Dualsteric compounds modulate the signaling pattern of muscarinic M1 acetylcholine receptors

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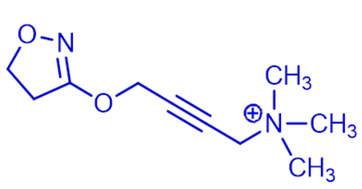
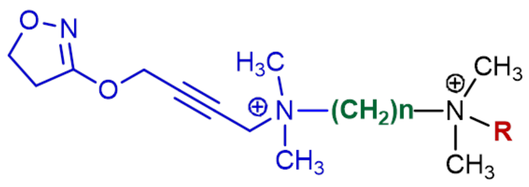
Introduction

G protein-coupled receptors (GPCRs) are cell surface receptors which, upon a conformational change in the receptor protein induced by an extracellular stimulus, can transduce the signal onto intracellular adaptor proteins such as heterotrimeric G proteins [1]. GPCR-induced cell signaling can be rather complex as several GPCRs may activate multiple different adaptor proteins and can additionally be activated via distinct binding sites, i.e. the orthosteric transmitter binding site and other "allosteric" binding sites [2].
In the present work, we investigated the influence of an allosteric binding site on receptor activation of muscarinic acetylcholine receptors (mAChRs). To this end, we employed the orthosteric full agonists acetylcholine (ACh) and iperoxo, as well as several dualsteric compounds consisting of iperoxo linked to an allosteric phthalimide (phth) or naphthalimide (naph) moiety through alkyl chains of different length, and investigated compound-induced activation of the human muscarinic M1 receptor. The M1 receptor preferentially activates $G_{q/11}$ proteins, but can also promiscuously stimulate G_s proteins [3].
Here we show that the orthosteric full agonists ACh and iperoxo can lead to a M1 receptor-mediated increase in intracellular IP1 and cAMP which reflects $G_{q/11}$ and G_s protein activation, respectively. Previous studies with the muscarinic M2 receptor revealed that the restriction of conformational flexibility by allosteric moieties that bind to the extracellular loops of the M2 receptor modulates the signaling pattern of the promiscuous M2 receptor [2]. In line with this we present that different allosteric moieties, i.e. phth or naph, in combination with various alkyl linker lengths can lead to a differential signaling pattern of the M1 receptor. For instance the bulkier naph residue compromised M1-induced $G_{q/11}$ and G_s signaling to a greater extent than the smaller phth moiety.
Furthermore, we show that M1 receptor-mediated increase in intracellular cAMP is not only due to G_s activation but that also $G_{q/11}$-induced elevation of intracellular Ca^{2+} contributes to the activation of adenylyl cyclases.

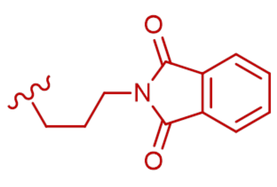
Compounds



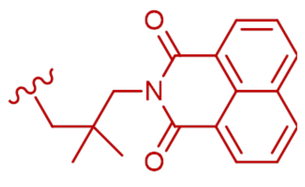
iperoxo



hybrids n = 6, 7, 8, 10



R_1 = phth



R_2 = naph

Methods

Chinese hamster ovary (CHO) cells stably transfected with the human muscarinic M1 acetylcholine receptor (CHO-M1) were cultured in Ham's nutrient mixture F-12 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM L-glutamine, and 0.2 mg/ml G418. The cells were grown at 37°C and 5% CO_2.
Second messenger assays were performed as described previously [5]. Briefly, to quantify M1 receptor-mediated **$G_{q/11}$**-activation, increase in intracellular IP1 was detected by using the IP-One HTRF® assay kit (Cisbio, France). Therefore100.000 cells/7 µl were seeded in 384 well microtiter plates and stimulated with test compound for 30 min before IP1 was detected according to the manufacturer's instructions.
To quantify M1 receptor-mediated **G_s**-activation, increase in intracellular cAMP was detected using the cyclic AMP cell-based assay kit (Cisbio, France). Cells were pretreated overnight with either 50 ng/ml pertussis toxin (PTX), 100 ng/ml cholera toxin (CTX), or both. 50.000 cells/5 µl were seeded in 384 well microtiter plates and incubated for two hours with or without the $G_{q/11}$ inhibitor FR900359 (1 µM) [4]. Subsequent to compound stimulation of 30 min, cAMP was detected according to the manufacturers' instructions.
Data analysis was performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA). All experiments were carried out in triplicates. Data are means ± s.e.m. from at least two independent experiments and data points from individual experiments were fitted to a four-parameter logistic equation [6].

