Summary

*Cannabis sativa* has been used by humanity as a source of weaving material as well as a medicinal agent for thousands of years. In Chinese traditional medicine, cannabis has been used as a remedy for a variety of diseases, including asthma. This fact and other anecdotal reports suggested that (endo)cannabinoids might have a role in airway pathophysiology and furthermore, that they might have a therapeutic potential.

The identification of the first cannabis-derived compounds and its main psychoactive constituent ($\Delta^9$-tetrahydrocannabinol, $\Delta^9$-THC, dronabinol) in the 60’s re-initiated wide interest in the effects and actions of cannabis, primarily because of its use as a drug of abuse. However, soon afterwards, interest moved to the potential medical uses of the drug. In recent years, the discovery of the existence and expression of two cannabinoid receptors (CB$_1$-receptor and CB$_2$-receptor) and of various endogenous cannabinoids, like anandamide and virodhamine, acting on these receptors, suggested a much wider role in (patho-)physiology than ever suspected before. Both cannabinoid receptors have been shown to couple primarily to $G_{i/o}$-proteins and to activate MAPK and PLC signalling pathways. In addition, CB$_1$-receptor has been shown to couple to $G_s$ as well.

The wide range of effects of cannabinoids on intact organisms makes these studies complex to perform. Therefore, we aimed to investigate the effects of endocannabinoids and related synthetic compounds on cultured cells, simplifying the study models.

Smooth muscle and epithelial cells are prime structural cells of the airways. The airway diameter is controlled by the smooth muscle tone and by the thickness of the epithelial layer. In addition, epithelial cells are an important source of a variety of cytokines and chemokines, as well as of bronchodilatory nitric oxide. For these reasons the present thesis aimed to investigate the effects of (endo)cannabinoids on these cell types.

Cannabinoid-mediated changes of the intracellular $\text{Ca}^{2+}$-concentration ([Ca$^{2+}$]$_i$) via CB$_1$-receptors have been described in several cell types. In chapter 2 we attempted to identify the $\text{Ca}^{2+}$-influx mechanisms responsible for the CB$_1$-receptor-induced increase in cytoplasmatic [Ca$^{2+}$] observed in DDT$_1$ MF-2 cells,
an established smooth muscle cell line, following application of the synthetic cannabinoid CP55,940.

Previous studies on this cell had already shown that the phytocannabinoid Δ9-THC increased \([\text{Ca}^{2+}]\), presumably via stimulation of CB₁-receptors. It had also been shown that the synthetic cannabinoid CP55,940 evoked a transient outward current, as assessed by a whole cell version of the patch clamp technique, which was completely abolished by the removal of Ca\(^{2+}\) from the bathing solution, suggesting Ca\(^{2+}\)-influx. The CP55,940-induced Ca\(^{2+}\)-response appeared to be partly dependent on release from thapsigargin-sensitive intracellular stores as well, implying the involvement of a capacitative calcium entry mechanism (CCE).

We found that CP55,940 evoked a transient outward current which was very similar to histamine, which releases Ca\(^{2+}\) from InsP₃-sensitive stores and consequently induces CCE. In addition, CP55,940 concentration-dependently increased \([\text{Ca}^{2+}]\) in DDT₁ MF-2 cells, which was abolished by removal of extracellular Ca\(^{2+}\). Application of the InsP₃ receptor antagonist 2-APB, at a concentration which inhibits histamine-evoked currents, had no effect on the CP55,940-evoked outward current, suggesting that the CP55,940-induced increase of \([\text{Ca}^{2+}]\) was independent of InsP₃-sensitive stores. Similarly, another CCE inhibitor, SKF 96365, did not affect the CP55,940-evoked current either.

Recently, a non-capacitive calcium-entry (NCCE) pathway has been described, through which intracellular messengers such as arachidonic acid can mediate Ca\(^{2+}\)-influx. In DDT₁ MF-2 cells, arachidonic acid concentration-dependently increased \([\text{Ca}^{2+}]\). Pre-exposure of the cells to arachidonic acid inhibited the CP55,940-evoked current and the CP55,940-evoked current was independent of arachidonic acid metabolites as well, as revealed by inhibitors of the cyclooxygenase and 5-lipoxygenase pathways. Furthermore, we found that arachidonic acid is released in response to CB₁-receptor activation by CP55,940. This release was fully inhibited by the CB₁-antagonist SR141716A, the phospholipase A₂ (PLA₂) inhibitor quinacrine and the MAPK inhibitor PD98059, indicating that arachidonic acid is produced downstream of the CB₁-receptor, phospholipase A₂ and MAP kinases.

