DISCUSSION

The major active component of marijuana, Δ^9–THC has been found to have acute bronchodilator and anti-asthmatic activity. Since this observation carried out in the seventies, there have not been any major clinical advances in the area. Only two decades later when the discovery of the CB receptors, their endogenous ligands, and the machinery for endocannabinoid metabolism and additionally the development of synthetic cannabinoid ligands, paved the way for new drug interventions. The number of studies related to the endocannabinoid and respiratory system is small which means that the future of cannabinoids in the airways is uncertain and it is of interest to further investigate their respiratory pharmacology and physiology. In this regard, this study used isolated guinea-pig bronchial preparation and human bronchial epithelial cell line, two different assays with emphasis on targets at tissue, cellular and molecular level.

The results of systems used are discussed separately in two following sections. Firstly, data gained at the tissue level describe pre- and postsynaptic effects of cannabinoids under physiological and pathological conditions. Secondly, data gained at both cellular and molecular level describe the identification of the CB receptors and assess their potential physiological role to induce signal transduction. The last section discusses the implications of all of the results as a whole.

4.1 Isolated guinea-pig bronchial preparation

4.1.1 Could the cannabinoid system be involved in neurogenic inflammation?

In the respiratory system C-fibres releasing sensory neuropeptides induce neurogenic inflammation which might contribute to the pathophysiology of bronchial asthma. The initial objective was to re-evaluate the possible modulation of this process by cannabinoids using EFS in the GPBP. The first evidence of cannabinoid-mediated modulation of neuropeptide release in airways has been reported by Yoshihara et al. (2004). They showed that in guinea-pig bronchi the non-selective CB agonist, WIN55212-2 and the selective CB_2 agonist, JWH133 dose-dependently inhibited EFS-evoked contraction in a CB_2 selective antagonist (SR144528)-sensitive manner whereas the selective CB_1 antagonist, SR141716A had no effect (Yoshihara et al., 2004). Moreover, WIN55212-2 inhibited CPS-induced bronchial smooth muscle contraction, but not the NKA-induced contraction. Both agonists, WIN55212-2 and JWH133 reduced the CPS-induced release of SP-like immunoreactivity from guinea-pig airway tissues. The inhibitory effect of WIN55212-2 on NANC contraction was reduced by Maxi-K^+ channel blockers, iberiotoxin and charybdotoxin, while the Maxi-K^+ channel opener, NS1619, inhibited NANC contractile responses to EFS. These observations suggest that WIN55212-2 inhibits the activation of C-fibres via CB_2 receptors and Maxi-K^+ channels in guinea-pig bronchi (Yoshihara et al., 2004). Consistent with this study, in the present study we have shown that WIN55212-2 at the single concentration of 1 μM inhibited NANC responses in guinea-pig bronchi. The 38 % inhibition by WIN55212-2 was significantly different from the control

NANC contraction. Additionally, its inactive isomer, WIN55212-3 (an important pharmacological tool to investigate whether a response is mediated or not mediated via activation of CB receptors), at the concentration of 1 μM failed to alter EFS contractions. This indicates a cannabinoid receptor-dependent action of WIN55212-2. In order to investigate the CB receptor subtype involved in the action of WIN55212-2 in our preparation, we also used the same CB receptor selective antagonists. In contrast to Yoshihara et al. (2004), the chosen concentration for the antagonists was 10-fold higher (100 nM), concentration which is still selective for the CB_2 versus the CB_1 receptor blockade (Rinaldi-Carmona et al., 1995; Rinaldi-Carmona et al., 1998). The inhibitory effect of WIN55212-2 was significantly reduced by SR144528 but not by SR141716A at 100 nM. On one hand, SR144528 significantly reduced the inhibitory effect of WIN55212-2 (1 μM) to 14 %. On the other hand, SR141716A also reduced the inhibitory effect of WIN55212-2 (1 μM) but the 26 % reduction of the inhibition was not significantly different from the control response induced by WIN55212-2 (1 μM) alone. Although we did not study higher concentration of WIN55212-2 (>1 μM), Yoshihara et al. (2004) showed that the maximum concentration of WIN55212-2 used (19.1 μM), produced 75 % inhibition of control contraction, the EFS inhibition nearly equal to the inhibited contractile response to CPS caused by WIN55212-2 (73 %) at the same concentration (19.1 μM). In contrast, WIN55212-2 did not influence NKA-induced guinea-pig bronchial smooth muscle contraction, excluding its postsynaptic action in this preparation (Yoshihara et al., 2004). Indeed, WIN55212-2 at the concentration of 1 μM did not contract lung parenchymal strips of the guinea-pig (Andersson et al., 2002). No postjunctional role for WIN55212-2 has also been confirmed in isolated guinea-pig trachea by Nieri et al. (2003). They demonstrated that WIN55212-2 did not change either the tracheal

smooth muscle tone under basal conditions or the histamine-induced contraction. In addition, the relaxant activity of the NO donor, sodium nitroprusside (on histamine-contracted tissue) was not influenced by WIN55212-2 (10 μM) (Nieri et al., 2003). Taken together, our data in the present study might suggest that in guinea-pig bronchi WIN55212-2 exerts its inhibitory effect on sensory nerves through presynaptic CB_2-like receptors. To the best of our knowledge, our results show for the first time a CB_2-like receptor activity of this synthetic cannabinoid agonist, WIN55212-2. The reason for this assumption is the non-significant effect of the CB_1 antagonist, SR141716A on EFS response to WIN55212-2. If the action of WIN55212-2 is purely CB_2 receptor-mediated, the 26 % reduced inhibition by SR141716A presumably would not occur in our GPBP.

However, a CB_2-like receptor response was suggested in the isolated nerve-smooth muscle preparation of the mouse vas deferens which was used to examine the effect of JWH015 and JWH051 (Griffin et al., 1997). These two compounds with higher affinities for CB_2 than CB_1 receptors were potent inhibitors of electrically evoked contractions of the mouse vas deferens which were not attenuated by CB_1 receptor specific concentrations (submicromolar) of SR141716A or AM630. In contrast, SR141716A produced a parallel rightward shift of concentration-response curves for both agonists, behaving as a competitive surmountable antagonist in the preparation of guinea-pig myenteric plexus longitudinal smooth muscle which was studied in parallel (Griffin et al., 1997). PCR analysis revealed a mouse vas deferens CB_2 mRNA variant which showed >95 % homology to that previously published for the mouse CB_2 receptor and >88 % homology with the published human sequence (Griffin et al., 1997). The so-called CB_2–like receptor mRNA could explain the significant discrepancy between the CB_2 receptor binding affinities of JWH015 and

JWH051 and the EC_{50} values of these ligands for inhibition of the twitch response in the mouse vas deferens preparation. However, the EC_{50} values of JWH015 and JWH051 for inhibition of the twitch response of the myenteric plexus were of the same order as their CB_1 receptor dissociation constants, indicating CB_1-mediated inhibition of electrically evoked contractions in the longitudinal smooth muscle preparation. Data suggested the expression of CB_2-like receptors in the mouse vas deferens which could mediate the inhibition of EFS-evoked contractions (Griffin et al., 1997).

In order to further clarify the possible involvement of the cannabinoid system in neurogenic inflammation, the role of the endogenous cannabinoids, AEA and PEA was investigated using the same experimental model as Yoshihara et al. (2005). They demonstrated that the mechanism of action of these endocannabinoids was similar to the action of synthetic cannabinoids seen in the previous study of Yoshihara et al. (2004) which included the activation of CB_2 receptors and opening of Maxi-K^+ channels. Additionally the CPS-induced SP-like immunoreactivity was significantly reduced by both endocannabinoids indicating the inhibition of neuropeptide release from C-fibre endings in guinea-pig airways (Yoshihara et al., 2005). These findings also support the idea that CB_2 receptors might play a role in cannabinoid-induced inhibition of sensory nerve function in guinea-pig bronchi. We could not corroborate this observation because under our conditions AEA (3 μM) evoked an inhibition of the response to EFS of 15 % which was not statistically significant. Similarly, a non-significant inhibition of 22 % was obtained to PEA. These results with AEA and PEA on EFS-induced NANC contractions were different from the published observation where AEA (2.88 μM) produced inhibition of 61 % and PEA (the maximal concentration of 3.3 μM) 98 % respectively. For this reason the possible involvement

of CB_2 receptors using these endocannabinoids has not been tested in the present study. The differences are difficult to interpret despite the subtle differences in methodology.

Yoshihara's results (Yoshihara et al., 2004) are in line with recent data on the effects of CB receptor agonists, WIN55212-2 and JWH133 on sensory nerves in the airways (Cui et al., 2007). In this *in vivo* study the neurogenic inflammation was manifested by HCl-induced bronchoconstriction (monitored as an increase in the airway resistance) and plasma extravasation (Evans blue reaction) in the trachea and main bronchi of guinea-pigs which was prevented by both agonists. WIN55212-2 and JWH133 dose-dependently (0.3-3 mg/ kg) inhibited both phenomena. While maximal inhibition of the HCl-induced increase in lung resistance produced by WIN55212-2 was at the dose of 1 mg/ kg, maximal inhibition of the HCl-induced microvascular leakage by both cannabinoids was reached at 3 mg/ kg. The protective effects of WIN55212-2 and JWH133 tested at 1 mg/ kg were abolished by the SR144528 (1 mg/ kg) but not the SR141716A (1 mg/ kg) treatment (Cui et al., 2007). Not surprisingly, WIN55212-2 (1 mg/ kg) was not able to inhibit tachykinin-induced effects at postsynaptic level because SP-induced bronchoconstriction and microvascular leakage were not altered in the presence of this CB agonist (Cui et al., 2007). Under our experimental conditions, cannabinoids play a role in neurogenic inflammation, a conclusion in line with the results of Yoshihara et al. (2004, 2005) and Cui et al. (2007).

However, the subtype of the CB receptor involved in the control of pulmonary neural pathway still remains a debated question. All experiments carried out by Cui et al. (2007) and Yoshihara et al. (2004, 2005) suggest that CB_2 receptors decrease the activation of CPS-sensitive afferent sensory nerves (C-fibres) in airways. In addition,

these vagal sensory nerves (i.e. afferent fibers) expressing $TRPV_1$ receptors are involved in cough associated with asthma. Apart from C-fibres with bronchial and pulmonary endings, the cough reflex is also under the control of RARs (Chung, 2005; Barnes, 2007). In support of the hypothesis about the involvement of CB_2 receptors in the inhibition of sensory nerve activation, and the CB_2 receptor-mediated cough reduction, there are two studies using an *in vitro* model of sensory nerve activation by nerve depolarization and an *in vivo* model of cough (Patel et al., 2003; Belvisi et al., 2008). In the guinea-pig vagus preparation, JWH133 dose-dependently (0.3-100 μM) inhibited sensory nerve depolarisation induced by hypertonic saline, CPS or PGE_2, and in the human vagus nerve, the same CB_2 agonist (10 μM) inhibited CPS-induced nerve depolarisation. These effects of JWH133 were abolished by the CB_2 antagonist SR144528 (10 nM) but not by the CB_1 antagonist, SR141716A at the same concentration of 10 nM (Patel et al., 2003). In addition, using a guinea-pig *in vivo* model of cough, JWH133 (10 mg/ kg) markedly reduced (by >50 %) the citric acid (0.3 M)-induced cough compared to vehicle control guinea-pigs (Patel et al., 2003). Only recently a similar study has been performed in the same laboratory (Belvisi et al., 2008) to confirm their previous finding (Patel et al., 2003) and rebut the finding of Calignano et al. (2000). The controversy was related to the potential role of the CB_2 versus the CB_1 receptor in the antitussive effects of cannabinoids. On one hand, Calignano et al. (2000) showed that in guinea-pigs, aerosolized AEA at a higher concentration (10 mg/ ml) suppressed cough induced by inhaled CPS (0.3 mM), an effect antagonized by SR141716A (0.5 mg/ kg) but not by SR144528 (0.3 mg/ kg), indicating CB_1 receptor-mediated antitussive action including sedation as an adverse effect. On the other hand, Belvisi et al. (2008) used the selective CB_2 agonist GW833972A which appeared to have >1000-fold selectivity for the CB_2 over the CB_1

receptor. Similarly to JWH133, GW833972A also inhibited CPS-induced guinea-pig and human vagal sensory nerve activation *in vitro*. Surprisingly, the more selective GW833972A was less potent (EC_{50}=33 μM) than JWH133 (EC_{50}=3 μM) in the inhibition of CPS-induced depolarization of the guinea-pig vagus. The effects of hypertonic saline, CPS or PGE_2, in the presence of GW833972A (10 μM) were also abrogated by SR144528 treatment (10 nM) and not affected by SR141716A (10 nM) (Belvisi et al., 2008). Encouragingly, the citric acid-induced cough (0.3 M) was also reduced by GW833972A (30 mk/ kg) by 88 %. In addition, this recent study validated the CB_2 receptor-mediated antitussive action by using the CB receptor antagonists. SR144528 (10 mg/ kg) but not SR141716A (10 mg/ kg) blocked the antitussive activity of GW833972A (Belvisi et al., 2008).

As mentioned above, the $TRPV_1$ receptors of sensory nerves partly contribute to the cough reflex (Chung, 2005; Barnes, 2007). In this regard, it has been demonstrated that AEA given by aerosol concentration-dependently (0.3-3 mg/ ml) increased the cough reflex via activation of $TRPV_1$ receptors (using capsazepine 0.3 mM as an antagonist of this receptor) in guinea-pigs (Jia et al., 2002) and also in mice at a high concentration (3 mg/ ml) (Kamei et al., 2006). In guinea-pigs, SR141716A (0.5 mg/ kg) and SR144528 (0.3 mg/ kg) had no effect on AEA-induced cough (0.3-3 mg/ ml) and there was no effect of inhaled AEA (3 mg/ ml) on CPS-induced cough (0.3 mM) (Jia et al., 2002). These findings do not agree with the results reported by Calignano et al. (2000). However, there is a consistency with data suggesting that AEA at a higher concentration (10 μM) can activate $TRPV_1$ receptors (capsazepine 10 μM) excluding activation of CB_1 (SR141716A 1 μM) or CB_2 receptors (SR144528 1 μM) by its direct ability to depolarize isolated guinea-pig vagus nerve preparations (Kagaya et al., 2002). The contrasting data from these studies may be due to the different

experimental conditions employed (e.g. the duration of AEA treatment or the size of aerosol particles), and in particular the doses of AEA used. Noteworthy is the differential distribution of these receptors in the airways. While CB_1 receptors in the rat lung were detected on axon terminals of sympathetic nerves in close proximity to bronchial smooth muscle cells and additionally co-localized with NPY (a co-transmitter in sympathetic neurons) (Calignano et al., 2000), $TRPV_1$ receptors are known to be expressed mainly on sensory nerves (De Petrocellis et al., 2001). There is immunohistochemical evidence of $TRPV_1$ positive neurons expressing SP but also $TRPV_1$ positive neurons without SP immunoreactivity suggesting that the distribution of $TRPV_1$ receptors can be also independent of sensory neuropeptides in the guinea-pig respiratory system (Kagaya et al., 2002; Watanabe, 2005). Thus, it is unlikely that AEA directly activates CB_1 receptors mediating its antitussive action and it seems more likely that the tussigenic effect of AEA is mediated through the activation of $TRPV_1$ receptors in guinea-pigs. Although, the expression of CB_2 receptors has not, to date, been directly detected in the airways, the studies carried out by Patel et al. (2003) and Belvisi et al. (2008) confirm the potential of the CB_2 receptor as a target for pathologies involving enhanced sensory nerve function (e.g. asthma, cough, COPD), without the unwanted sedative effects of the CB_1 receptor activation.

