Allosteric Small Molecules Unveil a Role of an Extracellular E2/Transmembrane Helix 7 Junction for G Protein-coupled Receptor Activation*

Received for publication, July 6, 2007, and in revised form, September 4, 2007 Published, JBC Papers in Press, September 21, 2007, DOI 10.1074/jbc.M705563200

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G protein-coupled receptors represent the largest superfamily of cell membrane-spanning receptors. We used allosteric small molecules as a novel approach to better understand conformational changes underlying the inactive-to-active switch in native receptors. Allosteric molecules bind outside the orthosteric area for the endogenous receptor activator. The human muscarinic M2 acetylcholine receptor is prototypal for the study of allosteric interactions. We measured receptor-mediated G protein activation, applied a series of structurally diverse muscarinic allosteric agents, and analyzed their cooperative effects with orthosteric receptor agonists. A strong negative cooperativity of receptor binding was observed with acetylcholine and other full agonists, whereas a pronounced negative cooperativity of receptor activation was observed with the partial agonist pilocarpine. Applying a newly synthesized allosteric tool, point mutated receptors, radioligand binding, and a three-dimensional receptor model, we found that the deviating allosteric/orthosteric interactions are mediated through the core region of the allosteric site. A key epitope is M2-Trp522 in position 7.35 that is located at the extracellular top of transmembrane helix 7 and that contacts, in the inactive receptor, the extracellular loop E2. Trp 7.35 is critically involved in the divergent allosteric/orthosteric cooperativities with acetylcholine and pilocarpine, respectively. In the absence of allosteric agents, Trp 7.35 is essential for receptor binding of the full agonist and for receptor activation by the partial agonist. This study provides first evidence for a role of an allosteric E2/transmembrane helix 7 contact region for muscarinic receptor activation by orthosteric agonists.

G protein-coupled receptors (GPCRs)4 have outstanding importance as targets for drug action (1, 2). Conformational changes underlying the inactive-to-active receptor switch in GPCRs are in the focus of current research. In general, the receptor transmembrane helices (TM) rearrange, allowing the intracellular loop region to unfold and to stimulate neighboring G proteins (3, 4). Conformational changes include extracellular receptor regions, and a critical role of the second extracellular loop (E2) for GPCR activation and ligand binding has emerged (5–9). Rational development of agonistic drugs for GPCR activation requires deeper insight into such conformational changes. Because GPCRs are hardly accessible for crystallization, indirect approaches are applied that often involve modification of the receptor protein such as receptor mutagenesis, introduction of metal ion sites or disulfide bridges, or covalent linkage of moieties for fluorescence resonance energy transfer.

Allosteric small molecules allow the study of native receptors. An increasing number of GPCRs is known to contain allosteric sites (10, 11); cinacalcet is the first allosteric GPCR modulator that has recently entered the market (12). Allosteric sites are located outside the orthosteric area that is occupied by the endogenous transmitter. Our strategy is based on the hypothesis that activation-related three-dimensional changes involve the whole receptor protein and thus include allosteric sites. Therefore, allosteric ligands should be useful to probe activation-related spatial rearrangement of the receptor protein outside the orthosteric binding area.

Muscarinic acetylcholine receptors, especially the M2 subtype, have served as an excellent model system to study cooperative interactions that result from simultaneous binding of allosteric and orthosteric agents (11). The so-called common allosteric site of muscarinic receptors resides extracellular to the orthosteric binding site for the endogenous activator acetylcholine. Until now, the consequences of muscarinic allosteric-orthosteric interactions have been studied with a focus on binding affinity. Affinity shifts are cooperative, i.e. the allosteric and the orthosteric ligand change each other’s binding affinity toward the same direction and to the same extent. Depending

