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Conformation of Ligands Bound to the Muscarinic Acetylcholine Receptor

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ABSTRACT

Many biogenic amines evoke a variety of physiological responses by acting on G protein-coupled receptors. We have determined the conformation of two acetylcholine analogs, (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine, bound to the M_2 muscarinic acetylcholine receptor (M_2 mAChR) by NMR spectroscopy. The analysis of the transferred nuclear Overhauser effect indicated that the receptor selectively recognized the conformers of (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine with the *gauche* O-C2-C1-N dihedral angle at +60°. This is distinct from the predominant conformations of these ligands in solution with O-C2-C1-N dihedral angle (+80∼85°) in the absence of the M_2 mAChR, as assessed by analyses of the coupling constants and nuclear Overhauser effect spectroscopy. We have also built a molecular model of the M_2 mAChR-(*S*)-methacholine complex, based on the X-ray crystallographic structure of rhodopsin. This model indicated that the conformation with the *gauche* O-C2-C1-N dihedral angle at +55.5°, which is similar to the one determined by NMR measurement, is energetically favored in the binding of (*S*)-methacholine to the receptor. We suggest that this conformation represents the binding of the agonist to the M_2 mAChR in the absence of G protein.

The muscarinic acetylcholine receptors (mAChRs) belong to the family of G protein-coupled receptors (GPCRs). Upon binding to acetylcholine, mAChR transmit signals via the activation of G proteins. Five subtypes of mAChRs (M_1–M_5) have been identified and found to be broadly expressed in the central nervous system and in the peripheral tissues (Kubo et al., 1986; Bonner et al., 1987, 1988; Peralta et al., 1987). In the central nervous system, the mAChRs are known to modulate learning and memory and to regulate the sensory, motor, and autonomic systems. In peripheral tissues, the mAChRs mediate parasympathetic activities, such as ileum muscle contraction.

GPCRs comprise one of the largest superfamilies. Because many of them have important pharmacological roles, their characteristics, such as the recognition of ligands, are now the targets of pharmaceutical interests (Flower, 1999). No detailed structural information has been available for the GPCRs, except for rhodopsin (Palczewski et al., 2000) and the ligand binding domain of metabotropic glutamate receptor (Kunishima et al., 2000). The receptor-bound conformations of pituitary adenylate cyclase activating polypeptide (Inooka et al., 2001) and glutamate (Kunishima et al., 2000) have recently become available but there is no direct information on how biogenic amines, such as acetylcholine, are recognized by GPCRs, such as mAChRs. To determine the nature of the ligand-receptor interaction, an understanding of the conformations of the interacting ligands as well as the architecture of their binding pocket would be necessary.

The mutagenesis studies of the mAChRs have revealed some key residues for acetylcholine binding, including Asp 103, and Tyr 104 and Tyr 403, located in the third and sixth transmembrane regions, respectively (Wess et al., 1991; Page et al., 1995; Vogel et al., 1997; Ward et al., 1999). The Asp 103 residue is likely to interact with the quaternary amine group of acetylcholine through charges and Tyr 104 and Tyr 403 may interact with the ester group of acetylcholine through hydrogen bonds or π interaction involving the benzene group. Thus, the binding of acetylcholine and other agonists seems

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ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; GPCR, G protein-coupled receptor; TRNOE, transferred nuclear Overhauser effect; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TRNOESY, transferred nuclear Overhauser effect spectroscopy; NMS, *N*-methylscopolamine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Sf9, *Sporodoptera frugiperda*; PAGE, polyacrylamide gel electrophoresis.

to be governed by the orientation of their quaternary amine and ester groups, and this orientation is defined mainly by the dihedral angles of O-C2-C1-N (Fig. 1).

Attempts have been made to determine the conformation of the muscarinic agonists producing the physiological effects by synthesizing and examining the diastereomers of conformationally rigid acetylcholine analogs (Portoghese, 1970; Casy, 1975). In the majority of the cases, the *trans* isomers of the rigid analogs were more potent than the *cis* isomers in inducing the muscarinic activities. Therefore, the pharmacologically active conformation of the muscarinic agonists has been assumed to have the *trans* O-C2-C1-N dihedral angle (~180°) (Taylor and Insel, 1990).