The next chapter discusses the data which have been gained under the studies of the functional pharmacology and mechanisms of actions of two endocannabinoids, AEA and VIR.

4.1.2 The effect of AEA and VIR on isolated guinea-pig bronchi

Tucker et al. (2001) demonstrated very clearly that the mechanism of AEA action in guinea-pig isolated main bronchi includes the activation of excitatory $TRPV_1$ receptors (excluding CB_1 receptors) on sensory nerve endings which in turn leads to the release of tachykinins (SP and NKA) and bronchoconstriction. In agreement with previous studies (Craib et al., 2001; De Petrocellis et al., 2001; Tucker et al., 2001; Andersson et al., 2002), we found that AEA is a weak bronchoconstrictor in comparison to CPS. This powerful C-fibre stimulant activating $TRPV_1$ receptors appeared to be equally potent (EC_{50}=10 nM) in both tissues of the guinea-pig bronchus (causing bronchoconstriction) and mesenteric artery [causing vasorelaxation of the pre-contracted tissue by U46619, a derivative of $PGF_{2\alpha}$ (Coleman et al., 1981; Morinelli et al., 1987; Miggin and Kinsella, 2002)] (Andersson et al., 2002). In contrast, AEA also as a $TRPV_1$ receptor active drug behaved differently in various in vitro bioassay systems. In HEK293 cells transfected with the $hTRPV_1$ receptor (but not in parental cells), AEA at the concentration of 10 μM (CPS at 1 μM) produced capsazepine (1 μM)-sensitive inward currents (Smart et al., 2000). In guinea-pig mesenteric arteries, AEA evoked concentration-dependent and complete relaxation (0.3-3 μM) which was inhibited by capsazepine (300 nM) (Andersson et al., 2002). Moreover, the potent CB agonists, HU210 and WIN55212-2, both at 1 μM had no effect on the U46619-evoked vasoconstriction (Andersson et al., 2002). These data obtained with AEA indicating full agonism at the $TRPV_1$ receptor, are not consistent with our data obtained in isolated GPBP expressing the $TRPV_1$ receptor (Kagaya et al., 2002; Watanabe et al., 2005). The possible explanation for this discrepancy could

be in the $TRPV_1$ receptor heterogeneity between the systems under study (Andersson et al., 2002; Watanabe et al., 2005).

However, our interest was more centred on the functional pharmacology of VIR, an endocannabinoid ligand structurally similar to AEA which at higher concentrations (30 μM and 100 μM) showed some effect on the cell membrane of 16HBE cells in our patch clamp study (Dudášová, unpublished observation). In the EFS study using the GPBP, VIR at the concentration of 1 and 10 μM failed to exhibit any effect, indicating no prejunctional role in the inhibition of sensory nerve function. This observation partly corresponds to the negative effect of AEA (3 μM) on eNANC responses in our hands but contradicts to Yoshihara's finding (Yoshihara et al., 2005) which was demonstrated by the ability of AEA to inhibit EFS contraction in a concentration-dependent manner (0.0288-28.8 μM) in isolated guinea-pig bronchi. The discrepancies related to AEA are difficult to explain because we tried to establish the same technical conditions as described by Yoshihara et al. (2004, 2005). Nevertheless, VIR showed a postjunctional action and we are the first to demonstrate its mechanism of action in this preparation. Similarly to AEA, VIR exhibited an excitatory ability to activate $TRPV_1$ receptors on tachykinin releasing sensory nerve endings. Its dose-dependent (1-100 μM) contraction of guinea-pig isolated bronchi was slightly higher than the contraction to AEA at the same concentrations. The concentrations of antagonists employed in the present study were equivalent to the concentrations used by Tucker et al. (2001). The $TRPV_1$ antagonist, capsazepine significantly attenuated the contractions induced by VIR leaving a small component resistant to capsazepine pretreatment. The NK_1 receptor antagonist, SR140333B failed to significantly inhibit VIR evoked responses. In contrast, the NK_2 receptor antagonist, SR48968C significantly reduced the contractile responses to VIR but did

not completely abolish it. However, although Tucker et al. (2001) reported that the excitatory action of AEA is not mediated via CB_1 receptors, the selective CB_1 or CB_2 antagonists have not been tested against the VIR-induced bronchoconstriction in our conditions. Evidence for the $TRPV_1$ activation by VIR is also supported by our finding that VIR is inactive in $TRPV_1$ receptor desensitized tissues pretreated with CPS. Similarly to AEA (Craib et al., 2001; Tucker et al., 2001), neuropeptide release to exogenously applied VIR was potentiated in the presence of the NEP inhibitor, phosphoramidon (10 μM). Although Tucker et al. (2001) used another NEP inhibitor (tiorphan), at the same concentration as phosphoramidon (10 μM), and this augmented the contractile response to AEA, confirming neuropeptide release from sensory nerve endings (Tucker et al., 2001). The non-specific action of phosphoramidon on the smooth muscle contraction (Chiba and Misawa, 1995) was ruled out because this NEP inhibitor failed to increase the CCh-induced bronchoconstriction. In addition, we confirmed the specificity of the selective NK_2 antagonist by demonstrating the failure of SR48968C to affect the CCh-induced bronchoconstriction. We can conclude that the mechanism of action of VIR in isolated guinea-pig bronchi corresponds to the effect of AEA in sensory nerves which was first published by Tucker et al. (2001). VIR produced dose-dependent bronchoconstriction by stimulating the $TRPV_1$ receptor, followed by release of neuropeptides that can activate NK_2 receptors in the GPBP. In the present bioassay studying bronchial contractility, the rank order of potency was CPS>AEA>VIR.

These mechanisms of actions of AEA and VIR studies were followed by a study assessing their possible interaction with the proteins that inactivate the endogenous cannabinoids which is discussed in the following two chapters 4.1.3 and 4.1.4.

4.1.3 Is FAAH constitutively active in isolated guinea-pig bronchi?

The biological effects of AEA can be terminated by either FAAH or the putative AMT, and alternative catabolic routes employing enzymes such as COX or lipoxygenase. There is pharmacological evidence that the AMT is not present in the guinea-pig bronchi because its inhibitor, VDM13 did not affect the constrictor response to AEA, underlining the possible importance of rapid enzymatic degradation via FAAH (Andersson et al., 2002). The inactivation of endocannabinoid signalling by FAAH is questionable in the isolated guinea-pig bronchi. While Tucker et al. (2001) and Andersson et al. (2002) reported no enhancement of AEA-induced bronchoconstriction in the presence of the FAAH inhibitor, PMSF (at the concentration of 100 μM and 50 μM, respectively), Craib et al. (2001) showed that PMSF at 20 μM significantly increased the bronchoconstriction to AEA implicating its active metabolism via FAAH in this guinea-pig tissue (Craib et al., 2001). Under our experimental conditions, the contractile responses to AEA were not significantly altered in the presence of PMSF at 20 μM, a finding in line with the results of Tucker et al. (2001) and Andersson et al. (2002). Taken together, rapid enzymatic degradation or metabolism via FAAH or COX can not explain the weak effect of AEA in guinea-pig main bronchi because PMSF (20 μM) failed to significantly increase the AEA response and the COX inhibitor, indomethacin (10 μM) was present in all experiments. By contrast, in isolated lung parenchymal strips of guinea-pigs, Andersson et al. (2002) demonstrated that PMSF at a higher concentration (100 μM) strongly inhibited and indomethacin at 10 μM prevented the contractile response to a single concentration of AEA (100 μM). They concluded that there was hydrolysis of AEA via FAAH to arachidonic acid and subsequent COX-dependent formation of

contractile eicosanoids. Indeed, they found that arachidonic acid-induced contractions were inhibited by indomethacin (Andersson et al., 2002). However, indomethacin may direct arachidonic acid to pass through the lipoxygenase pathway, and there is a possibility that the high concentration of AEA required to activate $TRPV_1$ receptors might be a consequence of the metabolism of AEA by lipoxygenase or the generation of lipoxygenase products. Therefore, the lipoxygenase metabolites might contribute to the AEA-induced bronchoconstriction in the GPBP. In support of this hypothesis, Craib et al. (2001) tested the influence of non-specific lipoxygenase inhibitors, ETYA and ETI on AEA-evoked responses. ETYA and ETI markedly attenuated the contractile action of AEA but had no effect on responses to SP. Nonetheless, non-specific effects of ETYA and ETI were not excluded because their effects were not examined on the contractile action of NKA in the GPBP (Craib et al., 2001). This hypothesis originated from a report published by Hwang et al. (2000) who showed that products of lipoxygenase such as hydroperoxyderivatives of arachidonic acid and LTs directly activate the $TRPV_1$ receptor in a capsazepine-sensitive manner, whereas, prostaglandins such as PGE_2, PGD_2, PGI_2 had no channel activity in sensory neurons from rat dorsal root ganglia or in transfected HEK293 cells. PGE_2 as a major COX metabolite producing contraction via activation of prostanoid EP_1 receptors and EP_2 receptor-mediated relaxation of histamine-induced contraction of the guinea-pig trachea (Ross et al., 2002), might be involved in the AEA-induced bronchoconstriction through an unknown mechanism for two reasons. First, in the absence of indomethacin, AEA caused relaxation of guinea-pig bronchi. Second, the EP_1 antagonist, SC-51089 failed to affect the contractile action of AEA in the GPBP (Craib et al., 2001). This may be indicative of the conversion of AEA to active COX metabolites in the GPBP.

To further clarify the possible active metabolism of AEA via FAAH in the GPBP, we decided to undertake an investigation with the more specific and the more potent FAAH inhibitor, URB597 (Tarzia et al., 2003; Fegley et al., 2005). Despite its selectivity, URB597 has been shown to act differently in different tissues. While in rat isolated small mesenteric arteries, URB597 potentiated the relaxation evoked by AEA (Ho and Randall, 2007), in rat aorta it caused reduction of the AEA vasorelaxation (Herradón et al., 2007). Also, in rat isolated urinary bladder, the same compound at the same concentration attenuated the AEA-induced contraction of the muscle strips (Saitoh et al., 2007). This might suggest that mechanisms by which AEA induces biological effects could differ between tissues and/or animal species. As previously mentioned, we found that the contractile response to AEA was not significantly altered in the presence of the non-specific serine hydrolase inhibitor, PMSF. In contrast, the more selective amidase inhibitor, URB597 evoked significant inhibition of the response to AEA, indicating that FAAH has an impact on AEA metabolism in the GPBP. All our experiments were carried out in the presence of indomethacin to block the production of prostaglandins such as PGE_2 which is able to contract guinea-pig bronchi and trachea through EP_1 receptors (Craib et al., 2001; Ross et al., 2002). In guinea-pig bronchi the possible contractile action of AEA on EP_1 receptors was excluded (Craib et al., 2001) which is in opposition to the data found in the rat urinary bladder by Saitoh et al. (2007). While the EP_1 antagonist, ONO8130 produced a significant parallel rightward shift of the concentration-response to AEA, URB597 markedly inhibited the response in a non-competitive manner and indomethacin nearly abolished it. The authors concluded that AEA might be degraded by FAAH to arachidonic acid which can be a substrate for COX to yield prostaglandins acting on EP_1 receptors and partly mediating the AEA-induced contraction in the rat bladder

(Saitoh et al., 2007). None of the authors (Herradón et al., 2007; Ho and Randall, 2007; Saitoh et al., 2007) evaluated the potential activation of CB_1/CB_2 receptors as a mechanism underlying the effects of FAAH inhibition. Although URB597 is claimed to have no affinity for CB receptors (Lambert and Fowler, 2005), we showed for the first time that the suppression of AEA-induced bronchoconstriction by URB597 was partially reversed by pretreatment with the CB_2 antagonist, SR144528, whilst the CB_1 antagonist, SR141716A had no effect. This suggests a CB_2 mechanism of action in the FAAH inhibition in the GPBP. In conclusion, we can conclude that the inhibition of FAAH has complex effects. While PMSF did not cause enhancement of the responses to AEA, the action of the more selective URB597 might be physiologically relevant involving CB_2 receptors in isolated guinea-pig bronchi. FAAH as a metabolic enzyme of AEA signalling might not be important but can not be excluded in the GPBP. Further work applying URB597 on its own to EFS-evoked responses could unveil whether there is an endocannabinoid tone and possible modulation of NANC contraction in the GPBP.

4.1.4 Are VIR and its vehicle absolute ethanol metabolized in isolated guinea-pig bronchi?

Structurally, VIR is an arachidonic acid and ethanolamine joined by an esther linkage which can easily undergo enzymatic degradation. To reduce the possible hydrolysis of VIR, we pretreated the tissue with PMSF which also possesses anti-esterase activity. This amidase inhibitor did not produce any change in VIR-evoked responses, the result being in line with AEA, but contradictory to a finding in human neocortical synaptosomes by Steffens et al. (2005). In their study, the presence of PMSF has changed the VIR-induced inhibition of [^{3}H]-CP55940 binding. A ~50 % reduction in the binding affinity of VIR after PMSF treatment indicates the possible degradation of this endocannabinoid via FAAH in human brain (Steffens et al., 2005). This finding is not surprising because of the high FAAH level in brain and also the existence of VIR in human hippocampus (Porter et al., 2002). Although we excluded the active metabolism of VIR (using PMSF only) through FAAH in our preparation, the conversion of VIR to active metabolites was taken into consideration for two reasons. First, the onset of the $TRPV_1$-and NK_2-mediated action of VIR was markedly slow (>10 min). This might indicate a possible role of lipoxygenase metabolites in the bronchomotor effect of VIR. Second, the omission of indomethacin from the Krebs solution caused a significantly lower concentration-related effect to exogenously applied VIR. The explanation might be due to the generation of inhibitory PGE_2 which can modulate the effects of exogenous and endogenous tachykinins in the airways (Frossard et al., 1989, Johansson-Rydberg et al., 1992).

