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MODULATION OF THE ENDOCANNABINOID SYSTEM BY THE FATTY ACID AMIDE HYDROLASE, MONOACYLGLYCEROL AND DIACYLGLYCEROL LIPASE INHIBITORS AS AN ATTRACTIVE TARGET FOR SECRETORY DIARRHOEA THERAPY

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Secretory diarrhoea is a leading cause of mortality and morbidity worldwide. Our aim was to characterize the effect of inhibition of selected enzymes involved in the synthesis or degradation of endocannabinoids on electrolyte equilibrium in the mouse colonic tissue. The aim of this study was to evaluate the effects of PF-3845, JZL-184 and RHC-80267, as inhibitors of fatty acid amide hydrolase (FAAH), monoacylglycerol (MAGL) and diacylglycerol lipase (DAGL), respectively on epithelial ion transport in isolated mouse colon stimulated by forskolin (FSK), veratridine (VER) and bethanechol (BET). Next, colonic tissue was co-incubated with selected inhibitors and cannabinoid receptor antagonists: AM 251 and AM 630 (CB_1 and CB_2 antagonists, respectively). We found that PF-3845 induced antisecretory effect in FSK-stimulated colonic tissue ($P < 0.01$), which was significantly reversed by AM 251 ($P < 0.001$) and AM 630 ($P < 0.01$). JZL-184 significantly reduced ΔI_{sc} ($P < 0.05$) in FSK-stimulated conditions and co-incubation with AM 630, but not AM 251 reversed this effect when compared to JZL-184 alone ($P < 0.05$). After addition of PF-3845 and JZL-184 to colon tissue stimulated by VER, we did not observe any significant effect on ΔI_{sc}. PF-3845, JZL-184 or RHC-80267 were without any statistically significant effect on BET-evoked ion transport when compared to control. Our findings showed that indirect modulation of the endocannabinoid system could be an attractive target for novel effective treatment of secretory diarrhoea, which is devoid of side effects on the central nervous system caused by direct administration of cannabinoid receptor agonists.

Key words: *endocannabinoid system, ion transport, secretory diarrhea, cannabinoid receptor antagonists, cannabinoid synthesis and degradation enzymes inhibitors, inhibitors of fatty acid amide hydrolase, forskolin, veratridine, bethanechol*

INTRODUCTION

Diarrheal diseases remain a major global public health problem and are the second leading cause of death, particularly in children and the elderly (1). Secretory diarrhoea can be the result of many factors, including bacterial (such as enterotoxigenic *Vibrio cholerae* and *Escherichia coli*), viral (rotavirus) and parasitic organisms (e.g. *Entamoeba histolytica* and *Cryptosporidium parvum*) (2). Bacterial toxins, such as cholera toxin, activate unregulated production of cyclic adenosine monophosphate (cAMP), what results in blocking Na^+ absorption and stimulates Cl^- secretion by the enterocytes (3). Non-infectious diarrhoeas are caused by bile acids, fatty acids, and some laxatives which can act as luminal secretagogues (4). Moreover, secretory diarrhoea is associated with intestinal inflammatory and autoimmune conditions, such as Crohn's disease or ulcerative colitis (5) and mucosal disorders such as celiac disease or Whipple's disease (6), in which the immune system modulates absorption of electrolytes by release of cytokines and by influence on enteric nervous system (7).

The movement of fluid between the intestinal lumen and blood is driven by the active transport of ions, mainly Na^+, Cl^-, HCO^{3-}, and K^+. Fluid absorption and secretion involves the coordinated activity of membrane transporters located on the apical and basolateral epithelial membranes (8). Secretory diarrhoea occurs when secretion of water and electrolytes into the intestinal lumen exceeds its absorption. One of the mechanisms involved in secretory diarrhoea is *via* increase in the intracellular levels of cAMP and cGMP, what activates protein kinase A (PKA), leading to an opening of the apical anion channel, and thereby initiation of secretion (9). An increase in Ca^{2+} levels also activates the calcium activated Cl^- channel (CaCCs), what increases fluid secretion (10).