Results

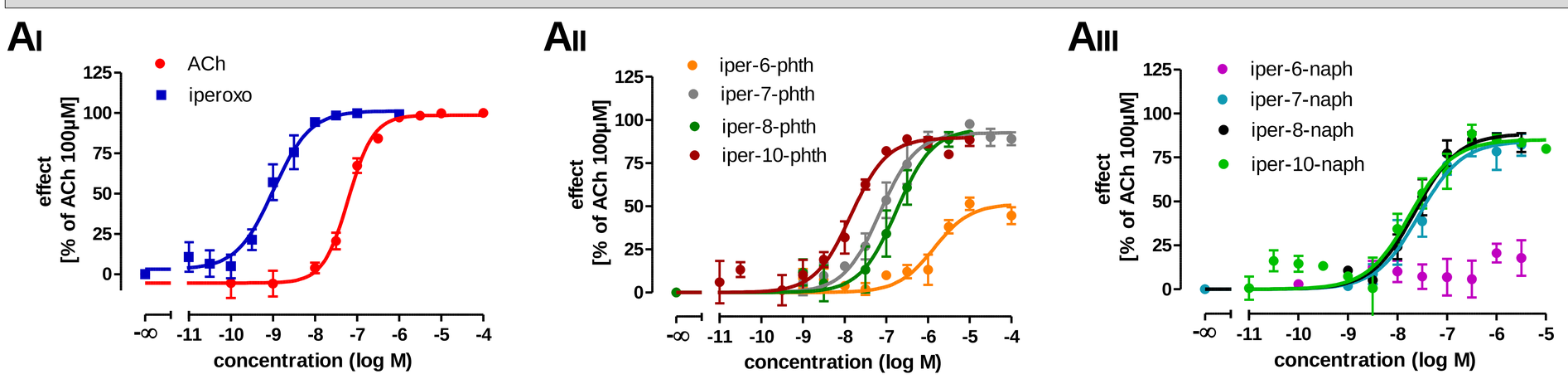


Figure 1 | CHO-M1-mediated $G_{q/11}$-activation: The bulkier allosteric moiety naph disturbed $G_{q/11}$-mediated signaling to a greater extent than phth.
(A) Agonist-induced $G_{q/11}$-activation in CHO-M1 cells quantified by intracellular IP1.
(A_I) Iperoxo was 100 fold more potent for M1-mediated $G_{q/11}$-activation than the endogenous agonist ACh. **(A_{II}, A_{III})** IP1 production was less compromised by inclusion of a phth residue **(A_{II})** than a naph moiety **(A_{III})** as allosteric building block in orthosteric-allosteric hybrid compounds.

