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Title page

A new molecular mechanism to engineer protean agonism at a G protein-coupled receptor

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Running Title Page

Designed dualsteric ligands act as protean agonists at M_2.

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Discussion: 1553 words

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List of nonstandard abbreviations:

[^{35}S]GTPγS: Guanosine 5'-O-(gamma-[^{35}S]thio)triphosphate)

[^{3}H]NMS: [^{3}H]*N*-methylscopolamine

5-HT$_{1B}$R: serotonin receptor 1B

ACh: Acetylcholine

Atr: atropine sulfate

B_2R: bradykinin receptor 2

BSA: bovine serum albumine

CB_2R: cannabinoid receptor 2

CHO-hM_2: CHO stably expressing the human muscarinic acetylcholine M_2 receptor

DTT: DL-dithiothreitol

FCS: fetal calf serum

Flp-In-CHO: Flp-In-Chinese hamster ovary cells

G proteins: guanine nucleotide-binding proteins

GPCRs: G protein-coupled receptors

H_3R: histamine receptor 3

Ham's F-12: Ham's nutrient mixture F-12

iper: iperoxo

M_2AChR: muscarinic acetylcholine receptor 2

mAChR: muscarinic acetylcholine receptor

NMDG: *N*-methyl-D-glucamine

OOM: oxo-oxotremorine M

α_{2A}AR: alpha-2A adrenergic receptor

β_2AR: beta-2 adrenergic receptor

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Abstract

Protean agonists are of great pharmacological interest as their behavior may change in magnitude and direction depending on the constitutive activity of a receptor. Yet, this intriguing phenomenon has been poorly described and understood, due to the lack of stable experimental systems and design strategies. Here, we overcome both limitations: First, we demonstrate that modulation of the ionic strength in a defined experimental set-up allows for analysis of GPCR activation in the absence and presence of a specific amount of spontaneous receptor activity using the muscarinic M_2 acetylcholine receptor as a model. Second, we employ this assay system to show that a dualsteric design principle, i.e. molecular probes, carrying two pharmacophores to simultaneously adopt orthosteric and allosteric topography within a G protein-coupled receptor, may represent a novel approach to achieve protean agonism. We pinpoint three molecular requirements within dualsteric compounds that elicit protean agonism at the muscarinic M_2 acetylcholine receptor. Using radioligand binding and functional assays we posit that dynamic ligand binding may be the mechanism underlying protean agonism of dualsteric ligands. Our findings provide both new mechanistic insights into the still enigmatic phenomenon of protean agonism and a rationale for the design of such compounds for a G protein-coupled receptor.

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Introduction

G protein-coupled receptors (GPCRs) comprise the largest superfamily of cell-surface receptors, accounting for 3% of all genes in the human genome (Fredriksson and Schiöth, 2005). Upon activation, GPCRs undergo a global conformational rearrangement to transduce their signals from extracellular stimuli into the intracellular environment. *Via* this mechanism, binding sites for cytosolic adaptor proteins, for instance heterotrimeric G proteins, become exposed (Rasmussen, et al., 2011; Kruse et al., 2013), which then serve to propagate the signaling within the cell (Pierce et al., 2002; Lefkowitz, 2004). GPCRs are versatile membrane proteins that exist in ensembles of possible conformations (Manglik and Kobilka, 2014) and ligands can be classified according to their ability to stabilize subsets of these conformations (Rosenbaum et al., 2009). For instance, agonists favor active receptor conformations over inactive states. Of note, receptors can also spontaneously transit from inactive to active states and *vice versa* (Costa and Herz, 1989; Leff, 1995).

In addition to the binding site for the endogenous messenger, GPCRs carry distinct druggable sites, which are designated as *allosteric* sites. Compounds binding to these allosteric binding sites may alter the efficacy and/or binding affinity of orthosteric ligands but may also exhibit intrinsic efficacy for receptor activation/inactivation in their own right (Christopoulos, 2002). Allosteric modulation has extensively been studied at muscarinic acetylcholine receptors (mAChRs) which are prototypal class A ("Rhodopsin-like") GPCRs. Recent studies have also described *dualsteric* ligands which can simultaneously occupy the orthosteric and an allosteric binding site of the M_2 subtype of mAChRs. This results in a unique receptor binding profile and signaling pattern (Steinfeld et al., 2007; Gregory et al., 2010; Bock et al., 2012; Schrage and Kostenis, 2016). Dualsteric/bitopic ligands can switch between two binding poses, i.e. either a dualsteric or a purely allosteric binding pose (Bock et al., 2014; Bock et al., 2016) and thus may stabilize not only one but at least *two* distinct subsets of receptor conformations (dynamic ligand binding) (Bock et al., 2014).

A particularly fascinating, albeit seldom described, class of GPCR ligands are protean agonists, i.e. compounds which display agonism in quiescent receptor systems with low levels of spontaneous receptor activity and inverse agonism in constitutively active systems (Kenakin, 1995). One explanation for this phenomenon may be that protean agonists stabilize a receptor conformation with lower efficacy for a certain signaling pathway than the spontaneously active conformation of a given GPCR. Thus, even though the intrinsic efficacy of the ligand does not vary, its effect changes with the amount of spontaneously active receptors that are present within the experimental system (Kenakin, 1995, 1997).

Up to now, protean agonists have only been identified for a handful of receptors, for instance cannabinoid receptor 2 (CB_2R) (Yao et al., 2006; Mancini et al., 2009; Xu et al., 2010; Bolognini et al., 2012), histamine receptor 3 (H_3R) (Gbahou et al., 2003), alpha-2A adrenergic receptor ($\alpha_{2A}AR$) (Jansson et al., 1998; Pauwels et al., 2002), serotonin receptor 1B ($5\text{-}HT_{1B}R$) (Newman-Tancredi et al., 2003), bradykinin receptor 2 (B_2R) (Fathy et al., 1999; Marie et al., 1999) and beta-2 adrenergic receptor (β_2AR) (Chidiac et al., 1994; Chidiac et al., 1996). Nevertheless, protean agonists are highly valuable tools as they might be most useful for the identification of ligand-specific active states of GPCRs (Kenakin, 2001). Furthermore, protean agonists may represent a promising new class of pharmacologically active compounds in targeted drug therapy for those cases in which receptor mutations cause constitutive activity (Tao, 2008). Under these conditions, protean agonists can re-establish the original tone of receptor activation (Jansson et al., 1998; Mancini et al., 2009). Moreover, the classification of ligands as protean agonists is of significant importance and not trouble-free, because they may be mistaken for inverse agonists in *in vitro* assays with high levels of constitutive activity (Chidiac et al., 1994), but act as agonists *in vivo* (Yao et al., 2006; Mancini et al., 2009; Xu et al., 2010).

