

**Analysis of the role of cannabinoid receptors and  $\alpha_2$ -adrenoceptor subtypes in gastrointestinal functions**

Ph.D. doctoral thesis

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# **1. Introduction**

## **1.1. Gastric mucosal integrity**

The development of gastric ulcers has been explained by the imbalance between the aggressive (e.g. hydrochloric acid, pepsin, gastrin, proteases, *Helicobacter pylori*) and defensive (e.g. barrier mucus, bicarbonate, mucosal microcirculation) factors. The current therapy of gastric ulcers includes inhibition of gastric acid secretion and eradication of *Helicobacter pylori*. However, acidity is just only one of the risk factors that lead to development of gastric ulcers and better therapeutic advances may be expected by focusing on augmentation of gastric mucosal defense.

Gastric mucosal defense can be initiated both peripherally and centrally. The most important regions of the central nervous system (CNS), that play role in the maintenance of gastric mucosal integrity and/or stimulation of gastric mucosal defense are the hypothalamus and the dorsal vagal complex (DVC). Several neuropeptides and their receptors were identified in DVC and they can influence gastric mucosal protection after central administration. For example, intracisternal injection of TRH, peptide YY and adrenomedullin as well as intracerebroventricularly (i.c.v.) administered amylin decreased the ethanol-induced gastric mucosal lesions in the rat. Moreover, different opioid peptides, ghrelin, orexin, nociceptin and nocistatin also induced mucosal protection against ethanol induced mucosal lesions following central administration.

The first part of this work focus mainly on the central role of endocannabinoid system in the regulation of gastric mucosal defense mechanisms in the ethanol ulcer model, which is an acid-independent ulcer model suitable, quick and simple for the evaluation of gastroprotective action of drugs.

### **1.1.1. Endocannabinoid system**

The endocannabinoid system consists of cannabinoid receptor-1 (CB<sub>1</sub>), cannabinoid receptor-2 (CB<sub>2</sub>), and their endogenous ligands. CB<sub>1</sub> receptors are expressed by central and peripheral neurons while, CB<sub>2</sub> receptors are mainly expressed by immune cells. In neurons, the

pre-synaptic CB<sub>1</sub> receptors mediate the effects of exogenous and endogenous cannabinoids primarily by modulating the transmission of different neurotransmitters. To date, the best characterized endogenous agonists for cannabinoid receptors are arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG). Both are produced on demand and they can undergo depolarization-induced release from neurons. The physiological effects of endocannabinoids (anandamide and 2-AG) are terminated by specific degradation systems involving the uptake of endocannabinoids into the cell by a facilitated endocannabinoid transport mechanism and the hydrolysis by fatty acid amide hydrolase (FAAH) (for both anandamide and 2-AG) and by monoacylglycerol lipase (for 2-AG). Anandamide, beside its action at cannabinoid receptors, is also able to activate transient receptor potential vanilloid receptor 1 (TRPV1).

### **1.1.2. Endocannabinoid system in the gastrointestinal tract**

The presence of CB<sub>1</sub> receptors in the neurons of the enteric nervous system (ENS) in different species has been demonstrated. In particular, immunohistochemical studies have shown the localization of CB<sub>1</sub> receptors on acetylcholine-containing neurons that innervate smooth muscle, mucosa and submucosal blood vessels of the rat stomach. Beside the presence of CB<sub>1</sub> receptors in the neurons of the ENS, CB<sub>1</sub> receptors are also identified in brain regions playing regulatory role in controlling GI functions, like in the DVC. The endocannabinoids (anandamide and 2-AG) were measured and detected in the GI tract together with high activity of FAAH.

### **1.1.3 Effects of cannabinoids on GI tract**

Peripheral administration of cannabinoids delays gastric emptying inhibits gastric motility and intestinal motility as well as inhibits pentagastrin stimulated gastric acid secretion. Cannabinoids were shown to inhibit the formation of gastric ulcers in acid dependent ulcer models (restraint stress, water immersion/restraint, aspirin-induced, and cold/restrain stress ulcer models), where the acid was the main causative agent of mucosal injuries. Similarly, central administration of cannabinoids inhibited gastric motility and gastrointestinal transit in the rat and mice respectively. In contrast,

stimulated gastric acid secretion was not affected by central activation of CB<sub>1</sub> receptors. In addition, activation of CB<sub>1</sub> receptors in the DVC mediates the antiemetic effect of cannabinoids. The role of endocannabinoids in the regulation of gastrointestinal motility has been demonstrated since the FAAH inhibitor N-arachidonyl-5-hydroxytryptamine and the selective anandamide inhibitor VDM11 delay gastric emptying and colonic propulsions in mice respectively.