Interestingly, the vehicle of VIR, absolute ethanol (1.1 %), slightly relaxed the bronchial tissue in the absence of indomethacin but produced a small contraction

when indomethacin was present in the Krebs solution. Indeed, ethanol (0.1-3 %) has been found to stimulate native and recombinant $TRPV_1$–mediated responses in rat primary neurons isolated from trigeminal and dorsal root ganglia and also in transfected HEK293 cells (Trevisani et al., 2002). These $TRPV_1$–dependent effects were capsazepine sensitive (10 μM). Electrophysiological whole cell recordings revealed the potentiating effect of ethanol (0.3-3 %) in $hTRPV_1$-expressing HEK293 cells. At the concentration of 3 % ethanol enhanced the AEA (1 μM)- and CPS (500 nM)-induced inward currents. The ethanol- and CPS-induced current responses were antagonized by the competitive $TRPV_1$ antagonist, capsazepine (10 μM) and also by the non-competitive $TRPV_1$ antagonist, ruthenium red (10 μM). In addition reversal potentials for currents elicited by CPS alone or CPS and ethanol were close to 0 mV which is a characteristic feature of the $TRPV_1$-mediated conductance (Trevisani et al., 2002). In light of this, the action of VIR and AEA might be potentiated by their vehicle, ethanol in isolated guinea-pig bronchi. This proposition is consistent with the observation by Trevisani et al. (2004) who showed that ethanol (0.3-3 %) contracted isolated guinea-pig bronchi. The difference between our and Trevisani's studies (Trevisani et al., 2004) lies in the mediation of ethanol-induced bronchoconstriction. While in our hands the contractile effect of ethanol at 1.1 % was not abolished by capsazepine pretreatment (10 μM), Trevisani et al. (2004) demonstrated $TRPV_1$-dependent ethanol-induced bronchoconstriction. Also they showed that capsazepine was able to markedly inhibit (10 μM) and significantly reduce (10 μM/ kg and 0.1 mM, respectively) two major *in vivo* proinflammatory responses, such as bronchoconstriction and plasma extravasation, produced by intravenous and intragastric administration of ethanol and CPS in anesthetized guinea-pigs. In contrast to ethanol and CPS, SP-evoked bronchoconstriction and plasma extravasation was not

altered by capsazepine pretreatment, clearly indicating the $TRPV_1$-dependent mechanism of ethanol action in guinea-pig airways (Trevisani et al., 2004). There is a hypothesis that the oxidative metabolite of ethanol, acetaldehyde, inducing histamine release from mast cells, is responsible for alcohol-induced asthma (Fujimura et al., 1999; Kawano et al., 2004, Matsuse et al., 2007). To test this assumption, the bronchial responsiveness to inhaled acetaldehyde and methacholine was compared in alcohol-sensitive asthmatics and nonalcohol-sensitive asthmatics. The former subjects showed selective hyper-responsiveness to acetaldehyde (Fujimura et al., 1999). Indeed, the direct effect of acetaldehyde on mast cells to release histamine was also confirmed *in vitro* in isolated human bronchi (Matsuse et al., 2007). Acetaldehyde also contracted isolated guinea-pig bronchi but its action was about three times more potent than the bronchocontractile effect of ethanol and was not sensitive to CPS pretreatment, to $TRPV_1$ receptor antagonism or to tachykinin receptor blockade, suggesting that the neurogenic inflammatory action of ethanol was distinct from that of acetaldehyde (Trevisani et al., 2004). This mechanism might be involved in the ethanol-induced asthma of susceptible individuals. These individuals include approximately half of the Japanese population who have difficulties in metabolizing alcohol because of genetic reduction of acetaldehyde dehydrogenase activity (Myou et al., 1996; Saito et al., 2001; Sisson, 2007). These findings raise a question about the residual component of VIR-evoked bronchoconstriction in the presence of capsazepine because the contractile effect of VIR vehicle, absolute ethanol at 1.1 % was not abolished by capsazepine pretreatment. Thus, could ethanol be locally metabolized to acetaldehyde in guinea-pig airways? However, it has been shown that in the canine respiratory tract only the trachea revealed alcohol dehydrogenase activity which was low in comparison to the liver parenchyma (Maier et al., 1999).

Cannabinoids mentioned in this discussion have so far been shown to evoke actions which were mediated by three specific receptor targets: the CB_1, the CB_2 and the $TRPV_1$ receptor. The next chapter studies the pharmacology of the putative CB receptor GPR55 in the GPBP.

4.1.5 Is GPR55 present and activated in isolated guinea-pig bronchi?

It is well known that not all the biological effects of cannabinoids are mediated through the established CB_1 and CB_2 receptors. Growing evidence suggests the orphan receptor GPR55 for the candidacy of an additional CB receptor (Ryberg et al., 2007). The pharmacological research in cannabinoid activity at GPR55 was initiated by two pharmaceutical companies, GlaxoSmithKline and AstraZeneca. Primary investigation by both companies focused on ligand fishing which revealed that GPR55 is activated by a range of plant, synthetic and endogenous cannabinoids and blocked by CBD. In GTPγS assays presented by Dr. Peter Greasly (AstraZeneca) at the Oxford Meeting of the British Pharmacological Society in December 2006, VIR appeared to have the greatest intrinsic activity among other endocannabinoid ligands (AEA, 2-AG and noladin ether). In addition, the phytocannabinoid with negligible affinity for CB receptors, CBD, was able to antagonize the agonist effect of CP55940 (IC_{50}= 445 nM) in HEK293 cells transfected with human GPR55. In contrast, GPR55-expressing yeast cells used by GlaxoSmithKline to identify endogenous and other ligands for orphan GPCRs, failed to display GPR55 activity to VIR and the activity of CBD was not measured (Brown, 2007). The expression profile of GPR55 was investigated in humans and rodents. Highest mRNA levels were detected in parts of the brain and gastrointestinal tract, and in spleen. In mouse lungs the expression level

of GPR55 mRNA was low (Drmota et al., 2004; Brown, 2007; Ryberg et al., 2007). Although the distribution of GPR55 mRNA has not been reported in the guinea-pig lung, we studied its possible functional expression in our system of isolated guinea-pig bronchi. We first showed that VIR evokes bronchoconstriction in a $TRPV_1$ receptor-sensitive manner. Based on our findings and the lack of information about VIR and the orphan receptor we put forward a hypothesis. What if VIR can also activate the putative GPR55 in guinea-pig bronchi? The next step which we attempted to do was to treat the guinea-pig tissue with CBD. In a GTPγS assay, the concentration range used to test the antagonist activity of CBD at GPR55, was from 1 nM to 30 μM with K_i=440 nM, parameters established in HEK293 cells (Greasly, personal communication). In our study the chosen concentration of 1 μM had no effect on the baseline tone, but the same concentration of CBD significantly attenuated the cumulative response to VIR in the GPBP. Noteworthy is the similar pattern of the residual contractile response to VIR in the presence of CBD on one hand, and the contractile response to ethanol, on the other hand. This might suggest that CBD was able to block the action of VIR completely and the residual response was due to the vehicle. Furthermore, three facts led us to investigate the possible interaction of CBD and CPS at the same $TRPV_1$ receptor in the GPBP. First, the elevation in $[Ca^{2+}]_i$ via activation of $TRPV_1$ receptors (over-expressed in HEK293 cells) caused by CBD (Bisogno et al., 2001). Second, the $TRPV_1$ receptor-mediated antihyperalgesic and anti-inflammatory effect of CBD in rat models of pain and inflammation (Costa et al., 2004, Costa et al., 2007). Third, the natural expression of the $TRPV_1$ receptor in guinea-pig airways (Kagaya et al., 2002; Watanabe et al., 2005). We found no differences between two concentration-response curves obtained with CPS only and in combination with CBD. The efficacy and potency of both

responses were very similar, indicating no action of CBD at a single concentration of 1 μM on $TRPV_1$ receptors in isolated guinea-pig bronchi. This conclusion is strengthened by the full although weak agonism of CBD at $TRPV_1$ receptors over-expressed in HEK293 cells (Bisogno et al., 2001). In their $[Ca^{2+}]_i$ assay CPS was a potent stimulant of the cytosolic Ca^{2+} concentration, whilst CBD showed >100 fold lower potency but almost identical efficacy compared to CPS. In addition, capsazepine abolished the effect of CBD and also in the binding assay of the same cells, CBD was able to displace the potent $TRPV_1$ agonist, [^{3}H]-resiniferatoxin (with K_i values similar to the EC_{50} values for the effect on $[Ca^{2+}]_i$), confirming the action of CBD at the $TRPV_1$ receptor (Bisogno et al., 2001). It is important to bear in mind the difference between the model assays and results obtained with artificial systems expressing higher level of receptors should be interpreted with caution. Our GPBP might contain a lower number of $TRPV_1$ receptors and the 1 μM concentration of CBD chosen by us might have not been sufficient to activate the $TRPV_1$ receptor or even displace CPS from the $TRPV_1$ receptor. It would be interesting to find out whether a 10 or 100 fold higher concentration of CBD would produce any difference either on the baseline tone or on the CPS-induced bronchoconstriction. However, the 1 μM concentration of CBD was effective in significantly reducing the VIR-induced bronchoconstriction, indicating a different mechanism of CBD action against CPS and VIR. Returning to the idea of whether GPR55 is present and activated in isolated guinea-pig bronchi, we were not able to unequivocally test this, and the possible function of GPR55 in isolated guinea-pig bronchi is still not clear. The detection of GPR55 expression in guinea-pig lung using quantitative PCR might give a qualitative answer but not to function.

This raises the question of what now CBD is acting in isolated guinea-pig bronchi? This non-psychoactive marijuana constituent with low affinity to CB_1/CB_2 receptors, has shown antagonistic properties in different assays. At the concentration of 10 μM, CBD antagonized CP55940-induced stimulation of [^{35}S]-GTPγS binding to rat cerebellar membranes (Petitet et al., 1998). In the low nanomolar range (K_B= 34 and 120.3 nM, respectively), CBD antagonized CP55940- and WIN55212-2-induced inhibition of EFS contractions, independently of CB_1 receptors in isolated mouse vas deferens (Pertwee et al., 2002). Also, in Pertwee's laboratory it has been demonstrated that CBD at the concentration of 1 μM behaves as an antagonist of CP55940- and WIN55212-2-induced stimulation of [^{35}S]-GTPγS binding to mouse brain membranes. The CB_1 antagonist/inverse agonist, SR141716 showed similar ability to produce a rightward shift in the log concentration-response curve of CP55940. In addition, CBD on its own at the same concentration (1 μM) reduced [^{35}S]-GTPγS binding to mouse brain membranes. CBD-induced inhibition of [^{35}S]-GTPγS binding was also detected in CHO cells transfected with human CB_1 receptors, indicating inverse agonism of CBD at CB_1 receptors (importantly, this inhibition was not observed in untransfected CHO cells). No such inhibition was found in CB_1 knockout mouse brain membranes, suggesting that this inverse effect might have a CB_1 receptor-independent component (Thomas et al., 2007). In experiments with CHO cell membranes transfected with human CB_2 receptors, CBD also antagonized CP55940 induced stimulation of [^{35}S]-GTPγS binding. The CB_2 antagonist/inverse agonist, SR144528 again resembled CBD in antagonizing CP55940-induced inhibition of [^{35}S]-GTPγS binding. Additionally, CBD and SR144528 by themselves inhibited [^{35}S]-GTPγS binding to CHO-hCB_2 cell membranes. In contrast, CP55940, CBD or SR144528 did not modulate [^{35}S]-GTPγS binding in untransfected cells. This might

suggest inverse agonism of CBD at CB_2 receptors (Pertwee, 2007a; Thomas et al., 2007). Thomas' report provided the first evidence of inverse agonism of CBD at CB_1 and CB_2 receptors and could pave the way to novel studies. It would be essential to target systems naturally expressing CB_2 receptors and to examine whether CBD can behave as a CB_2 receptor inverse agonist. This strategy might be beneficial in reducing the signs of inflammation. Clearly, additional research is needed to establish whether CBD could contribute to its potential anti-inflammatory effects in this regard and whether there is a functional association between CBD and GPR55.

However, CBD seems to be a cannabinoid ligand with multiple mechanisms of actions in the GPBP. The next chapter is a follow up of findings discussed in chapters 4.1.3 and 4.1.5.

4.1.6 Might CBD and URB597 act through the same mechanism in AEA-induced bronchoconstriction of isolated guinea-pig bronchi?

We decided to test the hypothesis that AEA and VIR as endocannabinoids with similar structures might behave similarly in the functional assay in the presence of CBD. We showed that CBD was able to antagonize the response to VIR in a non-competitive manner. The antagonistic activity of CBD was confirmed in isolated guinea-pig bronchi because CBD also produced significant attenuation of AEA responses in a non-competitive manner. Importantly, some of the pharmacological actions of CBD might be due to modulation of the endocannabinoid system by its ability to regulate the activity of FAAH (Watanabe et al., 1996; Massi et al., 2007; De Filippis et al., 2008b). It has been shown that CBD enhances the level of AEA by reducing its degradation and inhibiting its uptake (Watanabe et al., 1996; Rakhshan et

al., 2000; Bisogno et al., 2001). Mouse brain microsomes, a preparation rich in FAAH, was used to demonstrate the inhibition of AEA hydrolysis by CBD at high concentrations (>30 μM) (Watanabe et al., 1996). The inhibited hydrolytic degradation caused by CBD was measured as [^{14}C]-ethanolamine, the metabolite of [^{14}C]-AEA, also in N18TG2 cells (Bisogno et al., 2001). The same method was used to measure the residual [^{14}C]-AEA, in RBL2H3 cells in which CBD reduced AEA uptake via the putative AMT (Rakhshan et al., 2000; Bisogno et al., 2001). However, in guinea-pig bronchi, the expression of AMT was excluded (Andersson et al., 2002) and in our hands, FAAH as a metabolic enzyme of AEA signalling was found not to be constitutively active. Surprisingly, CBD-induced inhibition of bronchoconstriction was sensitive to PMSF. We demonstrated for the first time that the inhibitory action of CBD was reversed by PMSF, and there was no significant difference between contractile responses to AEA and PMSF, and contractile responses to CBD and AEA in the presence of PMSF. We suggest that PMSF might enhance the action of CBD which is considered as a potent modulator of the endocannabinoid system with subsequent enhancement of the endogenous level of AEA (Massi et al., 2007; De Filippis et al., 2008b). CBD alone at higher concentrations or in combination with PMSF might behave as an inhibitor of FAAH activity in isolated guinea-pig bronchi. To further confirm the involvement of the FAAH enzyme in the effect of CBD, additional pharmacological studies are needed to be performed. It would be interesting to find out whether the more selective FAAH inhibitor, URB597 would produce a similar effect to PMSF and prevent the antagonistic activity of CBD in AEA-induced bronchoconstriction. However, URB597 by itself caused significant attenuation of AEA-induced bronchoconstriction. Its inhibitory pattern was comparable with the inhibition produced by CBD. Based on these results, we posed

the question of whether CBD and URB597 might act through the same mechanism in AEA-induced bronchoconstriction of isolated guinea-pig bronchi. We showed that in the suppression of AEA-induced bronchoconstriction caused by URB597, CB_2 receptors might be involved because the response was partially reversed by pretreatment with the CB_2 antagonist, SR144528, whilst the CB_1 antagonist, SR141716A had no effect. To ascertain whether the action of CBD would also be affected by the selective CB_2 antagonist treatment, requires further investigation. This would clarify the question about CBD and URB597 possibly acting through the same mechanism in AEA-induced bronchoconstriction of isolated guinea-pig bronchi.

