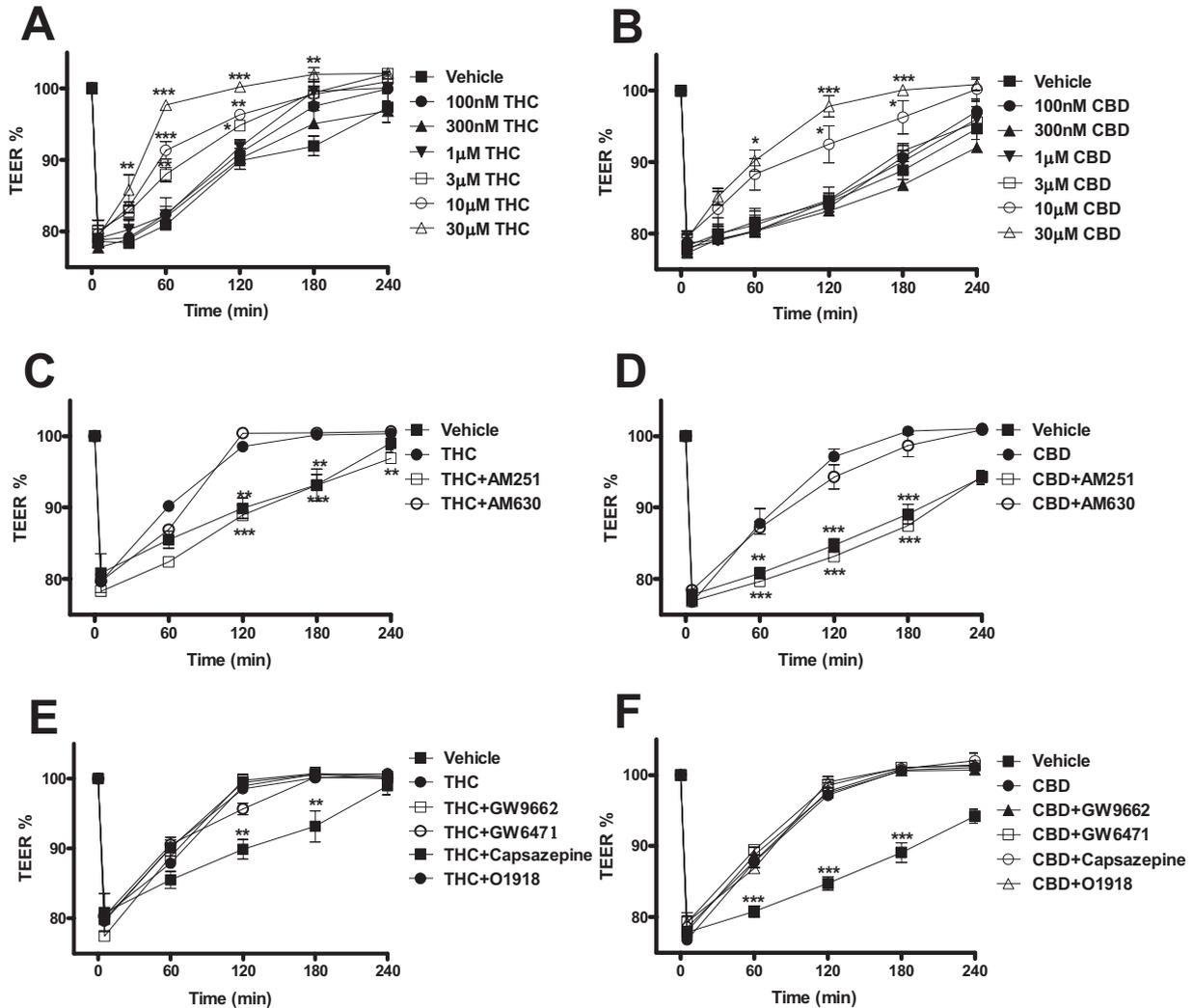


Fig. 3. Concentration-response curves to THC (A) and CBD (B) applied apically on the fall of TEER caused by apical EDTA (50 μM) application to Caco-2 cells. Effects of receptor antagonists on THC (C and E) and CBD (D and F) in Caco-2 cells treated with EDTA. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA).

action, the effects of THC (10 μM) and CBD (10 μM) were investigated when coapplied with several antagonists. The effects of THC on TEER were significantly inhibited by the cannabinoid CB₁ receptor antagonist AM251 (1 μM) only (Fig. 3C; Table 2). Likewise, the effects of CBD on TEER were only inhibited by AM251 (Fig. 3D; Table 2).

Because AEA and 2-AG were found to cause further decreases in TEER in addition to EDTA, we investigated

whether these endocannabinoids might themselves cause increases in permeability when applied to Caco-2 cells (without EDTA application). Endocannabinoid treatment of cells resulted in a concentration-dependent decrease in TEER, indicating increased permeability, which was significantly different to vehicle at 10 and 30 μM AEA (Fig. 4A) and 3, 10, and 30 μM 2-AG (Fig. 4B; Table 1). To establish the potential receptor sites of action for endocannabinoids, several antag-

TABLE 1

Area under the curve values (% · min⁻¹) for the effects of cannabinoids on TEER in the presence of EDTA (THC and CBD) or on TEER alone (AEA and 2-AG)

Data are means ± S.E.M.

