Summary

α- And ß-adrenoceptors are members of a large family of cell surface receptors that, upon agonist-stimulation, couple with G-proteins to initiate the subsequent signal transduction pathway. Following the initial classification of adrenoceptors into α- and ß-subtypes in 1948, ß-adrenoceptors were further subdivided into $ß_1$- and $ß_2$-subtypes in 1967. This subdivision, however, soon became insufficient to account for the nature of the ß-adrenoceptors in rat adipose tissue, which appeared to comprise properties of both $ß_1$- and $ß_2$-adrenoceptors and hence was designated as a hybrid, or atypical, ß-adrenoceptor in 1974. It was only with the introduction of adipocyte-selective ß-adrenoceptor agonists in 1984 that the existence of atypical, or $ß_3$-type, receptors gained wide acceptance which ultimately led to the identification and cloning of the human $ß_3$-adrenoceptor gene in 1989. At present, atypical or $ß_3$-adrenoceptors have been described not only in white and brown adipose tissue, but also in smooth muscle from various parts of the gastrointestinal tract, as well as in heart, skeletal muscle, airways and blood vessels.

The molecular structure of the human $ß_3$-adrenoceptor corresponded for 50.7 and 45.5% to the human $ß_1$- and $ß_2$-adrenoceptor-structure, respectively, with the highest degree of homology within the seven membrane-spanning domains. Interestingly, differences with the 'classical' $ß_1$- and $ß_2$-adrenoceptors were most prominent in regions of the third cytoplasmic loop and the carboxy-terminal tail, regions which are involved in G-protein coupling and receptor regulation (desensitization). Due to the lack of recognition sites for phosphorylation by protein kinase A (PKA) and the presence of only few phosphorylation sites for ß-adrenoceptor kinase (ßARK), the $ß_3$-adrenoceptor was found to be relatively resistant to short-term agonist-induced desensitization. Reports on long-term regulation though have been less unequivocal, but recent studies showed a marked influence of the cellular background in which the receptor is expressed on the pattern of desensitization and downregulation.

Another challenging topic within the field of $ß_3$-adrenoceptor research comprises the occurrence of functional (and structural) differences among the $ß_3$-adrenoceptors (cloned and native) of various species. Whereas rodent $ß_3$-adrenoceptors generally

CL 316,243 and SR 58611, their efficacy in human tissues is usually low. Whether these differences originate from the - species-related and/or organ-related - existence of multiple $ß_3$-adrenoceptor subtypes, differences in (efficiency of) signal transduction (e.g. the activation of different adenylyl cyclases) and/or the different levels of receptor expression remains a matter of debate until the present day and a direct challenge for future research.

This thesis focusses on the $ß_3$-adrenoceptors in gastrointestinal smooth muscle of rat, guinea pig, and human origin: functional characterization and regulation are central themes which have been dealt with in the preceding chapters:

At first, the ß-adrenoceptor population mediating relaxation of rat oesophagus smooth muscle (muscularis mucosae) was investigated. The selective $ß_3$-agonist BRL 37,344 was the most potent of the agonists used and relaxations were not antagonized by micromolar concentrations of either the highly selective $ß_1$-adrenoceptor antagonist CGP 20712A nor the highly $ß_2$-selective antagonist ICI 118,551. In contrast, ICI 118,551 already at 100 nM caused moderate rightward shifts of the concentration-response curves (CRCs) to (-)-isoprenaline and the $ß_2$-selective agonists fenoterol and clenbuterol (accompanied by a steepening of the CRC with the latter two agonists), which however did not further increase at higher concentrations, indicating only a minor contribution of $ß_2$-adrenoceptors. CRCs to all agonists were markedly further shifted to the right at the high concentration of 100 µM ICI 118,551, indicating a major role for $ß_3$-adrenoceptors. Conclusively, it was shown that BRL 37,344-induced relaxations were mediated exclusively through $ß_3$-adrenoceptors, whereas (-)-isoprenaline-, fenoterol- and clenbuterol-induced relaxations involved predominantly $ß_3$-, but also $ß_2$-adrenoceptors (Chapter 2).