Chapters 4.1.7 and 4.1.8 discuss results obtained with CBD and Δ^9-THC, assessing their possible interaction with the tachykininergic system in isolated guinea-pig bronchi.

4.1.7 Is there an interaction between CBD and NK_2 receptors on isolated guinea-pig bronchial smooth muscle?

Based on the established mechanism of VIR action to activate $TRPV_1$ receptors on the sensory nerves and the ability of CBD to antagonize VIR-evoked bronchoconstriction in isolated guinea-pig bronchi, we hypothesised that CBD might have an influence on NKA effects in the same bioassay. Indeed, we confirmed the observation that the NK_2 receptors of the isolated guinea-pig bronchi mediate the NKA-induced bronchoconstriction (Maggi et al., 1991; Corboz et al., 2003). The competitive antagonism was demonstrated by a parallel rightward shift of the response to NKA in the presence of the selective NK_2 receptor antagonist, SR48968C which is constistent with data obtained by Corboz et al. (2003). The reduced effect in

the presence of the antagonist assumed as a maximal effect achieved at the agonist concentration of 10 μM might be the result of methodological differences. While Maggi et al. (1991) studied the contractility to NKA in isolated guinea-pig bronchus with denuded epithelium, and in the presence of atropine, indomethacin and a NEP inhibitor, Corboz et al. (2003) excluded only atropine in their experiments. Our bronchial rings with intact epithelium were exposed only to indomethacin in order to avoid the possible influence of COX products on bronchial tone. Bronchial epithelium as the main source of NEP, the enzyme responsible for neurokinin breakdown, was left intact in our experiments which might have an impact on NKA responses (at 10 μM) after SR48968C treatment. The observation of a NK_2-mediated NKA response allowed us to continue our studies focusing on the possible interaction of CBD and NK_2 receptors on the guinea-pig isolated bronchial smooth muscle. We chose the repetitive experimental design for economical reasons because at the time of experimentation the number of guinea-pigs available was not sufficient. We tested CBD (1 μM) against NKA-induced bronchial smooth muscle contraction and showed for the first time a significant parallel rightward shift of the concentration-response curve to NKA, suggesting competitive antagonism at NK_2 receptors in the GPBP. The possible tachyphylaxis to exogenous NKA in the presence of CBD was excluded because its vehicle, absolute ethanol (0.01 %) failed to alter the contractile response to NKA. This shows that there is no difference between the first and the second responses to NKA obtained in the repetitive experimental design. It is important to point out that in the presence of CBD (1 μM) the significant parallel rightward shift of the concentration-response curve favors the idea of competitive antagonism, whereas a decline in the maximum response opposes the idea. We did not assess whether the 10-fold higher concentration of NKA agonist (100 μM) would prevent the decline and

achieve the supposed maximal effect in the presence of CBD at the same concentration of 1 μM. In support of the possible competition between CBD and NKA at the NK_2 receptor on the bronchial smooth muscle, we carried out the same experiment with a ten-fold higher concentration of CBD. However, instead of further parallel rightward shift, CBD (10 μM) significantly inhibited the concentration-dependent NKA response in a non-competitive manner. In contrast to the previous finding that 0.01 % ethanol did not evoke tachyphylaxis, 0.1 % ethanol, the vehicle concentration at 10 μM CBD, evoked slight tachyphylaxis to NKA. We postulated that the observed tachyphylaxis after ethanol (0.1 %) might be due to the depletion of endogenous NKA from sensory nerves of the GPBP, or its increased metabolism through NEP. It has been reported that a NEP inhibitor, tiorphan, potentiated the contractile response to CPS produced by endogenous tachykinins in isolated bronchi with intact epithelium (Maggi et al., 1990). The same NEP inhibitor potentiated the response to exogenous NKA in the presence of the epithelium but the responsiveness was more pronounced in epithelium-denuded bronchi (Maggi et al., 1990; Maggi et al., 1991). Surprisingly, in our hands phosphoramidon evoked a reduction of the responses to NKA. This NEP inhibitor has been already used by us against VIR-evoked bronchoconstriction where its stimulatory effect (at 10 μM, treatment for 30 min) was demonstrated. In contrast, phosphoramidon applied before cumulative doses of NKA at the concentration of 1 μM (treatment for 20 min), not only reduced the NK_2-mediated contraction but also disrupted the tissue viability. There was no response to CCh (10 μM) used as an internal standard for the responses to NKA. KCl (90 mM) used as an internal control for the tissue viability was also unable to evoke a contraction. Although papers report its uses in the range of 1-10 μM in similar bioassays without indication of toxic effect (Frossard et al., 1989, Heavey et al., 1997;

Corboz et al., 2003; Yoshihara et al., 2004), we must consider the possible toxicity of phosphoramidon at 1 μM in our GPBP. The tissue sensitivity to phosphoramidon was not investigated further.

The results also showed that CBD has no effect on contractile cholinergic responses stimulated by CCh in ASM which excluded the direct action of CBD at the M_3 receptors of bronchial smooth muscle. This is in line with the observation that Δ^9-THC has no direct effect in bronchial smooth muscle because this cannabinoid did not alter the responses to CCh (Orzelek-O'Neil et al., 1980a; 1980b). Another control study was related to histamine and NKA. Although tachykinins cause bronchoconstriction mainly by a direct action on the bronchial smooth muscle (Corboz et al., 2003), it has been demonstrated that tachykinins (NK_1 and NK_2 agonists) may also release histamine (Lilly et al., 1995). Tracheally injected CPS and SP into tracheally perfused guinea-pig lungs liberated histamine, most likely from airway mast cells, by a mechanism predominantly dependent on the activation of NK_1 and NK_2 receptors (Lilly et al., 1995). The H_1 antagonist, mepyramine at the concentration of 1 μM was able to reduce NKA release from bronchial tubes of sensitized guinea-pigs (Lindström and Andersson, 1997). In addition, mepyramine at the concentration of 1 μM produced an inhibitory effect on CPS-induced contractions in bronchial tube preparations (Lindström and Andersson, 1997). In agreement with these results, we found that mepyramine also at 1 μM produced a parallel rightward shift of the NKA-induced bronchoconstriction. Mepyramine treatment resulted in a slight though non-significant attenuation of control NKA responses, indicating a possible interaction between histamine and NKA in a sense that the exogenously applied NKA might induce NK_2-mediated histamine release from mast cells in the GPBP. This hypothesis is strengthened by a finding that NKA evoked concentration-

dependent histamine release through its respective NK_2 receptor on a murine liver derived mast cell line (Krumins and Broomfield, 1992). This suggests that a NK_2 receptor-dependent pathway might exist for the release of histamine.

The discovery of the rightward displacement of the response to NKA due to CBD (1 μM) treatment found in the repetitive experimental design, needed to be confirmed in the non-repetitive experimental design. It was doubtful that a true competitive antagonism could occur and more an indirect antagonism was assumed in our preparation. Considering SR48968C as a competitive antagonist of NKA at the NK_2 receptors in the non-repetitive experimental design of isolated guinea-pig bronchi, it was important to establish and compare these phenomena. In these experiments only one concentration-response curve per bronchus was constructed. Disappointingly, neither CBD at the concentration of 1 μM evoked any rightward shift of the cumulative concentration-response curve to NKA, nor CBD at 10 μM produced any inhibition of NKA responses. In contrast, CBD at both concentrations slightly enhanced NKA-induced bronchoconstriction, indicating no inhibitory effect of CBD in this design. The direct effect of CBD at M_3 receptors of the bronchial smooth muscle was also excluded in the non-repetitive experimental design because CBD (1μM) failed to alter the cumulative concentration-responses to CCh. Furthermore, the possible hypothesis that the NK_2-mediated histamine release takes place in response to exogenously applied NKA was not confirmed in the non-repetitive experimental design. The slight enhancement of NKA-induced bronchoconstriction might indicate that the action of mepyramine at 100 nM is non-specific and the exogenous NKA does not cause histamine release in the GPBP. It would be interesting to study the responsiveness of NKA in the presence of mepyramine at 1 μM. In addition to these studies we tested the effect of CBD (1 μM) against SP-evoked bronchoconstriction in

the non-repetitive experimental design. Corboz et al. (2003) have demonstrated that in isolated guinea-pig bronchi, CP99994, the selective NK_1 antagonist produced a parallel rightward shift in the concentration–response curve for SP whereas the selective NK_2 antagonist had no effect (Corboz et al., 2003). In line with this study our study showed that SP as a NK_1 receptor agonist produced smaller contractile response than NKA, the more potent bronchoconstrictor. These control responses were not markedly affected by CBD, indicating no interaction of CBD with NK_1 receptors in the GPBP.

In conclusion, we have shown for the first time that CBD exerted a clear inhibition on NKA-induced bronchial smooth muscle contraction which could only be demonstrated in the repetitive experimental design. To the best of our knowledge, there is only one research group who has published that the non-selective cannabinoid agonist WIN55212-2 failed to influence NKA-induced guinea-pig bronchial smooth muscle contraction (Yoshihara et al., 2004). This is in line with the *in vivo* results showing that intravenously applied endocannabinoids, AEA and PEA did not alter NKA-induced guinea-pig bronchoconstriction (Yoshihara et al., 2005). These results also agree with our observation that CBD failed to produce any significant change of the NKA-induced guinea-pig bronchial smooth muscle contraction in the non-repetitive experimental design. In contrast, CPS-induced bronchoconstriction was reduced by intravenous PEA, and all the cannabinoids, WIN55212-2, AEA and PEA were able to significantly inhibit CPS-induced SP release from guinea-pig airway tissues (Yoshihara et al., 2005). The authors concluded that on one hand, these cannabinoids might affect tachykinin release from sensory nerve endings but on the other hand, they do not antagonize the interaction of tachykinins on the NK receptors (Yoshihara et al., 2004; Yoshihara et al., 2005). The different results concerning the

repetitive and the non-repetitive protocols used by us are difficult to interpret. We assume that in the repetitive experimental design CBD acts indirectly on bronchial smooth muscle and its action is non-specific on the contractile NKA response. The possible tachyphylaxis to exogenous NKA in the presence of CBD at 1 μM was excluded because its vehicle, absolute ethanol (0.01 %) failed to alter the contractile response to NKA (i.e. the repeated NKA concentration-response curves were identical). The possibility of mast cell involvement was substantiated firstly by our finding that CBD inhibited bronchial contraction evoked by the mast cell secretagogue, compound 48/80 (see pages 264-266) and secondly by a report that NKA can activate rat lung mast cells *in vivo* and cause histamine release in BAL fluid of rats (Joos and Pauwels, 1993). Thirdly, tracheally injected CPS and NK receptor agonists (SP and [β-Ala^8]-NKA) into tracheally perfused guinea-pig lungs could liberate histamine, most likely from airway mast cells, by a mechanism predominantly dependent on the activation of NK_1 and NK_2 receptors (Lilly et al., 1995). Taken together, we suggest that CBD in the repetitive experimental design might target mast cells by inhibiting their histamine release stimulated by the second cumulative application of NKA in the GPBP.

4.1.8 Is there an indirect effect of Δ^9-THC on sensory nerves of isolated guinea-pig bronchi?

In comparison to CBD, more airway related research has been done with its psychotropic counterpart, Δ^9-THC. However, research investigation revealed that Δ^9-THC has no direct bronchodilating effect and its *in vivo* bronchoactivity observed in humans might be of an indirect or central origin (Ackerman, 1977; Orzelek-O'Neil et

al., 1980a; 1980b). In recent years, Δ^9-THC has also been shown to possess vasoactive properties through a mechanism involving sensory nerves in the rat mesenteric arteries (Zygmunt et al., 2002; O'Sullivan et al., 2005; Wilkinson et al., 2007). Nevertheless, there is no study investigating the possibility of sensory nerve activation by Δ^9-THC in the airways. The lack of information and our finding that CBD at 1 μM produced a rightward shift of the concentration-response curve to NKA in the repetitive experimental design, prompted us to carry out the same investigation with Δ^9-THC. Although Δ^9-THC at 1 μM did not significantly alter the repetitive responses to NKA, in the non-repetitive experimental design Δ^9-THC (1 μM) produced a marked potentiation of NKA-induced bronchoconstriction in the GPBP. Following this discovery we tested whether the effect of Δ^9-THC on NKA-induced bronchoconstriction was through sensitization of NK_2 receptors. Interestingly, the combination of Δ^9-THC and the NK_2 antagonist SR48968C evoked significant inhibition of NKA responses, indicating non-competitive antagonism. This also suggested that NK_2 receptors might be involved in the action of Δ^9-THC. The results obtained were not sufficient to support this conclusion and we assumed an indirect effect of Δ^9-THC on sensory nerves, excluding its direct action on the smooth muscle of isolated guinea-pig bronchi. The desensitization study (applied to desensitize the sensory nerves) partly revealed the answer, producing an opposite phenomenon to control responses. NKA produced a concentration-dependent contraction which was similar to the contraction evoked by NKA in the presence of Δ^9-THC under control conditions. This reflects a NK_2 receptor-mediated effect on the bronchial smooth muscle. After CPS treatment, the response due to combination of Δ^9-THC and NKA was smaller than to NKA alone as expected, indicating that Δ^9-THC under control conditions might sensitize the tissue via an unknown mechanism. Also noteworthy is

the similar responsiveness of combined Δ^9-THC and NKA desensitization treatment and the NKA response in non-desensitized tissues. We can conclude that Δ^9-THC might activate sensory nerves without a direct action on the guinea-pig bronchial smooth muscle.