#### **1.1.4. Interaction between the opioid and cannabinoid systems**

Both opioid and cannabinoid receptors modulate several functions of the gastrointestinal (GI) tract including motility, acid secretion, intestinal secretion and gastric mucosal protection. Moreover, the similar distribution of opioid and cannabinoid receptors in the DVC and the ENS provide the basis for possible interactions between the two systems. The strong co-localization of CB<sub>1</sub> and  $\mu$ -opioid receptors in the dorsal horn of the rat spinal cord, explained the interaction between cannabinoids and opioids in the control of nociception at spinal level. Activation of cannabinoid receptors may induce the release of opioid peptides. E.g. intrathecal administration of anandamide,  $\Delta^9$ -THC and CP55,940 induced spinal antinociception accompanied by differential kappa-opioid receptor involvement and dynorphin A peptide release. In addition,  $\Delta^9$ -THC synergizes the analgesic effect of morphine and codeine by releasing endogenous opioids.

### **1.2. Gastric motility**

It is well known that functional gastrointestinal disorders (FGID) like irritable bowel syndrome (IBD) and functional dyspepsia are associated with dysfunction of motor and/or sensory apparatus of the gastrointestinal tract. Current therapy for these conditions is unsatisfactory and there is still a need for the development of more selective therapeutics. Moreover, Correlation between gastric motility and gastric mucosal damage has been well documented, and increased gastric motor activity plays a role in the development of gastric mucosal lesions consequently, inhibition of gastrointestinal motility may contribute to gastroprotective action.

The inhibitory effects of different  $\alpha_2$ -adrenoceptor agonists on the gastrointestinal motility have been demonstrated in different species, for example in rat, mice as well as in human, which could provide a new therapeutical approach to management of patients with FGID and gastric ulcers as well.

In the second part of this work I analyzed the  $\alpha_2$ -adrenoceptor subtype(s) involved in the regulation of gastric motility by mean of in vitro pharmacological analysis of the effect of different  $\alpha_2$ -adrenoceptors stimulants on electrically evoked contractions in isolated gastric fundus strip of the rat and in the mice.

### **1.2.1. $\alpha_2$ -Adrenoceptors**

$\alpha_2$ -Adrenoceptors represent a group of heterogeneous receptors; three subtypes have been identified and named ( $\alpha_{2A}$ ,  $\alpha_{2B}$ ,  $\alpha_{2C}$ ) on the basis of their different affinities for many pharmacological agents and on molecular cloning evidences. Several functional studies aimed to investigate the role of different  $\alpha_2$ -adrenoceptor subtypes in mediating physiological function in different mammals, but the limited selectivity of  $\alpha_2$ -adrenoceptor agonists and antagonists left a doubt behind about defining a specific function for each subtype. On the other hand, the generation of mice with targeted deletions of the genes coding  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptors subtypes, offer a good opportunity for the evaluation of a specific physiological role for  $\alpha_2$ -adrenoceptor subtypes.

Functional and pharmacological analysis preformed with  $\alpha_2$ -adrenoceptors subtype selective drugs suggested, that of  $\alpha_{2A}$  subtype plays a dominant role in the inhibition of pre-synaptic neurotransmission, gastric acid secretion and gastrointestinal motility. Studies on mice lacking  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptor subtypes have shown that all the antihypertensive, bradycardic, sedative and antinociceptive effects of the  $\alpha_2$ -adrenoceptor agonists were found to be dependent entirely on  $\alpha_{2A}$ -adrenoceptor subtype. Moreover, in a study with mice lacking  $\alpha_{2A}$  subtype, an evidence of the involvement of  $\alpha_{2A}$  subtype in the regulation of intestinal motility was provided too.

## **1.2.2. Effects of $\alpha_2$ -adrenoceptors in the gastrointestinal tract**

Activation of pre-synaptic  $\alpha_2$ -adrenoceptors has been known to modulate several responses in GI tract and  $\alpha_{2A}$ -adrenoceptor subtype is likely to mediate most of the effects of  $\alpha_2$ -adrenoceptor stimulants. For example,  $\alpha_{2A}$ -adrenoceptors were shown to regulate stimulated gastric acid secretion, ion transport and fluid secretion in the small intestine. Also the dominant role of  $\alpha_{2A}$  subtype in the regulation of gastrointestinal motility has been demonstrated by both in vivo and in vitro studies.