The rather few examples of GPCR protean agonists are, at least in part, due to the lack of experimental systems in which the amount of spontaneous GPCR activity is reliably and reproducibly controlled. Moreover, specific design strategies to generate protean ligands are missing. In this study, we overcome both limitations: we have developed an assay system to enable such control and employ it here to

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demonstrate that the dualsteric design approach may serve as a novel means to rationally create protean agonists using M_2AChR as a model system.

In particular, we establish and validate experimental conditions that permit analysis of M_2 receptor-mediated G_i protein activation, in the absence and presence of a defined amount of spontaneous receptor activity. We found that: first, dualsteric ligands were able to induce protean agonism at M_2AChR. Second, we identified specific pharmacophoric elements which have to be combined to induce protean agonism at this receptor subtype. Third, we propose that dynamic ligand binding (Bock et al., 2014; Bock et al., 2016) may be the underlying molecular mechanism of protean agonism of dualsteric compounds targeting M_2AChRs.

Therefore, our findings deliver a new experimental system to study spontaneous activity of GPCRs, two new protean agonists for M_2AChR, and a new strategy along with a potential molecular mechanism to achieve protean agonism at this receptor subtype, which may pinpoint to the design of protean agonists at other GPCRs.

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Materials and Methods

Materials and reagents

Acetylcholine (ACh) iodide, atropine sulfate (Atr), and pilocarpine hydrochloride were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Guanosine 5'-O-(gamma-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) and [^{3}H]*N*-methylscopolamine bromide ([^{3}H]NMS) were from PerkinElmer Life and Analytical Sciences (Homburg, Germany). Cell culture media, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), *N*-methyl-D-glucamine (NMDG), sodium bromide (NaBr), bovine serum albumine (BSA), guanosine 5′-diphosphate (GDP) and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Magnesium chloride (MgCl) and sodium chloride (NaCl) were acquired from Acros Organics (Geel, Belgium). Ethylenediaminotetraacetic acid disodium salt (Na_2EDTA) was purchased from Applichem (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane (Tris) and potassium chloride (KCl) were acquired from Carl Roth GmbH (Karlsruhe, Germany).

Chemical synthesis

The synthesis of all compounds which are not commercially available has been performed following the protocols described elsewhere: iperoxo (iper) (Klöckner et al., 2010), isox and oxo-oxotremorine M (OOM) (Dallanoce et al., 1999), isox-6-naph (Disingrini et al., 2006), isox-8-naph and 8-naph (Bock et al., 2014), iper-6-naph, 6-naph and 6-phth (Antony et al., 2009), iper-8-naph (Bock et al., 2012), isox-6-phth (Disingrini et al., 2006), iper-6-phth (Antony et al., 2009), OOM-6-naph and OOM-6-phth (Disingrini et al., 2006). All derivatives were obtained with comparable yields and the same analytical purity as reported in the literature.

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Cell culture

Flp-In-Chinese hamster ovary cells (Flp-In-CHO) stably expressing the human muscarinic acetylcholine M_2 receptor (CHO-hM_2 cells) were cultured in Ham's nutrient mixture F-12 (Ham's F-12) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37 °C in a 5% CO_2 humidified incubator. The cells were passaged by trypsinization at nearly confluence.

Membrane preparation

CHO-hM_2 cells were grown to 90% confluence and treated with fresh medium supplemented with 5 mM sodium butyrate for 18–20 h. On the day of the membrane preparation, medium was aspirated and 2.4 ml ice-cold harvesting buffer (20 mM HEPES, 10 mM Na_2EDTA, pH 7.4) was added. Cells were mechanically detached with a cell scraper (Sarstedt AG & CO, Nümbrecht, Germany), before the cell suspension was homogenized using a Polytron homogenizer (1 × 25 s and 1 × 20 s, level 6). The suspension of lysed cells was centrifuged (10 min, 40,000 × *g*, 2 °C), and the pellet was resuspended in storage buffer (20 mM HEPES, 0.1 mM Na_2EDTA, pH 7.4). This centrifugation step was repeated twice. The remaining pellet was resuspended in an adequate amount of [^{35}S]GTPγS assay buffer (50 mM Tris, 2 mM MgCl, 1 mM Na_2EDTA, 1 mM DTT, pH 7.4) and stored at -80 °C (Schrage et al., 2013).

Equilibrium binding assays

To estimate apparent agonist binding affinities, membranes from CHO-hM_2 cells were diluted in assay buffer, i.e. Tris buffer (50 mM Tris, 2 mM MgCl, 1 mM Na_2EDTA, 1 mM DTT, pH 7.4) or Tris NaCl buffer (50 mM Tris, 2 mM MgCl, 1 mM Na_2EDTA, 1 mM DTT, 200 mM NaCl pH 7.4), supplemented with 100 µM GDP. 30 µg/ml membrane suspensions were then incubated with 0.2 nM [^{3}H]NMS and different concentrations of test compound in a 96-well microtiterplate (Thermo Scientific ABgene, Germany) in assay buffer in a final volume of 500 µl at 24 °C for 3 h to reach equilibrium conditions. Experiments were terminated by rapid vacuum filtration using a Tomtec Harvester (Tomtec Inc., Hamden,

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USA), and filter-bound radioactivity was calculated by solid scintillation. Non-specific binding was determined in the presence of 10 µM atropine.

Dissociation binding assays

To estimate ligand affinity to the allosteric binding site, [^{3}H]NMS dissociation binding assays were conducted. 30 µg/ml membranes were pre-incubated with 2 nM [^{3}H]NMS in assay buffer (Tris or Tris NaCl buffer (see above)) supplemented with 0.5% BSA and 1 µM GDP) for 45 min at 24 °C. Net dissociation of [^{3}H]NMS was initiated by the addition of 10 µM atropine, with or without the indicated concentrations of test compound. After the appropriate time interval (12 and 9 min in Tris and 6 and 3 min in Tris NaCl buffer), the dissociation was terminated by rapid vacuum filtration with a Tomtec Harvester (Tomtec Inc., Hamden, USA) and filter-bound radioactivity was calculated by solid scintillation. Nonspecific binding was defined as the radioactivity bound in the presence of 10 µM atropine.

[^{35}S]GTPγS binding assay

40 µg/ml CHO-hM_2 membranes were incubated with 0.07 nM [^{35}S]GTPγS, 1 µM GDP, 0.5% BSA and test compound in Tris buffer (50 mM Tris, 2 mM MgCl, 1 mM Na_2EDTA, 1 mM DTT, pH 7.4) or Tris NaCl buffer (50 mM Tris, 2 mM MgCl, 1 mM Na_2EDTA, 1 mM DTT, 200 mM NaCl pH 7.4) for 60 min at 24 °C. Experiments were terminated by rapid filtration a Tomtec Harvester (Tomtec Inc., Hamden, USA) and filter-bound radioactivity was measured by solid scintillation counting.