Cannabinoid receptors (CB_1 (11) and CB_2 (12)), endocannabinoids (anandamide (AEA) and 2-arachidonoylglycerol (2-AG)), and the enzymes responsible for their synthesis and degradation represent the elements of the endocannabinoid system (ECS). AEA is mainly synthesized from N-arachidonoyl phosphatidylethanolamine (NAPE) by phospholipase D, and degraded by fatty acid amide hydrolase

(FAAH) into arachidonic acid and ethanolamine (13), whereas 2-AG is synthesized from membrane phospholipids by phospholipase Cβ and diacylglycerol lipase (DAGL), and undergoes degradation by monoacylglycerol lipase (MAGL) (14). ECS is involved in several functions in the GI tract, including motility and secretion (15). However, cannabinoids have also an adverse effect on the central nervous system. Therefore, one of the proposed approaches to overcome this obstacle when applying ECS as therapeutic agents focuses on site-specific inhibition of enzymes involved in their synthesis and degradation that may be less aggressive than systemic administration of CB agonists (16).

The aim of this study was to investigate the effect of inhibitors of the enzymes involved in the synthesis (RHC-80267) and degradation (JZL-184 and PF-3845) of AEA and 2-AG on the FSK, VER, and BET-evoked epithelial ion transport in the mouse distal colon to provide potentially novel treatment of secretory diarrhoea. Additionally, to explore the mechanisms of action of selected enzyme inhibitors, we used the CB_1 and CB_2 receptor antagonists, AM 251 and AM 630.

MATERIALS AND METHODS

Animals

Male Swiss-Webster mice (Institute of Genetics and Animal Breeding of the Polish Academy of Sciences in Jastrzebiec, Poland), weighing from 26 – 30 g, were used for the study. Animals were housed at a constant temperature (22 – 23°C) and maintained under a 12-h light/dark (lights on at 6:00 a.m.). Mice were group-housed in sawdust coated transparent cages and had a free access to chow and tap water. The study was carried out in strict accordance with institutional animal ethics committee guidelines and approved by the Local Ethics Committee at the Medical University of Lodz with the following number: 11/£B735/2015.

Chemicals

Unless otherwise indicated, all reagents and drugs were purchased from Sigma-Aldrich, Inc. (St. Louis, Mo, USA). PF-3845, JZL-184, RHC-80267 (fatty acid amide hydrolase (FAAH), mono- (MAGL) and diacylglycerol lipase (DAGL) inhibitors, respectively), AM 251 and AM 630 (CB_1 and CB_2 cannabinoid receptors antagonist, respectively) were purchased from Tocris Bioscience (Ellisville, MO, USA). All drugs were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%.

Ussing chamber experiments

Epithelial ion transport was assessed according to the method described earlier (17). Mice were sacrificed by cervical dislocation. Subsequently, full-wall thickness segments (0.5 – 1 cm) of the distal colon were isolated and immediately placed in Ussing chamber (Physiologic Instruments, Inc., San Diego, CA, USA) containing 6 ml of Krebs solution of the following ionic composition (mM): NaCl, 115; KH_2PO_4, 2; $MgCl_2$, 2.4; $NaHCO_3$, 25; KCl, 8; $CaCl_2$, 1.3. Krebs solution was oxygenated with 95% O_2 and 5% CO_2 and contained glucose (10 mM) and mannitol (10 mM) added to the basolateral and mucosal side, respectively. The bath temperature was maintained at 37°C. The exposed surface of the tissue was 0.3 cm^2. Tissues were voltage clamped to zero, using the WPI EVC-4000 voltage clamp apparatus (World Precision Instruments, Sarasota, FL, USA) with Ag/AgCl electrode and 3 M KCl agar bridge. Once a stable baseline in short circuit current (I_{sc}, mA/cm^2) was achieved (15 – 30 min), tested drug previously dissolved in DMSO (PF-3845, JZL-184 or RHC-80267; final concentration: 10^{-6} M) or an equal volume of vehicle (DMSO, final concentration: 0.1%) was added to the basolateral side. Ten minutes later, preparations were challenged with either FSK (cAMP-dependent secretagogue, 10^{-5} M), VER (voltage-dependent Na^+ channel activator, 3×10^{-5} M) or BET (cholinergic receptor agonist, resistant to the action of cholinesterases, 10^{-4} M). To assess the