Because Δ^9-THC displays partial agonistic activity at both CB_1 and CB_2 receptors (Pertwee, 2007a), it was important to assess whether the stimulatory effect of Δ^9-THC was through sensitization of CB receptors. Unexpectedly, the selective CB_2 antagonist, SR144528 restored the efficacy of combined NKA and Δ^9-THC response while the CB_1 selective antagonist, SR141716A did not alter the efficacy of the NKA response. These results exclude the role of CB_2 receptors which are more likely to be present on the sensory nerves of guinea-pig airways (Yoshihara et al., 2004; Yoshihara et al., 2005). Results also suggest that CB_1 receptors might be involved in Δ^9-THC-induced potentiation of contractile responses to NKA in the GPBP. Taken together, Δ^9-THC (1 μM) stimulated NKA-induced bronchoconstriction in a way that, is independent of CB_2 receptors but might be dependent on CB_1 receptors involving sensory nerves of isolated guinea-pig bronchi. This result required confirmation using the same batch of Δ^9-THC because we suspected there may be decreased stability of Δ^9-THC diluted in absolute ethanol. Δ^9-THC originally purchased from Sigma was not available in time for experimentation using the desensitization protocol and CB antagonists. We decided to verify the effectiveness of the old batch of Δ^9-THC diluted in ethanol for 5 months. The chosen preparation was the guinea-pig whole ileum in which Δ^9-THC could inhibit EFS-evoked contractions (Gill et al., 1970; Layman and Milton, 1971). Neither of the reports published by Gill et al. (1970) or Layman and Milton (1971) mentioned the actual EFS parameters, employed to stimulate cholinergic responses of the ileum. For this reason we employed the EFS parameters

used in the myenteric plexus-longitudinal smooth muscle preparation of the guinea-pig ileum, a model used to study the agonist and antagonist activities of drugs acting on CB_1 receptors (Pertwee et al., 1996; Coutts and Pertwee, 1998). At these stimulatory parameters Δ^9-THC evoked a nearly significant (P=0.08) potentiation of EFS contractions in the isolated guinea-pig ileum. The parallel ethanol (0.01 %) control had no effect on EFS-evoked contractile responses. Our result is partly contradictory to the early studies by Gill et al. (1970) and Layman and Milton (1971) who were the first to show that Δ^9-THC reduced the twitch response of transmurally stimulated guinea-pig ileum, but evoked no change or sometimes even potentiation of the response to ACh (Gill et al., 1970). In addition, in the superfused guinea-pig ileum Δ^9-THC caused a non-parallel dextral shift of the concentration-response curve to histamine with a decreased size of its maximal effect, suggesting an interaction with H_1 receptors (Tűrker et al., 1975). Later Pertwee et al. (1996) provided further evidence (using the myenteric plexus-longitudinal smooth muscle preparation) that Δ^9-THC can induce CB_1 receptor-mediated inhibition of intestinal contraction excluding its direct action on the intestinal smooth muscle and supporting its prejunctional action on ACh release. They showed that Δ^9-THC reduced electrically evoked cholinergic contractions by decreasing ACh release in the guinea-pig myenteric-plexus longitudinal muscle preparation (Pertwee et al., 1996). Moreover, this was supported by *in vivo* studies where intravenous Δ^9-THC administration inhibited intestinal motility in rodents and humans (Massa et al., 2005). Indeed, it is generally accepted that CB_1 receptors in the gut control gastrointestinal motility (Coutts and Izzo, 2004), while other findings regarding the activation of gastrointestinal CB_2 receptors appear to be relevant in pathophysiological conditions, counteracting hypermotility (Izzo, 2007). It is noteworthy, however, that, Δ^9-THC can

exhibit both excitant and depressant effects in behavioral bioassays. It has been found that Δ^9-THC administered *in vivo* displayed mixed stimulatory-inhibitory effect on central neutransmitter release resulting in anxiolytic but also anxiogenic activity (Pertwee, 2007). Biphasic responses to Δ^9-THC have also been reported in the rat mesenteric arterial bed, with a concentration-dependent vasocontraction followed by vasorelaxation (Wilkinson et al., 2007). Diverse vasomotor effects of Δ^9-THC have also been described by Zygmunt et al. (2002) and O'Sullivan et al. (2005). Δ^9-THC produced vasoconstriction in the rat superior mesenteric artery, and vasorelaxation or no effect in smaller mesenteric arteries, implying that the size of the vessel studied might play a role in the effects observed (Zygmunt et al., 2002; O'Sullivan et al., 2005). Our findings with the guinea-pig ileum is difficult to interpret since the number of experiments is small. Overall, it would suggest that the 5 months-old batch of Δ^9-THC (0.01 M stock, diluted in absolute ethanol) underwent degradation. Results obtained from the desensitization studies and studies of the possible involvement of CB receptors in the action of Δ^9-THC against NKA-induced bronchoconstriction would require the repetition of the experiments with a new batch of Δ^9-THC. Having seen that Δ^9-THC potentiated EFS-induced contractions of isolated guinea-pig ileum, it was unfortunately not feasible to continue the investigation because our Δ^9-THC sample had run out. The possible mediation of the stimulatory effect of Δ^9-THC through the proposed CB_1 receptors in the guinea-pig ileum is therefore questionable.

In terms of the therapeutic potential, Δ^9-THC has shown bronchorelaxant activity both in normal and asthmatic subjects by a mechanism different from β-adrenergic agonists or anticholinergic agents (Shapiro et al., 1977; Tashkin et al., 1977; Hartley et al., 1978). Despite this fact, Δ^9-THC has not been marketed as an anti-asthma drug because of greater risks than benefits (Graham, 1986). Also the basic pharmacological

research carried out on Δ^9-THC by Orzelek-O'Neil et al. (1980a) was not satisfactory and excluded its significant direct effect on the bronchial smooth muscle. Using isolated human bronchioles the authors showed that Δ^9-THC (500 μM, treatment for 10 min) did not antagonize histamine- or $PGF_{2\alpha}$-induced contractions. In addition, there was a non-significant depression of CCh-evoked constrictive effects and isoprenaline-evoked relaxant effects (Orzelek-O'Neil et al., 1980a). Although there is a similarity in responsiveness of guinea-pig and human bronchial smooth musculature to pharmacologic agents, in guinea-pigs the intravenous administration of Δ^9-THC lacked the pulmonary action (Ackerman, 1977) of aerosolized Δ^9-THC (Tashkin et al., 1977) or smoked marijuana in human (Vachon et al., 1973; Tashkin et al., 1975). The difference in results obtained might be due to a variety of factors, such as species differences, experimental conditions or the vehicle used. However, the negative effects of Δ^9-THC found in isolated human bronchioles (Orzelek-O'Neil et al., 1980a) have been also confirmed in isolated guinea-pig tracheal and bronchial preparations (Ackerman, 1977; Orzelek-O'Neil et al., 1980b). There is also a negative evidence observed in sensitized guinea-pigs, used as a model of asthma where Δ^9-THC and nabilone did not alter antigen-induced responses of isolated bronchi (Orzelek O'Neil et al., 1980b). The next section discusses the implications of all of the results regarding the potential therapeutic utility of cannabinoids against asthma.

4.1.9 Might cannabinoids be beneficial in the treatment of allergic asthma?

Considering asthma as a complex inflammatory disorder, cannabinoids (in animal models) were able to modulate immunologic and pathologic features associated with this disease. In a murine model of asthma Δ^9-THC and another plant-derived

cannabinoid, cannabinol effectively attenuated OVA-induced allergic airway responses, including IL-2 and Th2 cytokine (IL-4, IL-5, and IL-13) mRNA expression in lung tissue, serum IgE production and overproduction of mucus in the lungs (Jan et al., 2003). However, Δ^9-THC being a potent psychoactive drug has a shortcoming that prevents its therapeutic employment as an anti-inflammatory agent (Costa, 2007). It is known, that its non-psychotropic counterpart, CBD shares many of the immunomodulatory effects with Δ^9-THC, suggesting that CBD and Δ^9-THC have complex lineage- and derivative-specific effects on cytokine production (Baczynsky and Zimmerman, 1983; Srivastava et al., 1998; Costa, 2007). In murine macrophages, apart from suppressing the production of the cytokine IL-10, CBD has been shown to increase the production of IL-12, therefore favouring the development of the pro-inflammatory Th1 response, implying its possible pro-inflammatory effect (Sacerdote et al., 2005). In contrast, many *in vivo* studies have described CBD as a potent anti-inflammatory agent. In a murine model of arthritis CBD reduced oedema caused by carrageenan (Lodzki et al., 2003). In a rat model of acute and chronic inflammation, CBD-induced anti-hyperalgesia was prevented by the $TRPV_1$ antagonist, capsazepine, but not by CB_1/CB_2 antagonists (Costa et al., 2004; Costa et al., 2007). In addition, Costa et al. (2007) demonstrated that CBD counteracted the progression of neuropathic pain and chronic inflammation in rats by reduction of plasma PGE_2, tissue NO production and lipid peroxide overproduction. CBD also attenuated the incidence of diabetes in non-obese diabetic mice associated with suppressed production of Th1 cytokines (IL-2, IFN-γ and TNF-α) and enhanced production of Th2 cytokines (IL-4 and IL-10) (Weiss et al., 2006). Moreover, CBD treatment inhibited insulitis caused by pancreatic beta cell destruction. The authors suggested that CBD instigated a possible deviation from destructive Th1 immunity to protective

Th2 immunity in diabetes (Weiss et al., 2006). In mice sensitized with OVA, humoral immune responses were examined focusing on antigen-specific antibody and cytokine production (Jan et al., 2007). In this study the serum level of OVA-specific antibodies, IgM, IgG_1 and IgG_{2a} was attenuated after CBD treatment (5-20 mg/ kg). In the same study using murine splenocytes, CBD (5 and 20 mg/ kg) reduced T cell proliferation and production of cytokines IL-2, IL-4 and IFN-γ. Attenuation of IL-4 and IFN-γ production was confirmed by direct treatment of splenocytes with CBD (2-8 μM) *in vitro* (Jan et al., 2007). In contrast, it was demonstrated recently that CBD at higher concentrations (3-10 μM) has pro-inflammatory potential associated with mast cell activation *in vitro*. This effect was not mediated by the known CB receptors but involved a rise in intracellular Ca^{2+} in a $TRPV_1$ receptor-insensitive manner (Del Giudice et al., 2007). The plethora of mechanisms of CBD actions sheds light on this compound, making it as a highly attractive therapeutic entity (Mechoulam et al., 2007). These findings raise a question of whether cannabinoids might be beneficial in the treatment of allergic asthma. The OVA-sensitized and challenged guinea-pig has been widely used as a model for asthma in man because of similarities between human asthma and allergen-induced changes in the guinea-pig (Muccitelli et al., 1987; Whelan, personal communication). An allergen exposure in individuals with allergic asthma results in an EAR followed by a LAR (Cockcroft, 1983). Similarly, in sensitized guinea-pigs, an antigen challenge can initiate an early bronchoconstrictor response due to release of pharmacological mediators (histamine, tachykinins, LTs and prostaglandins) from cells such as macrophages and mast cells which act on smooth muscle to cause bronchospasm. The late bronchoconstrictor response is associated with infiltration of key inflammatory cells into the airways, their activation and subsequent chronic inflammation (Smith and Johnson, 2005; Smith and Broadley,

2007). In collaboration with Dr. Cliff Whelan (University of Hertfordshire, U.K.), we used a well established asthma model in order to examine the effect of CBD on mast cell-driven antigen-induced contraction which represented the early allergic airway response in humans (Whelan, personal communication). The study was performed in bronchi obtained from actively sensitized guinea-pigs. Non-allergic control responses were conducted in parallel using the mast cell secretagogue, compound 48/80. In the present study guinea-pig bronchi were cumulatively challenged by the allergen OVA which caused concentration-dependent contractions. This confirmed that OVA-induced bronchial contraction is due to an immune response. Moreover, CBD at concentrations of 100 nM and 1 μM significantly inhibited the bronchoconstriction evoked by OVA. In contrast, CBD at 10 μM produced significant potentiation of the immune response. As far as we are aware, we have shown for the first time that a cannabinoid could alter antigen-induced responses of isolated bronchi. In terms of other cannabinoids, there is only one report documenting a negative effect of Δ^9-THC and nabilone in a similar model of asthma (Orzelek O'Neil et al., 1980b). This negative effect of at least Δ^9-THC was not confirmed in our asthma model. We decided to investigate the major mediators which are known to be cysLTs and histamine in the EAR of humans (Björck and Dahlén, 1993; Dahlén et al., 1983; Roquet et al., 1997). Similarly, in isolated guinea-pig tracheal and bronchial preparations, antigen-induced contractions were mediated by histamine and LTs (Muccitelli et al., 1987; Lindström et al., 1992; Chen et al., 1998). By using the H_1 antagonist, mepyramine at 1 μM, it was found that the histamine component was most pronounced in preparations with the epithelium removed. The intact epithelium contributed to the complexity of antigen-induced contractions, both COX and 5-LO products being involved (Lindström et al., 1992). While the 5-LO inhibitor, MK886

(Rouzer et al., 1990) significantly inhibited the egg albumin-induced response in sensitized guinea-pig airways, the COX inhibitor, indomethacin showed potentiation of the contraction. This indicated that, in intact epithelium, LTs are the main components of allergic contraction and the production of inhibitory prostaglandins (e.g. PGE_2) might be inhibited (Lindström et al., 1992). In accordance with this report, we used only preparations with intact epithelium and excluded the possible role of COX products by the presence of indomethacin in Krebs solution. Mepyramine at the concentration of 1 μM failed to produce inhibition of antigen-induced bronchoconstriction (data not shown) which is in contrast with the results published by Lindström et al. (1992) in guinea-pig trachea. This contradiction might be related to variations in the experimental methodology and the type of tissue used. Interestingly, a ten-fold lower concentration of mepyramine (100 nM) could produce significant inhibition of OVA responses in our study. The greater antagonism by mepyramine in the dose of 100 nM used by us may be due to a more specific modulation in terms of histamine release during an antigen-induced response. In fact, Antonissen et al. (1980) demonstrated the role of histamine as a primary mediator in an OVA-sensitized canine model of allergic asthma in which mepyramine at the concentration of 10 nM blocked the tracheal contraction to OVA. Surprisingly, the 5-LO inhibitor, MK886 at the same concentration as was used by Lindström et al. (1992), produced a dual result in our hands. Firstly, significant inhibition and secondly, a potentiation in sensitized GPBPs. This discrepancy is difficult to explain and the stimulatory tendency of MK886 might be the consequence of the difference in the group of sensitized guinea-pigs. In this particular group the control responses to OVA were lower (0.29 g ±0.07) in comparison to the average of control responses to OVA (0.38 g ±0.05) in the presence of other drugs (CBD, mepyramine, and MK886

with the inhibitory tendency). We speculate that the stimulatory tendency of MK886 on OVA-induced responses might reflect a non-specific action on mast cell function under our conditions. Taken together, we showed that mepyramine inhibited the OVA-induced contraction by 47.80 % ±7.84 and MK886 inhibited the contraction by 36.44 % ±9.92, whereas CBD at the concentration of 1 μM produced the greatest inhibitory effect of 77.31 % ±14.84. Our data confirm the dependence of the early phase of contraction on the release of histamine and 5-LO products. Additionally, they show subsequent inhibition of release from mast cells after CBD treatment at least at the concentration of 1 μM. The remaining question is whether the combination of both mediator antagonists (mepyramine and MK886) would result in a greater inhibitory effect than the inhibition produced by CBD itself. If a synergistic inhibitory action was absent, it would indicate that CBD may inhibit mast cell mediators other than histamine and 5-LO products. However, there have been reports showing that contractions produced by antigen challenge in tracheal preparations from sensitized guinea-pigs appear to be mediated by histamine and LTs released from mast cells because the combination of antagonists of these mediators nearly abolished the responses from asthmatic airways (Muccitelli et al., 1987; Lindström et al., 1992; Chen et al., 1998). Moreover, our data also points out an interesting fact that a higher concentration of CBD evoked an opposite effect. This phytocannabinoid at 10 μM markedly potentiated the responses to OVA (109.13 % ±37.07). In agreement with a previous report (Del Giudice et al., 2007) dealing with mast cell activation *in vitro*, the present study corroborates the pro-inflammatory potential of CBD. Similar profile was observed in the rat mast cell line, RBL-2H3, measuring β-hexosaminidase activity under basal and antigen-stimulated conditions. In both cases CBD dose-dependently (3-10 μM) increased β-hexosaminidase release which reflected mast cell

activation. In addition, an influx of extracellular Ca^{2+} considered as a crucial feature for IgE-dependent mast cell degranulation (Bradding, 2005), has been partly implicated in the CBD-mediated action. Although both CB receptors are present in the RBL-2H3 cell line, CBD-induced β-hexosaminidase release was independent of CB receptors activation in these cells (Del Giudice et al., 2007). On one hand CBD-induced intracellular Ca^{2+} rise was insensitive to CPS excluding interaction with the $TRPV_1$ receptors. On the other hand, it was inhibited by the cationic channel blockers, clotrimazol and nitrendipine. In addition, the dependence on extracellular Ca^{2+} was confirmed by chelation of extracellular Ca^{2+} when the elevated $[Ca^{2+}]_i$ due to CBD was nearly abolished. The authors suggested that Ca^{2+} influx channels on the mast cell surface or other signalling pathways are likely to be essential for the effect of CBD on mast cell activation (Del Giudice et al., 2007). In line with this, CBD has been shown to act as a potent modulator of intracellular Ca^{2+} homeostasis in neuronal cells (Ryan et al., 2007), although the precise mechanism is presently unclear.