Our previous results showed that  $\alpha_2$ -adrenoceptor agonists induced gastric mucosal protection given both centrally and peripherally, the effect was likely to be mediated by the  $\alpha_{2B/2C}$ -like-adrenoceptor subtypes. Since correlation between gastric motility and gastric mucosal damage has been well documented, we examined the effect of  $\alpha_2$ -adrenoceptor agonists and antagonists on gastric motility of the rat in vivo and the results showed the predominant role of  $\alpha_{2A}$ -adrenoceptor subtype in the action. However, the involvement of the  $\alpha_{2B}$ - and  $\alpha_{2C}$ - subtypes in the regulation of gastric motility has not been ruled out.

## **2. Aims of the study**

### **2.1. Gastric mucosal integrity**

The main aim of the study is to clarify, whether cannabinoids can inhibit gastric mucosal lesions induced in ethanol ulcer model, in which the gastric mucosal lesions are independent on acid secretion and related to impairment of gastric mucosal defense. The experiments aimed:

- To analyze the role of cannabinoid receptor agonists in the regulation of gastric mucosal integrity in ethanol ulcer model after both peripheral and central administration.
- To clarify the role of both central and peripheral  $CB_1$  receptors in the regulation of gastric mucosal integrity.
- To investigate whether endogenous cannabinoids are involved in the regulation of gastric mucosal protection.

- To examine whether a potential interaction between cannabinoid and opioid systems can be demonstrated in the gastric mucosal protection.
- To analyze whether TRPV1 receptors may contribute to the gastroprotective effect of cannabinoids.
- To study the possible role of nitric oxide (NO) and prostaglandins (PGs) in the mechanism of cannabinoid-induced gastroprotective effect.

## **2.2. Gastric motor activity**

This study is designed to examine the role  $\alpha_2$ -adrenoceptor subtypes in the regulation of gastric motor activity and to determine whether the inhibition of gastric motor activity by  $\alpha_2$ -adrenoceptor stimulants is mediated exclusively by  $\alpha_{2A}$ -adrenoceptors or the  $\alpha_{2B}$ - and  $\alpha_{2C}$ - subtypes may also contribute to the inhibitory effect of  $\alpha_2$ -adrenoceptor stimulants on gastric motor activity. In order to answer the questions experiments were carried out;

- To determine the effect of different  $\alpha_2$ -adrenoceptor agonists/antagonist on contractions evoked by electrical field stimulation (EFS) in isolated gastric fundus strips of rats and NMRI mice.
- To investigate the effect of  $\alpha_2$ -adrenoceptor agonists/antagonists on the contractions induced by EFS in isolated gastric fundus strips prepared from C57/6L wild type,  $\alpha_{2A}^-$ ,  $\alpha_{2B}^-$  and  $\alpha_{2C}^-$ -adrenoceptor subtypes deficient mice.

## **3. Materials and Methods**

### **3.1. Animals**

For ethanol ulcer experiments male Wistar rats (150-170g) were used. For in vitro analysis of gastric motor activity male Wistar rats (250–350g), NMRI mice, C57BL/6 wild type, and  $\alpha_{2A}^-$ ,  $\alpha_{2B}^-$ ,  $\alpha_{2C}^-$ -adrenoceptor subtype deficient mice of 20–30g weight were used.

### **3.2. Ethanol ulcer model**

Rats were fasted for 24 hours before experiments with free access to tap water. Then they were given orally 0.5 ml acidified ethanol (2 ml cc.HCl added to 100 ml absolute ethanol). One hour later the animals were killed by overdose of ether, the stomachs were excised, opened along the greater curvature, rinsed with saline and examined for lesions. Total number of mucosal lesions was assessed in blinded manner by calculation of lesion index based on a 0 - 4 scoring system. The numbers of different severity lesions were multiplied by the respective severity factors and finally the sum of them was taken as ulcer index. The percentage of inhibition of mucosal damage was calculated as follows:  $100 - [(\text{ulcer index in treated group} / \text{ulcer index in control group}) \times 100]$ .