Data analysis

Equilibrium binding data were analyzed by a four-parameter logistic equation (Barlow and Blake, 1989) yielding IC_{50} values which were subsequently converted into apparent equilibrium dissociation constants (K_A) using the Cheng-Prusoff correction (Cheng and Prusoff, 1973). Data from two-point kinetic dissociation experiments were analyzed using a one-phase exponential decay as described elsewhere (Voigtländer et al., 2003). Data obtained from [^{35}S]GPTγS binding assays were fitted to a four-parameter

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logistic equation yielding measures for agonist potency (pEC_{50}) and maximum effect (E_{max}). All nonlinear regression analyses were performed using Prism 5.03 (GraphPad Software, San Diego, CA).

Statistical analyses

Shown are mean values ± standard error mean (SEM). Comparison of two single means was performed using an unpaired two sample Student t-test. In case the number of data sets was equal or superior to three, a one-way ANOVA test with Bonferroni's post-test was used.

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Results

Distinct binding poses of dualsteric ligands may result in protean agonism

To unravel potential protean agonism at the muscarinic M_2 receptor, we applied a variety of orthosteric agonists, allosteric fragments, and dualsteric ligands (i.e. iper-6-naph, iper-8-naph, iper-6-phth, isox-6-naph, isox-8-naph, isox-6-phth, OOM-6-naph, OOM-6-phth**,** Fig. 1A) and investigated receptor-mediated activation of inhibitory G_i proteins in [^{35}S]GTPγS binding experiments.

The dualsteric ligands tested in the present study consist of an orthosteric part, i.e. iperoxo (iper), isox, or oxo-oxotremorine M (OOM), and an allosteric moiety, i.e. either the naphthalimide (naph) or phthalimide (phth) group. The two pharmacophores are covalently connected *via* a hexa- or octamethylene linker. The orthosteric building block provides high affinity for interaction with the binding site of the endogenous messenger, whereas the allosteric moiety has affinity for the allosteric binding site, which is located on top of the orthosteric site in the extracellular part of mAChRs (Prilla et al., 2006; Bock et al., 2012; Haga et al., 2012). In consequence, these "bipharmacophoric" ligands are able to bind in two orientations to a receptor protein (Bock et al., 2014): either both pharmacophores bind to their designated binding sites, yielding a *dualsteric* binding pose, or the compound only interacts with the allosteric site of the receptor, yielding a purely *allosteric* binding pose (Bock et al., 2016) (Fig. 1B). As the allosteric fragments are derived from allosteric antagonists/inverse agonists, the purely allosteric binding pose stabilizes an inactive receptor conformation (Tränkle et al., 1998; Disingrini et al., 2006; Bock et al., 2014; Matera et al., 2014). In contrast, the dualsteric binding pose stabilizes active receptor conformations, because the orthosteric moieties are agonists (Dallanoce et al., 1999; Schrage et al., 2013; Bock et al., 2014).

The unique ability of dualsteric compounds to bind in two different binding poses leads to a potential scenario in which these model derivatives may act as protean agonists because the affinities of the two pharmacophores may change in different systems (Bock et al., 2014). A dualsteric compound which prefers binding in an allosteric binding topography will result in inverse agonism in a system with

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pronounced spontaneous receptor activity (red curve in lower panel of Fig. 1B). However, if this compound is also able to bind in a dualsteric binding pose, functional agonism may occur in a quiescent receptor system (blue curve in lower panel of Fig. 1B). Thus, the versatile behavior of dualsteric ligands may be an avenue towards the design of protean agonists.

Establishment of an experimental system with a stable amount of constitutive activity of the muscarinic M_2 receptor

The investigation of protean agonism is technically challenging because it requires a stable spontaneously active receptor system which is difficult to achieve for several GPCRs (Kenakin, 2001). To establish a system which displays a robust and substantial amount of spontaneous activity for the M_2AChR, we investigated the M_2 receptor-mediated binding of [^{35}S]GTPγS in CHO-M_2 membranes triggered by the endogenous agonist ACh or the inverse agonist atropine (Atr) in the presence of various concentrations of NaCl in a Tris buffer. NaCl was chosen as a buffer supplement because sodium ions are known to be important to maintain the inactive receptor conformation of class A GPCRs (Liu et al., 2012; Katritch et al., 2014; Miller-Gallacher et al., 2014). In line with this, an increase of NaCl led to a reduction in basal [^{35}S]GTPγS binding to CHO-M_2 membranes as well as to a decrease in spontaneous receptor activity (estimated in presence of Atr) and ACh-induced [^{35}S]GTPγS (Fig. 2A(i)). To explore whether alternative conditions with an ionic strength equivalent to 200 mM NaCl may also be suitable to titrate spontaneous activity of the M_2AChR, we analyzed the effect of the inverse agonist Atr on M_2-mediated [^{35}S]GTPγS binding in a Tris buffer with 2 mM sodium ions (low ionic strength Tris, *Tris*) in comparison to a Tris buffer substituted with 200 mM NaCl or equimolar concentrations of potassium chloride (KCl), sodium bromide (NaBr), or *N*-methyl-D-glucamine chloride (NMDGCl). NMDGCl is often used as a sodium substitute as it was found to be osmotically equivalent (Pihlavisto et al., 1998; Barann et al., 2004; Billups et al., 2006; Vivo et al., 2006). Interestingly, any increase in ionic strength led to a complete abolishment of spontaneous receptor activity regardless of whether sodium or potassium salts were applied, or

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NMDGCl was used as a substitute (Fig. 2A(ii)). We conclude that conditions with an ionic strength equivalent to 200mM NaCl may also serve to keep the receptor in the inactive state.

Next, we were interested in the nature of the M_2 receptor system with high rates of spontaneous activity (low ionic strength Tris, *Tris*) in comparison to a quiescent M_2 receptor system (Tris supplemented with 200 mM NaCl, *Tris NaCl*, or KCl, *Tris KCl*). We hypothesized that high spontaneous M_2 activity may either (i) increase the basal level of [^{35}S]GTPγS binding without altering the maximum inducible effect of the system (Supplemental Figure 1A) or (ii) lead to an increase in basal [^{35}S]GTPγS binding and also a higher potential E_{max} of the system (Supplemental Figure 1B). To approach these hypotheses experimentally, we measured [^{35}S]GTPγS binding in CHO-M_2 membranes induced by the endogenous agonist ACh (Fig. 2B(i)) and the muscarinic superagonist iperoxo (Fig. 2B(ii)) in one system where the receptor was spontaneously active (red, *Tris*) and two systems where no spontaneous activity was apparent (light blue, *Tris KCl*, and blue, *Tris NaCl*). In line with our hypothesis number two (Supplemental Figure 1B), both basal and ligand-induced receptor activity was enhanced in the spontaneously active system. We next investigated [^{35}S]GTPγS binding to CHO-M_2 membranes induced by five different orthosteric muscarinic agonists in a Tris NaCl buffer (Fig. 2C(i), quiescent receptor) and a low ionic strength Tris buffer (Fig. 2C(ii), spontaneously active receptor). Agonists may prefer binding to spontaneously active receptors as this enhances the probability of ternary complex formation of agonist, receptor and G protein (De Lean et al., 1980; Strange, 2008). Accordingly, all muscarinic agonists displayed increased potency for M_2 receptor activation in a low ionic strength Tris buffer (Fig. 2C(i,ii), Supplemental Table 1), accompanied with higher apparent agonist binding affinities (Supplemental Figure 2, Supplemental Table 1).