We hypothesize that inhibition of the 5-LO pathway might be one mechanism responsible for the inhibitory effect of CBD on mediator release from mast cells in the sensitized GPBP. The reason for our postulation is a report published only recently by Massi et al. (2007). They demonstrated that CBD applied to nude mice decreased 5-LO activity and its content using glioma tumor tissues excised from these mice. Thus, there is a suggestion that CBD may induce tumor and cell growth inhibition through the modulation of the 5-LO pathway. The authors ruled out any direct effect of CBD on enzyme activity because *in vitro* exposure of human glioma U87 cell to CBD did not modify the activity of purified 5-LO (Massi et al., 2007). Important evidence brought forward by Massi et al. (2007) is the synergism between CBD and the 5-LO inhibitor MK886, as the antiproliferative action of CBD was enhanced by MK886.

Based on this finding we assume that in our mast cell-driven bronchoconstriction representing the EAR, CBD may behave as a more potent LT biosynthesis inhibitor causing markedly attenuated OVA responses in bronchial preparations from sensitized guinea-pigs. Similarly to the mechanism of action of MK886, CBD might block the membrane translocation of 5-LO from the cytosol to the membrane, thus preventing its membrane association and subsequent activation of the enzyme (Rouzer et al., 1990). This proposed mechanism of MK886 has been shown in human leukocytes stimulated by the Ca^{2+} ionophore A23187 which evoked synthesis of LTs and membrane translocation of 5-LO. In contrast, LT synthesis by inhibitor-treated leukocytes was undetectable and additionally there was an inhibition of membrane translocation as measured by inhibition of membrane–associated enzyme protein in MK886-treated cells. Importantly, MK886 failed to affect 5-LO activity or its subcellular localization in the absence of ionophore challenge (Rouzer et al., 1990). CBD with negligible affinity for CB receptors may possess membrane perturbing effects (Jan et al., 2007) whereby it might bind to a protein which would inhibit its function and thereby block the translocation of the enzyme and its subsequent activation. As a matter of fact, a 18,000-dalton protein in leukocytes membranes has been proposed to interact with MK886 resulting in the block of 5-LO mediated action (Rouzer et al., 1990). Overall, the remaining question which has to be addressed is whether the combination of CBD and MK886 would cause synergistic action and totally block the responses to OVA in bronchial preparations from sensitized guinea-pigs, used as a model of asthma.

Alternatively, inhibition of an equilibrative nucleoside transporter has been implicated in CBD-induced anti-inflammatory and/or immunosuppressive effects which might be related to asthma (Carrier et al., 2006). In this context we have not investigated

anything but it should be recalled that enhancement of adenosine signalling by adenosine agonists or adenosine uptake inhibitors is attributed to reduced inflammation. The anti-inflammatory effects of adenosine agonists and adenosine uptake inhibitors are similar to the effects of plant-derived cannabinoids, e.g. reduction of serum TNF-α in lipopolysaccharide treated mice or suppression of a pro-inflammatory Th1 response while enhancing the Th2 response (Carrier et al., 2006). Evidence of these actions is the inhibition of [^{3}H]-adenosine transport into murine microglial cells by CBD. Furthermore, CBD showed competitive inhibition of adenosine transport by its five-fold higher affinity for the equilibrative nucleoside transporter than the potent inhibitor of this transporter, S-(4-nitrobenzyl)-6-thioinosine (NBMPR) (Carrier et al., 2006). *In vivo* treatment with CBD decreased TNF-α induced by lipopolysaccharide in mice which was reversed by an adenosine A_{2A} antagonist. Adenosine mediation of CBD effect on TNF-α through the A_{2A} receptor was confirmed in A_{2A} receptor knockout mice in which the effect of CBD on TNF-α was abolished (Carrier et al., 2006). Regarding the airway inflammation, adenosine is a pro-inflammatory mediator involved in the pathogenesis of asthma and other respiratory disorders. In asthmatics in contrast to normal subjects inhalation of adenosine monophosphate which is a degradation product of adenosine, induced airway obstruction. Since there are four distinct types of adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) in airways, a great amount of research had to be done to understand their physiological role. Nowadays, as presented by Dr. Mike Trevethick from the Pfizer group at the 5th James Black Conference of the British Pharmacological Society (Cutting Edge Concepts in Lung Pharmacology) in October 2007, ligands acting at the A_{2A} receptor hold the potential to treat lung inflammation (Trevethick et al., 2008). Although GlaxoSmithKline discontinued the development of an A_{2A} receptor

agonist, GW 328267, because of tachycardia, there is an opinion that selective A_{2B} receptor antagonists might show a higher therapeutic index (Wilson, 2007). In accordance with this view, a number of studies indicate that targeting A_{2B} receptors might be a particularly beneficial approach (Spicuzza et al., 2006; Brown et al., 2008). Given the role of mast cells and adenosine in the pathophysiology of asthma, the interest in these attributes has increased. As adenosine A_{2A} and A_{2B} receptors are co-expressed in human mast cells, it is important to bear in mind their distinct function in these cells and variable distribution among species. While in humans activation of A_{2A} receptors reduces histamine release, A_{2B} receptors oppose their action and adenosine via A_{2B} receptors induces mast cells degranulation and increases the release of pro-inflammatory cytokines (Spicuzza et al., 2006; Brown et al., 2008). Apart from mast cells, A_{2B} receptors are also expressed on the ASM mediating adenosine-induced bronchoconstriction (Spicuzza et al., 2006; Brown et al., 2008). Only recently, increased expression of A_{2B} receptor mRNA has been detected in tracheal tissues from OVA-sensitized guinea-pigs (Breschi et al., 2007). There is a suggestion that the immediate bronchoconstrictor response (EAR) to adenosine is mast cell-dependent, and both A_{2B} and A_3 receptors may be responsible for mast cell activation, depending on the species (Smith and Johnson, 2005). In allergic guinea pigs, there are conflicting reports on which adenosine receptors mediate adenosine-induced bronchoconstriction. In an *in vivo* model of allergen-induced airway obstruction measured as an increase in airway resistance, Keir et al. (2006) claimed A_1 receptor involvement, a mechanism unrelated to histamine release from mast cells because mepyramine did not block airway obstruction induced by adenosine monophosphate. In contrast, airway obstruction to OVA was significantly inhibited by mepyramine, confirming the importance of histamine in mediating bronchoconstriction to OVA in this model (Keir

et al., 2006). Notwithstanding this different responsiveness of adenosine monophosphate and OVA to mepyramine treatment demonstrated by Keir et al. (2006), the profile of activity after OVA and adenosine monophosphate inhalation appeared to be similar in a study reported by Smith and Broadley (2005). They showed that both compounds evoked an EAR, LAR, hyper-responsiveness to histamine and cellular infiltration to the airways in their asthma model of guinea-pigs (Smith and Broadley, 2005). Martin and Broadley (2002) suggested an A_3 receptor-dependent response to adenosine in sensitized guinea-pig tracheal preparation. Novel evidence has suggested that A_{2B} receptors were responsible for the relaxing effects of adenosine in guinea-pig airways (Breschi et al., 2007). There is a proposal that in allergic conditions, most likely due to accumulated adenosine, A_{2B} receptors are susceptible to desensitization that hampers the ability of these receptors to mediate relaxing responses (Breschi et al., 2007). Further studies are definitely needed to improve our understanding of how adenosine functions in airways. However, our hypothesis that CBD might be beneficial in allergic asthma is strenghtened by the fact that this phytocannabinoid markedly attenuated the mast cell-driven bronchoconstriction evoked by OVA in a model of asthma. In addition, the finding that CBD decreased lipopolysaccharide-induced TNF-α production in an A_{2A} receptor-sensitive manner, and that CBD binds to the equilibrative nucleoside transporter, implicates an interaction with adenosine signalling. In keeping in this notion, we may raise the following question. Does CBD interact with any of the above mentioned adenosine receptors or the putative equilibrative nucleoside transporter in the GPBP? If so, are these assumed targets responsible for the action of CBD in our model of asthma?

In an attempt to confirm that mast cells are the site of CBD action, we set out to examine the effect of CBD on mast cells in response to non-allergic challenge in the GPBP. The phenomenon of the mast cell-driven bronchoconstriction from non-sensitized guinea-pigs was provided by a non-immunological stimulus, the mast cell degranulating agent, compound 48/80. The findings of the present study demonstrated that the cumulative challenge with compound 48/80 elicited concentration-related contractions in the GPBP. The contractile responses to immunological stimuli and non-immunological stimuli showed relatively similar profiles. There was an initial rapid increase in contraction that reached a maximum after approximately 2 to 3 min, followed by a sustained contraction that lasted up to 5-10 min. OVA (100 μg/ ml) elicited 79.16 % ±3.23 (n=28) of the maximal contraction induced by CCh (10 μM), whereas compound 48/80 (300 μg/ ml) yielded 72.78 % ±7.84 (n=16) of the maximal contraction induced by this muscarinic agonist on the ASM (data not shown). In preparations challenged with OVA, the response to 1 μg/ ml was immediate. In contrast, the same concentration of compound 48/80 appeared to be ineffective, only the concentration of 30 μg/ ml gave a detectable level of contraction. Furthermore, in preparations challenged with compound 48/80 only two concentrations of CBD were employed. While CBD at 1 μM evoked significant reduction, 10 μM CBD treatment resulted in a slight though non-significant enhancement of the contraction to compound 48/80 at the concentration of 300 μg/ ml. In order to identify the mediators released from mast cells in response to compound 48/80 in our GPBP, we tested the tissues in the presence of the H_1 antagonist, mepyramine and the non-selective 5-$HT_{1/2}$ receptor antagonist, methysergide. Our data show significant and parallel rightward shift of the concentration-response curve of compound 48/80 produced by mepyramine, indicating that the mast cell-mediated response to compound 48/80 is

due to histamine release in the guinea-pig bronchi. This is in accordance with a study of compound 48/80-induced H_1 and H_2 receptor antagonists-sensitive contraction in isolated guinea-pig bronchi (Mapp et al., 1993). We attempted to confirm the action of CBD at mast cells by studying the possible interaction between CBD and H_1 receptors in isolated guinea-pig bronchi. In this investigation reproducible concentration-response curves to histamine were only slightly reduced in the presence of CBD thus excluding a direct action of CBD at H_1 receptors in the GPBP. In addition, the present study showed a failure of methysergide in preventing bronchoconstriction that might be related to the fact that 5-HT is not the main mast cell mediator in guinea-pigs. In contrast, it has been reported that in isolated rat trachea compound 48/80-induced contraction was abolished in the presence of ketanserin, a 5-HT_{2A} receptor antagonist (Ikawati et al., 2000). Also in sensitized rat parenchymal strips, the mast cell-mediated OVA response was due to release of 5-HT and LTs (Wolber et al., 2004). Taking into account the species difference, the action of 5-HT in guinea-pig airways is bimodal. It can cause both constriction and relaxation of tracheal strips (via 5-HT_2 receptors), depending on the concentration used [lower concentration (0.1-10 μM) produced contraction and higher concentration (10-300 μM) produced relaxation]. This might be the consequence of 5-HT_2 receptor coupling to different effectors with different efficacies (Baumgartner et al., 1990).

Overall, our data with OVA and compound 48/80 provide further support for an involvement of mast cells in response to CBD. The similarity between OVA and compound 48/80 in attenuating and enhancing responses to CBD imply common mechanisms which are likely to be in airway mast cell inhibition and activation respectively. The mediator released in response to both agents is histamine. In addition, 5-LO products are mediators in the antigen-induced contraction of the

GPBP. The observed effects of CBD may have implications for the development of cannabinoid-based treatments of asthma. This idea needs to undergo more detailed investigation and the ability of CBD to modulate mast cell behaviour might offer clinical benefit. Indeed, the basic pharmacological research focusing on mast cells and cannabinoids may result in a new pharmacological approach to treat inflammatory events. There is much evidence regarding cannabinoids affecting mast cell function which is sometimes controversial. The reason for the discrepancies in results might be the species-dependent heterogeneity between mast cell phenotypes and special caution is needed in data interpretation and drawing conclusions.