The cannabinoid receptor agonists, the anandamide uptake inhibitor, the selective inhibitor of fatty acid amide hydrolyase (FAAH) and the opioid receptor agonists, were injected 15 min intravenously (i.v., 0.5 ml/100g) and 10 min intracerebroventricularly (i.c.v., 10  $\mu$ l/rat) prior to the ethanol challenge. The different antagonists, the cannabinoid receptor antagonists, the opioid receptor antagonists and the transient receptor potential vanilloid type1 (TRPV1) receptor antagonist were injected i.c.v. 10 min before the administration of the cannabinoid receptor agonists. The endomorphin-2 antiserum was injected intracisternally (i.c., 5  $\mu$ l/rat) 10 min before the administration of the cannabinoid receptor agonists.

### **3.3. In vitro analysis of gastric motor activity**

#### **3.3.1. Tissue Preparation**

Animals were fasted for 24 h, with free access to tap water. At the time of the experiment they were killed by cervical dislocation, their stomachs were removed and the grey fundal part was separated from the pink pyloric part. Then, a strip of about 2 - 4 cm long was prepared. A thread was attached to each end of the strip and then the strip was suspended vertically between upper circular and lower straight platinum electrodes in organ bath of 35 ml and 5 ml for strips of rat and mice respectively. The organ bath was filled

with Krebs solution of the following composition (mM/L: NaCl, 118.0; NaHCO<sub>3</sub>, 25.0; KCl, 4.7; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 11.0; CaCl<sub>2</sub>, 2.5; MgSO<sub>4</sub>, 1.2) and aerated with carbogen (gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>) at 37°C. The upper thread was attached to a transducer connected by an amplifier to computer system which recorded the contractile activity of the strips. The strips were allowed to equilibrate for 30 - 45 min under tension of 1g for strips of rat and 0.5g for strips of mice respectively.

### **3.3.2. Electrical field stimulation**

In the rat, contractions were elicited by applying EFS of the following parameters (200 ms pulse distance, 1 ms pulse width, 50 shocks, 9 V/cm i.e. supramaximal intensity of 5Hz repeated every 60 s). In the mouse, contractions were induced by applying EFS of the following parameters (200 ms pulse distance, 1 ms pulse width, 25 shocks, 9 V/cm i.e. supramaximal intensity of 5Hz repeated every 60 s). These contractions were inhibited by atropine (1000 nM) which indicates that the EFS-induced contractions are mediated via cholinergic mechanisms.

### **3.3.3. Experimental protocol**

After application of EFS a period of 20 – 30 min was allowed to reach stable contractions, then the  $\alpha_2$ -adrenoceptor agonists were added cumulatively into the organ bath. A period of 5 min long was allowed to elapse between agonists concentration increment. Cumulative concentration-response curves for the  $\alpha_2$ -adrenoceptor agonists were built up from 4 - 5 doses, and the 50 % effective concentration EC<sub>50</sub> (nM) and the maximal effect E<sub>max</sub> (%) values were calculated. When the effect of  $\alpha_2$ -adrenoceptor antagonists on the inhibitory effect of  $\alpha_2$ -adrenoceptor agonists was assessed, the antagonists were added into the organ containing the agonist and left to equilibrate for another 20 min.

## **3.4. Drugs**

### **Drugs acting on the endocannabinoid system**

Non-selective cannabinoid receptor agonist; anandamide, methanandamide, WIN55,212-2, selective CB<sub>1</sub> receptor agonist;

ACEA, anandamide uptake inhibitor; AM 404, FAAH inhibitor; URB-597, selective CB<sub>1</sub> receptor antagonist; SR141716A and AM-251. SR141716A was a generous gift from T. Freund. All the other drugs were purchased from Tocris Bioscience.

### **Drugs acting on the opioid system**

Selective  $\mu$ -opioid receptor DAGO (A. Magyar, Eotvos University, Budapest, Hungary), selective  $\delta$ -opioid receptor agonist Deltorphin II (G. Toth, Biological Research Centre of Hungarian Academy of Sciences, Szeged, Hungary), Naloxone (non-selective opioid receptor antagonist, sigma), Naltrindole (selective  $\delta$ -opioid receptor antagonist, sigma), Norbinaltorphimine (selective  $\kappa$ -opioid receptor antagonist, sigma) and Endomorphin-2 antiserum (I. Barna, and A. Rónai, Hungarian Academy of Sciences).