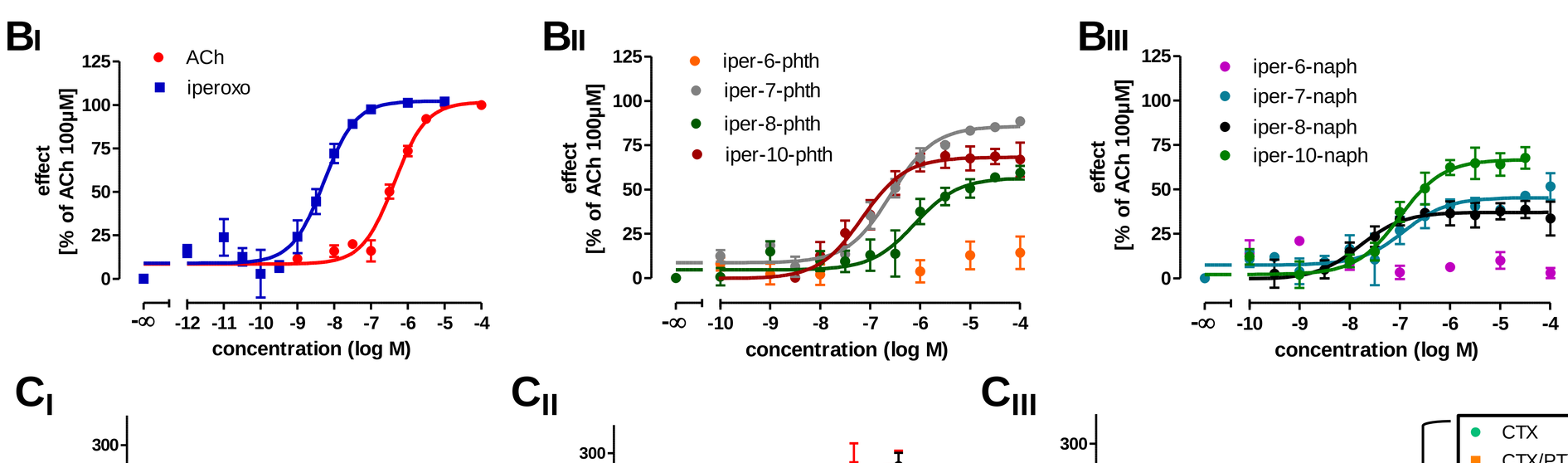


Figure 2 | CHO-M1-mediated G_s-activation:
(B) Agonist-induced G_s-activation in CHO-M1 cells quantified by intracellular cAMP.
(B_I) In line with the findings for $G_{q/11}$ (A), iperoxo was 100 fold more potent than ACh for M1-mediated G_s-activation and **(B_{II})** cAMP accumulation was less compromised by phth than by **(B_{III})** naph moieties. Interestingly, iper-6-phth did not show any G_s activation although this compound clearly activated $G_{q/11}$ (A_{II}).

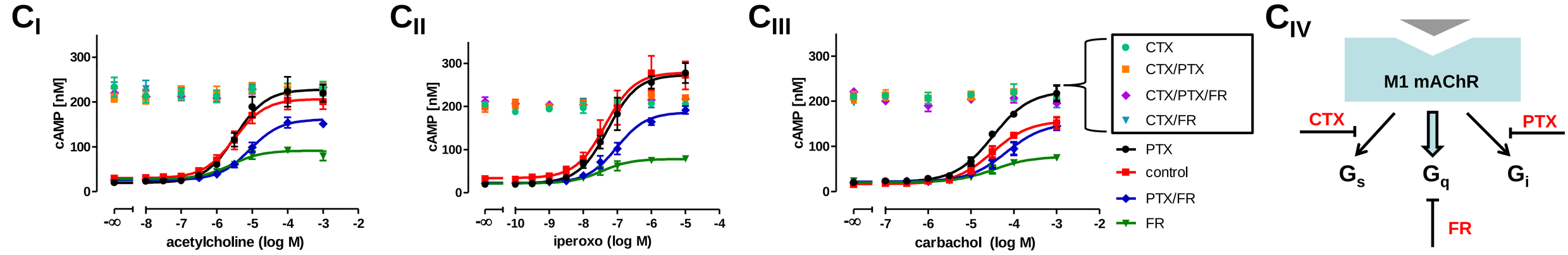


Figure 3 | CHO-M1-mediated activation of adenylyl cyclases can be facilitated by both G_s protein activation and $G_{q/11}$-mediated signaling.
(C) Agonist-induced increase in intracellular cAMP under control conditions, after permanent stimulation of G_s proteins (CTX), inhibition of G_i (PTX), and/or $G_{q/11}$ proteins (FR) [4]. All three muscarinic full agonists acetylcholine **(C_I)**, iperoxo **(C_{II}),** and carbachol **(C_{III})** induced profound M1-mediated increase in intracellular cAMP, which could be masked by the G_s activator CTX. Interestingly, the $G_{q/11}$ inhibitor FR was able to decrease cAMP production for all three agonists (green curves) and this effect could be attenuated by additional blocking G_i proteins (blue curves). **(C_{IV})** Schematic representation of the signaling repertoire of M1 mAChRs.

Conclusion

- M1 receptor activation was compromised by both allosteric volume and short linker length for G_s and $G_{q/11}$ signaling
- $G_{q/11}$-mediated signaling seemed to play an important role in adenylyl cyclase activation, since the intracellular cAMP increase was significantly diminished when the $G_{q/11}$ protein was blocked
- A combination of various toxins gave first evidence that there is crosstalk between G_i and $G_{q/11}$ proteins, i.e. M1 receptor-mediated G_i activation could only be detected when $G_{q/11}$ was blocked

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