The oesophagus receives a dense innervation of excitatory motor cholinergic nerves, but is, at least in the rat, only sparsely supplied with adrenergic fibres. Since it was not known whether the ß-adrenoceptors in rat oesophageal muscularis mucosae receive an adrenergic innervation or not, the effects of extraneuronal and neuronal uptake inhibition by corticosterone and cocaine, respectively, on the (-)-noradrenaline-induced relaxation of rat oesophageal smooth muscle were investigated (Chapter 3). Cocaine and - to a lesser extent - corticosterone markedly potentiated the (-)-noradrenaline-induced relaxations, which indicates the presence of an adrenergic innervation indeed. Furthermore, in the presence of 1 µM ICI 118,551, i.e. a concentration which blocks virtually all of the $ß_2$-adrenoceptors, the CRCs to (-)-noradrenaline were identical to CRCs without the antagonist. These observations demonstrate for the first time that in

rat oesophageal muscularis mucosae, (-)-noradrenaline acts solely through $ß_3$-adrenoceptors and that these receptors are in close proximity of the sympathetic nerves, thereby further supporting the physiological role of the $ß_3$-adrenoceptor.

Previous studies in rat white adipocytes have suggested the existence of different, i.e. functional and less functional, cyclic AMP pools generated by ß-adrenoceptor stimulation with the $ß_3$-adrenoceptor being able to generate cyclic AMP in a region of the cell where it can more efficiently activate PKA involved in triglyceride activation than typical $ß_1$- or $ß_2$-adrenoceptors. Alternatively, the coexistence of cyclic AMP dependent and non-cyclic AMP dependent mechanisms were proposed. To differentiate between these possibilities the effects of cyclic nucleotide phosphodiesterase (PDE) inhibition on the ß-adrenoceptor mediated relaxation of rat oesophageal muscularis mucosae were investigated. After having established the principal PDE-isoenzyme in the oesophagus to be of the PDE IV-type, its selective inhibition by a low concentration of rolipram (10 nM) was investigated. Rolipram was expected to potentiate the $ß_3$-adrenoceptor mediated relaxation less than that of the $ß_2$-adrenoceptor in case of a non-cyclic AMP dependent mechanism being preferentially involved; alternatively with a more efficient cyclic AMP pool being involved, a stronger susceptibility towards inhibition of cyclic AMP breakdown was expected with the $ß_3$-adrenoceptor mediated response. However, both responses were equally potentiated thereby precluding a definite conclusion whether or not a different and/or a more efficient coupling between $ß_3$-adrenoceptor activation and functional response compared with the $ß_2$-adrenceptor may exist. An alternative explanation of the reported cAMP compartmentalization should be envisaged (Chapter 4).

Recent molecular and functional studies have indicated a relative resistance of the $ß_3$-adrenoceptor towards agonist-induced desensitization, when compared to $ß_1$- and $ß_2$-adrenoceptors. In view of these findings, it was highly interesting to investigate the adaptive effects of chronically elevated catecholamine levels, present in spontaneously hypertensive rats (SHR), on the functional responses mediated by $ß_2$- and $ß_3$-adrenoceptors which coexist in rat oesophageal muscularis mucosae. To this aim, relaxations to fenoterol and BRL 37,344 were performed on oesophageal smooth muscle strips from SHR with developing and established hypertension (i.e. at 8-10 and 22-24 weeks of age, respectively) and age-matched SHR, adrenodemedullated at 4 weeks of age. In 8-10 week old SHR, the typical shallow CRC of fenoterol, observed with normotensive Wistar rats - which results from the preferential stimulation of $ß_2$-adrenoceptors - was not found, but a steep CRC was observed instead. Furthermore,

low concentrations of ICI 118,551 (100 nM - 10 μM) hardly antagonized the fenoterol-induced relaxations, indicating the absence of a functional $ß_2$-adrenoceptor-response. In contrast, at 100 μM of the antagonist, a clear rightward shift was observed yielding a pA_2-value consistent with an unaltered nature of the $ß_3$-adrenoceptors. Apparently, the $ß_2$-adrenoceptors are desensitized in SHR, whereas the $ß_3$-adrenoceptors are unaffected as compared to Wistar rats. This was also reflected by the similar functional affinity of BRL 37,344 in normotensive Wistar rats and SHR. In the older animals, a similar picture was found, though also the $ß_3$-adrenoceptor response had decreased slightly; however, this was also found in adrenodemedullated animals of the same age indicating a mere age-related process. Interestingly, adrenal demedullation of SHR at 4 weeks of age (to deprive the animals from adrenal-derived adrenaline, which prevents hypertension development) completely prevented $ß_2$-adrenoceptor downregulation: both the shallow fenoterol-CRC, as well as substantial antagonism of ICI 118,551 at low concentrations were again observed, indicating a profound role for adrenaline in desensitizing the $ß_2$-adrenoceptor mediated responses. Thus, the $ß_3$-adrenoceptor remains refractory to desensitization, even under persistent elevated catecholamine levels, whereas the $ß_2$-adrenoceptor is readily downregulated (Chapter 5).