4 The abbreviations used are: GPCR, G protein-coupled receptor; [35S]GTPγS, guanosine 5′-γ-thio)triphosphate; NMS, N-methylscopolamine; [3H]NMS, [3H]N-methylscopolamine methylbromide; TM, transmembrane helix; CHO, Chinese hamster ovary.
on whether affinities are increased, decreased, or left unaltered, cooperativity is positive, negative, or neutral. The starting point of the present study was a previous observation that an allosteric-orthosteric interaction involved muscarinic receptor activation (13). We wondered whether allosteric interactions can be exploited as a new strategy to gain insight into activation related conformational changes. Applying allosteric small molecules as probes, we provide evidence that the full agonist acetylcholine and a partial agonist induce divergent conformational changes in the extracellular receptor region. We identified an amino acid, M2-Trp422, that plays a key role for allosteric/orthosteric binding and activation cooperativity. This amino acid forms a junction between E2 and TM7 that appears to be essentially involved in GPCR activation.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Test Compounds*—Acetylcholine iodide, pilocarpine hydrochloride, carbachol chloride, oxotremorine sesquifumarate, oxotremorine-M iodide, atropine sulfate, N-methylscopolamine bromide, and gallamine triethiodide were obtained from Sigma-Aldrich, and [35S]GTPγS and [3H]NMS were from PerkinElmer Life Sciences. Acruconium chloride was generously provided by Hoffmann-La Roche AG (Grenzach Wyhlen, Germany). Naphmethonium and W84 are commercially available through AXXORA Deutschland (Grünberg, Germany). The synthesis of propyl-semiW84 has been described elsewhere (14). The structural formulas of the test compounds applied in this study are shown in supplemental Fig. S1.

*Chemical Synthesis of Propyl-[3-[1,3-dioxo-1H,3H-benzo-deisoquinoline-2-yl]-2,2-dimethylpropyl][dimethyl ammonium bromide—*Seinnamin*—780 mg (0.5 mmol) of N-[3-[(N,N'-dimethylamino)-2,2-dimethylpropyl]naphthalimide (15) were dissolved in diethyl ether, and dried. Obtained precipitate was filtrated, washed three times with quinoline-2-yl]-2,2-dimethylpropyl]dimethyl ammonium bromide was applied to ensure equilibrium binding conditions in these experiments. Nonspecific [3H]NMS binding was determined in homologous [3H]NMS/NMS competition experiments. Allosteric probe binding affinity was determined in homologous [3H]NMS/NMS/allosteric agent interaction experiments. Apparent affinities of orthosteric agonists were deduced from [3H]NMS/agonist interaction experiments performed either in the absence or presence of a fixed concentration of allosteric probe. Filtration and measurement of membrane-bound radioactivity was carried out as described above. [3H]NMS dissociation experiments were performed as described previously (16).

**Three-dimensional Modeling and Molecular Dynamics Simulations**—The coordinates of the x-ray structure 1U19 (Protein Data Bank) of bovine rhodopsin in its inactive state with a resolution of 2.2 Å (17) were used as template for the homology model of the human muscarinic M2 receptor. Further details were as described previously (16).

**Receptor-Ligand Complex—Docking of N-methylscopolamine (NMS) has been described previously (16). The allosteric modulator seminaph was docked using the software package GOLD (18). The binding site was defined as a sphere with a diameter of 10 nm around the residue M2Asp175, which is located in the center of the allosteric binding pocket. 50 different complex geometries in three main clusters were written out and scored with GoldScore. The best scored protein-ligand complex, which was in accordance with mutation experiments, was used for the following molecular dynamics simulation.

**Molecular Dynamics Simulations**—For further refinement and structure validation, several molecular dynamics simulations were carried out using the software package GROMACS (19). The simulations were performed for the ligand-free and...
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the seminaph-occupied receptor and the ternary complex consisting of the $M_2$ receptor, NMS, and seminaph, respectively. Further details including the model quality check have been described previously (16).

Data and Statistical Analysis—Indicated are the mean values ± standard error. Nonlinear regression analysis was done using the software PRISM 4.03 and INSTAT 3.0 (Graph Pad, San Diego, CA). Data from [35S]GTPγS binding studies were analyzed according to a ternary complex model, which explicitly includes effects of the allosteric ligand on receptor activation (20).

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R_{\text{active}}/R_t = \frac{L \cdot (1 + \alpha \cdot K \cdot [A] + \beta \cdot M \cdot [B] (1 + \alpha \cdot \gamma \cdot K \cdot [A]))}{1 + L \cdot M \cdot [B] (1 + \beta \cdot L) + K \cdot [A] (1 + \alpha \cdot \gamma + L \cdot M \cdot [B] (1 + \alpha \cdot \beta \cdot L \cdot L))}
\]

(Eq. 1)

$R_{\text{active}}/R_t$ is the fraction of active receptors relative to the total population of receptors. $K$ and $M$ are the association constants of the orthosteric agonist A and the allosteric agent B, respectively. $L$ denotes the receptor isomerization constant. $\alpha$ and $\beta$ indicate the intrinsic efficacy of A and B, respectively. $\gamma$ represents the binding cooperativity between A and B, and $\delta$ represents the activation cooperativity between A and B. The zero level of muscarinic receptor dependent [35S]GTPγS binding was defined as the binding in the presence of 1 $\mu$M of the inverse agonist atropine, $R_{\text{active}}/R_t = 0.0$. The maximum effect of the full agonist acetylcholine on [35S]GTPγS observed under control conditions in the absence of another test compound was set as $R_{\text{active}}/R_t = 1.0$. Global nonlinear curve fitting of fractional receptor activation $R_{\text{active}}/R_t$ as the dependent variable with A and B as the independent variables yielded estimates for $L$, $K$, $M$, $\alpha$, $\beta$, $\gamma$, and $\delta$.