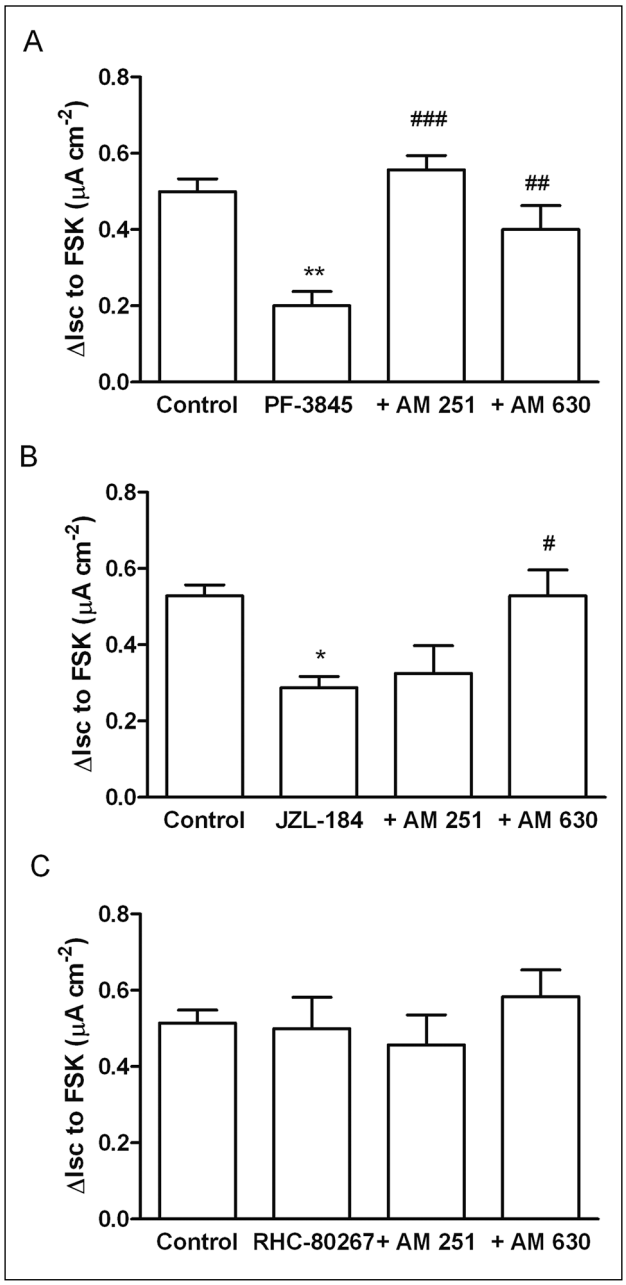

Fig. 1. Changes in forskolin (FSK, 10^{-5} M)-stimulated short-circuit current (I_{sc}) in mouse distal colon after basolateral application of FAAH inhibitor PF-3845 (10^{-6} M) (A), MAGL inhibitor JZL-184 (10^{-6} M) (B) and DAGL inhibitor RHC-80267 (10^{-6} M) (C) alone or together with selected antagonists: AM 251 (CB_1 receptor antagonist) (10^{-5} M), or AM 630 (CB_2) (10^{-5} M). Data represent mean ± S.E.M., n = 6. *P < 0.05, **P < 0.01, as compared to control. $^{\#}P < 0.05$, as compared to JZL-184. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, as compared to PF-3845.

involvement of cannabinoid receptors, the following antagonists were added 10 min prior to tested drugs: AM 251 (10^{-5} M, CB_1 antagonist) and AM 630 (10-5 M, CB_2 selective antagonist). For each challenge, the peak change in I_{sc} (ΔI_{sc}) was determined.