Interestingly, iperoxo shows a higher E_{max} value than ACh, isox, and OOM. This might indicate a lower stimulus-response coupling in the quiescent system which enables to directly visualize the higher efficacy of iper over the other investigated agonists. Of note, the “classical” muscarinic partial agonist pilocarpine

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is an exception to the observed trend, as the E_{max} value and the potency did not change with the spontaneous activity of the system.

Specific dualsteric compounds display protean agonism at muscarinic M_2 receptors

The ability to reliably fine-tune spontaneous M_2 receptor activity enabled us to investigate [^{35}S]GTPγS binding in CHO-M_2 membranes induced by dualsteric compounds in a quiescent receptor system (*Tris NaCl*) or a spontaneously active receptor system (*Tris*). When the muscarinic superagonist iper was incorporated as the orthosteric part into the dualsteric skeleton, the three tested compounds behaved as agonists in both buffer systems (Fig. 3A-C). Iper-6-naph (Fig. 3A) and iper-6-phth (Fig. 3B) displayed higher agonist efficacy and potency in the spontaneously active receptor system in comparison to the quiescent receptor state, whereas iper-8-naph only slightly gained potency in Tris buffer over Tris NaCl (Fig. 3C). Conversely, when isox or OOM, which possess "ACh-like" efficacy, were applied as orthosteric building blocks (Fig. 3D-H), the effect induced by isox-6-naph (Fig. 3D) and OOM-6-naph (Fig. 3G) changed its direction, depending on the activity state of the M_2 receptor: in an inactive M_2 receptor system, both compounds behaved as partial agonists (blue), whereas in a system with spontaneous activity of the receptor both displayed inverse agonism (red) as expected for protean agonists. In contrast to this and in line with the findings for iper-6-phth, isox-6-phth (Fig. 3E) and OOM-6-phth (Fig. 3H) had increased efficacy in Tris buffer as compared with Tris NaCl. Likewise, we noted higher efficacy for the isox-derived hybrid carrying an allosteric naphthalimide residue with a linker length of 8 carbon atoms (isox-8-naph) in the spontaneously active receptor system over the quiescent system (Fig. 3F). As predicted and in agreement with previous findings (Jäger et al., 2007; Antony et al., 2009; Bock et al., 2014), the allosteric fragments 6-naph, 8-naph, and 6-phth (Fig. 1A) alone behaved as allosteric inverse agonists in presence of high spontaneous M_2 receptor activity but were silent at the inactive receptor (Supplemental Figure 3).

Taken together, our data allow us to derive structure-activity relationships to induce protean agonism at muscarinic M_2 receptors: the orthosteric agonist should display an ACh-like efficacy for receptor

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activation, the allosteric residue should be bulky - the sterically demanding 1,8-naphthalimide moiety being superior to the less voluminous phthalimide analog - and the two pharmacophores should be connected by an alkyl chain of optimally 6 carbon atoms.

Dissociation binding experiments point to an increased allosteric affinity to M_2 receptors in Tris buffer of a protean agonist

To assess whether protean agonism of isox-6-naph and OOM-6-naph was due to an increase in allosteric binding to the [^{3}H]NMS-bound M_2 receptor in the low ionic strength buffer, we estimated binding affinities for selected compounds in the allosteric binding pose to M_2. To this end, [^{3}H]NMS dissociation experiments were performed in a low ionic strength Tris buffer (red curve) and Tris buffer supplemented with NaCl (blue curve) in order to measure allosteric binding affinities in the same ionic strength than the aforementioned [^{35}S]GTPγS binding experiments (Fig. 4A-E).

Both investigated dualsteric compounds carrying iper as the orthosteric building block, i.e. iper-6-naph and iper-8-naph, displayed a gain in affinity for allosteric binding (Fig. 4A,B, Supplemental Table 1) of about 3- and 7-fold, respectively, to M_2 receptor in Tris compared to Tris NaCl buffer. The protean agonist isox-6-naph displayed a greater increase in affinity, of about 20-fold, (Fig. 4C, Supplemental Table 1) in the low ionic strength over the NaCl supplemented buffer. In contrast to this, the affinity for the allosteric binding site of isox-8-naph appeared to be similar in both buffer systems (Fig. 4D). The allosteric affinity of the OOM-containing protean agonist OOM-6-naph was significantly increased in Tris buffer in comparison to Tris NaCl (6-fold), even though the gain in affinity was not as prominent as that reported for isox-6-naph (Fig. 4E). Taken together, these data indicate that both protean agonists may display increased allosteric binding under the assay conditions similar to the spontaneously active system compared with the conditions of the inactive system and that this may contribute to the inverse agonism we observed for isox-6-naph and OOM-6-naph.

Of note, we also observed an increase in allosteric binding for iper-6-naph and iper-8-naph to the M_2 receptor in Tris compared to Tris NaCl although these two compounds clearly did not display any protean

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agonism. This might be explained by the higher intrinsic efficacy for receptor activation of the orthosteric moiety iper in comparison to isox and OOM. In consequence, the beneficial effect of the spontaneously active M_2 receptor on the orthosteric superagonist iper (increase in binding affinity 10-fold in Tris *vs.* Tris NaCl) surpassed the increase in allosteric binding (increase in allosteric binding affinity 3.5-fold and 7-fold for iper-6-naph and iper-8-naph, respectively) and this precluded protean agonism.

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Discussion

Protean ligands induce opposing effects (E_{max}) depending on the level of spontaneous activity of a G protein-coupled receptor. This phenomenon was first reported in 1995 (Kenakin, 1995) and is based on the assumption that ligand–bound GPCRs may adopt not only one (fully) active and one inactive conformation, but also intermediate conformations engendering intermediate intrinsic efficacies for activation of intracellular signaling pathways. In this regard, receptor species with a lower efficacy than the spontaneously active one yield *positive* signaling in the presence of quiescent receptors and *negative* signaling when the majority of receptors is constitutively active (Kenakin, 1997, 2001; Chidiac, 2002). Experimentally, the discovery of protean ligands remains challenging for two reasons, i.e. the lack of (i) stable and reliable spontaneously active systems for several GPCRs and (ii) strategies for the rational design of this class of ligands.