Allosteric Probe Effects—The effect of naphmethonium on [3H]NMS equilibrium binding was analyzed as described (21, 22). [3H]NMS binding data derived from inhibition experiments with an agonist in the absence and presence of allosteric agent were fitted globally according to the extended allosteric ternary complex model using Equation 34 in Ref. 22. Specifically, fractional occupancy was fitted as the dependent variable with the concentrations of the agonist and the allosteric agent as two independent variables. After fixing the affinities of the radioligand and the allosteric agent to parameter values having been determined in separate experiments, curve fitting yielded estimates of agonist affinity and agonist cooperativity with naphmethonium.

RESULTS

Agonist-dependent Receptor Sensitivity to Allosteric Probes—We used $M_2$ receptors expressed in membranes from CHO cells transfected with the human receptor gene. Receptor activation leads to G protein stimulation, which was measured using the GTP analogue [35S]GTPγS that binds to an activated G protein but is not hydrolyzed (23). To induce receptor activation we applied the endogenous full agonist acetylcholine, the partial agonist pilocarpine, and additional agonists as specified below. As allosteric probes we chose the flexible “spaghetti-like” bis(ammonio)alkane-type compounds naphmethonium and W84, their shortened derivatives seminaph and propyl-semiW84, the rigid “disk-like” alcuronium, and the smaller gallamine (all structures shown in supplemental Fig. S1).

Naphmethonium (Fig. 1A) and the other probes hardly affected the basal, starting level of G protein activity but discriminated sensitively between pilocarpine- and acetylcholine-bound receptors (Fig. 1B, left and right panels, respectively). As expected, the partial agonist pilocarpine induced a significantly lower maximum response than did the endogenous full agonist acetylcholine. Naphmethonium reduced the maximum response of pilocarpine but not of acetylcholine. The potency as indicated by the inflection point of the curve was allosterically increased with pilocarpine but strongly decreased with acetylcholine (arrows in Fig. 1B indicate the inflection point shift to the left and right, respectively).

To parameterize the findings obtained with naphmethonium (Fig. 1C) and the other probes (supplemental Fig. S2), we applied Hall’s allosteric two-state model (20). The model assumes that receptors switch spontaneously between inactive and active (G protein stimulating) states (Fig. 2, gray and green panels, respectively). A ligand, either orthosteric or allosteric, may bind to these states with different affinities, thus affecting the equilibrium between inactive and active states. The model does not exclude that ligand-bound receptors differ in conformation depending on the type of bound ligand (20). When orthosteric ligand and allosteric probe are present simultaneously, reciprocal cooperative interactions may occur between these ligands with respect to both binding affinity and receptor activation (log $\gamma$ and log $\delta$ in Fig. 1C). Depending on whether these parameters are increased, decreased, or left unchanged, cooperativity is positive, negative, or neutral, respectively.

With pilocarpine, all probes except gallamine displayed negative cooperativity of receptor activation, whereas binding cooperativity varied depending on the probe (Table 1). With acetylcholine, however, there was no evidence for impaired receptor activation with any of the probes, whereas binding cooperativity was strongly negative with all probes. The divergent cooperativities with allosteric probes suggest a pronounced difference in three-dimensional shape of the allosteric site between the acetylcholine-bound and the pilocarpine-bound receptor.

To check that the divergent sensitivities between acetylcholine and pilocarpine to the allosteric probes do not merely depend on differences in the chemical nature of the agonists, we compared responses of a number of agonists to high concentrations of the probes naphmethonium and alcuronium. Agonist concentration-effect curves for $M_2$ receptor-mediated [35S]GTPγS binding were analyzed on a descriptive level with the inflection point and the upper plateau of the curve, indicating agonist potency and intrinsic efficacy, respectively. Control values obtained in the absence of allosteric probe are compiled in Table 2, allosteric changes of these parameters are illustrated in Fig. 3. The allosteric agents reduced the potency of the full agonists acetylcholine, carbachol, oxotremorine, and oxotremorine M to a similar extent by ~2–3 orders of magnitude, whereas the potency of
the partial agonist pilocarpine was not reduced or even increased (Fig. 3A). Vice versa, the maximum effect of the full agonists was hardly changed by the allosteric probes, whereas a strong reduction of efficacy was observed with the partial agonist (Fig. 3B). Therefore, we conclude that the difference in sensitivity between acetylcholine and pilocarpine to allosteric modulation is related to their full and partial agonist character, respectively.