Statistical analysis

All data were expressed as the mean ± standard error of the mean. One-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test was used for multiple comparisons. Statistical significance aimed at $P < 0.05$. All statistical calculations were performed with Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

PF-3845 and JZL-184 decreased forskolin-evoked epithelial ion transport in the mouse colon

First, we characterized the effect of selected inhibitors: PF-3845, JZL-184 and RHC-80267 (selective for FAAH, MAGL and DAGL, respectively) on the FSK-evoked epithelial ion transport in the distal colon. FSK exerts its activity by stimulation of adenylate cyclase, and thereby increasing cellular cAMP concentration leading to Cl^- and H_2O secretion through the colonic epithelium (18).

We observed that application of PF-3845 (10^{-6} M) to the basolateral side of FSK-stimulated colonic tissue caused a statistically significant decrease in ΔI_{sc} compared with control ($P < 0.01$, 0.20 ± 0.04 versus 0.50 ± 0.03 mA/cm², respectively) (*Fig. 1A*). Moreover, pretreatment of the colonic tissue with the CB_1 antagonist AM 251 (10^{-5} M), followed by the incubation with PF-3845 caused a significant increase in ΔI_{sc} compared with PF-3845 alone ($P < 0.001$, 0.56 ± 0.04 versus 0.20 ± 0.04 mA/cm², respectively). As a result of application of the CB_2 antagonist AM 630 (10^{-5} M), we found a statistically significant increase in ΔI_{sc} compared with PF-3845 alone ($P < 0.01$, 0.40 ± 0.07 versus 0.20 ± 0.04 mA/cm², respectively).

Addition of JZL-184 (10^{-6} M) to the basolateral side of colonic tissue under FSK-stimulated conditions caused a significant decrease in ΔI_{sc} when compared to control ($P < 0.05$, 0.29 ± 0.03 versus 0.53 ± 0.03 mA/cm², respectively) (*Fig. 1B*). Furthermore, application of AM 630 reversed this effect leading to an increase in ion transport in the colonic segments treated subsequently with JZL-184 when compared to JZL-184 alone ($P < 0.05$, 0.53 ± 0.07 versus 0.29 ± 0.03 mA/cm², respectively). However, the ΔI_{sc} response of the colonic tissue to pre-treatment with AM 251 was not significantly different from that upon the treatment with JZL-184 alone.

We did not observe any significant effect of FSK on epithelial ion transport in the colon exposed to RHC-80267 alone (10^{-6} M), and co-incubated with AM 251 or AM 630 (*Fig. 1C*).

AM 251 and AM 630 increased VER-evoked epithelial ion transport in JZL-184-stimulated mouse colon

Next, we determined the effect of selected inhibitors and CB receptor antagonists under VER-stimulated conditions (*Fig. 2*). VER is a voltage-dependent Na^+ channel activator and leads to enteric neurons depolarization and induction of Cl^- secretion into the lumen (19).

Application of PF-3845 alone or PF-3845 together with AM 251 or AM 630 had no statistically significant effect on colonic ΔI_{sc} (*Fig. 2A*). Similarly, after basolateral addition of JZL-184 alone, we did not observe any significant effect on colonic ΔI_{sc}. However, application of AM 630 to colonic tissue treated subsequently with JZL-184 led to a pronounced increase in ΔI_{sc} compared with JZL-184 alone ($P < 0.01$, 0.80 ± 0.08 versus 0.43 ± 0.06 mA/cm², respectively). We also observed a significant increase in ΔI_{sc} after addition of AM 251 ($P < 0.05$, 0.71 ± 0.08 versus 0.43 ± 0.06 mA/cm², for JZL-184 with AM 251 and JZL-184 alone, respectively) (*Fig. 2B*).

Similarly to PF-3845, addition of RHC-80267 alone or together with AM 251 or AM 630 did not have any significant effect on VER-evoked transepithelial ion transport (*Fig. 2C*).