	THC (with EDTA)	CBD (with EDTA)	AEA (No EDTA)	2-AG (No EDTA)
Vehicle	3065 ± 202	3689 ± 149	712 ± 96	786 ± 115
100 nM	2499 ± 378	3382 ± 229	792 ± 21	1115 ± 125
300 nM	2852 ± 485	3453 ± 393	807 ± 65	1203 ± 138
1 μM	2350 ± 151	3206 ± 314	1066 ± 197	1695 ± 137*
3 μM	1856 ± 157*	3131 ± 58	1271 ± 162**	1945 ± 145**
10 μM	1545 ± 52**	1965 ± 498**	1498 ± 51***	2849 ± 131***
30 μM	1027 ± 33***	1308 ± 172***	2714 ± 82***	4356 ± 384***

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with vehicle values (ANOVA with Dunnett's post hoc test).

TABLE 2

Area under the curve values (% · min⁻¹) for the effects of cannabinoids plus various antagonists on TEER in the presence of EDTA (THC and CBD) or on TEER alone (AEA and 2-AG)

Data are means ± S.E.M.

	THC	CBD	AEA	2-AG
Vehicle	2353 ± 242	3514 ± 217	775 ± 32	713 ± 101
Cannabinoid (10 μM)	1293 ± 101***	1623 ± 221***	2416 ± 28***	2205 ± 415***
+ AM251	2832 ± 205	3791 ± 162	679 ± 21	751 ± 125
+ AM630	1430 ± 17**	1843 ± 163***	2260 ± 77***	2378 ± 325***
+ GW9662	1423 ± 48**	1500 ± 86***	2251 ± 117***	1998 ± 161***
+ GW6471	1177 ± 94***	1392 ± 26***	2208 ± 48***	2211 ± 272***
+ capsazepine	1258 ± 109***	1570 ± 75***	2283 ± 217***	2301 ± 219***
+ O-1918	1417 ± 133**	1527 ± 23***	2107 ± 169***	2245 ± 246***

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with vehicle values (ANOVA with Dunnett's post hoc test).

onists were investigated. Only AM251 (1 μM) significantly inhibited the fall in TEER caused by either AEA (10 μM; Fig. 4C; Table 2) or 2-AG (10 μM; Fig. 4D; Table 2).

We investigated whether AM251 itself had any effect on TEER, either alone or after the application of EDTA. In these experiments, we found that on its own, AM251 at 1 and 10 μM but not 100 nM caused a significant increase in TEER in Caco-2 cells (i.e., a decrease in permeability; Fig. 5A). After a fall in TEER was induced by EDTA, AM251 significantly enhanced the recovery of TEER at 1 and 10 μM but not 100 nM (Fig. 5B). For these reasons, in future experiment 100 nM AM251 was used. The experiments investigating the effects of AM251 on endocannabinoid reduction in TEER were repeated at the lower concentration of AM251 (100 nM), which was found to similarly inhibit the fall in TEER caused by AEA or 2-AG (data not shown).

We also investigated the effects of THC and CBD on the increased permeability caused by endocannabinoids and found that both (10 μM) completely inhibited the fall in TEER caused by AEA (10 μM; Fig. 6A) or 2-AG (10 μM; Fig. 6B).

Effects of Cannabinoids on Caco-2 Cell Monolayer Integrity (Basolateral Application). In a second set of experiments, we assessed the effects of cannabinoids (10 μM) on the fall in TEER caused by EDTA when cannabinoids were applied basolaterally. In this situation, phytocannabinoids and endocannabinoids tended to cause a more rapid recovery of TEER values (Fig. 7, A and B); however, this was only significantly different from vehicle controls for CBD and AEA.

Figure 7A shows that there was a concentration-dependent recovery of TEER values with increasing concentrations of THC and CBD applied basolaterally, as observed previously when THC and CBD were applied to the apical membrane (Fig. 8B). As observed when THC and CBD were applied apically, only AM251 significantly inhibited

the effects of THC (Fig. 8C) or CBD (Fig. 8D) on the fall in TEER caused by EDTA.

Basolateral application of endocannabinoids (AEA and 2-AG; 10 μM) also caused a concentration-dependent recovery of TEER values after the fall in TEER caused by EDTA (Fig. 9, A and B). The effect of basolateral application of AEA (10 μM) on the fall in TEER caused by EDTA was significantly inhibited by the TRPV1 antagonist capsazepine (1 μM) and the cannabinoid CB₁ receptor antagonist AM251 (100 nM; Fig. 9, C and E). The effect of basolateral application of 2-AG (10 μM) on the fall in TEER caused by EDTA was only inhibited by AM251 (Fig. 9D).