In guinea pig oesophagus smooth muscle, the presence of both α- and ß-adrenoceptors was acknowledged in 1965, but until now no attempts were made to characterize the ß-adrenoceptor subtype(s) involved. In the presence of prazosin and corticosterone (to block α-adrenoceptors and extraneuronal uptake, respectively), relaxations of a methacholine-induced contraction by (-)-isoprenaline, (-)-noradrenaline, fenoterol and Cc25 were all found to be mediated predominantly by $ß_1$-type adrenoceptors. The high (and similar) pA_2-values for the selective $ß_1$-antagonist ICI 89,406, competitively antagonizing (-)-isoprenaline- and (-)-noradrenaline-induced relaxations as well as the low pA_2-value (7.06 against (-)-isoprenaline) for ICI 118,551 would indicate the presence of a single population of $ß_1$-adrenoceptors. However, (-)-noradrenaline behaved only as a partial agonist. With fenoterol and its catecholamine isomer Cc25 biphasic CRCs were obtained and antagonism by ICI 118,551 resulted in Schild plots with slopes significantly different from unity, which most likely indicate the presence of a heterogeneous ß-adrenoceptor population. Interestingly, against a prostaglandin $F_{2\alpha}$-induced contraction of similar magnitude, Cc25-induced relaxations became monophasic and complete (like (-)-noradrenaline) and they were found to involve only $ß_1$-adrenoceptors. To investigate the putative involvement of $ß_3$-type adrenoceptors, a series of selective $ß_3$-adrenoceptor agonists were compared for their ability to induce

relaxation of guinea pig oesophageal smooth muscle. Their functional affinities decreased in the order CGP 12,177 > BRL 37,344 > SR 58611 > ZD 2079, whereas CL 316,243 and octopamine were inactive, indicative of at least some involvement of a $ß_3$-type adrenoceptor. In contrast, on rat oesophagus, the affinity order was found to be: CL 316,243=BRL 37,344 > SR 58611 > ZD 2079 > octopamine > CGP 12,177, which strongly suggests marked differences in the nature of the $ß_3$-adrenoceptors of rat and guinea pig oesophagus. This is supported by the observations that the putative $ß_3$-antagonist/partial agonist ZD 7114 and the non-selective ß-adrenoceptor antagonist carazolol, antagonizing CL 316,243 and BRL 37,344-induced relaxations of rat oesophagus smooth muscle, yet were found hardly active against CGP 12,177 and BRL 37,344-induced relaxations of guinea pig oesophageal muscularis mucosae (Chapter 6).

In Chapter 7, the nature of the ß-adrenoceptors mediating relaxation of human colon circular smooth muscle was investigated, performing cumulative concentration-response curves to (-)-isoprenaline, (-)-noradrenaline and a series of selective $ß_3$-adrenoceptor agonists on KCl- or $PGF_{2\alpha}$-contracted smooth muscle strips. Against a 30 mM KCl-induced contraction, (-)-isoprenaline behaved as a partial agonist; its relaxant responses being only slightly antagonized by the $ß_1$-selective antagonist CGP 20712A (10 nM to 100 µM) and to a limited extent by the $ß_2$-selective antagonist ICI 118,551 (10 nM to 10 µM), indicating at most a minor and limited involvement of $ß_1$- and $ß_2$-adrenoceptors, respectively. Relaxations to (-)-noradrenaline were antagonized by 1 µM CGP 20712A, yielding an apparent pK_B-value of 6.89, well below reported pA_2-values for this antagonist on $ß_1$-adrenoceptor preparations. On 10 µM $PGF_{2\alpha}$-contracted smooth muscle strips, however, (-)-isoprenaline and (-)-noradrenaline behaved almost as full agonists with a concomittant increase in pD_2-values. The rightward shifts induced by CGP 20712A were clearly increased with both agonists, indicating that the relative contribution of $ß_1$-adrenoceptors in the response had increased, whereas the role of the $ß_2$-adrenoceptors appeared to be diminished.

In the presence of CGP 20712A (1µM) and ICI 118,551 (10 µM), i.e. under virtually complete blockade of $ß_1$- and $ß_2$-adrenoceptors, respectively, both (-)-noradrenaline and (-)-isoprenaline were still able to induce complete relaxation of $PGF_{2\alpha}$-contracted smooth muscle. The $ß_3$-adrenoceptor agonists CL 316,243; CGP 12,177; SR 58611A; ZD 7114 and octopamine were inactive as smooth muscle relaxants of human colon. BRL 37,344 was inactive against a KCl-induced contraction, but induced some relaxation from a methacholine- or $PGF_{2\alpha}$-induced tone. ZD 2079 was the most potent $ß_3$-agonist studied, though pD_2-values were below 4.5.