Because of the recent development of the Sf9/baculovirus system and its culturing techniques, sufficient quantities of many GPCRs (milligram order) have become available for biophysical studies, such as NMR. The conformation of small molecules interacting with large molecules such as proteins can be determined using the TRNOE method (Clore and Gronenborn, 1982). The intensities of negative NOE signals (TRNOE), observed because of protein-ligand interaction provide the spatial information between protons of the bound state of the ligands. By using this NMR method and an energy calculation, the conformation of acetylcholine bound to the nicotinic acetylcholine receptor has been suggested (Behling et al., 1988). However, the receptor-bound confor-

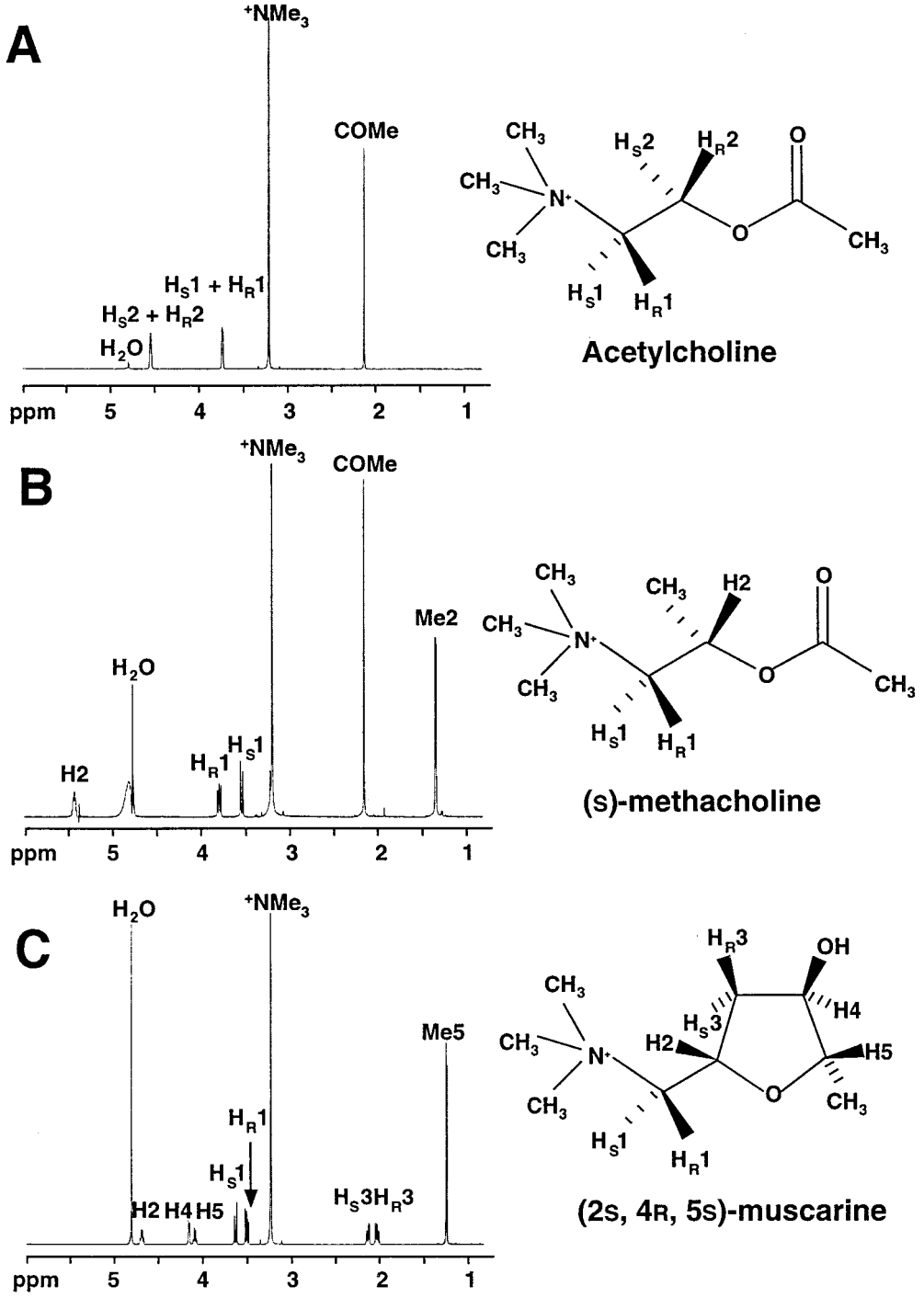

Fig. 1. ^{1}H-NMR spectrum of the muscarinic ligands at 600 MHz. The two protons on C1 or C2 are not distinguished in acetylcholine (A), whereas the corresponding protons are distinguished, conferring distinct signals in (*S*)-methacholine (B) or (2*S*,4*R*,5*S*)-muscarine (C) (Me, methyl group).

mation of acetylcholine cannot be determined only by NMR data because the four methylene protons cannot be distinguished on an NMR spectrum, due to the chemical equivalency of the two sets of methylene protons (Fig. 1). The acetylcholine analogs methacholine and muscarine show an individual signal for each proton due to the difference in the chemical shifts. The full assignment of the protons enables us to determine the receptor-bound conformation based solely on the TRNOE intensities.

In this article, we report the conformations of (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine bound to the mAChR and discuss the physiological significance. The preliminary results have been reported elsewhere (Furukawa et al., 2001).

Materials and Methods