The first study which demonstrated the expression of binding sites for cannabinoids and the gene encoding the CB_2 receptor in rat RBL-2H3 cells and rat peritoneal mast cells was published by an Italian group (Facci et al., 1995). Additionally, they observed that PEA but not AEA inhibited [^{3}H]-5-HT release from RBL-2H3 cells. The authors concluded that mast cell CB_2 receptors might inhibit mast cell activation, and thus inflammation (Facci et al., 1995). In support of the observation that rat peritoneal mast cells express CB_2 mRNA, Bueb et al. assessed the capacity of natural, endogenous and synthetic cannabinoids (Δ^9-THC, Δ^8-THC, PEA and their derivatives, AEA, WIN55212-2, SR141716A and SR144528) to induce histamine release from these cells. They showed that only Δ^9-THC and Δ^8-THC (10-100 μM) were able to release histamine from rat peritoneal mast cells in a CB receptor-independent manner, indicating non-specific effects of cannabinoids (Bueb et al., 2001). Similarly, the presence of functional CB_1 and CB_2 receptors in rat peritoneal mast cells was not supported by a study from Lau and Chow (2003). They demonstrated that AEA only at concentrations higher than 1 μM significantly induced histamine release but that anti-IgE induced histamine release was not affected by

AEA treatment (Lau and Chow, 2003). In contrast, the synthetic cannabinoids, WIN55212-2 and HU-210 (both at 10 μM) enhanced anti-IgE-induced histamine release. All these effects of cannabinoids were not reversed by CB_1 and CB_2 antagonists, suggesting that cannabinoids might not influence mast cell activation through CB receptors (Lau and Chow, 2003). Subsequent studies from other groups demonstrated co-expression of CB_1 and CB_2 receptors modulating different signalling pathways in RBL-2H3 cells (Samson et al., 2003). While the CB_1 receptor mediated the suppression of 5-HT release, CB_2 receptor appeared to mediate activation of extracellular signal-regulated kinases, most likely linked to multiple transcription factor genes in RBL-2H3 cells (Samson et al., 2003). In the same model of RBL-2H3 cells, Small-Howard et al. (2005) suggested that the anti-inflammatory effects of CB_1 ligands after long-term stimulation (over a 60-120 min time course) may be due to cAMP elevation which in turn can cause suppression of mast cell degranulation, while CB_2 ligands caused the opposite, and decreased cAMP levels. Importantly, the short-term exposure (<60 min) of RBL-2H3 mast cells to CP55940 (100 nM) that bind to both CB_1 and CB_2 receptors showed suppression of mast cell activation measured by 5-HT release (Samson et al., 2003). Indeed, the non-selective cannabinoid agonists, CP55940 and WIN55212-2 concentration-dependently (0.1-10 μM) reduced the activation of RBL-2H3 cells measured by β-hexosaminidase release after 1 hour of exposure to these cannabinoids (Del Giudice et al., 2007). In guinea-pig mast cells, Vannacci et al. put forward a hypothesis of a down-regulation of the immunological response by CB_2 receptors (Vannacci et al., 2002; Vannacci et al., 2003; Vannacci et al., 2004). *In vitro*, 2-AG- and CP55940-mediated suppression of histamine release from guinea-pig mast cells was reversed by the non-selective NO synthase inhibitor, L-NAME and the selective CB_2 receptor antagonist, SR144528 (Vannacci et al.,

2004). They suggested that endogenous 2-AG and exogenous CP55940 might evoke generation of NO and PGE_2 which in turn elevates the intracellular level of cGMP resulting in the inhibition of antigen-induced increase in intracellular Ca^{2+}, the key feature of mast cell degranulation (Vannacci et al., 2004). An early *in vitro* study showed that the human mast cell line HMC-1 did not express functional CB receptors and neither PEA nor AEA could affect tryptase release from these cells. However, HMC-1 cells were able to transport and hydrolyze AEA by the action of FAAH, indicating functional endocannabinoid metabolism in human mast cells (Maccarrone et al., 2000). Cannabinoids have also been shown to influence mast cell function *in vivo*. The selective CB_2 receptor agonist, JWH133 reduced mast cell oedema induced by the non-antigenic mast cell degranulator, compound 48/80 in the model of plasma extravasation in the mouse ear pinna (Jonsson et al., 2006). Furthermore, both, the selective CB_1 agonist, ACEA and the selective CB_2 agonist, JWH015 attenuated mast cell function in a model of λ-carrageenan-induced granuloma formation in rats (De Filippis et al., 2008c). Whether CB receptors mediated the above effects of cannabinoids is not clear, and non-CB receptor-mediated effects were not excluded in these reports (Jonsson et al., 2006; De Filippis et al., 2008c). In the light of studies conducted with PEA monitoring its anti-inflammatory potential, this cannabinomimetic compound might have a clinical benefit. The evidence for this is a new drug containing PEA approved by the Food and Drug Administration for the treatment of dermatitis (De Filippis et al., 2008a). A pilot study assessing 20 pediatric subjects who suffered atopic dermatitis used twice daily a topical emulsion containing 2 % Adelmidrol (PEA analogue) and had a positive clinical response with complete resolution in 80 % of patients (Pulvirenti et al., 2007).

Thus, cannabinoids with the potential to regulate mast cell behaviour represent possible candidates for treating several inflammatory diseases and definitely more investigation is needed in this area. Unlike cannabinoid research focusing on mast cells, less attention has been paid to airway epithelial cells. There is a deficit of information regarding the possible cannabinoid-mediated effects on these peripheral cells in airways. Through secretion of cytokines, chemokines and growth factors airway epithelial cells are emerging to take centre stage in asthma and may offer novel targets for the development of new anti-asthma drugs which might be more selective than corticosteroids or immunosuppressants (Holgate, 2007b). The next chapter of this discussion covers the data obtained with the human bronchial epithelial cell line.

4.2 Human bronchial epithelial cells 16HBE

4.2.1 Identification of ion channel activity in response to cannabinoids in 16HBE cells

Our collaborator, Dr. Ad Nelemans and his research group (University of Groningen, The Netherlands) provided the first evidence that cannabinoids can trigger CB receptor-mediated effects in the human bronchial epithelium. Using the human bronchial epithelial cell line, 16HBE, they demonstrated that the endocannabinoid, VIR and the synthetic non-selective cannabinoid agonist, CP55940 can affect cAMP accumulation and IL-8 release. In addition, they were the first to identify the expression of CB_1 and CB_2 receptors, both at the level of mRNA and as proteins in 16HBE cells (Gkoumassi et al., 2007). In their investigation both CB_1 and CB_2 receptors were differentially coupled to AC and hence differentially coupled to cAMP. Both VIR and CP55940 decreased forskolin stimulated cAMP accumulation in a CB_2 receptor-sensitive manner. The involvement of $G_{i/o}$-proteins in CB_2 receptor-mediated inhibition of cAMP formation was tested by PTX which enhanced the forskolin-induced cAMP accumulation in response to both cannabinoids. In contrast, the stimulatory response in the presence of PTX was prevented by SR141716A, indicating a CB_1 receptor-mediated increase of cAMP formation (Gkoumassi et al., 2007). Furthermore, they reported that VIR significantly and CP55940 to a lesser extent inhibited TNF-α-induced IL-8 release. The responses were not affected by the CB_1 antagonist, SR141716A. Because the CB_2 antagonist, SR144528 on its own markedly reduced the TNF-α-induced IL-8 release from 16HBE cells, it was not possible to confirm the identity of the CB_2 receptor involved in the inhibition of TNF-

α-induced IL-8 release. Basal IL-8 release was not influenced by either antagonist (Gkoumassi et al., 2007). They concluded that VIR might exert anti-inflammatory effects in the airways by CB_2 receptor-mediated modulation of cytokine release from the bronchial epithelium (Gkoumassi et al., 2007). In our attempt to analyze the possible cannabinoid signal transduction in 16HBE cells by using the patch clamp technique, Nelemans' research group kindly provided us with a batch of these epithelial cells. In order to create the same conditions for cell growth, we also obtained a protocol from our collaborator. After the establishment of standard cell growth, we decided to test the basic electrophysiological properties of the 16HBE cell line under our conditions. In the whole-cell patch clamp configuration, a voltage step protocol was used to determine the voltage-dependent ion channels present in epithelial cells. Applied membrane potentials evoked outward currents. These were blocked by a combination of Cs^+ and TEA, suggesting that these currents were mediated by voltage-dependent K^+ channels. Importantly, the value of the membrane potential for resting cells was in line with the already published value by Koslowsky et al. (1994) and Kunzelmann et al. (1994) who established the 16HBE cell line as an appropriate model for the investigation of the pathophysiology of cystic fibrosis.

Before testing cannabinoids on 16HBE cells, we chose ATP as a positive control. It has been shown that this nucleotide evoked significant and reversible hyperpolarization, a response due to an increase of the K^+ conductance and most likely mediated via activation of P2Y2 receptors (Koslowsky et al., 1994). In the present study ATP produced an oscillating outward current which was recorded in voltage clamp. Under current clamp conditions, ATP hyperpolarized the cell membrane probably by stimulation of K^+_{Ca}. The reason for this assumption is that oscillations are visible under voltage clamp conditions produced by extracellular ATP

in 16HBE cells. Moreover, Koslowsky et al. (1994) indicated that the ATP-induced increase of the K^+ conductance is mediated by an increase in $[Ca^{2+}]_i$ because the Ca^{2+} ionophore ionomycin mimicked the action of extracellular ATP in 16HBE cells. In our cells, the ATP-evoked hyperpolarization was in line with the hyperpolarization published by Koslowsky et al. (1994). We concluded that the 16HBE cell line might provide an appropriate model for the study of cannabinoid signal transduction. Therefore, cannabinoids were applied to bronchial epithelial cells in order to determine any change either in membrane current or membrane potential. We found, firstly, CP55940 and AEA had no effect on membrane potentials measured in current clamp. Secondly, CP55940 and WIN55212-2 failed to affect membrane currents of 16HBE cells measured in voltage clamp. Thirdly, only VIR at concentrations of 30 µM and 100 µM evoked a delayed hyperpolarization of the cell membrane, suggesting the opening of K^+_{Ca} channels. Noteworthy was the different nature of the membrane potential change evoked by ATP and VIR. While the changes in membrane potential occurred within 1 min after the application of ATP and they were reversible when ATP was removed, this was not the case for VIR. The membrane potential change to VIR was delayed (2-3 min) and was not fully reversible. The proposed involvement of CB receptors and P2Y2 receptors could not be tested because of intermittent (50 %) responsiveness of 16HBE cells to VIR. In light of this, we raise the question whether VIR could trigger a non-CB receptor signalling pathway via activation of P2Y2 receptors in 16HBE cells.

Despite the presence of CB receptors in the 16HBE cell line sent by Nelemans' research group (Gkoumassi, personal communication), the negative data obtained with AEA and two potent synthetic cannabinoid agonists, CP55940 and WIN55212-2, and poor responsiveness to VIR compelled us to question the functional expression of

CB receptors in 16HBE cells under our conditions. The first approach to this problem was taken by testing the possible adverse effect of FBS on the electrophysiological profile of 16HBE cells. On one hand, FBS is considered to be an essential cellular growth factor, but on the other hand, it might cause unpredictable culture growth and contamination with viruses or mycoplasmas (Barnes, 1985). With this in mind, experiments were designed to compare electrical properties of cells growing in serum-containing and serum-free media. Under these different conditions, there were no changes in electrical properties of patched cells. In addition, the membrane potential was tested to exogenously applied ATP, AEA and VIR, but there was no change in the responsiveness to any of these three drugs. ATP as a positive control produced hyperpolarization, AEA did not evoke any change of the membrane potential and VIR was able to hyperpolarize the cell membrane of 2 cells out 5 cells. In contrast, the yield of healthy cells was decreased in the absence of FBS from the culture medium. Although the composition of the culture medium and the culturing surface were the same as in Nelemans' laboratory, there are many factors that might affect the cell properties including the passage number of the cells, cell seeding density, and time in culture (Forbes et al., 2003; Forbes and Ehrhardt, 2005). The issue was not further investigated, and we hypothesized that our culture conditions might negatively influence the expression of CB receptors in 16HBE cells supplied by our collaborators. In their hands these cells showed positive expression of both CB receptors (Gkoumassi et al., 2007). For this reason the second approach was taken to target the expression of CB receptors on both transcript and protein level in 16HBE cells.

4.2.2 Identification of CB_1/CB_2 receptors in 16HBE cells

The investigation of the possible cannabinoid signalling on the cellular level was followed by a study on the molecular level. The objective was to confirm or exclude the presence of CB_1/CB_2 receptors in our 16HBE cells and compare the result with the result of our collaborators who were the first to identify the expression of CB_1 and CB_2 receptors, both at the level of mRNA and as proteins in 16HBE cells (Gkoumassi et al., 2007). In our hands PCR analysis demonstrated the presence of CB_1 mRNA and GAPDH as a housekeeping gene provided a viability test for our 16HBE cells. However, the detected transcript obtained with the CB_2 specific primers was 37 bp larger than the expected 263 bp and thus we questioned the presence of the CB_2 receptor mRNA in 16HBE cells. Using western blot analysis we assessed the expression of CB receptor proteins and the results confirmed the presence of the CB_1 and the absence of the CB_2 receptor protein in the 16HBE cell line. Our western blot data is in line with PCR data. Like Gkoumassi et al. (2007), we found CB_1 receptors to be expressed in these cells, both at the level of mRNA and as proteins. Noteworthy is that the different experimental methods between two laboratories led to the same conclusion regarding the expression of CB_1 receptors in the 16HBE cell line. In contrast, it was not case for the expression of CB_2 receptors in these cells. The larger size (>300 bp) of the proposed CB_2 transcript and the larger size (>60 kDa) of the protein for the proposed CB_2 receptor in 16HBE cells, strengthen the validity of our findings and counter the findings obtained by Nelemans' research group. One shortcoming of their study was the omission of positive controls which are important for validating the signal specificity. While PCR analysis and western blot analysis studies performed in our conditions involved CHO-hCB_1/CB_2 cells used as positive

controls, studies by Gkoumassi et al. (2007) did not include any positive controls or negative controls which were included in our western blot studies. We believe that this difference increased the reliability of our results clearly showing positive expression of CB_1 and negative expression of CB_2 receptors in the 16HBE cell line. Taken together, the negative effect of CP55940, WIN55212-2 and AEA and the evidence of CB_1 receptors would suggest that the cannabinoid ligands examined might not be functionally coupled to a mechanism which influences the membrane potential or the membrane current, and VIR might trigger a CB_1 receptor-independent signalling pathway in 16HBE cells. Although the study of a possible induction of signal transduction by cannabinoids in human bronchial epithelial cells using the patch clamp technique was not successful, our intention was to find a technique which would enable us to study the proposed cannabinoid signalling in these non-excitable cells. The chosen method was the Ca^{2+} assay employing the FLIPR technique.