Effects of Cannabinoids on the Expression of mRNA of Tight Junction Proteins. The apical junctional complex maintains epithelial cohesion and polarity, and the TJ is the most apical factor within this complex, which is rate-limiting for the paracellular permeation of molecules. Expression and differential assembly of TJ proteins contribute to the dynamic changes in small-molecule paracellular flux. Altered expression of proteins involved in the assembly of the intestinal epithelial tight junction might explain the differential ability of cannabinoids to alter TEER. We therefore explored cannabinoid-induced mRNA changes in critical components of the TJ, namely, ZO-1, claudin-1, and occludin. After treatment with THC, CBD, AEA, or 2-AG, there was a significant up-regulation of the expression of the mRNA of the tight junction protein ZO-1 (Fig. 10A). In the presence of the CB₁ receptor antagonist AM251 (100 nM), this effect of cannabinoids was abolished. After treatment with endocannabinoids AEA and 2-AG, but not the phytocannabinoids THC and CBD, the expression of the mRNA of the tight junction protein claudin-1 was significantly down-regulated (Fig. 10B). This effect was also inhibited by the CB₁ receptor antagonist AM251. There was no effect on the expression of occludin by cannabinoids (Fig. 10C).

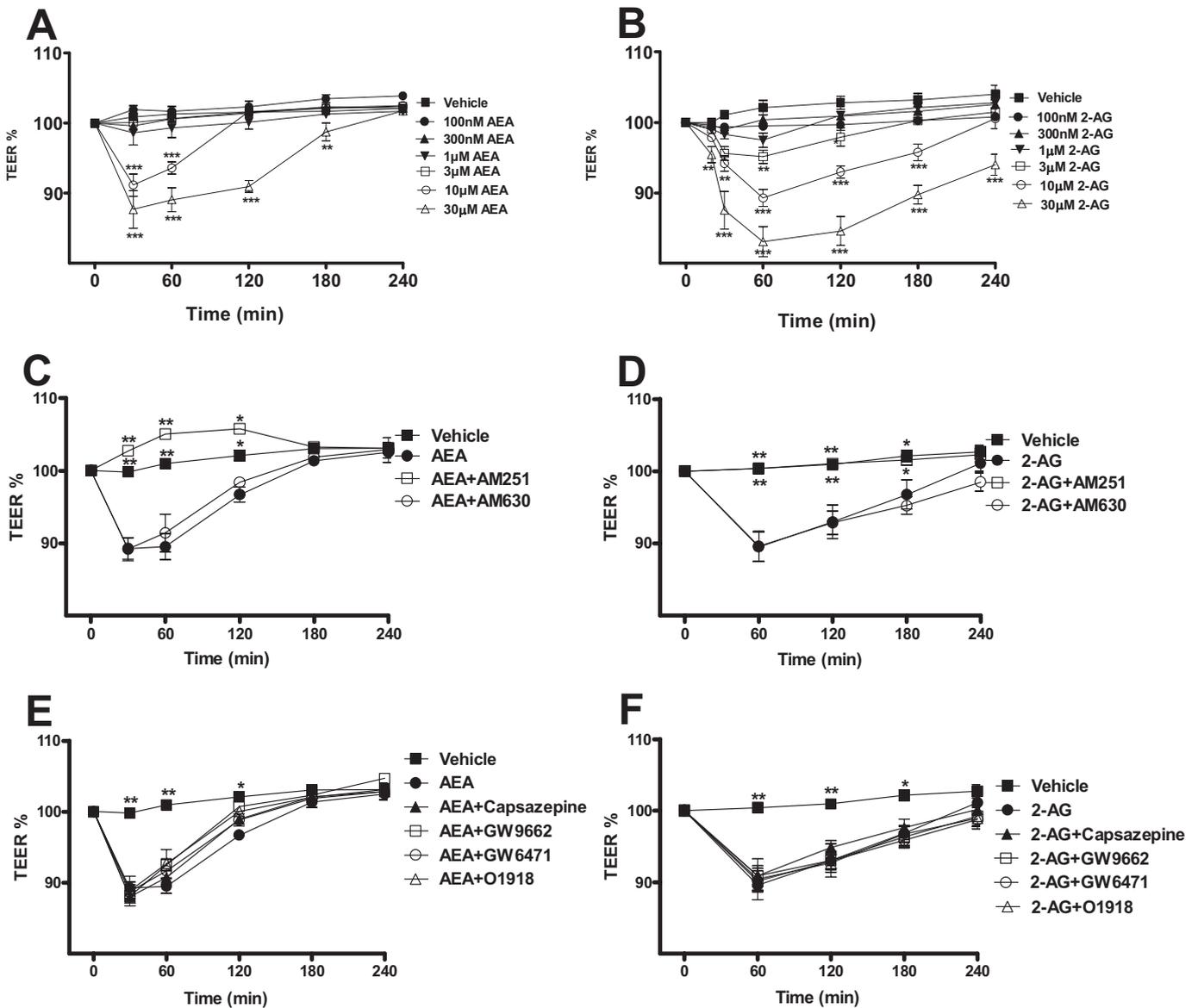


Fig. 4. Concentration-response curves to AEA (A) and 2-AG (B) applied apically in Caco-2 cells. Effects of receptor antagonists on the fall in TEER caused by AEA (C and E) and 2-AG (D and F) applied apically to Caco-2 cells. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA).