In the present study, we show that spontaneous activity of the muscarinic M_2 receptor can reliably be titrated by changing the ionic strength in CHO-M_2 [^{35}S]GTPγS binding experiments. We used this system to identify dualsteric compounds as protean agonists at M_2AChR. Structure-activity relationships allowed us to define the molecular features required for protean agonism at M_2 receptors among a set of dualsteric compounds and to introduce a new molecular mechanism for protean agonism.

In particular, we present two dualsteric ligands, i.e. isox-6-naph and OOM-6-naph, as new protean agonists at the muscarinic M_2 receptor. Interestingly, only slight alterations of the three chemical moieties were sufficient to shift the profile from protean agonism to “classical” agonism (Fig. 3). The molecular features required for protean agonism at the muscarinic M_2 receptor are: an orthosteric part endowed with an acetylcholine-like efficacy, a bulky allosteric part to impair the flexibility of the extracellular loop area, and a flexible linker chain of 6 carbon atoms.

Up until now, protean agonism was thought to reside in the intrinsic efficacy of a ligand (Kenakin, 1997). Our results point to an additional mechanism that may underlie this phenomenon at GPCRs in case a ligand can simultaneously occupy both the binding site for the endogenous messenger and an allosteric

binding site of the receptor protein. These dualsteric compounds may bind to a receptor protein in two distinct binding modes (Bock et al., 2014; Bock et al., 2016): a purely allosteric and a dualsteric mode (Fig. 1B) and may thus have two distinct efficacies for receptor activation. Our findings presented here go in line with the idea that the two protean agonists isox-6-naph and OOM-6-naph prefer binding in the purely allosteric binding pose, as has already been demonstrated for isox-6-naph in a previous study (Bock et al., 2014). Both compounds display functional inverse agonism under conditions in which the M_2 receptor displays a substantial amount of spontaneous activity (Fig. 3D,G). However, at least under conditions in which the receptor is silent, a significant fraction of receptors must be bound in a dualsteric binding pose, as we observed functional agonism in the quiescent M_2 system. This indicates dynamic ligand binding as the molecular mechanism of protean agonism of isox-6-naph and OOM-6-naph.

Spontaneous activity of the M_2AChR could robustly be fine-tuned by changing the ionic strength of the applied buffer system, such as presence/absence of sodium ions. To test whether isox-6-naph and OOM-6-naph swap their binding pose depending on the buffer system used, we performed radioligand binding experiments and estimated the allosteric binding affinity ($EC_{50,diss}$ values) of several dualsteric ligands included in this study. The allosteric affinity measure of isox-6-naph was 20-times higher in Tris than in Tris NaCl buffer, whereas the orthosteric building block isox gained only 3.5-fold in affinity under these conditions (Fig. 4C, Supplemental Figure 2). Assuming that the affinities of the allosteric and of the orthosteric moiety determine the fraction of ligand bound in one or the other pose (Bock et al., 2014), this result supports the idea that isox-6-naph increases in allosteric binding pose in the low ionic strength buffer. Yet, OOM-6-naph displayed only a slight increase in allosteric binding affinity in Tris *vs.* Tris NaCl buffer. This effect was comparable to the increase in orthosteric affinity of the orthosteric building block OOM. Even if these data indicate that OOM-6-naph does not display increased allosteric binding in Tris buffer, the dominant binding pose under these conditions must be the allosteric pose, as we clearly see inverse agonism at the spontaneously active M_2AChR (Fig. 3G). Of note, changes in the ionic strength of the buffer may not only alter the spontaneous activity of the M_2AChR (see Fig. 2B, increase of the lower curve plateau), but may also affect the micro environment in the orthosteric and allosteric binding

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pocket. Therefore, ligand binding behavior may be altered not only by the functional state of the receptor protein, but additionally be influenced by the presence/absence of ions in the binding pockets. Interestingly, all dualsteric compounds share a common bis-ammoniumalkane structure which is known to engage with the allosteric M_2AChR binding site via polar cation-π interactions (Dror et al., 2013), which are susceptible to variation of ionic strength (Papaneophytou et al., 2014).

However, our study clearly indicates a major impact of ionic strength on the macro level, i.e. the functional state of the receptor. A recent study by Yuan et al. (2016) has shown that a continuous channel of water molecules inside the receptor protein is an essential feature of the functionally active state of the adenosine A_1 receptor, another class A GPCR which likely shares a common activation mechanism with the M_2AChR. Therefore, one might speculate that ionic strength fine-tunes receptor activity by determining the amount of water molecules available within the binding pocket. Yet, albeit tempting, ultimate proof for such a hypothesis remains to be provided by crystallographic studies.

One might argue that protean agonism of isox-6-naph and OOM-6-naph resides in the rather low efficacy of the two ligands and is not necessarily due to dynamic ligand binding. However, dualsteric ligands like isox-6-phth and isox-8-naph did not display any protean agonism although the efficacy of these compounds did not differ significantly from the efficacy of the two protean agonists at the quiescent M_2AChR. Moreover, it has been demonstrated previously that a purely allosteric binding pose predominates for isox-6-naph (Bock et al., 2014). In sum, our data indicate that dynamic ligand binding (Bock et al., 2014) is the underlying molecular mechanism for protean agonism of isox-6-naph and OOM-6-naph at M_2AChR.

Protean agonism is still a seldom described phenomenon. Studies which identified protean agonists by comparison of agonist-induced signaling in two different functional assay systems may be compromised by biased signaling (Jansson et al., 1998; Gbahou et al., 2003). Therefore, two experimental systems which differ only in the amount of spontaneous activity are essential for the identification of protean agonists. To this end, several strategies have been employed to study agonism in presence and absence of spontaneously active receptors. For instance, constitutively activating receptor mutations (Ganguli et al.,

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1998; Fathy et al., 1999; Pauwels et al., 2002), an increased expression of receptor/G protein (Jakubík et al., 1998), or a change in buffer composition (Newman-Tancredi et al., 2003), have been applied. Here, we choose to alter M_2 wild type receptor activity by variation in the concentration of sodium ions, because previous studies demonstrated that sodium ions can keep class A GPCRs in an inactive conformation (Liu et al., 2012; Katritch et al., 2014). Yet, our results indicate that inactivation of the M_2AChR by a high concentration of sodium ions depends on high ionic strength in the buffer rather than the nature and/or composition of a particular salt. In a low ionic strength Tris buffer, basal as well as agonist-induced [^{35}S]GTPγS incorporation was increased in CHO-M_2 membranes in comparison with a buffer system with high concentrations of sodium or potassium ions. This is in line with previous findings (Tian et al., 1994) for the adrenergic α_2-AR and reflects a stronger stimulus-response-coupling and ternary complex formation of agonist, receptor and G protein of the spontaneously active M_2 receptor compared to quiescent M_2. Accordingly, the distinct efficacies of ACh and iper could only be detected in the inactive receptor system (Fig. 2C). Moreover, almost all dualsteric compounds which did not display protean agonism showed increased E_{max} values and all orthosteric agonists gained binding affinity and potency in Tris compared to Tris NaCl buffer, as agonists prefer binding to an active rather than an inactive receptor (De Lean et al., 1980). The classical muscarinic partial agonist pilocarpine is an exception to this behavior as the compound changed neither in potency nor in efficacy depending on the buffer composition. A previous work (Tota and Schimerlik, 1990) reported that pilocarpine does not distinguish between active and inactive receptors. However, direct labeling of active M_2 receptors with a radioagonist indicated higher apparent affinity of pilocarpine for this receptor population in comparison to inactive receptors, albeit the affinity difference was lower than the respective difference for full agonists (Schrage et al., 2014). Together with the finding that pilocarpine activates M_2 receptors in native tissue as well as in recombinant cells (Seemann et al., 2016), this implicates that pilocarpine has higher affinity for the active M_2 receptor relative to the inactive form, although the nominal difference in affinity appears to be lower than for full agonists.