Previous binding experiments suggested that the allosteric site of the M₂ receptor changes considerably in shape when the orthosteric site is occupied by an agonist instead of an antagonist (24). In that study we compared structure-binding relationships for stepwise shortened allosteric agents in M₂ receptors whose orthosteric site was occupied by either the radioantagonist [³H]NMS or the radioagonist [³H]oxotremorine M. Being an inverse agonist NMS stabilizes the receptor in an inactive conformation. We predicted that the binding cooperativities found in the present study with acetylcholine should differ from binding cooperativities with the antagonist NMS, and we wondered how these cooperativities would compare with those found for pilocarpine.

As shown in Fig. 4, there is no correlation between the binding cooperativities of the probes with acetylcholine and NMS, respectively. In contrast, probe/NMS binding cooperativities strongly correlate with probe/pilocarpine binding cooperativities. This suggests that the conformation of the allosteric site is similar between the inactive pilocarpine-bound state and the inactive NMS-bound state. Notably, this nicely corresponds with the finding that the conformation of the intracellular loop region of a GPCR is less divergent from the inactive state when a partial agonist is bound to the receptor instead of a full agonist (25). Thus, conformational changes in the extracellular receptor region seem to mirror spatial changes occurring in the intracellular region.

**Pinpointing the Critical Receptor Region for Cooperativity—**
Probes that have a strong negative binding cooperativity with acetylcholine but a neutral or even positive cooperativity with pilocarpine (i.e. naphmethonium, seminaph, alcuronium; cf. Fig. 4) are useful to discriminate between the three-dimensional forms of acetylcholine-bound and pilocarpine-bound receptors. To narrow down the allosteric domain whose occu-
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TABLE 1
Test compound actions in terms of the allosteric two state model of receptor activation

Underlying experimental data are displayed in supplemental Fig. S2. The parameter values were obtained as described in the legend of Fig. 1.

| Spontaneous receptor activity: receptor isomerization constant (log L) | Agonist/receptor interaction | Allosteric probe/receptor interaction | Probe/agonist/receptor interaction |
|---------------------------------------------------------------|--------------------------------|---------------------------------|----------------------------------|
|                                                               | Agonist affinity for R (log K) | Agonist intrinsic efficacy (log α) | Allosteric probe affinity for R (log M) | Allosteric probe intrinsic efficacy (log β) | Cooperativity of binding to inactive receptors (log γ) | Cooperativity of receptor activation (log δ) |
| Experiments with the partial agonist pilocarpine             |                                |                                 |                                  |                                  |                                           |                                        |
| W84                                                          | −0.80 ± 0.04                   | 5.76 ± 0.06                     | 1.08 ± 0.04                       | 6.54 ± 0.10                         | −0.34 ± 0.08                      | −0.89 ± 0.14                            | −0.64 ± 0.11b                          |
| Propyl-semiW84                                               | −0.80 ± 0.04                   | 5.92 ± 0.06                     | 1.06 ± 0.04                       | 6.58 ± 0.16                         | 0.01 ± 0.05                      | −1.77 ± 0.13                            | −0.79 ± 0.07b                          |
| Seminaph                                                     | −0.83 ± 0.02                   | 6.12 ± 0.03                     | 1.09 ± 0.02                       | 5.66 ± 0.11                         | 0.07 ± 0.03                      | 0.11 ± 0.12                             | −0.89 ± 0.03b                          |
| Alcuronium                                                   | −0.82 ± 0.02                   | 5.97 ± 0.06                     | 1.08 ± 0.03                       | 6.37 ± 0.12                         | −0.10 ± 0.03                     | 0.14 ± 0.14                             | −0.84 ± 0.04b                          |
| Gallamine                                                    | −0.84 ± 0.04                   | 5.99 ± 0.05                     | 1.09 ± 0.04                       | 6.18 ± 0.08                         | 0.03 ± 0.05                      | −2.06 ± 0.14                            | −0.15 ± 0.07                           |

* No significant difference in any pair of log M values for each given allosteric probe (t test, p > 0.05).
* Significantly smaller than zero indicating negative activation cooperativity (t test, p < 0.05).