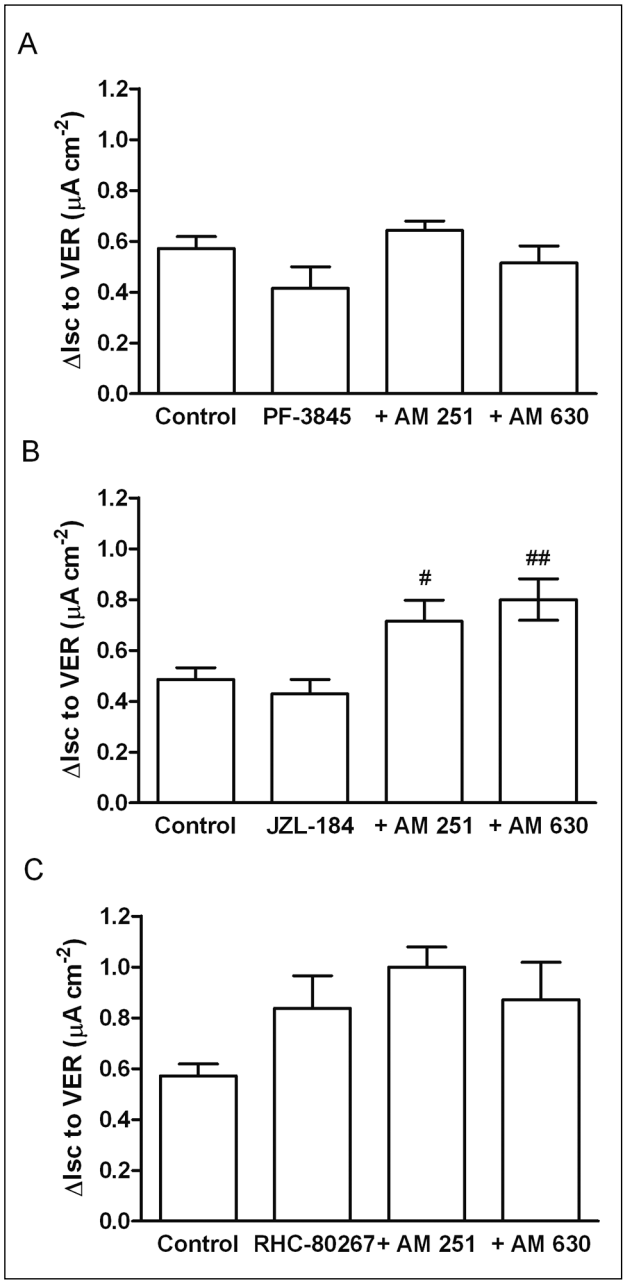

Fig. 2. Changes in veratridine (VER, 3×10^{-5} M)-stimulated short-circuit current (I_{sc}) in mouse distal colon after basolateral application of FAAH inhibitor PF-3845 (10^{-6} M) (A), MAGL inhibitor JZL-184 (10^{-6} M) (B) and DAGL inhibitor RHC-80267 (10^{-6} M) (C) alone or together with selected antagonists: AM 251 (CB_1 receptor antagonist) (10^{-5} M), or AM 630 (CB_2) (10^{-5} M). Data represent mean ± S.E.M., n = 6. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, as compared to JZL-184.

PF-3845, JZL-184 and RHC-80267 did not affect bethanechol-stimulated epithelial ion transport

Finally, we investigated the changes in BET-stimulated I_{sc}. BET is a selective M3 muscarinic acetylcholine receptor agonist that is responsible for downstream effects associated with activation of phospholipase C. This activation results in releasing of intracellular Ca^{2+} and leads to apical Cl^- efflux *via* Ca^{2+} activated Cl^- channels (CaCC) (20).

Basolateral addition of PF-3845, JZL-184 or RHC-80267 was without any statistically significant effect on BET (10^{-4} M)-evoked ion transport when compared to control (*Fig. 3*). Furthermore, we did not detect any significant differences between effects of PF-3845, JZL-184 or RHC-80267 alone and together with AM 251 or AM 630 in BET-stimulated colonic tissue.