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On the whole, we established an experimental system which allowed us to identify two new protean agonists at M_2 muscarinic acetylcholine receptors. We propose an unprecedented molecular mechanism by which protean agonism is induced at a class A GPCR, i.e. the M_2AChR, which may virtually be extended to generate protean agonists for other class A GPCRs harboring allosteric binding sites such as adenosine or dopamine receptors. It will be interesting to see if this dualsteric approach will furnish protean ligands for other receptor types.

Similar to the great potential of biased GPCR signaling, improved understanding of protean agonism may provide another level towards the targeted exploitation of the GPCR signaling machinery, which could be relevant to the knowledge-based design of innovative drug candidates.

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Author contributions

Participated in research design: De Min, Schrage, Bock and Mohr.

Conducted experiments: De Min.

Contributed new reagents or analytical tools: Matera, Dallanoce, De Amici, Muth, Kloeckner and Holzgrabe.

Performed data analysis: De Min, Bock, Traenkle and Holze.

Wrote or contributed to the writing of the manuscript: Schrage, De Min, Kostenis, Kenakin, Bock, De Amici, Holzgrabe and Mohr.

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Footnotes

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Figure legends

Figure 1. Test compounds and proposed mechanism of action of dualsteric ligands acting as protean agonists. (A) Structures of the dualsteric ligands examined in this study, their respective orthosteric moieties isox, iperoxo (iper), and oxo-oxotremorine M (OOM), and the allosteric fragments C6-naphthalimide (6-naph), C6-phthalimide (6-phth) and C8-naphthalimide (8-naph). The classical muscarinic partial agonist pilocarpine and the endogenous agonist acetylcholine (ACh) were used as reference compounds. **(B)** Proposed model to clarify the mode of action of dualsteric protean agonists at the M_2 receptor. Dualsteric ligands may potentially bind either in a dualsteric binding pose, stimulating receptor signaling, or in a purely allosteric pose, inactivating the receptor (Bock et al., 2014). In this sense, dualsteric binding of protean agonists may lead to a positive signaling output in an inactive receptor system (hypothetical blue curve), whereas, the compound may behave as an inverse agonist in a receptor system with spontaneous activity (hypothetical red curve).

Figure 2. Establishment of a robust assay system with substantial amount of spontaneous activity for the muscarinic M_2 receptor. (A_i) Increasing concentrations of NaCl affecting basal, atropine-inhibited, and ACh-induced [^{35}S]GTPγS binding. **(A_{ii})** Effect of NaCl, KCl, NaBr, or NMDGCl (200 mM each) on the spontaneous activity of M_2 receptors in [^{35}S]GTPγS experiments. ***: $P<0.001$, significantly different from Tris according to one-way ANOVA test with Bonferroni's post-test. **(B)** Experimental dose-response curves of **(B_i)** ACh and **(B_{ii})** iper obtained in presence (Tris) or absence (Tris NaCl, Tris KCl) of constitutive activity of the M_2 receptor. In the spontaneously active system, the whole curve was translated to higher values, as proposed in Supplemental Figure 1B. **(C)** Orthosteric compounds-induced [^{35}S]GTPγS binding in CHO-hM_2 membranes in absence (Tris NaCl, **C_i**) or presence (Tris, **C_{ii}**) of spontaneous receptor activity. While the maximum inducible effect (E_{max}) of isox and OOM were equal to that of ACh in both systems, iperoxo showed a superior E_{max} than ACh in a silent system. (**A-C)** Shown are mean values ± SEM of three to seven independent experiments each performed in triplicate.

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Figure 3. Isox-6-naph and OOM-6-naph display protean agonism in CHO-hM_2 membranes in absence (Tris NaCl) or presence (Tris) of spontaneous receptor activity. (A,B,C) Dualsteric ligands which carry iperoxo as the orthosteric building block displayed partial agonism in both the silent and the spontaneously active set-up regardless of their C6-naph **(A)**, C6-phth **(B),** or C8-naph **(C)** allosteric moiety. Noteworthy, iper-6-naph and iper-6-phth displayed significantly higher E_{max} values in the spontaneously active system. **(D,E,F,G,H)** Isox-6-naph, isox-6-phth, isox-8-naph, OOM-6-naph and OOM-6-phth were all weak partial agonists in the quiescent system (blue curve) but changed their E_{max} in the presence of constitutive receptor activity (red curve). Isox-8-naph displayed increased receptor activation (**F**), isox-6-phth (**E**) and OOM-6-phth (**H**) showed no difference in effect, whereas the two compounds with a C6-naph moiety, i.e. isox-6-naph **(D)** and OOM-6-naph **(G)**, switched from partial to inverse agonism and were thus classified as protean ligands. **(A-G)** Shown are mean values ± SEM of three to seven independent experiments each performed in triplicate.

Figure 4. Isox-6-naph displayed enhanced affinity to the allosteric binding site of the [^{3}H]NMS-bound M_2 receptor in Tris. (A-E) [^{3}H]NMS dissociation experiments performed in presence of **(A)** iper-6-naph, **(B)** iper-8-naph, **(C)** isox-6-naph, **(D)** isox-8-naph and **(E)** OOM-6-naph in Tris or Tris NaCl buffer. Depicted are concentration-effect curves which show the delay of [^{3}H]NMS dissociation rate (k_{-1}) induced by the dualsteric test compounds in two point kinetic experiments (Kostenis and Mohr, 1996). The inflection point of the curves represents the allosteric affinity of the tested compounds for the [^{3}H]NMS-bound receptor. **(A-E)** Shown are mean values ± SEM of three to five independent experiments each performed in duplicate.