TABLE 2
Potency and efficacy of the orthosteric agonists under control conditions in [35S]GTPγS binding experiments

Pairwise recording of concentration-effect curves of the agonist under study and of acetylcholine for comparison. ACh, acetylcholine; CCh, carbachol; Oxo, oxotremorine; OxoM, oxotremorine M; Pilo, pilocarpine. Potency is the −log value of the inflection points of the curves. Efficacy is the maximum agonist effect expressed as percentages of the maximum effect of acetylcholine set as 100%. The data indicate the means ± S.E. of 2–27 experiments performed in quadruplicate determinations.

| Agonist/receptor interaction | Allosteric probe/receptor interaction | Probe/agonist/receptor interaction |
|-----------------------------|-------------------------------------|----------------------------------|
| Ach                         | CCh                                 | Oxo                             |
|                             |                                     | Oxo-M                           | Pilo |
| Potency                     | 7.66 ± 0.03                         | 7.11 ± 0.14                     | 8.01 ± 0.15                     | 8.13 ± 0.10 | 6.35 ± 0.09 |
| Efficacy (%)                | 100                                 | 102 ± 6                         | 108 ± 4                         | 108 ± 4    | 106 ± 4    | 79 ± 3a  |

* Significantly different from acetylcholine efficacy (t test, p < 0.05).

pacity is sufficient to differentiate between pilocarpine-bound receptors and acetylcholine-bound receptors, we synthesized seminaph (Fig. 5A), which represents the essential half of naphmethonium (15). Binding experiments were carried out with seminaph in wild-type and selected point mutated human M₂ receptors. Muscarinic allosteric agonists typically reveal the highest affinity for the M₂ subtype and the lowest affinity for M₃. Previous studies starting with M₂/M₃ chimeric receptors and followed by the mutual exchange of corresponding single amino acids between these subtypes led to the identification of M₂Tyr¹⁷⁷ and M₂Thr⁴²³ as key epitopes for the M₂ selectivity of structurally diverse allosteric agents (26, 27). Subsequently, M₂Trp⁴²² was found to provide subtype-independent base-line affinity for allosteric agents (16). These three amino acids line the core region of the allosteric site (16). As revealed by [³H]NMS binding experiments, seminaph has a clearly higher affinity for M₂ than for M₃, and its binding to M₂ depends on each of the three key epitopes of the allosteric site of the M₂ receptor (supplemental Fig. S3). We conclude that seminaph binds to the core region of the M₂ receptor.

We applied a three-dimensional model of the M₂ receptor (16), which is based on the crystal structure of inactive bovine rhodopsin, and molecular dynamics simulations performed in a virtual membrane comprising lipid bilayer and aqueous phases to gain insight into the topography of seminaph binding. In the

FIGURE 3. Allosteric effects on the action of full agonists and the partial agonist pilocarpine explored in [³²P]GTPγS binding experiments as shown in Fig. 1. A, difference in agonist potency (log concentration at the agonist curve inflection point) in the presence and absence of allosteric probe. B, difference in maximum effect (upper curve plateau) in the presence and absence of allosteric probe expressed in percentages of the maximum effect of acetylcholine set as 100%. In case of pilocarpine because of pronounced efficacy loss. The data indicate the means ± S.E. derived from two to 14 concentration-effect-curves determined in quadruplicate. ACh, acetylcholine; CCh, carbachol; Oxo, oxotremorine; Oxo-M, oxotremorine M; Pilo, pilocarpine.

inactive NMS-bound receptor, seminaph undergoes an aromatic interaction with the side chains of M₂Tyr¹⁷⁷ and M₂Trp⁴²² (Fig. 5, B and C). The longer molecules naphmetho-
The inactive-to-active switch of a GPCR is thought to involve the movement of TM6 and TM7 relative to TM3 (3, 4) and a conformational change between E2 and TM7, whereas E2 is fixed to TM3 through the disulfide bond (Fig. 5). Without NMS, a very similar receptor conformation is stabilized by seminaph (Fig. 1D). In the absence of any ligand, the interaction between M2Tyr177 and M2Trp422 is still present (Fig. 5E), thus forming a junction between E2 and TM7, but the receptor does not reach an energetically stable state within the applied simulation period of 6,000 ps. This might reflect that the unliganded receptor protein is free to switch into an active conformation.