The non-selective Ca\(^{2+}\)-antagonists La\(^{3+}\) and Gd\(^{3+}\) inhibited the CP55,940-evoked currents at concentrations that had no effect on thapsigargin-evoked CCE. La\(^{3+}\) also inhibited the outward currents evoked by arachidonic almost completely, indicating that arachidonic acid production occurs downstream of Ca\(^{2+}\)-entry.
Quinacrine also inhibited the CP55,940-induced outward current, putting PLA₂ activation downstream of the Ca^{2+}-entry. The conclusion of this chapter was that in DDT₁ MF2 cells, arachidonic acid is an integral part of the CB₁-receptor signaling pathway, which acts upstream of NCCE, and downstream of MAP kinase-activated cPLA₂.  

In chapter 3 we aimed to identify the Ca^{2+} signaling pathways initiated in human bronchial epithelial cells (16HBE14o⁻ cells), following the administration of the phytocannabinoid of Δ⁹-tetrahydrocannabinol (Δ⁹-THC), the synthetic cannabinoid CP55,940 and the endocannabinoid virodhamine.  

We found that all three cannabinoids increased [Ca^{2+}]_i in 16HBE14o⁻ cells. The phytocannabinoid Δ⁹-THC appears to mobilize Ca^{2+} from intracellular stores and to induce subsequently Ca^{2+}-entry from the extracellular space, suggesting the involvement of a CCE mechanism. The non-selective cannabinoid agonist CP55,940 also induced Ca^{2+}-entry from the extracellular space and release from intracellular stores but to a much smaller extent than Δ⁹-THC. The Ca^{2+}- influx was inhibited by Ni^{2+} indicating the involvement of a CCE mechanism, possibility via the transient receptor potential (TRP) channel TRPC1. The endocannabinoid virodhamine induced Ca^{2+}-entry in these cells by two distinct pathways. At concentrations below 30 µM virodhamine responses were found comparable to CP55,940. At higher concentrations, however, an additional robust Ca^{2+}-entry occurred. The finding that these responses required significantly higher concentrations of virodhamine than for CB₂-receptor-mediated inhibition of cAMP accumulation (chapter 4), in combination with the steep Ca^{2+}-entry observed with 100 µM virodhamine, suggest a sustained opening of a CB-receptor-independent ion channel. This notion is supported by the fact that both the CB₁-receptor antagonist SR141617A and the CB₂-receptor antagonist SR144528 increased rather than decreased the Ca^{2+}-influx.  

Recently, endogenous cannabinoids have been shown to exert some of their effects via opening of specific and aspecific ion-channels, such as the channels from the TRP family. We found that 16HBE14o⁻ cells express a variety of TRP channels indeed. The Ca^{2+}-influx, observed following 30 µM virodhamine, was partially inhibited by the TRPV1 blocker capsazepine, but no effects of capsazepine at lower virodhamine concentrations were found. The TRPV1/TRPV4-inhibitor ruthenium red also partially inhibited the Ca^{2+}-influx, while...
the CCE-inhibitor Ni\textsuperscript{2+} had no effects. Possible involvement of these channels was substantiated by the detection of mRNA encoding for TRPV1 as well as TRPV4 in these cells. TRPC6 might also be involved in this process as this channel mediates NCCE and is highly sensitive to low concentrations of Gd\textsuperscript{3+} and La\textsuperscript{3+}, fitting well with our results. Accordingly, TRPC6 mRNA was expressed in 16HBE14o\textsuperscript{-} cells indeed.

Since arachidonic acid-mediated Ca\textsuperscript{2+}-entry has been implicated in NCCE in DDT\textsubscript{1} MF-2 smooth muscle cells (chapter 2), we also investigated the effect of virodhamine on arachidonic acid release from the epithelial cells. Virodhamine does indeed stimulate arachidonic acid release from these cells, but in contrast to the smooth muscle cells, this effect was not mediated by either the CB\textsubscript{1}- or the CB\textsubscript{2}-receptor. Furthermore, the source of arachidonic acid was unclear, since the phospholipase A\textsubscript{2} inhibitor quinacrine only partially reduced the observed arachidonic acid release. Collectively, the results described in this chapter indicate that the cannabinoids \(\Delta^9\)-THC, CP55,940 and virodhamine use different mechanisms of action to elevate intracellular [Ca\textsuperscript{2+}]. \(\Delta^9\)-THC, CP55,940 and low concentrations of virodhamine increase [Ca\textsuperscript{2+}] via mobilization and subsequent CCE, whereas higher concentrations of virodhamine increase [Ca\textsuperscript{2+}], via a NCCE dependent pathway.