It was concluded, that both $ß_1$-, $ß_2$-, and $ß_3$-type adrenoceptors mediate relaxation of human colon circular smooth muscle. The relative contribution of each subtype was found to depend on the nature of the contractile agonist applied. The behaviour of selective $ß_3$-adrenoceptor suggests the $ß_3$-type adrenoceptor mediating relaxation of human colon to be different from rodent native $ß_3$-adrenoceptors.

Finally, in Chapter 8, the putative desensitization of the oesophageal $ß_3$-adrenoceptors after exposure to $ß_3$-selective agonists, as described in several gastrointestinal preparations for BRL 37,344, was investigated. To this aim, the effect of repeated agonist exposure on $ß_2$- and $ß_3$-adrenoceptor functions in rat oesophageal muscularis mucosae was investigated by performing two consecutive concentration response curves (CRCs) on the same preparation using (-)-isoprenaline, fenoterol, (-)-noradrenaline and a series of $ß_3$-selective agonists. A first CRC to (-)-isoprenaline and fenoterol induced a clear desensitization of the $ß_2$-adrenoceptor population as second CRCs were significantly shifted to the right; in the case of fenoterol, subsequent CRCs also steepened. In the presence of 1 µM ICI 118,551 however, first and second CRCs were virtually identical, indicating no impairment of the $ß_3$-adrenoceptor mediated response. Even prolonged (1h) exposure to 100 µM (-)-noradrenaline, an agonist that mediates relaxation of rat oesophagus smooth muscle solely through $ß_3$-adrenoceptors (Chapter 3), did not change the position and slope of subsequent CRCs at all.

In contrast, the most potent selective $ß_3$-agonists BRL 37344, Cl 316,243, BRL 35135, and SR 58611A, all having the same *m*-chloro substituted phenylethanolamine moiety, were found to impair both the methacholine-induced contraction upon commencing the second CRC, as well as the specific $ß_3$-adrenoceptor mediated relaxation. However, when structurally different $ß_3$-adrenoceptor agonists - i.e. agonists lacking the *m*-chloro-substituent (BRL 35113, BRL 28410, BRL 27466, ZD 7114, and ZD 2079) - were applied, no apparent desensitization was observed at all. These findings strongly argue in favour of a compound-related effect, rather than an inherent feature of the $ß_3$-adrenoceptor itself. Thus, conclusively, $ß_2$-adrenoceptors are readily desensitized after exposure to isoprenaline or fenoterol, whereas the $ß_3$-adrenoceptor population is resistant to short term agonist-induced desensitization. The desensitization-like effects observed with BRL 37344 and similar $ß_3$-adrenoceptor agonists most likely reside in the chemical structure and appear to be related to the presence of the *m*-chloro substituent.

In conclusion, the presence of functional $ß_3$-type adrenoceptors has been demonstrated in the oesophagus muscularis mucosae of rat and guinea pig, as well as in human colon

circular smooth muscle. Remarkable differences were found in the nature and the relative functional contribution of the ß$_3$-adrenoceptors among the different species. Relaxations of rat oesophageal muscularis mucosae are mediated predominantly through ß$_3$-adrenoceptors similar to rat white adipocyte ß$_3$-adrenoceptors, showing low affinities for selective ß$_1$- and ß$_2$-antagonists and high potencies of selective ß$_3$-agonists. Under conditions of repeated agonist exposure or prolonged elevated plasma catecholamine-levels, the coexisting ß$_2$-adrenoceptors rapidly desensitize, whereas the ß$_3$-adrenoceptors are almost completely refractory towards desensitization / downregulation. Hence, under conditions of downregulated ß$_1$- and ß$_2$-adrenoceptor functions, the ß$_3$-adrenoceptor can still be active as an 'emergency' receptor.

In contrast to the rat, the typical ß-adrenoceptor population of the guinea pig oesophagus smooth muscle was found ß$_1$ in nature. Additional ß$_3$-type adrenoceptors were found active, the nature of which appearing different from those of the rat.

Human colon circular smooth muscle is occupied with all three ß-adrenoceptor subtypes, each capable of contributing to relaxation depending on the nature of the contractile agonist applied. Again, the ß$_3$-type adrenoceptor clearly showed functional differences with that of the rat.

Although different levels of receptor expression may have attributed to part of the described differences in the contribution of the ß$_3$-adrenoceptor populations in the response of the smooth muscles explored, further subdivision of functional ß$_3$-adrenoceptors in ß$_{3A}$, ß$_{3B}$, ...? seems appropriate, which in turn offers perspectives for the development of new generation(s) of selective ß$_{3...}$-adrenoceptor agonists.