β-Agonist-induced constitutive β2-adrenoceptor activitiy and desensitization in airway smooth muscle.
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The β₂-adrenoceptor is probably the best studied member of the G-protein-coupled receptor (GPCR) family. In airway smooth muscle, stimulation of the receptor by an agonist leads primarily to activation of adenylyl cyclase and production of cAMP, which in turn induces smooth muscle relaxation via the activation of protein kinase A (PKA) and subsequent phosphorylation of several target proteins [1,21].

In recent years it has become clear that GPCRs, including the β₂-adrenoceptor, exist in an equilibrium between an inactive R and a constitutively active R* state, which may couple to the G protein in the absence of an agonist [2,12,13,19]. Under basal conditions, constitutive β₂-adrenoceptor activity is low due to a conformational constraint that keeps the receptor in the inactive R state by preventing its interaction with Gs. Agonist binding, as well as mutations in the third intracellular loop of the receptor, shift the equilibrium towards R* by relieving this constraint [12,18]. Constitutive activity of the wild type β₂-adrenoceptor in the absence of agonist has clearly been visualized by overexpression of the receptor, which increases the absolute amount of R* [2]. Constitutive receptor activity may be inhibited by antagonists with negative intrinsic efficacy ('inverse agonists'), which shift the receptor from the active R* to the inactive R conformation [2,20]. Interestingly, for the µ-opioid receptor, another GPCR, it has been demonstrated that prolonged agonist stimulation may lead to induction of R*, which remains after washout of the agonist [25].

Continuous stimulation of GPCRs, including the β₂-adrenoceptor, leads to attenuation of the agonist response ('desensitization') by rapidly inducing a reduced coupling of the receptor with its stimulatory G protein ('uncoupling'), subsequently followed by a reduction of the receptor number ('down-regulation') [1,10,11]. A possible association between constitutive β₂-adrenoceptor activity and β₂-adrenoceptor desensitization has been indicated by the observation that a constitutively active mutant of the β₂-adrenoceptor was tonically desensitized, presumably by tonic phosphorylation of the receptor by β-adrenergic receptor kinase (BARK) [17]. In some systems, it has been demonstrated that prolonged activation of the β₂-adrenoceptor may also increase the expression and function of receptors that functionally antagonize β₂-receptor function [6,7,14].

β-Adrenoceptor agonists are the most effective bronchodilators in the treatment of asthma [16]. However, several observations have suggested that there may be long-term adverse effects associated with frequent use of these drugs. These adverse effects include the development of tolerance to the bronchodilator action of the agonists, a reduced protection of agonists towards bronchoconstrictor stimuli and an increased bronchial reactivity after cessation of the therapy [4] (Chapter 1).

The investigations presented in this thesis have been focused on the molecular mechanisms involved in the changes in airway smooth muscle responsiveness to β₂-adrenoceptor and contractile agonists after acute and chronic β-adrenoceptor stimulation...
with special reference to the role of the constitutively activated β2-adrenoceptor in these processes.

In Chapters 2, 3 and 4, the development of constitutive β2-adrenoceptor activity upon acute and prolonged β-agonist stimulation is described. It was demonstrated that preincubation of bovine tracheal smooth muscle (BTSM) strips with various concentrations (0.1, 1 and 10 μM) of fenoterol for various periods of time (5 min, 30 min and 18 h), followed by extensive washout (3 h), caused a rapid (within 5 min), time- and concentration-dependent inhibition of KCl-induced contraction. After 18 h of 10 μM fenoterol incubation the 40 mM KCl-induced contraction was inhibited by approximately 55% and the sensitivity of the strips to KCl was significantly reduced.

The reduced KCl contractions were fully reversed in the presence of the inverse agonist timolol (1 μM). Remarkably, the sensitivity to KCl in the presence of timolol was even significantly enhanced after fenoterol incubation. This corresponds with a previous study in SF9 cells overexpressing the human β2-adrenoceptor, which showed that β-agonist-induced desensitization induced by prolonged receptor stimulation was associated with increased inverse agonism by several β-adrenoceptor antagonists [3]. As in overexpressed SF9 cells, inverse agonism in β-agonist-treated BTSM was also found for other β-adrenoceptor antagonists, with a rank order of efficacy of pindolol > timolol > propranolol > alprenolol > sotalol > labetalol. Remarkably, this rank order did not correspond with the rank order of receptor occupancy by the antagonists used. At a 25 mM KCl-induced tone, the contraction induced by cumulative timolol administration was competitively antagonized by the less efficacious inverse agonist labetalol, clearly demonstrating that the fenoterol-induced effects cannot be explained by residual β-agonist binding.

Based on our findings and those of others, we proposed a theoretical model describing the sequential reactions involved in the β-agonist-induced development of constitutive β2-adrenoceptor activity, enhanced inverse agonism and desensitization. In this model, it is hypothesized that all these processes are initiated by rapid phosphorylation of the β2-adrenoceptor by βARK (Chapter 2).