A main signaling pathway used by cannabinoïd receptors is modulation of adenylyl cyclase activity. Both the CB\textsubscript{1}- and CB\textsubscript{2}-receptor are classified as pertussis toxin (PTX) sensitive and to couple to G\textsubscript{i/o}-proteins. However, in some systems cannabinoids have been shown to increase cAMP production through G\textsubscript{s}-linkage. To unravel the role of these signaling pathways in human bronchial epithelial cells we therefore investigated the effects of the endocannabinoid virodhamine and the synthetic cannabinoid CP55,940 on forskolin-induced cAMP accumulation in 16HBE14o\textsuperscript{-} cells in chapter 4.

We found that in this cell line both cannabinoid receptors were co-expressed, both on the mRNA and on the protein level. Virodhamine and CP55,940 inhibited forskolin-induced cAMP accumulation, although, unexpectedly, the action of CP55,940 was less pronounced compared to virodhamine. Furthermore, the inhibitory responses were mediated via the CB\textsubscript{2}-receptor, since they were blocked by the selective CB\textsubscript{2}-receptor antagonist SR144528. As the inhibition was PTX-sensitive, G\textsubscript{i/o}-proteins are involved. Interestingly, treatment with PTX
unmasked, at higher concentrations, a stimulatory component for both agonists. The latter effect was mediated via CB$_1$-receptors as it could be prevented by the selective antagonist SR141617A and most likely, involved coupling of the receptor with G$_s$-proteins.

The pro-inflammatory cytokine IL-8 is an important chemoattractant of neutrophils and has been implicated in the pathophysiology of chronic obstructive pulmonary disease (COPD) and severe asthma. In human colonic epithelial cells the selective cannabinoid agonist WIN55212-2 has been shown to reduce tumor necrosis factor-α (TNF-α) induced release of IL-8. Therefore we proceeded by investigating the effects of the cannabinoids on TNFα-induced IL-8 release in the human bronchial cells. We observed that virodhamine and CP55,940 inhibited TNFα-induced IL-8 release in concentrations similar to those causing CB$_2$-receptor-mediated adenylyl cyclase inhibition. Accordingly, this inhibition of IL-8 production was resistant to selective blockade by the CB$_1$-receptor antagonist SR141716A, strongly suggesting that attenuation of cAMP production by CB$_2$-receptors was involved. The selective CB$_2$-receptor antagonist could not be used because this compound by itself markedly reduced TNFα-induced IL-8 release from 16HBE14o$^-$ cells by a thus far unknown mechanism. These dual effects of virodhamine and CP55,940, as observed in the current chapter, show a complex role for modulation of adenylylcyclase and IL-8 production by cannabinoids and their receptors. The findings also suggest that the endocannabinoid virodhamine, via stimulation of CB$_2$-receptors, may exert anti-inflammatory effects in the airways by modulation cytokine release from the epithelium.

Cannabinoids appear to have dual effects on cell survival and viability. In some cases they have been reported to induce apoptosis and necrosis and in other cases to be protective against apoptotic stimuli, including oxidative stress. Regulation of cellular Ca$^{2+}$, acting either alone or in concert with cAMP, is of major importance to control cell survival, in particular under conditions of cellular Ca$^{2+}$ overload, as has been described for higher concentrations of virodhamine in chapter 4. In chapter 5 we attempted to investigate the mechanisms by which survival of human bronchial epithelial cells (16HBE14o$^-$) could be modulated by (endo)cannabinoids. The exact mechanisms are far from clear, they could involve mitogen-activated protein kinases (MAPKs) since the modulation of MAPK pathways is a known property of cannabinoids.
We found that at high concentrations (above 30 μM) virodhamine decreased cell viability and survival in a concentration- and time-dependent manner; lower concentrations were inactive. This decrease in cell survival was not dependent on coupling of the CB-receptors to G_{i/o}-proteins as pretreatment with PTX did not profoundly change virodhamine-induced attenuation of cell survival. However, the applied virodhamine concentrations induced Ca^{2+}-overload in 16HBE14o- cells in a time- and concentration-dependent manner, indicating that cellular Ca^{2+} overload might be responsible for its effects on cell survival. Although the selective antagonists of CB_{1}- and CB_{2}-receptors did not reduce virodhamine-induced Ca^{2+}-alterations (chapter 3) we were curious to learn about the role of the endogenous expression of CB_{1}-receptor and the CB_{2}-receptor by using small interference RNA (siRNA). Silencing of CB_{1}- and CB_{2}-receptors, however, did not dramatically alter virodhamine-induced Ca^{2+} responses or inhibition of adenylyl cyclase, indicating that these effects of virodhamine are less relevant for 16HBE14o- cell survival. These results are in accordance with findings described in chapter 3, showing that the linear intracellular Ca^{2+} increase induced by the higher concentrations of virodhamine is mediated via a non-CB_{1}-receptor, non-CB_{2}-receptor mechanism.