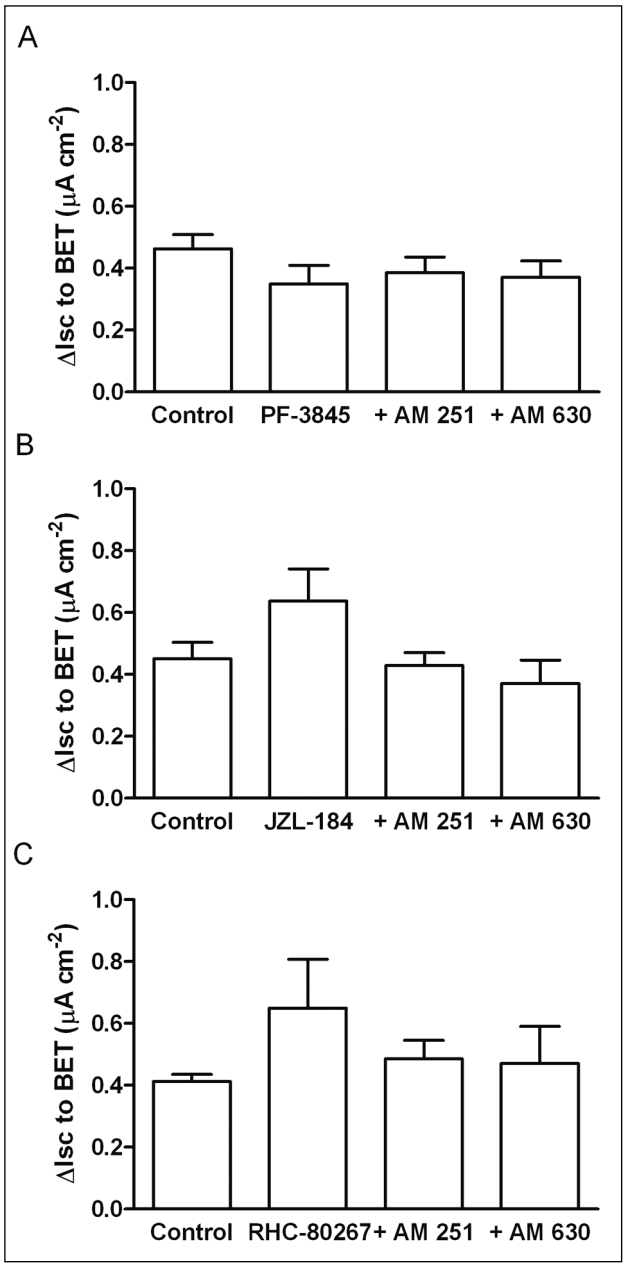

Fig. 3. Changes in bethanechol (BET, 10^{-4} M)-stimulated short-circuit current (I_{sc}) in mouse distal colon after basolateral application of FAAH inhibitor PF-3845 (10^{-6} M) (A), MAGL inhibitor JZL-184 (10^{-6} M) (B) and DAGL inhibitor RHC-80267 (10^{-6} M) (C) alone or together with selected antagonists: AM 251 (CB_1 receptor antagonist) (10^{-5} M), or AM 630 (CB_2) (10^{-5} M). Data represent mean ± S.E.M., n = 6.

DISCUSSION

In this report, we described the antisecretory effects of PF-3845 and JZL-184, selective inhibitors of FAAH and MAGL, respectively, in FSK-stimulated colonic tissue. We also observed that these effects were reversed by addition of selective CB_1 and CB_2 antagonists, AM 251 and AM 630. Furthermore, we found that application of JZL-184 together with AM 251 and AM 630 led to an increase in epithelial ion transport in VER-stimulated colonic tissue. PF-3845, JZL-184 or RHC-80267 were without any statistically significant effect on BET-evoked ion transport when compared to control.