### **Drugs acting on the $\alpha_2$ -adrenoceptors**

Clonidine (non-selective  $\alpha_2$ -adrenoceptors agonist, Sigma), oxymetazoline (selective  $\alpha_{2A}$ -adrenoceptor agonist, RBI Natick), ST-91 ( $\alpha_{2B/2C}$ -adrenoceptor preferring agonist, P. Matyus, Department of Organic Chemistry, Semmelweis university, Budapest, Hungary), idazoxan (non-selective  $\alpha_2$ -adrenoceptors antagonist, Sigma), BRL 44408 ( $\alpha_{2A}$ -adrenoceptor selective antagonist, Tocris Bioscience) and ARC-239 ( $\alpha_{2B/2C}$ -adrenoceptor antagonist, Tocris Bioscience).

### **Other drugs**

Capsazepine (transient receptor potential vanilloid receptor 1 antagonist, Tocris Bioscience), N<sup>G</sup>-nitro-L-arginine (LNNA, NO synthase inhibitor, Sigma), indomethacin (non selective cyclooxygenase inhibitor, Sigma) and atropine (anticholinergic, Sigma).

## **3.6. Statistical analysis**

The results were presented as means  $\pm$  S.E.M. (Standard Error of Mean). The ethanol induced gastric mucosal damage experiments results were evaluated by means of analysis of variance (ANOVA) followed by Newman-Keuls post hoc test for multiple comparison. Data of in vitro analysis of gastric motility were evaluated partly by mean of analysis of variance (ANOVA) for repeated measures followed by Newman-Keuls post-hoc test for multiple comparisons and partly by mean of paired Student's *t* test.

A probability value of  $p < 0.05$  was considered statistically significant.

## **4. RESULTS and DISCUSSION**

### **4.1. Gastric mucosal integrity**

#### **4.1.1. Effect of cannabinoids in the ethanol ulcer model in the rat**

Cannabinoids were shown to inhibit the mucosal lesions in acid-dependent ulcer models, after peripheral administration. The question has been raised whether cannabinoids are able to inhibit gastric mucosal damage induced by ethanol an acid-independent ulcer model, where the mechanism of mucosal damage have been described to be associated with disturbances of gastric mucosal microcirculation.

The present results demonstrated first that the endocannabinoid anandamide (0.28-5.6  $\mu\text{mol}$ ), its stable analogue methanandamide (0.7-5.6  $\mu\text{mol}$ ) and the synthetic non-selective cannabinoid WIN55,212-2 (0.05-0.2  $\mu\text{mol}$ ) prevented the ethanol-induced gastric mucosal damage in a dose dependent manner after i.v. administration. Similarly, i.c.v. injection of anandamide (2.9-115 nmol/rat), methanandamide (0.27-70 nmol/rat), WIN55,212-2 (1.9-38 nmol/rat) and the selective CB<sub>1</sub> receptor agonist ACEA (0.13-1.37 nmol/rat) significantly inhibited the gastric mucosal lesions induced by ethanol. This centrally induced gastroprotective effect of anandamide and methanandamide were reversed by a selective cannabinoid CB<sub>1</sub> receptor antagonist SR141716A (2.16 nmol/rat) injected centrally, indicating involvement of CB<sub>1</sub> receptor in their gastroprotective effect. Moreover, the gastroprotective effect of methanandamide given i.v. was reversed by SR141716A administered centrally suggesting the primary role of centrally located CB<sub>1</sub> receptors in the gastroprotective effect of methanandamide. The effective dose range of cannabinoids against ethanol-induced lesions given i.c.v. were much lower than that injected i.v., providing an additional evidence for the dominant role of central CB<sub>1</sub> receptors compared to the peripheral CB<sub>1</sub> receptors in

this action. Accordingly, since, cannabinoids agonist are lipophilic molecules which can easily cross blood brain barrier, their peripheral gastroprotective effect might be mediated via central CB<sub>1</sub> receptors. The gastroprotective effect of cannabinoids is further supported by the findings that CB<sub>1</sub> receptor and its mRNA were found throughout the central nervous system in particular, CB<sub>1</sub> receptors were co-localized in the DVC. In addition, the distribution of CB<sub>1</sub> receptor immunoreactivity in the cholinergic neural elements innervating smooth muscle, mucosa and submucosal blood vessels of rat stomach as well as in the enteric nervous system of different species was demonstrated.

