SUMMARY

G protein-coupled receptors (GPCRs) are essential in many physiological processes. Therefore, GPCR function is tightly controlled by a variety of mechanisms at various levels. In this thesis some of these regulatory mechanisms are studied, especially for some specific receptors important in the cardiovascular system.

In *chapter 1* the most important mechanisms to regulate GPCR function, including GPCR desensitization/resensitization and GPCR up-/down-regulation, are described with some specific examples of regulation in the cardiovascular system. In addition, this chapter gives an overview of a class of proteins important in the regulation of GPCR signalling, the RGS proteins, and their role in the cardiovascular system.

GPCR signalling is mostly initiated by binding of a ligand to the receptor. Alteration of ligand availability might be an important mechanism to control GPCR function. *Chapter 2* describes the impact of changes in S1P availability on S1P receptor-mediated signalling.

The S1P concentration was shown to decrease in time when added exogenously to cells and this decrease was dependent on the cell density. In addition, an incubation time of 60 minutes resulted in a lower potency for S1P to inhibit forskolin-induced cAMP accumulation than an incubation of 5 minutes in two different cell types. This time-dependent decrease in potency was not seen for the synthetic S1P receptor ligand FTY720-P. Lipid phosphate phosphatases might be partly responsible for the time-dependent decrease in S1P potency as inhibition by XY-14 reduced the potency shift, although it only slightly inhibited the decrease in S1P concentration in time. From these results we conclude that evaluation of the effects of S1P receptor signalling is affected by the decrease in S1P concentration when using long incubation times. Therefore, the S1P concentration has to be controlled properly in experiments with long incubation times.

Beside the ligand availability also the receptor availability is an important factor in GPCR function. Receptor availability can be changed by internalization and the subsequent recycling or degradation of the receptor. The immunosuppressive effect of the pro-drug FTY720 is thought to be the result of alterations in the S1P receptor availability.

In *chapter 3* internalization and reappearance at the membrane of $S1P_1$ and $S1P_3$ receptors is investigated. Both receptors, stably expressed in CHO cells, are internalized upon stimulation with S1P or FTY720-P in a time- and concentration-dependent manner. Similarly to previous studies in HEK293 cells, the $S1P_1$ receptor stably expressed in CHO cells is also differentially regulated by S1P and FTY720-P after internalization. S1P-induced internalization of the $S1P_1$ receptor is reversible whereas FTY720-P caused a sustained internalization. The

$S1P_3$ receptor, however, is not differentially regulated, as internalization of the $S1P_3$ receptor was reversible upon stimulation with either S1P or FTY720-P.

Another regulatory mechanism of GPCR function involves the regulation of signalling proteins, which might influence GPCR responsiveness. In *chapter 4* the role of Regulator of G protein Signalling (RGS) proteins in S1P receptor function is investigated in vascular smooth muscle cells (VSMCs) and transfected CHO cells. A down-regulation of RGS4, RGS16 and $S1P_1$ receptor mRNA was found in VSMCs during phenotypic modulation induced by culturing. Furthermore, RGS5 mRNA expression was transiently up-regulated whereas expression levels of RGS2, RGS3, the $S1P_2$ and $S1P_3$ receptor were unchanged. However, these major alterations in RGS expression did not affect the S1P-induced increase in $[Ca^{2+}]_i$ in VSMCs. Also, co-transfection of RGS2, RGS3, RGS4, RGS5 and RGS16 into cells stably expressing the $S1P_1$ or $S1P_3$ receptor did not alter the S1P-induced inhibition of cAMP accumulation. Similarly, the signalling induced by SEW2871, a selective $S1P_1$ receptor agonist, was not affected. The inhibition of cAMP accumulation by FTY720-P via the $S1P_1$ receptor, however, was significantly reduced by co-transfection with RGS5. From these data we conclude that the mRNA expression of the RGS proteins and S1P receptors are differentially regulated during phenotypic modulation of VSMCs. However, major alterations in RGS expression have only limited effects on S1P receptor function.

Chapter 5 describes the effect of the growth stimulatory factor S1P on mRNA expression levels of several RGS proteins in VSMCs. The expression of RGS2 and RGS16 mRNA was increased by S1P whereas FTY720-P stimulation only induced RGS16 mRNA expression. This indicates that the S1P-induced increase in RGS2 mRNA is mainly mediated by the $S1P_2$ receptor whereas the increase in RGS16 mRNA is not. Furthermore, the S1P-induced increase in RGS16 mRNA was $G_{i/o}$-dependent whereas the increase in RGS2 mRNA was not. These data indicate that S1P uses distinct S1P receptor subtypes and signalling proteins to alter RGS2 and RGS16 mRNA expression levels.

In *chapter 6* the effect of lipopolysaccharide (LPS) exposure on vascular contractility was studied as well as its effect on mRNA expression of RGS proteins. LPS exposure of rat aortic rings differentially affected contractile responses to phenylephrine, angiotensin II, endothelin-1 and serotonin. Phenylephrine- and angiotensin II-induced contraction was reduced upon LPS exposure whereas endothelin-1-induced contraction was unaffected. In contrast, serotonin-induced contraction was enhanced upon LPS exposure. Furthermore, LPS treatment caused a time-dependent increase in RGS16 mRNA expression in aortic rings and VSMCs, which was independent of the increased activity of iNOS. The expression of RGS2, RGS3, RGS4 and RGS5 mRNA was unaffected by LPS treatment. Since the differential regulation of contractile responses to vasoconstrictors upon LPS exposure cannot be explained completely by the

increased NO production, it is speculated that the increase in RGS16 probably contributes to these differential effects.

The final *chapter 7* places the findings described in this thesis into a broader context. Our studies have shown that GPCR signalling, in particular S1P receptor signalling, can be regulated by different mechanisms. Some of the regulatory mechanisms have been demonstrated to occur also under controlled experimental conditions and can have a great impact on the results. The importance of more insight in the regulatory mechanisms of GPCR signalling, especially in pathological conditions, is discussed.