CB_1 receptors are mainly expressed in the central nervous system (CNS) and, to a lesser extent, in some peripheral tissues, including adrenal gland, heart, lung, prostate, ovary and the gastrointestinal tract (21); CB_2 receptors are present almost exclusively in peripheral tissues (12). In gastrointestinal tract, CB_1 has been demonstrated to be expressed in normal colonic epithelium, smooth muscle, and the submucosal myenteric plexus. Furthermore, Wright *et al.* showed that CB_1 and CB_2 receptor expression was present on plasma cells in the *lamina propria*, whereas only CB_2 was present on macrophages (22).

Cannabinoid receptors play a role, among others, in depressing gastrointestinal motility and mediating the pharmacological effects of cannabinoids on food intake (23). CB_1 is involved in the release of neurotransmitters, including -aminobutyric acid, dopamine, noradrenaline, and serotonin, thereby determining the central action of cannabinoids (24). For example, it has been demonstrated that co-administration of leptin and CB_1 receptor antagonist AM 251 suppressed food intake and reduced body weight in rats. However, this effect was abolished by the injection of 5-HT_{2C} and 5-HT_{1B} receptor blockers indicating that serotonin might be the downstream mediator of the CB_1 and leptin-dependent effects on energy homeostasis (25). Furthermore, Merroun *et al.* (26) showed that intracerebral injection of AM 251 resulted in decrease in orexin expression and increase in c-Fos expression in the lateral hypothalamus, supporting hypothesis that the hypothalamic orexigenic neuropeptides are involved in the reduction of appetite and mediated by the cannabinoid receptor antagonists.

Cannabinoid receptors are also involved in the regulation of the intestinal water and electrolyte transport. For example, in a study by MacNaughton *et al.* CB_1 activation inhibited the responses to capsaicin which activated extrinsic primary afferents resulting in an increase in Isc. Addition of a cannabinoid receptor agonist, WIN 55,212-2, did not change ΔI_{sc} responses to FSK or BET. Thus, it has been concluded that cannabinoid agonists act on nerves rather than directly on the epithelium to attenuate stimulated ion transport (27).

Forskolin, a naturally occurring diterpene, causes rapid and reversible activation of adenylate cyclase through a direct action on the enzyme catalytic subunit and has been reported to influence cyclic AMP-dependent cellular actions (28). cAMP activates protein kinase A (PKA) leading to an increase in Cl^- and water secretion into the lumen through CFTR channels, and inhibits water absorption *via* phosphorylation of NHE2/3 regulatory proteins (29). FSK-activated intestinal chloride secretion mimics the mechanism underlying secretory diarrhoea. Here, we examined whether pharmacological blockade of FAAH by PF-3845 exerts antisecretory effect in FSK-stimulated tissue.

PF-3845 is a highly selective inhibitor for FAAH and has a longer duration of action than other FAAH inhibitors, such as URB597 and OL-135 (30). Previously FAAH was found to be a physiological regulator of intestinal motility; it has also been demonstrated that FAAH-deficient mice possessed approximately 2.8-fold higher levels of AEA in the small intestine than the wild-type littermates (31). In this study we observed that PF-3845 significantly reduced ion transport under FSK-stimulated conditions. Since FAAH hydrolyses not only the 'classical' endocannabinoids AEA and 2-AG, but also 'non-classical' endocannabinoids palmitoylethanolamide (PEA) and oleamide (32), we hypothesized that these cannabinoid receptor ligands are responsible for the pronounced decrease in the ion transport in the colonic tissue exposed to PF-3845 (32).

Previous study showed that WIN 55212-2 effectively inhibited neurally evoked ileal secretion, and this effect was reversed by the cannabinoid CB1 receptor selective antagonist SR141716A (33). Here, we observed that the inhibition of FAAH by PF-3845 in FSK-stimulated tissues was reversed by 'classical' cannabinoid receptor antagonists: AM 251 and AM 630. AM 251 is a potent CB_1 receptor antagonist that displays over 300-fold selectivity over CB_2 receptors (34), while AM 630 is a CB_2 antagonist/inverse agonist with 165-fold selectivity over CB_1 receptors (35). Our observations confirm that 'classical' cannabinoid receptor antagonists may play a role in PF-3845-mediated antisecretory actions in the mouse intestine. However, the involvement of other mechanisms cannot be excluded.