In Chapter 3, we have shown that constitutive β2-adrenoceptor activity in BTSM is not only induced by fenoterol, but also by several other β2-selective and nonselective β-agonists, including salbutamol, terbutaline, orciprenaline, Cc25, isoprenaline and dichloroisoprenaline. Very remarkably, the full β-agonist isoprenaline was hardly effective to induce constitutive β2-adrenoceptor activity. Moreover, for all agonists no relationship was observed between the efficacy of the agonists to activate adenylyl cyclase and their effectiveness to induce constitutive β2-adrenoceptor activity. As described above, a similar lack of relationship between direct receptor occupancy and inverse efficacy was found with antagonists (Chapter 2). The results from the agonist studies also indicated that the development of constitutive activity is independent of cAMP and subsequent activation of PKA. This was confirmed by demonstrating that inhibition of phosphodiesterase activity by 3-isobutyl-1-methylxanthine (IBMX) as well as direct activation of adenylyl cyclase by forskolin did not induce constitutive
β₂-adrenoceptor activity. With different enantiomers of salbutamol, it was demonstrated that the induction of constitutive activity was restricted to the active (−)-enantiomer of the agonist.

As described in Chapter 4, we subsequently determined the effects of fenoterol-induced constitutive β₂-adrenoceptor activity on muscarinic agonist- and histamine-induced BTSM contractions. To this aim, BTSM strips were incubated with 10 μM fenoterol for 5 min, 30 min and 18 h. After extensive washout of the β₂-agonist, contractions were measured to the full muscarinic agonist methacholine, the partial muscarinic agonist McN-A-343 and histamine.

Fenoterol treatment significantly reduced the sensitivity to methacholine in a time-dependent manner, without affecting maximal contraction. Fenoterol treatment similarly reduced the sensitivity of McN-A-343 and histamine; however, maximal contractions to these agents were also significantly reduced to approximately 70% of control after 18 h treatment. These results may well be explained by the considerable receptor (i.e. transduction) reserve of the full muscarinic agonist methacholine with respect to contraction of BTSM, while histamine and the partial agonist McN-A-343 have only a partial or no reserve, respectively [15,22-24].

The inverse agonist timolol consistently restored the reduced sensitivity and maximal contractions of the agonists and even significantly enhanced the sensitivity to McN-A-343 and histamine in the fenoterol-treated airways. The latter effect indicates that enhanced inverse agonism by β-blockers after β-agonist treatment may cause hyperreactivity to bronchoconstrictive stimuli, and could explain the earlier observation that asthmatic patients develop enhanced airway hyperreactivity to propranolol after prolonged terbutaline treatment [8,9].

In Chapter 5 we tried to determine the molecular level at which fenoterol-induced constitutive β₂-adrenoceptor activity affects methacholine- and histamine-induced contractions. To this aim, inositol phosphate (IP) responses to the contractile agonists were measured in BTSM slices pretreated with vehicle or fenoterol for 30 min or 18 h, after extensive washout of the β₂-agonist.

The IP response to methacholine was not affected by 30 min of fenoterol treatment. However, after 18 h of treatment the methacholine-induced IP response was reduced by about 70% of control, with a reduced sensitivity to the agonist. The reduced IP response was not restored by timolol, indicating that the observed effect was not caused by constitutive β₂-adrenoceptor activity. For histamine, the IP response tended to be reduced already after 30 min and was significantly reduced to about 75% of control after 18 h of fenoterol treatment. In contrast to the change in methacholine-induced IP response, timolol reversed the IP response to histamine in both 30 min and 18 h pretreated tissues, indicating that constitutive β₂-adrenoceptor activity does restrain histamine-induced phosphoinositide metabolism. Thus, prolonged treatment of BTSM with fenoterol may reduce both methacholine- and histamine-induced IP responses, however, by apparently different mechanisms. While the reduced IP response to histamine may be due to induction of constitutive β-adrenoceptor activity, the reduced response to methacholine may be due to antagonism of muscarinic receptors.

Prolonged response by both agonist and antagonist in other systems receiving prolonged treatment has been observed [25]. The possibility that fenoterol-induced inverse agonism at the functional antagonist in isolated BTSM mediates maximal effects on fenoterol-induced tone would explain reduced sensitivity and maximal contractions of methacholine and histamine in fenoterol-treated airways. The latter effect is due to antagonism of inverse agonist fenoterol and helps explain why fenoterol treatment does not cause hyperreactivity. Regular treatment of BTSM with fenoterol may reduce both methacholine- and histamine-induced contractions.