Expression and Purification of the M_2 mAChR Mutant (M_2 Mutant). A mutant of mAChR M_2 subtype (M_2 mutant) was constructed from the M_2 mAChR cDNA, as in the previous report (Hayashi and Haga, 1996). The mutation included the deletion of the central part of the protease-susceptible third intracellular loop (233–380), the replacement of the putative glycosylation residues Asn 2, 3, 6, and 9 with Asp to prevent molecular heterogeneity, and the addition of a hexa-histidine tag and a thrombin cleavage site at the C terminus for purification. The Sf9 cells, grown to a density of 3.0×10^6 cells/ml at 28° in Cell Master (Wakenyaku, Kyoto, Japan), were infected with the recombinant virus for the M_2 mutant at an m.o.i. of 5 to 10 in the presence of 1 μM atropine sulfate. The cells were harvested after 48 h of infection. The membrane fraction, prepared as described previously (Hayashi and Haga, 1996), was solubilized with 1% digitonin (Wako Pure Chemicals, Osaka, Japan) and 0.3% sodium cholate and was loaded onto 3-(2′-aminobenzhydryloxy)tropane (ABT)-agarose affinity chromatography gel (Haga and Haga, 1985). The eluted fraction was loaded onto a hydroxyl apatite column and was washed with 12 volumes of a buffer containing 10 mM potassium phosphate buffer, pH 7.4, 0.1 mM atropine sulfate, and 0.3% sodium cholate (Dojindo, Kumamoto, Japan). The M_2 mutant was eluted with a buffer containing 1 M potassium phosphate buffer, pH 7.4, 0.1 mM atropine sulfate, and 0.3% sodium cholate. The eluate was loaded onto a PD10 column (Sigma) equilibrated with a buffer containing 10 mM Tris(hydroxymethyl-*d3*)amino-*d2*-methane-deuterium chloride (Sigma-Aldrich, St. Louis, MO), pH 7.0, and 0.2% sodium cholate in D_2O. After the addition of 1.5 mM (*S*)-methacholine or (2*S*,4*R*,5*S*)-muscarine to the void volume fraction, the receptor was concentrated to 50 μM. Crude soybean phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) was added to a concentration of 1.5 mg/ml before NMR measurements.

NMR Measurement. The NMR measurements were performed on a Bruker ARX-600 spectrometer (Bruker, Osaka, Japan) at 296°K for the NOESY and TRNOESY experiments, and on a Bruker ARX-800 spectrometer at 296°K for the one-dimensional spectroscopy experiments. The assignment of the proton signals for (*S*)-methacholine was based on the previous reports (Casy et al., 1971), and that for (2*S*,4*R*,5*S*)-muscarine was based on correlation spectroscopy, total correlation spectroscopy, and NOESY spectra.