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Figures

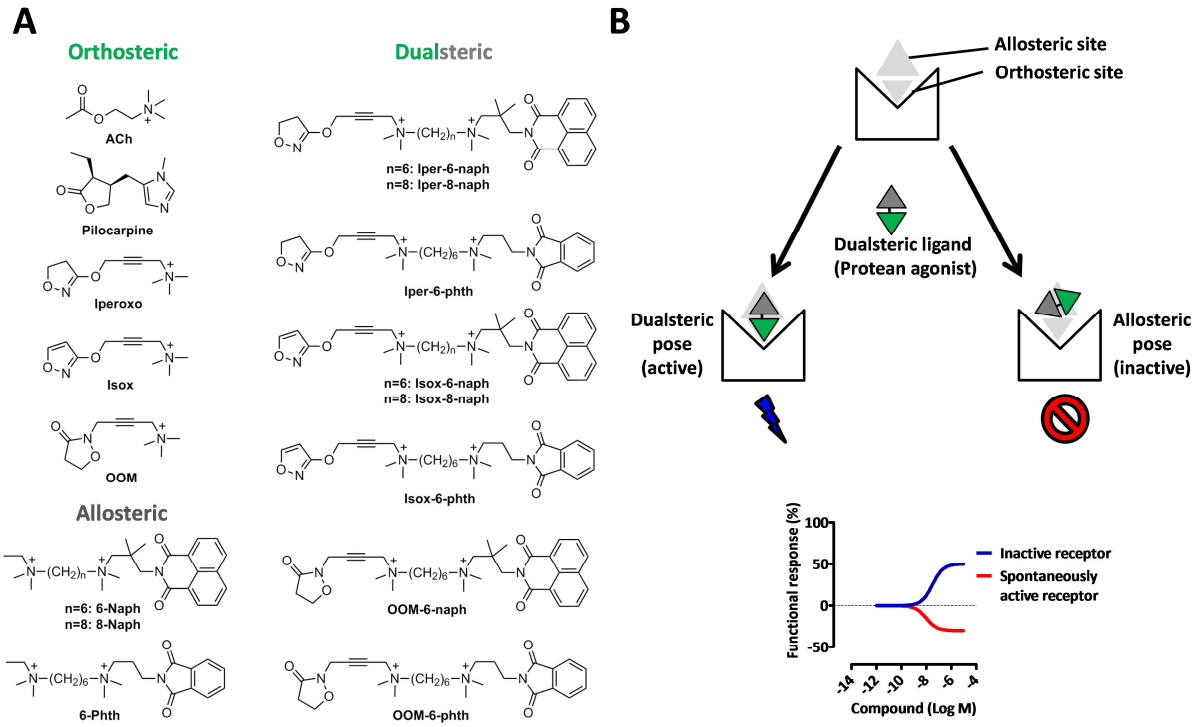

Figure 1

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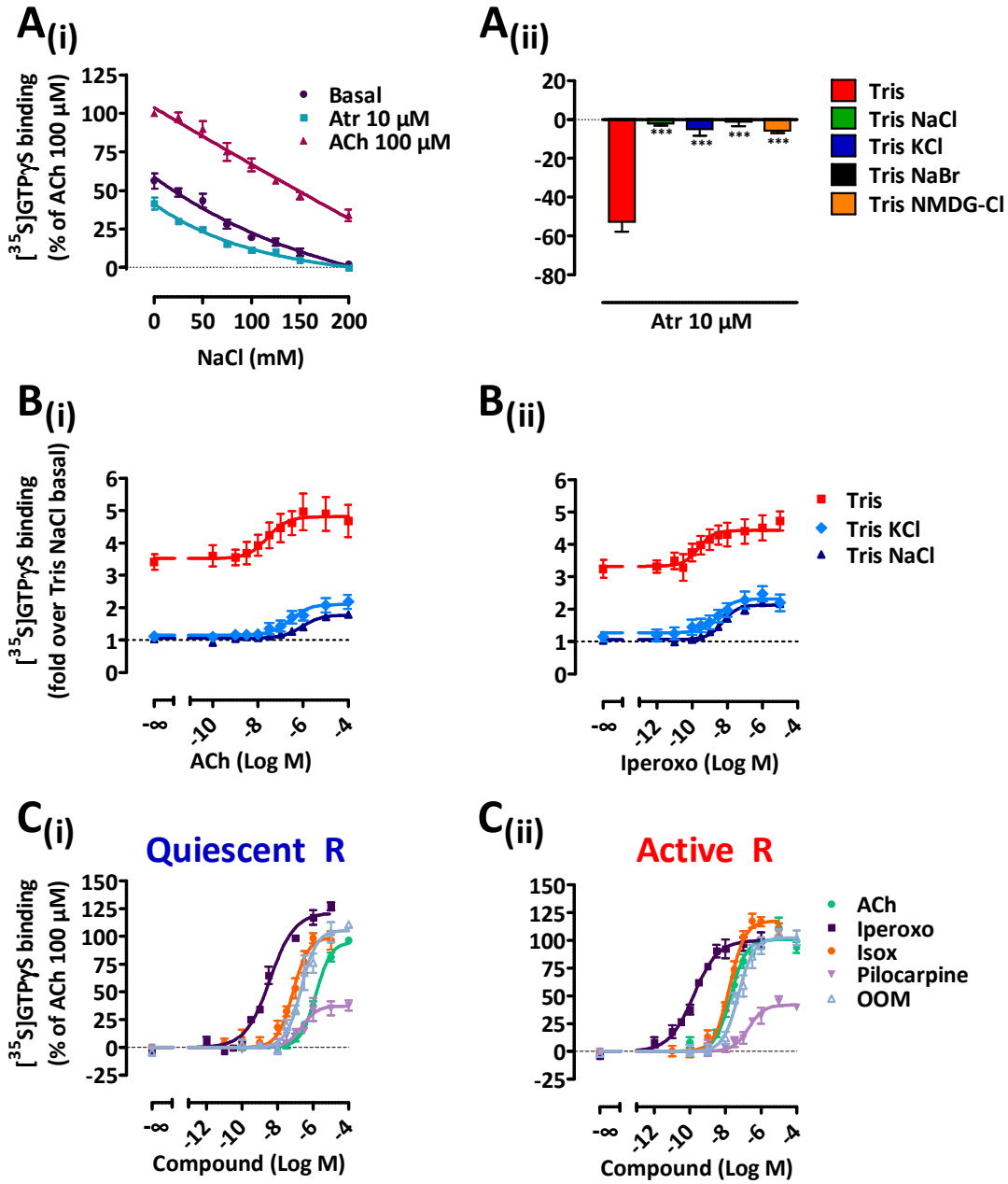