Based on the findings in Chapter 5, we investigated the possibility that fenoterol-induced inverse agonism may affect the IP response to histamine. The IP response to histamine was significantly reduced after 18 h of fenoterol treatment. The reduced IP response was not restored by timolol, indicating that the observed effect was not caused by constitutive β₂-adrenoceptor activity. For histamine, the IP response tended to be reduced already after 30 min and was significantly reduced to about 75% of control after 18 h of fenoterol treatment. In contrast to the change in methacholine-induced IP response, timolol reversed the IP response to histamine in both 30 min and 18 h pretreated tissues, indicating that constitutive β₂-adrenoceptor activity does restrain histamine-induced phosphoinositide metabolism. Thus, prolonged treatment of BTSM with fenoterol may reduce both methacholine- and histamine-induced IP responses, however, by apparently different mechanisms. While the reduced IP response to histamine may be due to induction of constitutive β-adrenoceptor activity, the reduced response to methacholine may be due to antagonism of muscarinic receptors.

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demonstrated that fenoterol-induced bronchial hyperreactivity to histamine was reduced by 25% after 30 min of fenoterol treatment, while histamine-induced bronchial hyperreactivity was not altered. Prolonged β-agonist treatment has since long been known to result in attenuation of the response by uncoupling or down-regulation of the β-adrenoceptor. Furthermore, in some systems reciprocal regulation of functionally antagonizing receptors and/or G-proteins has been observed, including the muscarinic M2 receptor and Gβδ [5,6,18]. In Chapter 6, we investigated fenoterol-induced desensitization in BTSM both at the level of adenylyl cyclase activation and at the level of smooth muscle relaxation. In addition, we investigated the possibility that enhanced muscarinic M2 receptor function could contribute to a reduced functional antagonism of cholinergic tone by isoprenaline. Long-term (18 h) incubation of isolated BTSM cells with 10 μM fenoterol induced a modest desensitization of isoprenaline-induced adenylyl cyclase activity in BTSM membranes, characterized by a 25% decrease in maximal effect. Maximal isoprenaline-induced relaxation of a submaximal methacholine-induced tone was similarly reduced by 25%, which was associated with a significantly reduced sensitivity to the β-agonist. As determined by gallamine-induced M2 receptor antagonism and pertussis toxin-induced inactivation of Gβδ, M2 receptor-mediated functional antagonism did not play a role in BTSM relaxation to isoprenaline, both in control and 18 h fenoterol-treated tissue. In line with these observations, we found no enhanced M2 receptor-mediated inhibition of 1 μM forskolin-stimulated adenylyl cyclase activity after 18 h fenoterol treatment. Thus, enhanced muscarinic M2 receptor function is not involved in the reduced β-agonist-induced relaxation of cholinergic BTSM tone after fenoterol treatment.

Regular fenoterol inhalation also affected the airway responses to β-agonists in conscious and unrestrained guinea pigs, both in vivo and ex vivo (Chapter 7). Inhalation of clinically relevant doses of fenoterol 3 times daily did not change the airway reactivity to histamine in vivo as well as histamine-induced tracheal smooth muscle contraction ex vivo after 1 and 10 days of treatment with the β2-agonist. However, the acute protective effect of fenoterol against histamine in vivo was considerably reduced already after 1 day of treatment (3 doses of the drug), which did not decrease further after 10 days. Remarkably, one day of fenoterol inhalation did not result in desensitization of isoprenaline-induced tracheal smooth muscle relaxation ex vivo, while after ten days of treatment this response was significantly reduced. The dissociation between the in vivo and ex vivo results indicates that desensitization of airway smooth muscle is not critically involved in the development of tolerance to β2-agonist-induced protection against histamine induced by fenoterol.

**Summary**

Based on the research described in this thesis, it can be concluded that β-agonist treatment of BTSM induces a constitutive, i.e. agonist-independent, β2-adrenoceptor activity in this tissue, causing functional antagonism of both receptor-independent (KCl) as well as...
Constitutive activity can be induced by a variety of β-agonists, in a cAMP-independent manner, and is inhibited by β-adrenoceptor antagonists with different efficacies of inverse agonism. Fenoterol-induced constitutive β2-adrenoceptor activity in BTSM does not affect the IP response to methacholine, but reduces the IP response to histamine. The same effects are observed for acute β-agonist administration. Prolonged (18 h) fenoterol treatment of BTSM does reduce the methacholine-induced IP response, presumably by down-regulation of (one of the) components of its signal transduction cascade.

Prolonged treatment of BTSM with fenoterol leads to desensitization of the β-adrenergic response in this tissue, causing reduced functional antagonism of methacholine-induced contraction. No evidence was found for a role of enhanced muscarinic M2 receptor function in the reduced relaxation of cholinergic tone.

Regular inhalation of clinically relevant doses of fenoterol by conscious, unrestrained guinea pigs during 1 to 10 days does not affect the airway reactivity to histamine, but rapidly reduces the protection against histamine-induced bronchoconstriction by the β-agonist. The development of reduced bronchoprotection against histamine is not paralleled by the development of reduced relaxability of airway smooth muscle, suggesting that induction of tolerance to mechanisms other than β-agonist-induced bronchodilation is more important.

**REFERENCES**