The proton two-dimensional NOESY or TRNOESY spectrum was acquired in a phase-sensitive mode using the time proportional phase increments method or the States-time proportional phase increments method with the water signal suppression by presaturation during the relaxation delay and the mixing time. The spectrum was baseline-corrected; multiplied by a π/2-shifted, squared, sine bell window function in the F1 and F2 dimensions; Fourier transformed; and zero-filled to confer the final data matrices.

For the NOESY experiments with the free (*S*)-methacholine and the (2*S*,4*R*,5*S*)-muscarine, a mixing time of 1.2 s was used. For the two-dimensional TRNOESY experiments of the ligands in the presence of the purified M_2 mutant, the mixing time was randomized at ± 10% from 50 and 150 ms for (*S*)-methacholine and for (2*S*,4*R*,5*S*)-muscarine, respectively, to cancel the correlation spectroscopy peaks. Two TRNOESY spectra were acquired in each experiment: one with the sample in the presence of the purified M_2 mutant (50 μM) and (*S*)-methacholine or (2*S*,4*R*,5*S*)-muscarine (1.5 mM) and the other with the same sample except for the addition of 1.5 mM atropine. The latter spectrum was subtracted from the former to acquire the spectrum representing the specific binding of (*S*)-methacholine or (2*S*,4*R*,5*S*)-muscarine to the M_2 mutant. The intensities of the TRNOE cross-peaks were roughly linear up to 100 ms, suggesting that the spin diffusion effect is negligible in this range.

The coupling constants of the ligands in solution were determined using high-resolution one-dimensional ^{1}H-NMR measurements with a Bruker ARX-800 spectrometer. The free induction decay was multiplied by the exponential or Gaussian function, and then was Fourier-transformed.

[^{3}H]NMS and [^{35}S]GTPγS Binding Assays. The binding assays were carried out on virus-infected Sf9 cell membrane fractions expressing the M_2 mutant or the M_2 mutant fused to the α subunit of the G protein G_{i1} at the C terminus (M_2-$G_{i1}\alpha$), as described in the previous studies (Furukawa and Haga, 2000; Guo et al., 2001). The [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) binding (1.5 nM) was displaced by acetylcholine, carbamylcholine, (*S*)-methacholine, (*R*)-methacholine, or atropine. The agonist-stimulated binding of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (50 nM) to the M_2-$G_{i1}\alpha$ was measured in the presence of 1 μM GDP and various concentrations of the ligands. The volume of the above reactions was 200 μl. After incubating the mixtures at 30°C for 1 h, the membrane fraction was trapped on a GF/B glass fiber filter and the radioactivity bound to the membrane was measured.

The M_2 mAChR Modeling. The model of photoactivated rhodopsin (metarhodopsin II) was constructed by adopting the rigid body movement of transmembrane segments 3 through 6 to the crystal structure of nonactivated rhodopsin (Palczewski et al., 2000), in accordance with Borhan et al. (1996) and Farrens et al. (2000). Then, the model of the activated form of the M_2 mAChR was built using the model of metarhodopsin II, employing the "Homology" module. The transmembrane bundles were built by replacing the amino acid residues of the helices of rhodopsin with the sequence of the M_2 mAChR and the loop structures were constructed by using the fragment library in the "Biopolymer" module of Insight II (Accelrys, Princeton, NJ). The structure was energy-minimized by the use of the DISCOVER 3 force field. The model of (*S*)-methacholine was docked to the putative binding site of the M_2 mAChR model, guided by the ligand-receptor interactions between the quaternary amine-Asp 103 and the carbonyl oxygen-Tyr 403. The molecular dynamics with simulated annealing, employing DISCOVER 3 force field, was carried out with the (*S*)-methacholine bound model involving the amino acid residues within 9 Å from the ligands.

Results

Preparation of the M_2 Mutant. Throughout this work, we have used the M_2 mutant without the putative glycosylation site and the central part of the third intracellular loop, and with the hexa-histidine tag at the C terminus, which has been previously shown to possess the intrinsic activities of the M_2 mAChR (ligand binding and G protein activation) (Hayashi and Haga, 1996; Furukawa and Haga, 2000).