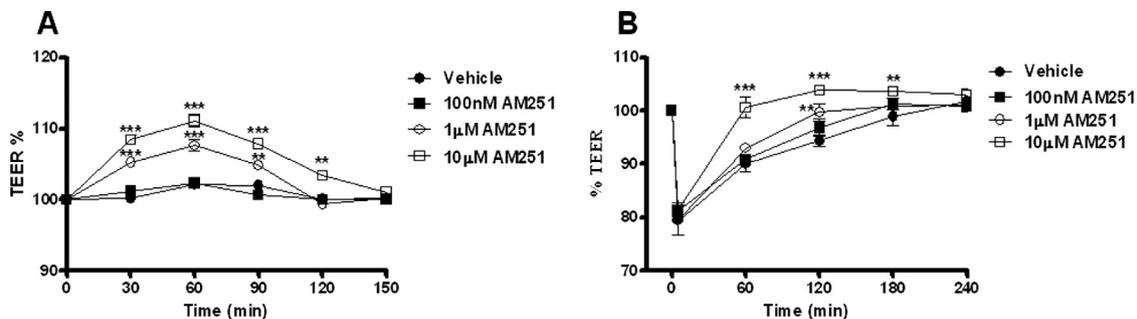


Fig. 5. Effects of the CB_1 antagonist AM251 (100 nM, 1 μ M, and 10 μ M) on TEER values in Caco-2 cells applied alone (A) or in cells treated with EDTA (50 μ M; B). Data are given as means with error bars representing S.E.M. (**, $P < 0.01$; ***, $P < 0.001$, ANOVA).

Discussion

Cannabinoid administration is associated with many beneficial effects in the gut, such as decreasing emesis, gastric acid secretion, inflammation, and intestinal motility (for re-

views, see Izzo, 2007; Aviello et al., 2008; Storr et al., 2008; Wright et al., 2008). These effects are primarily mediated by CB_1/CB_2 activation, although CBD, which is not thought to bind to cannabinoid receptors with high affinity, also has been shown to have numerous beneficial effects in the gut.

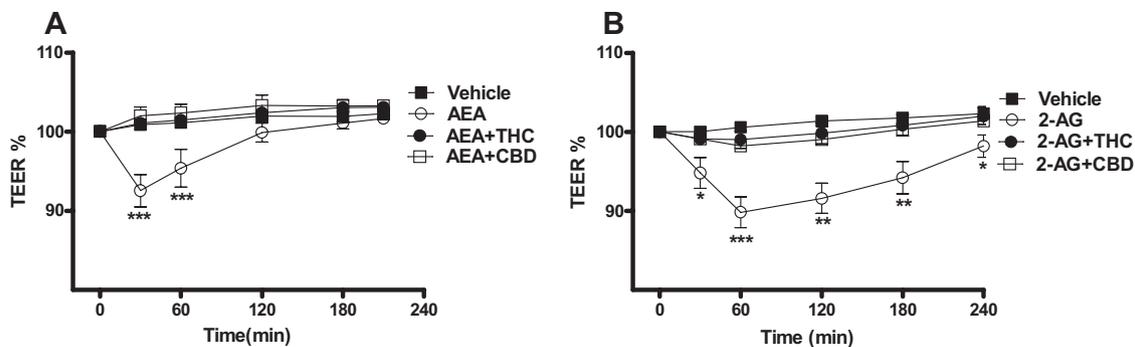


Fig. 6. Effects of THC and CBD on the fall in TEER caused by AEA (A) and 2-AG (B) in Caco-2 cells. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA).

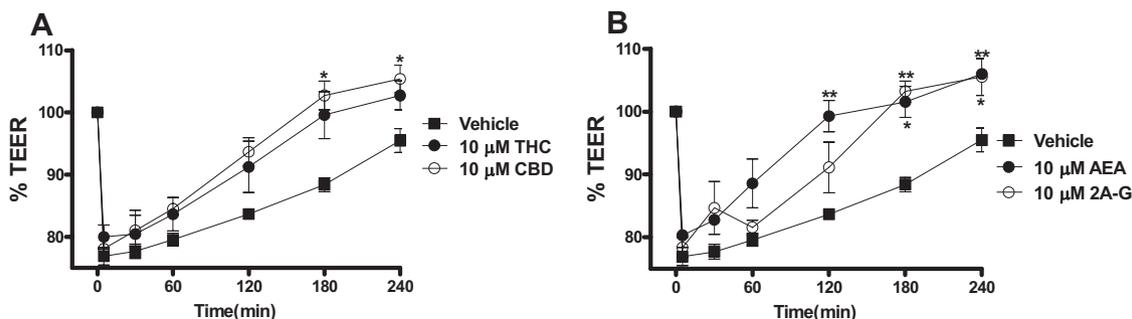


Fig. 7. Effects of THC and CBD (10 μM ; A) and endocannabinoids (AEA and 2-AG, 10 μM ; B) applied basolaterally on the fall in TEER caused by EDTA (50 μM) in Caco-2 cells. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$, ANOVA).