The inactive-to-active switch of a GPCR is thought to involve movement of TM6 and TM7 relative to TM3 (3, 4) and a conformational change between E2 (6–9). It is intriguing that the interaction between M2Trp422 and M2Tyr177 links the beginning of TM7 helix with E2, whereas E2 is fixed to TM3 through the disulfide bond (Fig. 5F). Thus, key amino acids of the allosteric site might help to hinder movement of TM7 relative to E2 and TM3 and might thus stabilize an inactive conformation. Remarkably, all muscarinic receptor subtypes except M5 contain an aromatic amino acid in the corresponding position to M2Tyr177, M2Phe222, M2Phe186, and M2Gln184. M2Trp422 is conserved among all five muscarinic receptor subtypes.

Role of TM7-M2Trp422 for Binding and Activation Cooperativity and for Agonist Action—The increase in pilocarpine potency and the strong decrease of acetylcholine potency seen in the G protein activation experiments under the influence of naphmethonium were interpreted in terms of corresponding changes in receptor binding affinity (see above). To check this interpretation, radioligand binding experiments with the orthosteric antagonist [3H]NMS in M5 wild-type receptors were carried out. Displacement of [3H]NMS by the respective agonist was measured in the absence and in the presence of naphmethonium, which is an enhancer of NMS binding in wild-type M2 receptors (supplemental Fig. S4). Apparent affinities of pilocarpine and acetylcholine in the absence of naphmethonium are illustrated in Fig. 6A (filled columns). Naphmethonium shifted agonist displacement curves to lower concentrations in the case of pilocarpine and to higher concentrations in the case of acetylcholine (supplemental Figs. S6 and S5, respectively). This indicates positive binding cooperativity with pilocarpine and negative binding cooperativity with acetylcholine (Fig. 6B, filled bars). Thus, it is validated that potency shifts observed in the [35S]GTPyS experiments can be interpreted in terms of shifts in binding affinity.

Among the allosteric core amino acids M2Tyr177, M2Trp422, and M2Thr423, only M2Trp422 is conserved in all five muscarinic receptor subtypes (position 7.35 according to Ref. 28). The corresponding M1Trp422 was reported to contribute to the binding of acetylcholine (29). We chose M2Trp422 to gain insight into the role of the allosteric core region for the observed cooperativities on the amino acid level.

[3H]NMS binding experiments with the receptor mutant M2Trp422→Ala revealed that M2Trp422 contributes more than one log unit of affinity to the binding of acetylcholine, whereas affinity remained unchanged for pilocarpine (Fig. 6A, open bars, right and left panels, respectively; supplemental Figs. S5 and S6).

M2Trp422 also strongly contributes to the affinity of naphmethonium (pK values, wild type = 7.69 ± 0.14, M2Trp422→Ala = 5.91 ± 0.18; supplemental Fig. S4). Therefore, the pronounced negative binding cooperativity between naphmethonium and acetylcholine appears to result, at least partially, from a contest between both ligands for recruiting M2Trp422, which is located at the interface between the allosteric and the orthosteric site as an affinity providing epitope. In line with this, strong negative binding cooperativity between naphmethonium and pilocarpine changes into the reverse direction, i.e. from positive in M2 wild type to slightly negative in the M2Trp422→Ala mutant (Fig. 6B, left panel, open bar). Thus, M2Trp422 like a pivot mediates the opposite binding cooperativities of naphmethonium with acetylcholine and pilocarpine.

To gain insight into the role of M2Trp422 for agonist-induced receptor activation, we carried out [35S]GTPγS binding experiments in the receptor containing CHO cell membranes under the conditions of the radioligand binding assays. As depicted in Fig. 6, pilocarpine looses much of its ability to stimulate [35S]GTPγS binding in the M2Trp422→Ala mutant (Fig. 6C, left panel, open bar). In contrast, the mutant is still sensitive to the full agonist acetylcholine (Fig. 6C, right panel, open bar). These findings show that M2Trp422 is critical for the binding affinity of the full agonist acetylcholine and for the receptor activation by the partial agonist pilocarpine.

**DISCUSSION**

The muscarinic acetylcholine receptor belongs to the rhodopsin type of GPCRs. The endogenous activator acetylcholine binds to the orthosteric site that is embedded in the depth of the ligand-binding cavity lined by the transmembrane helices of the receptor protein. The allosteric site is located above the orthosteric site in the extracellular loop region of the receptor. This...
study shows that allosteric modulators interact with orthosteric agonist-bound receptors in an agonist-dependent fashion. We conclude that allosteric modulators sense agonist-dependent differences in the three-dimensional arrangement of the extracellular loop region of the receptor. We localized the receptor region for modulator binding (which is the core region of the allosteric site) and mutated a conserved amino acid (M2-Trp422 → Ala) in this region. The mutant mimicked the consequences of allosteric modulation, i.e., pronounced loss of agonist affinity in case of acetylcholine but not pilocarpine and loss of agonist efficacy in case of pilocarpine but not acetylcholine. Thus, the amino acid (in the absence of allosteric agent) is critical for the binding event in the case of acetylcholine and for the receptor activation event in the case of pilocarpine.