#### **4.1.2. The role of endogenous cannabinoids in the gastric mucosal protection**

The physiological role of endocannabinoids has been raised in the regulation of gastrointestinal motility. Namely, the inhibitor of anandamide cellular uptake VDM11 decreased colonic propulsions in mice; the effect was counteracted by SR141716A. Also the fatty acid amide hydrolyase inhibitor N-arachidonyl-5-hydroxytryptamine inhibited gastric emptying and this effect was also counteracted by SR141716A. To evaluate the role of the endogenous cannabinoid in gastric mucosal protection, the effect of the selective anandamide uptake inhibitor AM 404 and the selective inhibitor of fatty acid amide hydrolyase URB-597 on gastric mucosal damage induced by ethanol was tested. Both AM 404 and URB-597 are known to increase the level of anandamide by interfering with anandamide uptake by the cells and its degradation by FAAH, respectively. It was found that i.c.v. injected anandamide uptake inhibitor AM 404 and the FAAH inhibitor URB-597 significantly inhibited the gastric mucosal lesions induced by ethanol. This effect was mediated via activation of CB<sub>1</sub> receptor, since the selective CB<sub>1</sub> receptor antagonist AM-251 reduced the gastroprotective effect of AM 404 and URB-597. This new finding provides the first evidence for the physiological role of endocannabinoids in gastric mucosal integrity.

### **4.1.3. Interactions between cannabinoid and opioid systems in gastroprotection**

Our present findings demonstrated first a cannabinoid-opioid interaction in centrally-induced gastric mucosal protection. It was shown that naloxone (27.5 nmol/rat) given centrally antagonized the gastroprotective effect of anandamide (115 nmol/rat) and WIN55,212-2 (38 nmol/rat) injected i.c.v., the effect of methanandamide (70 nmol/rat i.c.v.) was diminished, but the reduction was not statistically significant. Similar results were obtained when the effect of centrally injected naloxone was examined on the protective effect of anandamide (5.6  $\mu$ mol/kg), methanandamide (5.6  $\mu$ mol/kg) and WIN55,212-2 (0.2  $\mu$ mol/kg) given intravenously; naloxone antagonized the mucosal protective effect of anandamide and WIN55,212-2, and decreased the protective effect of methanandamide in a significant manner. Since the centrally injected naloxone also inhibited the mucosal protective effect of intravenously injected anandamide, methanandamide and WIN55,212-2, the interaction may be located primarily in the CNS. In addition, the gastroprotective of centrally injected anandamide was counteracted by  $\delta$ -opioid receptor antagonist naltrindole and  $\kappa$ -opioid receptor antagonist norbinorphimine (norBNI) as well. These data, strongly suggest the involvement of central  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors in mediating the mucosal protective effect of cannabinoids. Our findings confirmed that the interaction between cannabinoid and opioid system is likely to be indirect, namely endomorphin-2 antiserum given i.c. reduced the protective effect of i.c.v. injected anandamide in a significant manner, indicating that endomorphin-2 release may be crucial for anandamide-induced gastroprotective effect. Endomorphin-2 and endomorphin-1 are  $\mu$ -opioid receptor selective endogenous opioids; however, endomorphin-2 can induce the release of other endogenous opioids like dynorphine and [Met<sup>5</sup>]enkephalin. This may explain partly that both the  $\kappa$ -opioid receptor antagonist norBNI and the  $\delta$ -opioid receptor antagonist naltrindole reduced the gastroprotective effect of anandamide. We also investigated the effect of CB<sub>1</sub> receptor selective antagonist SR141716A on the gastroprotective effect of the  $\mu$ -opioid receptor selective agonist (DAGO) and  $\delta$ -opioid receptor

selective agonist deltorphin II after central administration. It was found that the gastroprotective effect of opioid peptides was attenuated but not completely abolished by SR141716A, indicating that endocannabinoids may partly contribute to the gastroprotective effect of opioid peptide. The above data suggested the crucial role of central CB<sub>1</sub> receptors as well as the cannabinoid-opioid interaction in the gastroprotective effect of cannabinoids.

#### **4.1.4. The role of TRPV1 receptors in the gastroprotective effect of cannabinoids**

The endogenous cannabinoid receptor ligand anandamide has been identified to function as an endogenous agonist at the TRPV1 receptor. To evaluate, whether the TRPV1 receptor is involved in central gastroprotective effect of endocannabinoid, we studied the effect of i.c.v. injected TRPV1 selective antagonist capsezepine on the gastroprotective effect of methanandamide given centrally. It was found that, the gastroprotective effect of methanandamide was reduced by capsazepine but not abolished completely. This result indicates that activation of central CB<sub>1</sub> and TRPV1 receptors induce convergent actions, and the TRPV1 induced effect may contribute to the gastroprotective effect of cannabinoids.