The aim of the present study was to investigate the effects of cannabinoids on intestinal permeability by using Caco-2 cells as a model of intestinal permeability. Our data have shown that cannabinoids have the ability to both positively and negatively modulate paracellular permeability. In our experiments, the decrease in TEER caused by EDTA (i.e., increased intestinal permeability) would involve the paracellular transport of substances up to 10 but not 20 kDa.

Our first finding was that the phytocannabinoids THC and CBD increase the recovery of TEER over time (i.e., reverse the increase in permeability) in a concentration-dependent manner. This is the first study to date to examine the effects of cannabinoids on gut permeability. In other models of altered cell permeability, cannabinoids have similarly been shown to reverse increases in permeability. For example, in a co-culture (endothelial cells and astrocytes) model of the blood-brain barrier, cannabinoids (CP 55,940 and arachidonyl-2-chloroethylamide, both CB_1 receptor agonists) inhibited HIV-1-induced or substance P-induced decreases in permeability (Lu et al., 2008). Likewise, Rajesh et al. (2007) found that CBD attenuates the effects of a high-glucose environment increasing cellular permeability in human coronary endothelial cells. CBD treatment also has been shown to significantly reduce vascular hyperpermeability in the diabetic retina (El-Remessy et al., 2006).

We proceeded to establish the receptor site of action through which THC and CBD may be acting. We found that the effect of THC and CBD in reversing the increase in permeability in our model was sensitive to antagonism of the CB_1 receptor but not the CB_2 receptor. We also examined many other potential sites of action at which cannabinoids are known to act, such as TRPV1 and the nuclear receptors PPARs, but we did not find any contribution of these target

sites. Our findings are consistent with the known expression of CB_1 in Caco-2 cells (Wright et al., 2005) and the variability of findings concerning CB_2 expression, where expression has not been observed previously (Ligresti et al., 2003) or is only evident during inflammation (Wright et al., 2005). Our findings also are consistent with a study by Lu et al. (2008) that showed the effects of cannabinoids in inhibiting decreases in permeability of the blood-brain barrier were inhibited by a CB_1 receptor antagonist. However, the effects of CBD on blood-brain barrier permeability were not found to be mediated by either CB_1 or CB_2 receptors (Rajesh et al., 2007). Although CBD is not thought to bind to cannabinoid receptors with high affinity (Pertwee, 2008), our finding that the effects of CBD on intestinal permeability were sensitive to CB_1 antagonism are not unusual. Capasso et al. (2008) and de Flippis et al. (2008) have similarly shown that the effects of CBD in inhibiting hypermotility in mice were sensitive to CB_1 antagonism, which might suggest that CBD agonizes CB_1 in the gut. However, another explanation of the effects of CBD in the present study could be that CBD is antagonizing CB_1 -mediated increases in permeability mediated by locally produced endocannabinoids, as was observed in the present study with AM251 administration alone.

Our second major finding is that the endocannabinoids anandamide and 2-AG cause a further fall in TEER when applied after EDTA; i.e., they cause a further increase in cell permeability. We then went on to show that endocannabinoid application alone also led to a concentration-dependent and reversible decrease in TEER (increased permeability) of approximately 15% at the highest concentrations. As with THC and CBD, this response was sensitive to antagonism of the CB_1 receptor but not other sites of action, such as CB_2 , TRPV1, or PPARs under these conditions. These data sug-