Interestingly, silencing of CB_{1}- and CB_{2}-receptor exerted opposite effects on virodhamine-induced attenuation of cell survival. CB_{1}-receptor silencing further decreased cell viability following virodhamine incubation, whereas CB_{2}-receptor silencing had the opposite effect. As it is generally accepted that members of the MAP-kinase family like ERK1/2 are involved in the control of cell survival, we studied ERK1/2 responses in 16HBE14o- cells as well. We found that, in accordance with previous studies suggesting that purinergic receptors control cell survival via ERK1/2, administration of ATP strongly increased ERK1/2 phosphorylation. Virodhamine alone, under basal conditions, did not induce ERK1/2 phosphorylation. However, virodhamine completely blocked ATP-induced ERK1/2 phosphorylation. Silencing of the CB_{1}- and CB_{2}-receptors reciprocally altered ERK1/2 responses. Silencing of the CB_{1}-receptor abolished ERK1/2 phosphorylation induced by all stimuli, while silencing of the CB_{2}-receptor unmasked a small stimulatory component of ERK1/2 phosphorylation by virodhamine, which was not dramatically altered in the presence of ATP. These results match the effects of virodhamine observed on cell viability, as described above.
Collectively, the results suggest that regulation of cell survival by virodhamine is independent of specific CB\textsubscript{1}-receptor- and CB\textsubscript{2}-receptor-induced changes in cAMP and [Ca\textsuperscript{2+}]. However, the opposite effects of CB\textsubscript{1} - and CB\textsubscript{2}-receptor mRNA silencing do indicate that CB\textsubscript{1} - and CB\textsubscript{2}-receptors reciprocally modulate virodhamine-induced attenuation of cell survival, and that such effects probably converge on the level of ERK1/2 activation.

The main conclusions of this thesis are the following:

- In DDT\textsubscript{1} MF2 smooth muscle cells the synthetic cannabinoid CP55,940 increases [Ca\textsuperscript{2+}] by a CB\textsubscript{1}-receptor-dependent signaling pathway which is mediated by arachidonic acid, causing a non-capacitive calcium entry (NCCE). The results also provided evidence for CB\textsubscript{1}-receptor mediated activation of PLA\textsubscript{2}, upstream of arachidonic acid generation and (presumably) downstream of MAP kinase activation.
- In 16HBE14o- bronchial epithelial cells, the cannabinoids Δ\textsuperscript{9}-THC, CP55,940 and (low concentrations of) virodhamine increase [Ca\textsuperscript{2+}], via mobilization from internal stores and subsequent capacitive calcium entry (CCE).
- Higher concentrations of virodhamine increase [Ca\textsuperscript{2+}], in these epithelial cells via a non-CB\textsubscript{1}-receptor, non-CB\textsubscript{2}-receptor dependent NCCE, presumably involving TRPV1, TRPV4 and TRPC6 channels.
- In these epithelial cells CB\textsubscript{1}- and CB\textsubscript{2}-receptors differentially modulate adenylyl cyclase. Virodhamine and CP55,940 predominantly inhibit cAMP accumulation, which is mediated by CB\textsubscript{2}-receptors, coupled to G\textsubscript{i/o}-proteins. After pertussis toxin treatment a G\textsubscript{s}-mediated activation of adenylyl cyclase was unmasked, enhancing cAMP accumulation.
- Activation of the dominant inhibitory CB\textsubscript{2}-receptor signaling pathway diminished cAMP accumulation and TNF\textgreek{a}-induced interleukin-8 (IL-8) release at similar cannabinoid concentrations.
- Cell survival of bronchial epithelial cells is attenuated by virodhamine, independent of CB\textsubscript{1}- and CB\textsubscript{2}-receptor mediated changes in cAMP levels and [Ca\textsuperscript{2+}].
- CB\textsubscript{1}- and CB\textsubscript{2}-receptors reciprocally modulate virodhamine-induced attenuation of cell survival; these effects converge on the level of ERK1/2 activation.