The M_2 mutant was expressed and purified to homogeneity without any degradation. The addition of the muscarinic antagonist, atropine sulfate, into the culturing medium improved the expression level in the Sf9/baculovirus by 1.5-fold. The yield of the M_2 mutant was from 1 to 1.5 mg/l of culture, which is one of the highest reported expression levels for

eukaryotic membrane proteins (Grisshammer and Tate, 1995). The membrane fraction was solubilized by the mixture of digitonin and sodium cholate, and was purified to homogeneity using ABT-agarose and hydroxyl apatite columns (Haga and Haga, 1985) (Fig. 2). The upper band in the SDS-PAGE was thought to represent the dimeric form of the M_2 mutant because it was stained with the anti-hexa-histidine tag polyclonal antibodies (data not shown). The washing of the hydroxyl apatite column with the sodium cholate-based buffer partially removed the digitonin bound to the receptor. This step was effective for avoiding nonspecific binding of (*S*)-methacholine or (2*S*,4*R*,5*S*)-muscarine to the digitonin micelles, which produced nonspecific TRNOE signals. Such signals were observed in the presence of more than 10 mg/ml digitonin. The concentrations of digitonin and sodium cholate in the sample containing 2 mg/ml M_2 mutant were approximately 5 mg/ml and 2 mg/ml, respectively, as determined by the band intensities in thin-layer chromatography (data not shown). The addition of soybean phosphatidylcholine at 1.5 mg/ml further diminished the nonspecific TRNOE and increased the stability at higher temperature (23°C). The M_2 mutant thus prepared retained approximately >95% and 90% of the L-[^{3}H]quinuclidinyl benzilate or [^{3}H]NMS binding activity in the presence of 1.5 mM of (*S*)-methacholine or (2*S*,4*R*,5*S*)-muscarine after three days at 4 and 23°C, respectively: the K_i values estimated under NMR conditions were essentially the same as the K_i and EC_{50} values estimated for M_2 mutants and M_2-$G_{i1}\alpha$ fusion proteins in the membranes (Table 1). The purified M_2 mutant did not show any sign of degradation after the NMR measurements, as assessed by SDS-PAGE (Fig. 2).

Characteristics of the Ligand Binding. The data points in the [^{3}H]NMS displacement curves were well fitted to the equation for a one-site model (data not shown). The affinities (in K_i) for acetylcholine, (*S*)-methacholine, and (2*S*,4*R*,5*S*)-muscarine were similar to each other (20–80 μM), whereas that for (*R*)-methacholine was approximately 20-fold lower than that of acetylcholine (Table 1), and are consistent with the previous reports for the M_1 mAChR (Page et al., 1995).

The M_2-$G_{i1}\alpha$ showed agonist-dependent [^{35}S]GTPγS binding in the presence of 1 μM GDP. The EC_{50} values as well as the [^{35}S]GTPγS binding maxima were similar between acetylcholine and (*S*)-methacholine (Table 1). Consistent with the previous report on the fusion protein of the β_2-adrenergic receptor and the α subunit of G protein G_s (Seifert et al., 1998), the addition of the G protein $\beta\gamma$ subunit was not necessary to observe agonist-dependent [^{35}S]GTPγS binding to the M_2-$G_{i1}\alpha$. The *S*-isomer of methacholine was chosen for the NMR analysis because its characteristics resembled those of the physiological ligand, acetylcholine. The EC_{50} and [^{35}S]GTPγS binding maximum values for (*R*)-methacholine were 100-fold higher and 2-fold lower than those for acetylcholine, respectively. Therefore, the *R*-isomer is a partial agonist of the M_2 mutant.