Figure 2

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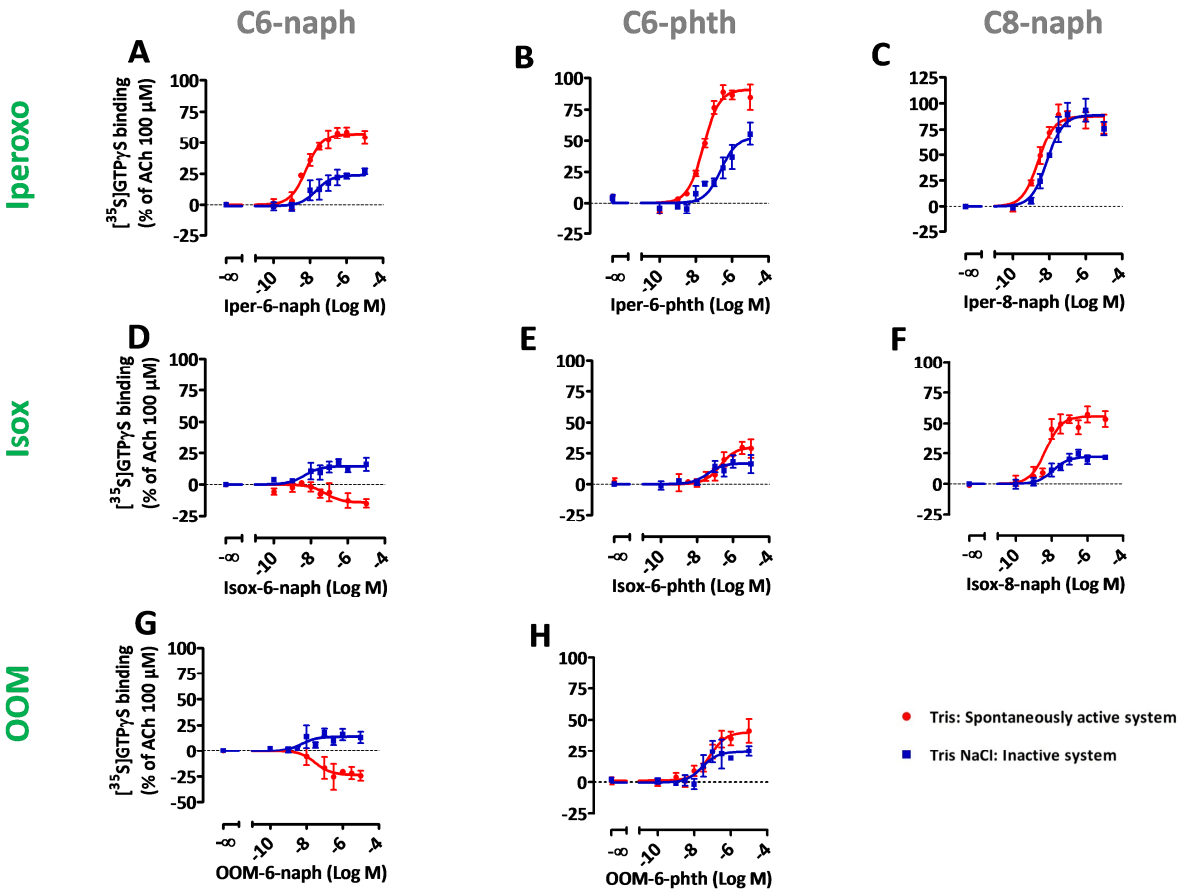

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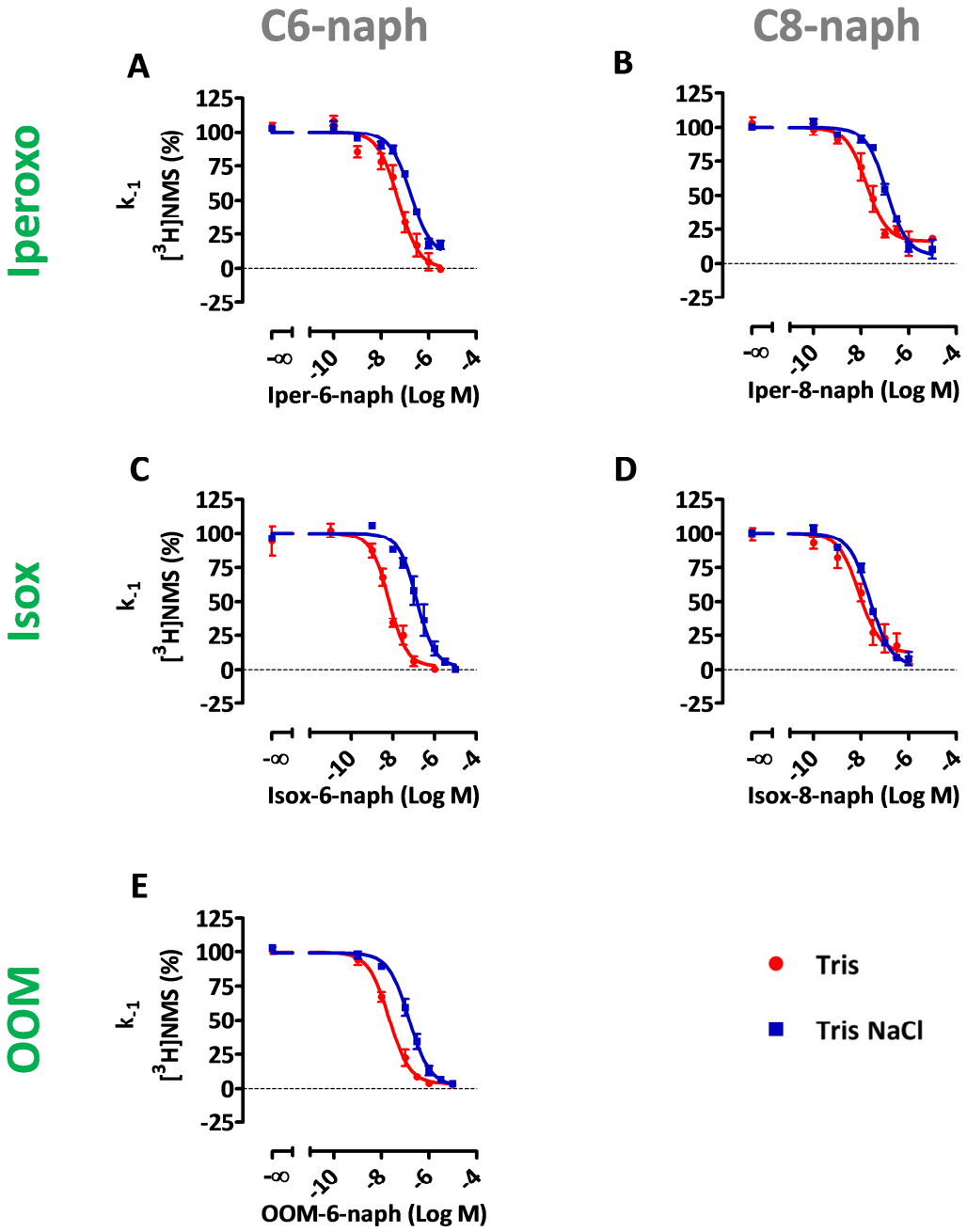

Figure 4