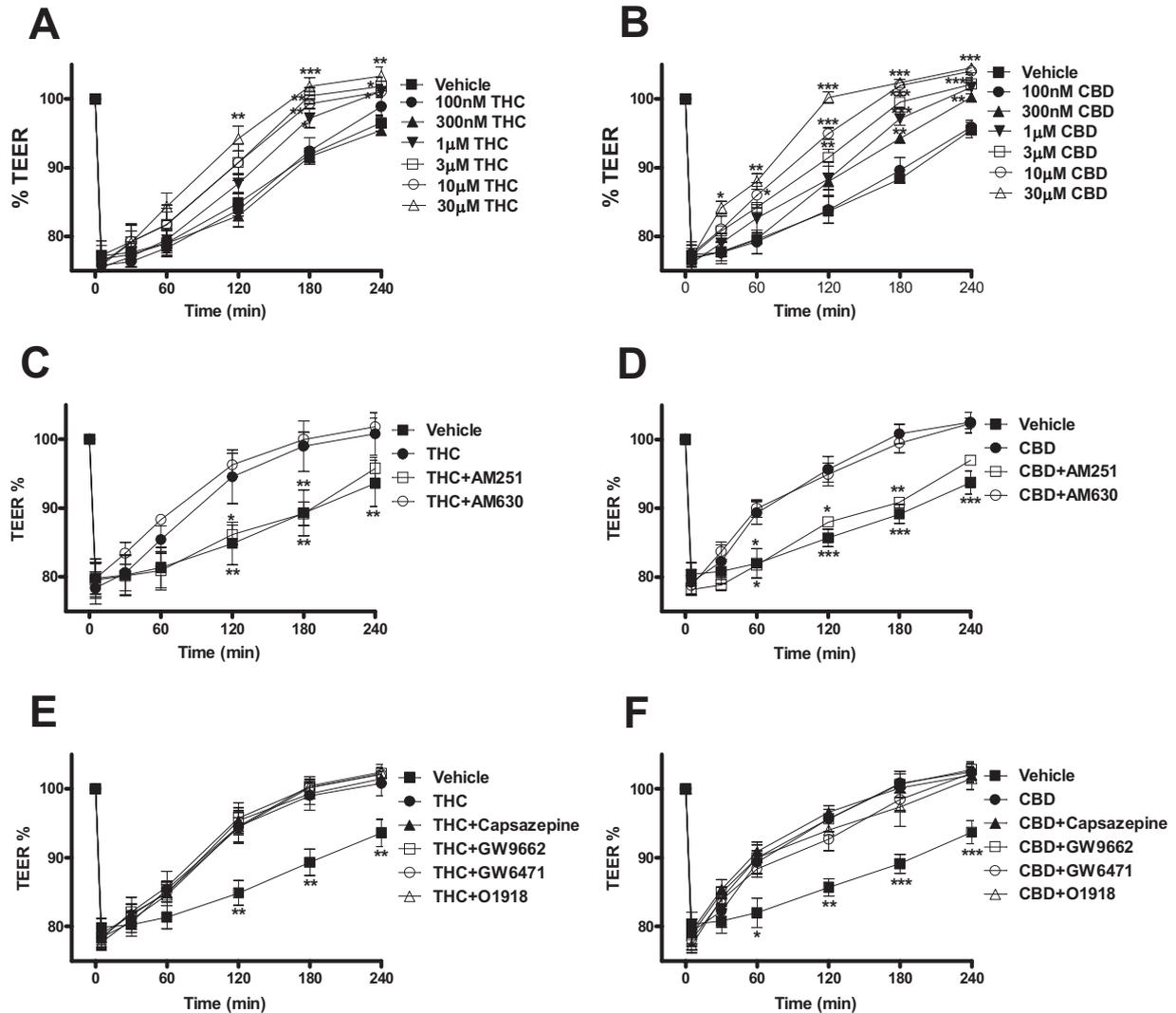


Fig. 8. Concentration-response curves to THC (A) and CBD (B) applied basolaterally on the fall in TEER caused by EDTA (50 μ M) application to Caco-2 cells. Effects of receptor antagonists on THC (C and E) and CBD (D and F) in Caco-2 cells treated with EDTA. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA).

gest that endocannabinoids may play a physiological role in the modulation of gut permeability. It also is possible that overproduction of endocannabinoids could lead to pathological changes in gut permeability, as has been suggested previously (Di Marzo and Izzo, 2006). It was interesting to observe that application of the CB₁ receptor antagonist alone, or in the presence of EDTA, increased TEER, i.e., decreased permeability, further suggesting that locally produced endocannabinoids, acting through the CB₁ receptor, increase gut permeability. However, given the effects of AM251 were only observed in the micromolar range, this also could suggest a nonspecific action of AM251.

In addition to reversing the fall in TEER caused by EDTA, we found that THC or CBD completely inhibited the fall in TEER caused by endocannabinoids. This suggests that either THC or CBD could be used to prevent increases in gut permeability.

Our third major finding is that the effects of endocannabinoids (but not THC and CBD) on Caco-2 cell permeability are different when applied to the apical versus the basolateral membrane. Specifically, when applied apically, endocanni-

noids cause a fall in TEER (increase permeability) and have the opposite effect (decrease permeability) when applied basolaterally. When applied basolaterally, there is also a contribution of TRPV1 activation by anandamide (but not 2-AG) in decreasing cell permeability. This is particularly interesting because it suggests that systemic (basolateral) or luminal (apical) stimulation of endocannabinoid production from cell membrane precursors leads to differential effects of endocannabinoids in polarized cells such as intestinal epithelium. D'Argenio et al. (2006) have shown that anandamide, but not 2-AG, is up-regulated in a suggested protective manner in mouse models of colon inflammation and in biopsies of patients with ulcerative colitis. Unfortunately, it is impossible to detect whether this is uniform across the epithelial cell or even uniform across the tissue, but it could potentially have significant implications on the effects of anandamide on intestinal permeability in inflammation according to our results.

Our final experiments investigated the effects of cannabinoids on the expression of several tight junction proteins involved in the regulation and maintenance of epithelial bar-