Next, we investigated the effect of JZL-184 on FSK-evoked ion transport. JZL-184 is a potent and selective MAGL inhibitor that blocks hydrolysis of 2-AG (36). We observed a significant decrease in ion transport when tissues were incubated with JZL-184. However, this effect was not as pronounced as it was after application of PF-3845. This may be due to the fact that the inhibition of MAGL raises only the level of 2-AG, but not AEA (37, 38), in contrast to inhibition of FAAH resulting in both 2-AG and AEA level increase. Of note, co-incubation of JZL-184 with AM 630, but not with AM 251, caused a significant increase in ion transport. This may be related to a higher affinity of 2-AG to CB_2 receptors. This is in contrast to the report of Karwad *et al.*, who showed that endogenous 2-AG and AEA synthesis and CB_1 activation play a key modulatory roles in normal intestinal mucosa permeability, and in inflammatory and hypoxic conditions in human tissue collected from colorectal resections (39). After basolateral application of JZL-184, permeability has been decreased *via* CB_1 receptor. Whether the antisecretory effect of JZL-184 is mediated by CB_2 receptors in healthy tissues and by CB_1 in inflamed intestine requires further investigation.

We also investigated the effects of RHC-80267 and selected antagonists under FSK-stimulated conditions. RHC-80267 is a DAGL inhibitor that enhances the activity of PKC indirectly by increasing the concentration of diacylglycerol (40). Here, we did not observe any significant effect of RHC-80267 on epithelial ion transport in the colon exposed to FSK alone, or co-incubated with AM 251 or AM 630. This is in line with the fact that 2-AG synthesis is blocked by RHC-80267. Moreover, it may be suggested that there were no compensatory mechanisms leading to changes in ion transport, which would counteract the lower concentration of 2-AG.

Next, the colonic tissues were stimulated by VER which is used to examine the effect of neural stimulation on the intestinal ion transport. After basolateral addition of PF-3845, JZL-184 or RHC-80267, we did not observe any significant effects. However, interestingly, co-incubation of JZL-184 and AM 251 or AM 630 led to increased ion transport. VER depolarizes enteric neurons as a result of increased voltage-sensitive Na^+ permeability, and consequently causes epithelial Cl^- secretion across the colonic mucosa (41). VER was shown to stimulate the release of enteric neurotransmitters, such as substance P, VIP (42) and acetylcholine (43). It is noteworthy that the concentrations of the antagonists used to block CB_1 and CB_2 receptors caused an effect *per se*, unlike in the case of FSK and BET (data not shown). However, the concentrations of AM251 and AM630 used in our study were similar to those applied in several other setups, where a blocking effect was obtained at the lowest concentration possible. We may thus suggest that it may be difficult to find a proper antagonist concentration that causes effective blocking of the receptors without producing an effect *per se*. Moreover, whether the effect observed in our study was driven by endocannabinoid-dependent pathways or another mechanism, still needs to be investigated.

In summary, this study provides strong evidence for the implication of the endocannabinoid system in the mechanisms underlying secretory diarrhoea. We demonstrated here that the modulation of the activity of the enzymes involved in the endocannabinoid degradation, FAAH and MAGL, may constitute a novel approach for development of effective anti-diarrheal strategies.

Authors contribution: J.F. and A.W. designed the research study; A.W. contributed to the acquisition of animal data; A.W. and J.F. analyzed the data; A.M., M.S. and J.F.. provided necessary tools and materials for the completion of the study; A.W. and J.F. drafted the manuscript; A.W., A.M., M.S. and J.F. critically reviewed content and approved the final version of the manuscript.

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Conflict of interests: None declared.

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