4.2.3 Identification of $[Ca^{2+}]_i$ elevation in 16HBE cells

Cannabinoids have been shown to modulate Ca^{2+} signalling in a variety of preparations (Demuth and Molleman, 2006). In 16HBE cells CP55940 and VIR, at high concentrations (10 μM-100 μM), exerted an increase in Ca^{2+} levels measured by Nelemans' research group using Fura-2 conventional fluorescence spectrometry. The study was only preliminary and did not test cannabinoid antagonists (unpublished data, Nelemans et al.). Based on this information we decided to examine the potential ability of selected cannabinoid ligands and a vanilloid, to raise $[Ca^{2+}]_i$ using a plate-based FLIPR Ca^{2+} assay in collaboration with Dr. Malcolm Begg (GlaxoSmithKline, Stevenage). In addition, the assay provided a test for evaluation of functional potency at CB_1 receptors, as detected by PCR and western blotting in 16HBE cells. In the concentration range of 0.1 nM-10 μM none of the cannabinoid ligands (AEA, CBD, CP55940, WIN55212-2, JWH133), and the vanilloid, CPS had effect on the intracellular Ca^{2+} in 16HBE cells. Only VIR at the maximal examined concentration (10 μM) evoked a very slight increase in $[Ca^{2+}]_i$. In contrast, exposure to ATP applied as a positive control evoked, at its maximal concentration (100 μM), a nearly 6 fold increase in $[Ca^{2+}]_i$ in the 16HBE cell line. This elevated $[Ca^{2+}]_i$ is in agreement with a report which used the 16HBE cell line as a model for studying ATP-induced Ca^{2+} signals (Sienaert et al., 1998). Single-cell $[Ca^{2+}]_i$ measurements revealed that only IP_3 receptors were involved in the intracellular Ca^{2+} release. Ryanodine receptors were not functionally important in these cells because caffeine, the pharmacological activator of the ryanodine receptor failed to induce Ca^{2+} release (Sienaert et al., 1998). Furthermore, it has been shown that ATP in the presence of the purinergic antagonist, suramin failed to increase intracellular Ca^{2+}, confirming the purinergic (P2Y2)

mediation of the response (Walsh et al., 2000). The inability of the cannabinoid ligands examined to induce an increase in $[Ca^{2+}]_i$ might indicate that CB_1 receptors are not functionally coupled to any Ca^{2+} signalling pathway in 16HBE cells. Our finding is not surprising because there is no published evidence for cannabinoid-mediated Ca^{2+} changes in airway epithelial cells. In contrast, in other non-excitable cell lines cannabinoids increased intracellular Ca^{2+} levels (Mombouli et al., 1999; Chou et al., 2001; Zoratti et al., 2003; Demuth et al., 2005). In the human umbilical vein-derived endothelial cell line the AEA (0.1 μM-10 μM)-induced Ca^{2+} increase was only marginally blocked by the CB_1 antagonist, SR141716, insensitive to pertussis toxin and blocked by caffeine, suggesting the release of Ca^{2+} from caffeine-sensitive intracellular stores (Mombouli et al., 1999). In calf pulmonary endothelial single cells, AEA (10 μM) could initiate Ca^{2+} elevation via CB_2 receptors linked to the activation of PLC, and formation of IP_3. The PLC inhibitor, U73122 and the IP_3 receptor inhibitor, 2-APB prevented the effect of AEA. While the Ca^{2+} signalling in response to AEA was not sensitive to the CB_1 antagonist SR141716, the CB_2 antagonist, SR144528 produced an inhibition of the response. The involvement of the CB_2 receptor in these endothelial cells was confirmed by molecular identification using partial sequencing (Zoratti et al., 2003). Although CP55940 had no effect in our cells, this drug (2-50 μM) induced concentration-dependent Ca^{2+} release in Madin-Darby canine kidney cells, insensitive to cannabinoid antagonists, AM251 and AM281 (structural analogues of SR141716A). CP55940 exerted its effect by discharging intracellular Ca^{2+} in an IP_3-independent manner, as the response was not altered by U73122 (PLC inhibitor, inhibitor of IP_3 synthesis), and by inducing extracellular Ca^{2+} influx, as the response was significantly reduced in Ca^{2+}-free medium (Chou et al., 2001). More recently, our group has shown that in hamster vas

deferens smooth muscle cells, CP55940 (0.01-100 μM) induced a rise in $[Ca^{2+}]_i$ which was dependent on extracellular Ca^{2+}. CB_1 receptor-mediated outward current produced by CP55940 (10 μM) was markedly inhibited by Ca^{2+} influx inhibitors, La^{3+} and Ga^{3+}. Thapsigargin-sensitive stores were not influenced by neither La^{3+} nor Ga^{3+}, implicating a CB_1 receptor-mediated Ca^{2+} influx distinct from CCE. In addition, CP55940 evoked a pathway involving arachidonic acid formation which was most likely followed by activation of non-capacitative Ca^{2+} entry, through which arachidonic acid mediates Ca^{2+} influx (Demuth et al., 2005). In the present study, the negative effect of CPS is not consistent with the idea that 16HBE cells might express functional $TRPV_1$ receptors. We postulated these channels on the basis of a report published by Agopyan et al. (2003). Using RT-PCR they revealed the presence of $TRPV_1$ receptors in three types of epithelial cells (immortalized human bronchial epithelial cells, normal human bronchial/tracheal epithelial cells, normal human small airway epithelial cells from the distal airways) which responded to CPS by elevation in $[Ca^{2+}]_i$. The response was abolished in the presence of capsazepine, indicating $TRPV_1$-mediated effect (Agopyan et al., 2003). The reason we used the high-affinity CB_2 selective agonist, JWH133 was to exclude CB_2 receptor-mediated Ca^{2+} increase in 16HBE cells. As predicted, this compound was inactive in our cells. However, the result of Rao and Kaminski (2006) who showed a failure of JWH133 to elevate $[Ca^{2+}]_i$ in the CB_2 receptor-expressing T cell line was unexpected. In contrast, Δ^9-THC, CBN and HU210 evoked robust Ca^{2+} rise but not CBD or CP55940. They concluded that only tricyclic cannabinoids possessing a pyran ring are able to induce $[Ca^{2+}]_i$ elevation in T cells (Rao and Kaminski, 2006). CBD, induced a CB and $TRPV_1$ receptor-insensitive and concentration-dependent rise in $[Ca^{2+}]_i$ in the RBL-2H3 mast cell line (measured by flow cytometry) (Del Giudice et al., 2007), a finding

contradictory to our observation in the 16HBE epithelial cell line. Whether the 10 fold higher (100 μM) exposure of cells to VIR could produce a greater stimulation in $[Ca^{2+}]_i$ remains an open question. If it is the case, we might suggest an association between VIR-induced hyperpolarization and the proposed VIR-evoked $[Ca^{2+}]_i$ increase.

In conclusion, cannabinoids might be linked to cAMP changes in 16HBE cells, suggesting their anti-inflammatory potential in the bronchial epithelium, but we could not corroborate cannabinoid signalling involving CB receptor-mediated membrane current/membrane potential changes or intracellular Ca^{2+}. However, we have shown for the first time that VIR-induced hyperpolarization might be due to an elevation of $[Ca^{2+}]_i$ in 16HBE cells.

4.3 Conclusion

The aim of this study was to elucidate the possible role of cannabinoids in the airways by approaching different targets at tissue, cellular and molecular level. The investigation using the isolated tissue focused on the diverse cannabinoid pharmacology under normal and pathological conditions. The investigation using the cell line was intended to study signal transduction mechanisms of cannabinoids and their possible physiological role. The main findings are:

- In isolated guinea-pig bronchi, the non-selective cannabinoid agonist, WIN55212-2 (1 μM) probably exerts its inhibitory effect on sensory nerves through CB_2-like receptors, confirming a role of the cannabinoid system in airway sensory nerve function.
- The excitatory action of the endocannabinoid, VIR is mediated by $TRPV_1$ receptors on tachykinin releasing sensory nerve endings, unveiling its mechanism of action which is not distinct from AEA in the GPBP.
- The main non-psychoactive constituent of cannabis, CBD revealed multiple mechanisms of actions in guinea-pig bronchi. At a single concentration (1 μM) CBD antagonized AEA- and VIR-induced bronchoconstriction, the effects mediated by $TRPV_1$ and NK_2 receptors in the GPBP. At the same concentration CBD indirectly influenced NKA-related effects. CBD concentration-dependently (0.1-10 μM) modulated mast cell function in isolated bronchi of guinea-pigs.
- The main psychoactive constituent of cannabis, Δ^9-THC might activate sensory nerves via an unknown mechanism in the GPBP.
- CBD markedly reduced antigen-induced bronchoconstriction in an *in vitro* model of bronchial asthma, indicating its anti-allergic activity.

- Cannabinoids examined in this study were ineffective to induce signal transduction which would be linked to ion channel activity or to intracellular Ca^{2+} changes in human bronchial epithelial 16HBE cells.
- 16HBE cells express the CB_1 receptor but not the CB_2 receptor.
- 16HBE cells responded only to VIR which might trigger a CB_1 receptor-independent signalling pathway in these cells.

4.4 Further work

Some of the experiments in this study provided novel and interesting data which should undergo further investigations. These are highlighted below.

- Current data obtained with CBD (1 μM) in sensitized guinea-pigs, used as an asthma model, need to be completed. Essential experiments include testing the effects of the combination of antagonists: firstly, mepyramine and MK886 and secondly, CBD and MK886 against OVA responses in bronchial preparations from sensitized guinea-pigs. These findings might clarify the proposed synergistic action of released mast cell mediators reflecting the EAR.
- Further work would involve an investigation related to the putative interaction of CBD with adenosine signalling in the isolated guinea-pig bronchi. These studies might help to improve the understanding of adenosine functions in airways.
- Our data showed that CBD was able to antagonize the VIR-induced bronchoconstriction, suggesting GPR55 activity in isolated guinea-pig bronchi. As there are no well-validated GPR55 tool compounds, it is difficult to assess the proposed functional pharmacology of GPR55 in this bioassay. The detection of GPR55 mRNA expression pattern in guinea-pig airways and lungs using *in situ* hybridization or quantitative real-time PCR analysis respectively might reveal the putative importance of this orphan receptor argued as an additional CB receptor subtype in the airways.
- We compared the effects of two FAAH inhibitors (PMSF and URB597) on AEA-induced bronchoconstriction which are considered as potential therapeutic agents against inflammation. In addition, we postulated that CBD as a potent modulator of the endocannabinoid system, may contribute to the complexity of FAAH

inhibition in the GPBP. Further studies are needed to unveil the relevance of mechanisms of action of these FAAH inhibitors which might help in the development of anti-inflammatory treatment for respiratory conditions.

- Although there is no direct morphological evidence concerning the presence of CB_2 receptors in the respiratory tract, in guinea-pig airways functional studies revealed their involvement in the inhibition of sensory nerve function. The biosynthesis of AEA has been detected in rat and guinea-pig lung tissue. Because the endocannabinoid system is targeted for new anti-inflammatory interventions, more attention should be paid for this area. Our unsuccessful attempt to reproduce Yoshihara's finding (Yoshihara et al. 2005) of the ability of two exogenously applied endocannabinoid ligands (AEA and PEA) to inhibit the function of C-fibres via presynaptic CB_2 receptors would require repeated investigation. Thus, it would be feasible to verify the possible existence of an endocannabinoid tone by determining the effects of compounds inhibiting the endocannabinoid degradation (e.g. FAAH inhibitors) by their own (in the absence of an exogenous agonist) on EFS-evoked NANC contractions in isolated guinea-pig bronchi.
- One obvious area of further work would be to repeat the study investigating the action of Δ^9-THC against NKA-induced bronchoconstriction, and the possible involvement of CB receptors using the selective CB_1/CB_2 antagonists. It would be important to carry out a control study related to the effects of these selective CB_1/CB_2 antagonists by their own on NKA responses.
- The latter work could be extented by an investigation to explore the possible abilities of Δ^9-THC and CBD to presynaptically modulate sensory nerve activation of C-fibres which are involved in neurogenic inflammation of airways. It is well known that both compounds have anti-inflammatory effects and the

research should be directed towards identifying the mechanisms that underlie potentially beneficial effects in airway diseases.

- In addition to study of the possible modulation of NANC contractile response by CBD, it would be worthwhile to assess whether CBD could affect NANC relaxant response mediated by VIP and NO.

Future work could be bestowed to the investigation of cannabinoids and two cell types, mast cells and epithelial cells.

- Since it was identified that mast cell ion channels offer a novel target for attenuation of allergic disease, it may be particularly attractive to examine these cells electrophysiologically using the patch clamp technique. In support of this idea, it is desirable to increase the understanding of possible interactions between the endocannabinoid system existing in mast cells and signalling pathways which might be activated by cannabinoids.
- Immunomodulatory studies could bring clarification to the relationship between cAMP levels and cytokines secreted by mast cells and in this regard the role of cannabinoids could be assessed under physiological and pathological conditions.
- Further elucidation is required in immunomodulatory actions of cannabinoids in airway epithelial cells, whether stimulated cannabinoid signalling may have an impact on inflammation.

The most pronounced discovery of this thesis, the positive anti-allergic activity of CBD in an *in vitro* model of acute antigen-induced airway constriction representing the EAR of human asthma, requires more investigation. There are many studies which could reinforce this finding and would allow further extension of this observation by investigating other potential cellular mechanisms of CBD in the airways.

- First, the effect of antigen challenge on pulmonary cell influx measured from BAL fluid samples would be feasible in our conditions. This method could examine whether there are any significant differences between sham and OVA-immunized guinea-pigs in terms of total and differential BAL cell counts (neutrophils, eosinophils, mononuclear cells).
- Second, if there was markedly increased number of BAL cells in the group of sensitized guinea-pigs, it would be of value to test whether CBD could attenuate proposed cell accumulation in BAL fluid after acute exposure to antigen in sensitized guinea-pigs.
- Third, guinea-pigs used for BAL experimentation could be also used for the study of possible hyperactivity of ASM to cholinergic stimulation alone and *in vitro*. The effectiveness of CBD in the case of enhanced contractile responses to a muscarinic agonist would be desirable.
- Fourth, it would be interesting to find out whether the assumed BAL eosinophilia is accompanied by eosinophil activation measured as eosinophil peroxidase activity from the BAL fluid following acute OVA challenge of immunized guinea-pigs. In the case of positive results, the effect of CBD against eosinophilia could be assessed.
- Fifth, in the current experiments to confirm the results of functional studies (OVA-induced bronchoconstriction), sampling of bath fluid effluents would

provide a direct reflection of mediator secretion after stimulation in subsets of allergic asthma. Using high performance liquid chromatography (HPLC) releasability of major mast cell mediators under control conditions and after CBD treatment could be detected. The measurement would focus on LTs, their possible identification and quantitation in bath fluid samples.

- Sixth, as an alternative of the above could be that the bronchial tissue bath fluid might be analyzed by enzyme immunoassay to determine whether some of the key interventions affected the amount of released mediators during antigen-induced challenges.
- Seventh, Jan et al. (2003) demonstrated that cannabinoid treatment (Δ^9-THC and CBN) robustly attenuated Th2 cytokine (IL-2 and IL-4) mRNA expression and mucus production in lung tissues, and serum IgE production in the murine OVA model of allergic airway disease. It would be of interest to investigate the ability of CBD to reduce these hallmarks of asthma in OVA-immunized guinea-pigs.
- Eighth, if possible, it would be worthwhile to put into correlation the BAL findings with other features of clinical relevance in asthma, such as the *in vivo* measurement of airway responsiveness (airway resistance or airway conductance) to a spasmogen (e.g. methacholine or histamine) which would reflect the possible degree of acute airway hyper-responsiveness following challenge with inhaled OVA in the model of allergic asthma. Obviously, the effect of CBD would stay as a main concern. This technique would require collaboration with other well experienced laboratory with facilities suitable for working *in vivo*.
- Ninth, this study has not investigated the antitussive role of cannabinoids. Recent data mentioned in the discussion (see pages 224-226) suggest new perspectives and potential targets for peripherally acting drugs with the CB_2 receptor

selectivity. In addition, Lai and Lin (2005) employing a guinea-pig model of cough put forward an interesting postulation. They suggested that mast cells are involved in citric acid-induced cough via their mediators. Compound 48/80, cromolyn sodium, MK886, histamine (but not indomethacin or methysergide) significantly attenuated the citric acid-induced cough (0.6 M). In fact, in compound 48/80-pretreated guinea-pigs exogenously applied LTC_4 and histamine replaced the depleted mast cell mediators and there was a significant augmentation of citric acid-induced cough. Importantly, compound 48/80 blocked the citric acid-evoked elevated histamine plasma concentration (Lai and Lin, 2005). Based on their investigation and our observation of the ability of CBD to act on mast cells, a similar cough study could be carried out and using CBD its proposed antitussive activity involving afferent C-fibres could be tested.