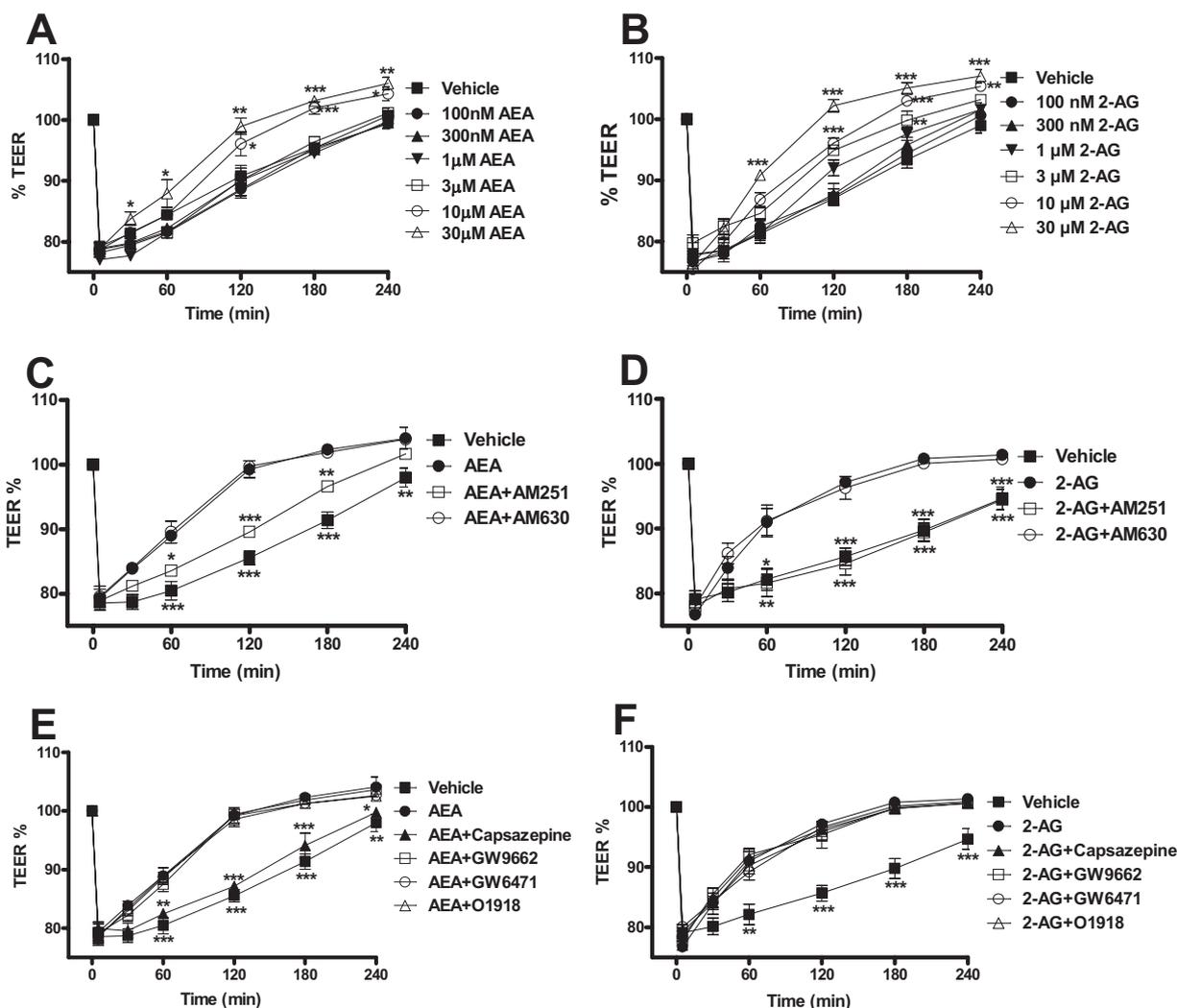


Fig. 9. Concentration-response curves to AEA (A) and 2-AG (B) applied basolaterally on the fall in TEER caused by EDTA (50 μM) application to Caco-2 cells. Effects of receptor antagonists on AEA (C and E) and 2-AG (D and F) applied basolaterally to Caco-2 cells. Data are given as means with error bars representing S.E.M. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ANOVA).

rier. We found that all the cannabinoids tested caused a significant increase in the mRNA expression of ZO-1. By contrast, claudin-1 expression was down-regulated only by the endocannabinoids anandamide and 2-AG but was not affected by THC or CBD. The mRNA expression of occludin was not affected by cannabinoid treatment. In all cases, the changes in tight junction mRNA brought about by cannabinoid treatment were sensitive to CB₁ receptor antagonism, confirming the role for this receptor in bringing about changes in intestinal permeability. Given that these gene expression changes are not a result of an inflammatory setting, would suggest that endogenous cannabinoids play a homeostatic regulatory role in the dynamic structure of the TJ. ZO-1 is integral to cytoskeletal rearrangements that contribute to make required changes in the TJ, whereas permutations of claudin and occludin assembly define the size of pore formation and determine charge selectivity (for review, see Ménard et al., 2010). Potentially, overproduction of the endocannabinoids could lead to a dysregulation of the structure of the TJ, particularly with regard to claudin-1, allowing incorrect passage of small molecules into the lamina propria. Equally, the up-regulation of ZO-1 by both plant and endog-

enous cannabinoids may explain their ability to restore permeability when applied basally. Further studies under inflammatory conditions are underway to dissect this scenario.

There are several anomalies within our data that warrant discussion. For example, that both increases and decreases in TEER appear to be mediated by the CB₁ receptor suggests different cannabinoid receptor ligands may initiate different signaling pathways. Our pilot experiments have indicated that the effects of THC and CBD appear to be mediated through mitogen-activated protein kinase, but the effects of endocannabinoids are mediated through nitric oxide signaling (data not shown). We also have shown in the present study that there are differential effects of endocannabinoids versus phytocannabinoids on changes in the expression of tight junction proteins, both of which are inhibited by CB₁ expression. It is likely that different signaling pathways are coupled to changes in different tight junction proteins, and this underlies the differential effects of cannabinoids on gut permeability.