Despite of the distance between the allosteric site and the orthosteric site in the inactive receptor (Fig. 5C), we cannot rule out the possibility that in the active conformation M2-Trp422 might be a direct contact point for orthosteric agonists. However, it seems more likely that this amino acid plays a mediating role for receptor binding by acetylcholine and receptor activation by pilocarpine when these agonists enter the orthosteric cage of hydrophobic amino acids that is thought to constrain upon agonist binding (3).

According to the three-dimensional model of the inactive receptor, M2-Trp422 is a direct contact point for allosteric agents such as naphmethonium. When this contact point is utilized by an allosteric probe, it appears to be unavailable as an affinity-providing epitope for acetylcholine and as a receptor activation-mediating epitope for pilocarpine. The applied allosteric probes did not induce receptor activation, and for some of them such as W84 and alcuronium, it has even been found that the receptor can be stabilized in an inactive conformation (13, 30).

The agonist type-dependent roles of M2-Trp422 for agonist binding and for the agonist-induced inactive (R) to active (R*) receptor switch could be tentatively explained as follows. Upon binding to the inactive receptor R, acetylcholine and other muscarinic
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agonists will form cation π-bonds with their positively charged nitrogen that attracts neighboring aromatic amino acid residues (3). This conformational change results in a concerted reorientation of amino acids and in stabilization of the active receptor conformation R*. We suggest that the E2/TM7 junction is maintained and M2Trp422 reoriented in the course of the inactive-to-active switch but in an agonist type-dependent fashion.

The affinity of the full agonist acetylcholine for the active receptor is ~100-fold higher than for the inactive receptor (agonist intrinsic efficacy log α ~2; Table 1). Correspondingly, the difference in three-dimensional shape between the acetylcholine-bound inactive and active receptor state should be more pronounced than in case of the partial agonist pilocarpine (log α ~1). If constriction of the hydrophobic cage is pivotal for the active receptor conformation, pilocarpine should have less power to promote receptor activation. This model is supported by divergent physicochemical properties of the two agonists.

In the acetylcholine molecule the positive charge is dispersed over the ammonium-methyl groups and the ethyl bridge, resulting a cloud of low charge density but high volume. Therefore, acetylcholine may interact simultaneously with various aromatic residues of the hydrophobic cage. In the protonized state of pilocarpine, the positive charge oscillates between the two nitrogens of the aromatic imidazole ring, resulting in a planar area of focused charge. Therefore, pilocarpine is likely to interact with a single aromatic residue. Additionally, the NH function of the imidazole may form a hydrogen bond. Taken together, the pattern of interactions with neighboring amino acid residues will widely differ between acetylcholine and pilocarpine. Acetylcholine seems to be better suited to induce a concerted reorientation of the orthosteric cage of aromatic amino acid residues.

In the case of acetylcholine, the strong negative binding cooperativity at inactive receptors (γ in Table 1 and Fig. 2) indicates a pronounced loss of affinity of the allosteric probes when the receptors are agonist-bound instead of free (γM versus M). This suggests that acetylcholine already in the inactive receptor state affects the E2/TM7 junction and moves M2Trp422 away from the allosteric site. In line with this, the binding experiments in the M2Trp422 → Ala mutant (Fig. 6A) show that Trp422 greatly contributes to the affinity of acetylcholine. The affinity of the allosteric probes for the acetylcholine-bound active receptor (βγδM; Fig. 2) is as low as for the inactive receptor (γM), because the parameter values for β (allosteric probe intrinsic efficacy) and δ (cooperativity of receptor activation) hardly differ from unity (Table 1). Thus, binding of the allosteric probes does not shift the equilibrium between acetylcholine-bound R and R*. Accordingly, acetylcholine efficacy for receptor activation is not influenced by the allosteric probes. The experiments carried out with the M2Trp422 → Ala mutant (Fig. 6C) reveal that receptor activation by acetylcholine does not depend on M2Trp422, which seems conceivable because reorientation of M2Trp422 occurs already in the inactive state (see above). In other words, acetylcholine appears to be able to stabilize the active receptor conformation without contribution of M2Trp422.

