Functional characterization of inverse agonists at the histamine H1 receptor

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The histamine H1 receptor (hH1r) is expressed on cells of the immune system like eosinophils and mast cells as well as neuronal cells [1,2] and is involved in the pathogenesis of pruritus [1-3]. Accordingly, hH1r antagonists may become important drugs for the therapy of pruritus [3]. When co-expressed with Gαq4 in Sf9 insect cells, the hH1r shows high-affinity inverse agonist activities in the steady-state GTPase assay. This suggests that inverse agonists rather than neutral antagonists should be used to treat hH1r-associated diseases. We investigated a series of hH1r antagonists (a, X = CH) and inverse agonists (b) that are derived from the prototypical hH1r antagonist JNJ-7771120 (1-(5-Chloro-1H-indol-2-yl)(carbonyl)-4-methyl-piperazine) [4]. We performed steady-state GTPase assays with Sf9 insect cell membranes co-expressing the hH1r with Gαq4 and Goαq4. Thienopyrrole, the most efficacious inverse hH1r antagonist known, was used as reference. Most compounds behaved as partial inverse agonists or dual agonists/antagonists in a range of 20-80% of the thienopyrrole effect. Interestingly, substitution of R1 by -Br in the indole compounds (R = H: JNJ-7781111) or of R3 by -CH3 in the thienopyrrole derivatives (R1 = H: JNJ-1806545, R3 = Cl: JNJ-1940637) resulted in neutral antagonism. Surprisingly, the Kd values of most inverse agonist compounds determined in the presence of histamine (100 nM) were much lower than the EC50 values determined in the absence of histamine. This indicates that the histamine-stabilized active hH1r state shows a higher affinity to inverse agonists than the constitutively active agonist-free hH1r state. Our results provide first hints for structure-activity-relationships of hH1r antagonists at the hH1r and show that the hH1r adopts distinct active states that can be differentiated from each other by their different affinity profiles.


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Effects of thiazolidinediones on S1P receptor expression in renal mesangial cells

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Since peroxisome proliferator-activated receptors (PPARs) were discovered, they have evolved from another member of the nuclear hormone receptor family to an extremely important set of targets for drug discovery. Three isoforms of PPAR, namely PPARα, PPARβ/δ and PPARγ are characterized. PPARγ is a ligand-induced transcription factor that can be activated by thiazolidinediones, fatty acids and eicosanoids and regulates the expression of target genes by binding to DNA sequence elements as a heterodimer with the 9-cis retinoic acid receptor. PPARα has been shown to play an essential role in the regulation of adipogenesis, immune response, insulin sensitivity, and glucose homeostasis. In addition to their classic role, PPARs are also capable of ameliorating glomerulosclerosis and kidney dysfunctions in diabetic nephropathy but also exert beneficial effects in non-diabetic chronic kidney disease. In addition, S1P receptor signaling in mesangial cells can be associated with progression of chronic kidney disease. Furthermore, renal mesangial cells play a central role in most pathological processes of the renal glomerulus. To evaluate a possible correlation between PPARγ activation and S1P receptor signaling in association with the development of chronic kidney diseases, we investigated the effect of PPARγ ligands (troglitazone, rosiglitazone) on the expression and relevant downstream effects of S1P receptors in mesangial cells. In this study, we show for the first time that thiazolidinediones are able to increase the relative mRNA levels of S1P receptors in rat mesangial cells, indicating a possible relationship between PPARγ activation and S1P receptor expression.

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36 Site-specific two color labeling of proteins with FlAsH and ReAsH living cells