We also have found that THC and CBD can inhibit the effects of endocannabinoids although both appear to be mediated through CB₁ activation. Likewise, several studies have shown that CBD is capable of antagonizing CB₁/CB₂

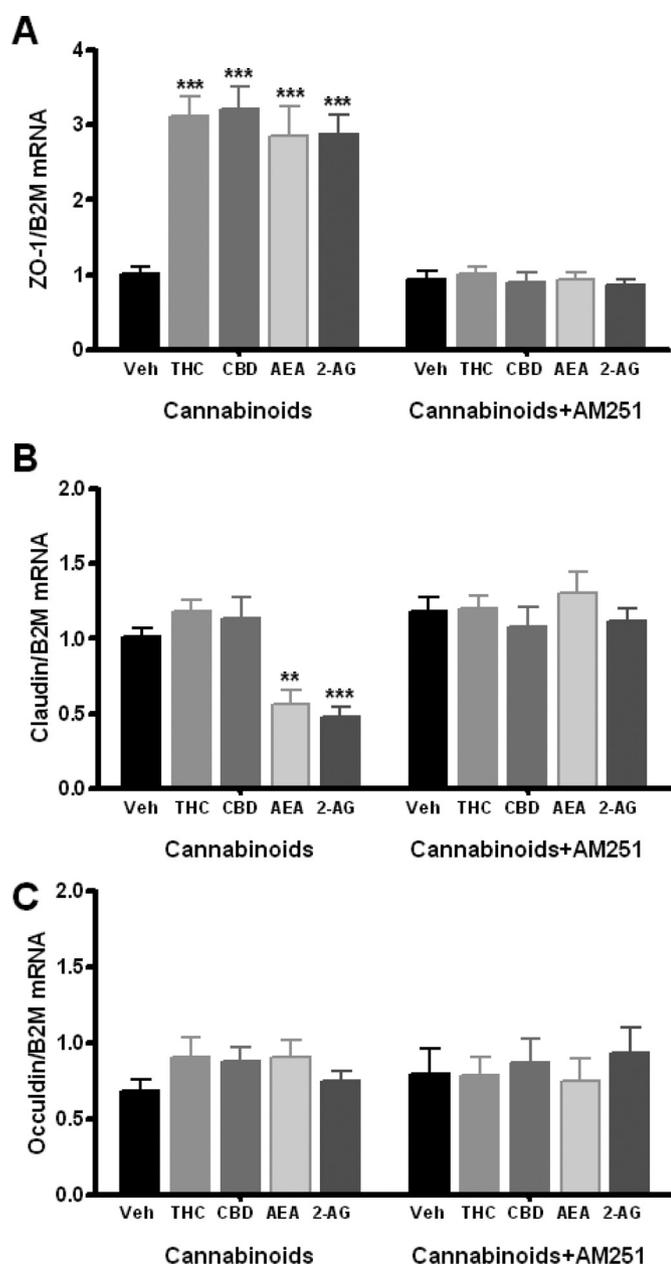


Fig. 10. Effects of cannabinoids (THC, CBD, AEA, and 2-AG; 10 μ M each) in the absence or presence of the CB₁ receptor antagonist AM251 (100 nM) on the mRNA expression of the tight junction proteins ZO-1, claudin-1, and occludin in Caco-2 cells. mRNA expression was detected by quantitative RT-polymerase chain reaction relative to the housekeeping gene B2M. Data are given as means with error bars representing S.E.M. (**, $P < 0.01$; ***, $P < 0.001$, ANOVA).

agonists, with K_B values in the low nanomolar range, and that THC can block the effects of CB₁ or CB₂ agonists (for review, see Pertwee, 2008). The mechanisms by which THC and CBD can act as agonists and antagonists of cannabinoid receptors are not clear but could include several possibilities, such as allosteric or orthosteric modulation CB₁, competitive antagonism, and inverse agonism, or they could be due to heterogeneity in the CB₁ population (Pertwee, 2008).

Conclusions

These data show that cannabinoids are capable of modulating intestinal permeability in an in vitro model. Endocan-

nabinoids cause an increase or decrease in permeability depending on whether they are acting at the apical or basolateral membrane. THC and CBD are capable of restoring increased permeability induced by either EDTA or endocannabinoids whether applied to the apical or basolateral membrane. These data suggest that endocannabinoids may play a role in the modulation of gut permeability and that cannabis-based medicines may possess therapeutic benefit in a variety of gastrointestinal diseases characterized by abnormal intestinal permeability, such as inflammatory bowel disease and shock. However, it is important to note that the EDTA-induced hyperpermeability-induced changes in epithelial cells in the present study are not likely to be observed in physiological conditions; but as a model of increased permeability, this study gives us an early indication of the pharmacological effects of cannabinoids on gut permeability. Further work is in progress to establish the role of cannabinoids on gut permeability in more physiological settings, such as an inflammatory setting.

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