In the case of pilocarpine, the allosteric probes have similar affinity for the inactive pilocarpine-bound receptor and for the inactive NMS-bound receptor (Fig. 4), suggesting that the E2/TM7 junction is maintained and M2Trp422 still available as a contact point for the probes. Correspondingly, the binding experiments in the M2Trp422 → Ala mutant (Fig. 6A) show no significant change in pilocarpine affinity. In the active state of the pilocarpine-bound receptor, the allosteric probes (except gallamine) have a lower affinity (βγδM; Fig. 2) than in the inactive state (γM), because δ is significantly lower than unity, whereas β is near unity (Table 1). Because of their preference

FIGURE 6. Consequences of the mutation M2Trp422 → Ala for receptor binding and receptor activation. Receptor containing membranes were derived from transiently transfected CHO cells, and measurements were made under the buffer conditions of the [35S]GTPγS binding experiments. A, agonist apparent affinities for wild-type M2 and the receptor mutant as deduced from [3H]NMS radioligand binding experiments. The data indicate the means ± S.E. of three to six separate experiments each carried out in triplicate. B, binding cooperativities between the indicated ligands and naphthylmethylamine as deduced from the [3H]NMS binding experiments. C, effects of pilocarpine (Pilo), 10−7 M, and acetylcholine (ACh), 10−7 M, on M2 receptor-dependent G protein activation in these membranes. Baseline binding of [35S]GTPγS in the absence of drug was set as 100%. The data indicate the means ± S.E. of 16–32 measurements derived from two to four separate experiments. Comparisons were performed by two-tailed t testing. n.s., not significant; *** p < 0.0001; ** p < 0.001.
for the inactive receptor, the allosteric probes shift the pilocarpine-bound R-R* equilibrium in favor of the inactive state and thereby reduce the maximum effect of pilocarpine.

The lower affinity of the probes for pilocarpine-bound active R* suggests that the E2/TM7 junction is altered and that M2Trp422 is reoriented to contribute to the active conformation. In the light of the weak power of pilocarpine to stabilize the active receptor conformation, the reoriented M2Trp422 might be a necessary component in the network of conformational changes that are required to maintain the active conformation. This would explain why pilocarpine looses efficacy in the M2Trp422 → Ala mutant (Fig. 6C). It should be mentioned that a possible contribution to the affinity of pilocarpine of the reoriented M2Trp422 in the active M2 wild-type receptor would not be seen in the binding studies with the M2Trp422 → Ala mutant (Fig. 6C), because the pilocarpine-receptor complex is almost inactive in this mutant and would therefore not utilize M2Trp422 as an affinity providing epitope (see above).

We are aware that our explanation for the findings of this study is hypothetical. One aspect is that we cannot fully exclude a direct interaction between acetylcholine and M2Trp422. Furthermore, during the association and dissociation reaction, orthosteric ligands have to pass the allosteric site, and it is thermore, during the association and dissociation reaction, the active receptor conformation, the reoriented M2Trp422 in the active M2 wild-type receptor would not be seen in the binding studies with the M2Trp422 → Ala mutant (Fig. 6C), because the pilocarpine-receptor complex is almost inactive in this mutant and would therefore not utilize M2Trp422 as an affinity providing epitope (see above).

Taken together, the allosteric core region of the muscarinic receptor is not an “innocent bystander” in activation related conformational changes but may even control receptor activity. In this view it is consequent that appropriate allosteric compounds may induce receptor activation (31).

We conclude that orthosteric agonist-induced inactive-to-active conformational changes involve spatial rearrangement within the allosteric core region, possibly including reorientation or even opening of the allosteric junction between E2 and the top of TM7. Notably, other receptor epitopes that have been implicated in the binding of allosteric agents are located in the close vicinity of the junction between M1Tyr177 and M2Trp422. This is the so called EDGE sequence present in E2 of M2 (Glu172, Asp173, Gly174, Glu175) and the stretch of M2 amino acids Asn419, Val421, and Thr423 at the beginning of TM7 (32–34). Thus, there are additional contact points for bridging E2 and the top of TM7 by allosteric molecules.

The presence of allosteric sites on GPCRs is increasingly recognized, and allosteric ligands have been developed for a number of GPCRs with the aim to exploit allosteric sites for therapeutic reasons (35). Here, we show that the allosteric instrumentation can successfully be used for gaining more insight into the conformational changes underlying receptor activation by the physiological transmitter and related agents. This novel methodological approach might be useful far beyond the muscarinic M2 receptor applied in the present study.

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