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Specific labeling of two proteins simultaneously in living cells with two different fluorophores, we observed a higher affinity for the FLNCCPGCCMEP motif. For both pairs, the Trp 7.35 pocket. Archetypal muscarinic allosteric modulators are subtype-selective with a high degree of subtype-specificity. We hypothesized that the interaction of the allosteric site with the orthosteric ligand may be important for the selectivity of muscarinic antagonists. To test this hypothesis, we investigated the interaction of thiazoline with the orthosteric GABA receptor (hM2). The orthosteric antagonist oxotremorine (OXO) and the inverse agonist N-methyl scopolamine. Receptor binding of the hM2 receptor was studied using radioligand-binding assays (0.07nM [35S]GTPγS-binding assay (0.07nM [35S]GTPγS, 10 µM GDP, 100 mM HEPS, 10 mM MgCl2, 100 mM NaCl, pH 7.4, 30°C). Incubation was terminated after 1 h by vacuum filtration and [35S]GTPγS-binding was measured. The potency of acetylcholine for G protein-activation amounted to pEC50 = 7.35 at the beginning of TM 7 provides subtype-independent baseline affinity for various allosteric agents [1]. In addition we found for the hM2 receptor that this epitope (M2-104Tyr→Ala) is sensitive to the allosteric double mutant M2-104Tyr→Ala, binding affinity of the antagonist hybrid was significantly reduced toward the wild-type M2. In conclusion, although sharing an orthosteric binding pocket with the agonist block, the antagonist hybrid fails to exploit M2 selectivity-providing amino acids of the allosteric site. [1] Antony et al. (2009) FASEB J. in press

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37 Muscarinic receptor interaction of novel allostERIC/orthostERIC antagonist ligands

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The ligand binding pocket of the muscarinic acetylcholine receptor subtypes contains a less well conserved allosteric domain that is located extracellularly to the highly conserved orthosteric binding site. Archetypal allosteric modulators such as bis(amino)alkyl-type compounds display high binding affinity for the allosteric site, whereas the orthosteric site is least preferred for M2. In an attempt to exploit the allosteric site for subtype-selectivity and the orthosteric site for high affinity, allomERIC/orthostERIC hybrid compounds were designed. In the present study we aimed at elucidating the role of Trp 7.35 for acetylcholine action on the M2 receptor. Trp 7.35 is critical for the activation of acetylcholine at the M2 subtype of the M2 receptor (hM2). The transcription factor NF-kappaB and the inhibition of NF-kappaB by the allomERIC/orthostERIC hybrid provides neuroprotection against glutamate-induced neurotoxicity to the expression of small conductance calcium-activated potassium (KCa) channels. Small conductance KCa channels are functionally coupled with the M2 subtype of the muscarinic acetylcholine receptor (hM1) and M3 receptors and their activity modulates the shape of excitory postsynaptic potentials (EPSPs). By regulating after-hyperpolarization currents and reducing NMDA-receptor activity in a

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38 Role of the muscarinic allostERIC site for receptor activation: the conserved orthosteric binding site


We have previously shown that tumor necrosis factor-alpha (TNF-α) induces neuroprotection against excitotoxic damage in primary cortical neurons via sustained nuclear factor-kappa B (NF-κB) activation. The transcription factor NF-κB can regulate the expression of small conductance calcium-activated potassium (KCa) channels. Small conductance KCa channels are functionally coupled with the M2 subtype of the muscarinic acetylcholine receptor (hM2) and M3 receptors and their activity modulated the shape of excitatory postsynaptic potentials (EPSPs). By regulating after-hyperpolarization currents and reducing NMDA-receptor activity in a

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variety of neurons, small conductance K<sub>a</sub> channels may reduce neuronal excitability and as such may yield neuroprotection against neuronal overstimulation. In the present study, we also show that activation of small conductance K<sub>a</sub> channels may also improve cell survival under stress-related circumstances. In addition we studied whether activation of TNF-α-mediated neuroprotective signaling is inducing changes in the expression of several known key neuronal and glial cell markers. To this end, we induced activation of small conductance K<sub>a</sub> channels by bath application of xanomeline-like moieties in addition to tacrine. [1] Tränkle et al., J Neurosci. 23:8903-10, 2003. In contrast, analysis of the P2X subunit expression revealed a significant upregulation of the P2X<sub>3</sub> receptor subunit in KO versus WT mice. In conclusion, the transcription factor CREB is critical for cardiac deterioration after β-adrenergic stimulation, suggesting CREB as a transcription factor involved in the pathophysiology of heart failure. (Supported by the [1]. Institute of Pharmacology and Toxicology, University Münster, Germany.

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Transcription factor CREB is critically implicated in β-adrenergic receptor-mediated detrimental cardiac effects. Small conductance K<sub>a</sub> receptor activation of primary cortical neurons with TNF-α increases K<sub>a</sub> channel expression, which renders neurons more resistant to excitotoxic cell death. Small conductance K<sub>a</sub>-mediated neuroprotection effect place small conductance K<sub>a</sub> channels as possible targets for the treatment of disorders linked to neuronal hyperexcitability.