#### **4.1.5. Peripheral factors involved in the central gastroprotective effect of cannabinoids**

Data of the literature suggest that nitric oxide, PGs and sensory neuropeptides are responsible for the gastric mucosal integrity in the periphery. Therefore, we investigated the involvement of these mediators in the central gastroprotection induced by cannabinoids. The present data showed that the inhibitor of NO synthase, N<sup>G</sup>-nitro-L-Arginine (L-NNA) given i.v., reduced the protective effect anandamide given i.c.v. This result indicates that mucosal NO is likely to be involved in the cannabinoids-induced gastroprotection. Moreover, inhibition of PGs synthesis by indomethacin also resulted in a significant reduction of the cannabinoids-induced mucosal protective effect, suggesting that in addition to NO, PGS may also participate in the mucosal protective mechanism of the cannabinoids.

## 4.2. Gastric motor activity

### 4.2.1. In vitro analysis of the role of $\alpha_2$ -adrenoceptor subtypes in the regulation of gastric motility in the rat

The non-selective  $\alpha_2$ -adrenoceptor agonist clonidine, the  $\alpha_{2A}$ -subtype selective agonist oxymetazoline and the  $\alpha_{2B/2C}$ -subtype preferring agonist ST-91 reduced the cholinergic contractions induced by EFS in a concentration dependent manner. The concentration of clonidine, oxymetazoline and ST-91 which inhibit 50% of maximal strip contractions ( $EC_{50}$ ), were ( $75 \pm 5.2$  nM,  $10.3 \pm 5.9$  nM,  $79.9 \pm 4.3$  nM, respectively). Oxymetazoline showed higher potency than clonidine and ST-91. The relative potency was taken 1 for clonidine and the relative potencies for oxymetazoline and ST-91 compared to that of clonidine were 7.3 and 0.9 respectively. This result indicates the involvement of  $\alpha_2$ -adrenoceptor in the control of gastric motility in the rat. To further characterize the  $\alpha_2$ -adrenoceptor subtype that mediate the inhibitory effect of  $\alpha_2$ -adrenoceptor agonists on EFS-induced gastric contractions, the effect of non-selective  $\alpha_2$ -adrenoceptor antagonist idazoxan, the  $\alpha_{2A}$ -adrenoceptor subtype selective antagonist BRL 44408 and the  $\alpha_{2B/2C}$ -adrenoceptor subtype selective antagonist ARC-239 was investigated. The inhibitory effect of clonidine (1000 nM), oxymetazoline (1000 nM) and ST-91 (1000 nM) on the EFS-induced contractions in gastric fundus stripe of rat were antagonized by idazoxan (10,000 nM). Similarly, the BRL 44408 (10,000 nM) significantly reversed the inhibitory effect of clonidine (1000 nM), oxymetazoline (1000 nM) and that of ST-91 (1000 nM) which is considered as  $\alpha_{2B/2C}$ -subtype preferring agonist. In contrast, the inhibitory effect of clonidine (1000 nM), oxymetazoline (1000 nM) and ST-91 (1000 nM) was not affected by the  $\alpha_{2B/2C}$  subtype selective antagonist ARC-239 (10,000 nM). The results indicate that  $\alpha_{2A}$ -adrenoceptor subtype may mediate the inhibitory action and  $\alpha_{2B}/\alpha_{2C}$  subtypes is not likely to be involved in the regulation of gastric motor activity of the rat.

#### **4.2.2. In vitro analysis of the role of $\alpha_2$ -adrenoceptor subtypes in the regulation of gastric motility in the NMRI mice**

Similar results were obtained in the NMRI mice. Clonidine, oxymetazoline and ST-91 inhibited the EFS-induced cholinergic contractions in a concentration-dependent manner. They showed approximately the same efficacy; their maximal inhibitory effect ( $E_{\max}$ ) was  $86.6 \pm 1.2 \%$ ,  $78.4 \pm 3.1 \%$  and  $75.7 \pm 2.4 \%$ , respectively. The relative potency was taken 1 for clonidine and the relative potencies for oxymetazoline and ST-91, compared to that of clonidine, were 5 and 0.7 respectively. The inhibitory effect of clonidine (1000 nM), oxymetazoline (100 nM) and ST-91 (1000 nM) against the EFS-induced contractions in gastric fundus stripe of NMRI mice were significantly reversed by idazoxan. BRL 44408 (10,000 nM), reduced the inhibitory effect of the clonidine (1000 nM), oxymetazoline (100 nM) and surprisingly, also that of ST-91 (1000 nM) in a significant manner. This suggests that  $\alpha_{2A}$ -adrenoceptor subtype is involved in the mediation of the effect in mice as well. ARC-239 (10,000 nM) failed to affect the inhibitory effect of clonidine (1000 nM), oxymetazoline (100 nM) and even that of ST-91 (1000 nM) indicating that  $\alpha_{2B/2C}$ -adrenoceptors are not likely to be involved in the mediation of the effect of  $\alpha_2$ -adrenoceptor agonists.

#### **4.2.3. Analysis in the $\alpha_{2A}$ -, $\alpha_{2B}$ - and $\alpha_{2C}$ -adrenoceptor subtype deficient mice**

The next series of experiments were carried out in  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptor subtype deficient mice and in the C57BL/6 wild type mice to confirm the role of  $\alpha_{2A}$ -adrenoceptor subtype in regulation of gastric motility gained by pharmacological analysis. First, the effect of clonidine and ST-91 on EFS-induced contractions of  $\alpha_{2A}$ -,  $\alpha_{2B}$ -,  $\alpha_{2C}$ -subtype deficient mice and C57BL/6 wild type mice were examined. It was found that, both clonidine and ST-91 failed to inhibit the EFS-induced contractions in  $\alpha_{2A}$  subtype deficient mice suggesting the crucial role of  $\alpha_{2A}$  subtype in the inhibitory effect of clonidine and ST91. To determine whether besides  $\alpha_{2A}$ -subtype, the  $\alpha_{2B}$ - and/or  $\alpha_{2C}$ -adrenoceptor subtypes may also contribute to the

inhibitory effect of clonidine and ST-91 on the EFS-induced contractions in  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptor subtype deficient mice and C57BL/6 wild type mice was tested. The results showed that, clonidine and ST-91 inhibited the contractions induced by EFS in the gastric fundus strips of wild type,  $\alpha_{2B}$  and  $\alpha_{2C}$  subtype deficient mice indicating that the deletion of either  $\alpha_{2B}$ -or  $\alpha_{2C}$ -subtypes had no effect on the inhibitory action of either clonidine or ST-91. Consequently, it was first demonstrated that exclusively  $\alpha_{2A}$ -adrenoceptor subtype mediates the regulatory effect of  $\alpha_2$ -adrenoceptor agonists on the gastric motor activity in the mouse.

## **5. Conclusions**

### **5.1. Gastroprotective effect of cannabinoids**

- **Both peripheral and central administration of cannabinoids induce gastric mucosal protection in acid independent, ethanol ulcer model**
- **The gastroprotective effect of cannabinoids is likely to be mediated by activation of central CB<sub>1</sub> receptor**
- **Inhibition of the uptake or degradation of endocannabinoids resulted in gastric mucosal protection, the effect was mediated by CB<sub>1</sub> receptors**
- **Interaction between cannabinoids and opioids system have been demonstrated in gastroprotection and the gastroprotective effect of cannabinoid may be mediated at least partly by release of endogenous opioids**
- **TRPV1 receptors may contribute to the gastroprotection-induced by cannabinoids**
- **NO and PGs are involved in the peripheral mechanisms of the gastroprotective action of cannabinoids**

### **5.2. Determination of $\alpha_2$ -adrenoceptor subtype responsible for inhibition of gastric motor activity**

- **Pharmacological analysis with  $\alpha_2$ -adrenoceptor agonist/antagonist suggested predominant role of  $\alpha_{2A}$ -subtype mediated inhibition of EFS-induced contractions in isolated gastric fundus strips of rat and mice**

- $\alpha_2$ -adrenoceptors agonist exerted inhibitory effect on the EFS evoked contractions in  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptor subtype deficient mice but not in the  $\alpha_{2A}$ -subtype deficient mice
- These results suggest that the  $\alpha_2$ -adrenoceptor agonist induced inhibition of EFS-evoked contractions is mediated purely by the  $\alpha_{2A}$ -subtype

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