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Diagnostics of cardio-stimulatory beta<sub>1</sub>-receptor antibodies and evaluation of their blockade by novel cyclopeptides using FRET-microscopy


Background: Dilated cardiomyopathy (DCM) is one of the main causes of severe heart failure in young adults, leading to progressive dilatation and pump failure of the heart. Up to 50% of DCM-patients develop agonist-like autoantibodies against the second extracellular loop of the β1-adrenergic receptor [β1-ECII-ABS]. These functionally active antibodies may cause DCM in the rat. Here we attempt to block their harmful stimulatory effect in vitro by different cyclopeptides mimicking β1-ECII. Methods: Recently, we developed a new diagnostic method to detect functionally active β1-ECII-ABS using HEK-293 cells stably expressing the human β1-adrenergic receptor and an Epac1-based cAMP-sensor. Upon β1-ECII-AB-induced stimulation, intracellular cAMP increases. Generated cAMP binds to the sensor resulting in conformational changes and thereby decreases fluorescence resonance energy transfer (FRET) between its chromophores CFP and YFP. The β1-ECII loop contains 3 cysteine residues. Two of them are in an intracytoplasmatic bridge, one creates a cysteine bridge between β1-ECII and the third transmembrane domain (TM3) of the receptor. Two different ECII homologous 18α (amino-acid) cyclopeptide-mutants (18αCys/Cys/Ser and 18αCys/Ser/Cys) have been tested for their capability of blocking activating antibodies. Results: 18αCys/Cys/Ser efficiently blocked β1-ECII-ABS in n=8/15 immunized rats. Only 4 animals exhibited β1-ECII-ABS that were blocked by either 18αCys/Cys/Ser or 18αCys/Ser/Cys, as determined by ELISA. In most animals with the Cys/Ser/Cys- cyclopeptide (CP) ELISA signals were blocked by 62±3% compared to only 2±1% with the Cys/Ser/CP. Regarding functional activity, 18αCys/Cys/Ser/CP also efficiently blocked the stimulatory effect of polyclonal rat and monomolecular mouse β1-ECII-ABS in our novel FRET assay, whereas a scrambled peptide (containing the same aa in a randomized order) had no significant antibody-blocking effect. Conclusion: 18αCys/Cys/Ser/cyclopeptides, containing an intraloop-bridge between the first and second cysteine of β1-ECII, exhibit a markedly superior blocking efficacy of functional β1-ECII-ABS compared to 18αCys/Ser/Cys/CP. Our results fit well with the recently published crystal structure of the β1-adrenergic receptor. Our data suggests that functionally active β1-ECII-ABS recognize conformational epitopes within the β1-ECII loop, which can be mimicked by specific cyclopeptides in order to scavenge such harmful antibodies.

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Defects in lung development of α2<sub>1</sub>-adrenergic-deficient mice

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α2<sub>1</sub>-adrenergic receptors are not only essential presynaptic regulators of norepinephrine release from sympathetic nerves but also influence developmental signaling pathways and angiogenesis. Previous studies have suggested that α2<sub>1</sub>-adrenergic receptors are essential for development of the placental vascular system between embryonic days E10.5 and E12.5. Thus this study was initiated to determine the significance of α2<sub>1</sub>-adrenergic receptors for embryonic and perinatal development in mice. α2<sub>1</sub>-deficient mice which survived the embryonic period were lost during the first 24 hours of life. These α2<sub>1</sub>-"mice" were characterized by reduced body weight and dwarfishness, immediately postnatally breathing rate, drinking behavior, cardiac rhythm and heart rate were not altered in α2<sub>1</sub> vs. α2<sub>1</sub> mice. Within the first hours after birth, α2<sub>1</sub> mice rapidly developed cyanosis. Histological analysis of heart revealed differences between genotypes. Thorough investigation of heart, liver, gastrointestinal system and kidney from α2<sub>1</sub> newborn mice did not reveal any morphological defects. Postnatal clorotic rates of the ductus arteriosus did not differ between the genotypes, microscopical analysis of the lung was severely altered in α2<sub>1</sub> newborn mice. Alveolar spaces were significantly reduced and alveolar septa were abnormally thickened, indicating that the final phase of alveolarization did not occur. Several key factors required for lung development were differentially expressed between newborn α2<sub>1</sub> and α2<sub>1</sub> mice. α-smooth muscle actin and sonic hedgehog (shh) were