The Conformations of (*S*)-Methacholine and (2*S*,4*R*,5*S*)-Muscarine in Solution. The coupling constants between H_S1 and H2 ($^3J_{HS1\text{-}H2}$) and between H_R1 and H2 ($^3J_{HR1\text{-}H2}$) were 1.3 and 9.4 Hz for (*S*)-methacholine and 1.4 and 9.4 Hz for (2*S*,4*R*,5*S*)-muscarine, respectively, in the ^{1}H NMR spectra at 800 MHz (Fig. 3). By using the modified Karplus equation (Haasnoot et al., 1980) for $^3J_{HS1\text{-}H2}$, the O-C-C-N dihedral angles of (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine were estimated to be +83° and +82°, respectively. The estimated angle for (*S*)-methacholine is essentially the same as those reported by Casey et al. (1971): approximately +90° for the O-C-C-N dihedral angle determined from the coupling constants of $^3J_{HS1\text{-}H2}$ (1.5 Hz) and $^3J_{HR1\text{-}H2}$ (8.8 Hz). On the other hand, Partington et al. (1972) proposed that β-methacholine exists as a 3:1 mixture of the two *gauche* conformers with O-C-C-N dihedral angles of +60° and −60°. Although it is theoretically possible to assume that β-methacholine and muscarine take two or more conformations that are in equilibrium, we consider it more probable that they exist predominantly in a single conformation rather than in the two *gauche* conformations with a ratio of 3 to 1 for the following reasons: 1) Molecular mechanics calculation (Discover III) indicated that the conformation with the O-C-C-N dihedral angles at +81° is energetically the most stable, and that the *trans* and the *gauche* conformations with +180° and −60° O-C-C-N dihedral angles is not energetically preferable because of the steric hindrance between the C2-methyl and *N*-methyl groups (data not shown). 2) The present results for O-C2-C1-N dihedral angles of (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine (+83° and +82°) are similar to those estimated form their crystal structures (+85° and +73°), which is consistent with the idea that they are stable in these conformations rather than in the *trans* or *gauche* conformations with +180° or −60° O-C-C-N dihedral angles. 3) The NOE signal between H_R1 and H2 was too weak to consider the presence of the *gauche* conformer with O-C-C-N dihedral angle at −60°.

The Conformations of the Receptor-Bound (*S*)-Methacholine and (2*S*,4*R*,5*S*)-Muscarine. The negative NOE signals for both (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine were best observed at the ligand-to-receptor ratio of

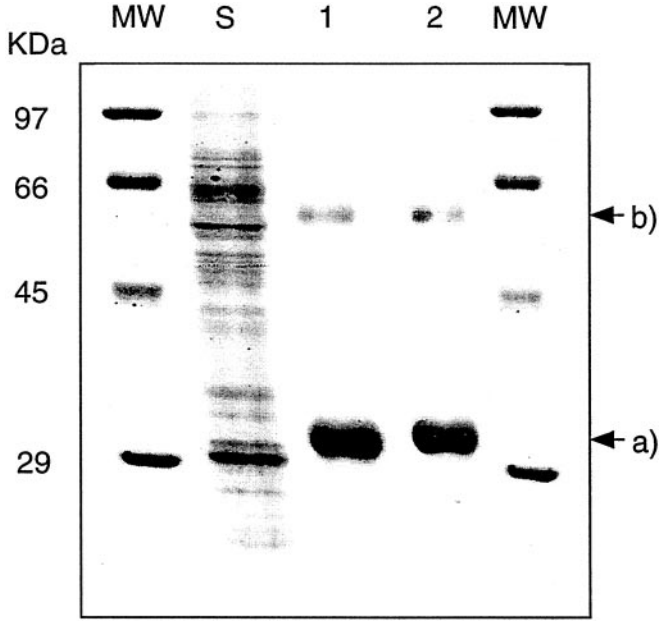

Fig. 2. Purification of the M_2 mutant. The solubilized M_2 mutant was purified with ABT-agarose and hydroxyl apatite column and then was subjected to 12.5% SDS-PAGE and Coomassie Brilliant Blue staining. Arrows a) and b) indicate monomeric and dimeric species of the M_2 mutant. MW, molecular mass marker; S, solubilized fraction; 1, purified sample; 2, purified sample after TRNOESY measurement.

30. No signal for either ligand was detected in the control buffer containing detergents (0.5% digitonin and 0.2% sodium cholate) and lipids but not the M_2 mutant. Furthermore, the negative NOE signals disappeared when atropine, an antagonist of the mAChRs, was added to the sample solution containing the receptor protein. These control experiments indicated that the negative intramolecular NOE signals observed were produced by the specific ligand-receptor interaction. The TRNOESY spectra were obtained in the presence and absence of atropine. Then, the former spectrum was subtracted from the latter to confer the final spectrum representing the specific binding of the above agonists to the M_2 mutant. No proton peak, besides the one for the acetyl protons in (*S*)-methacholine, overlapped with those for atropine in the ^{1}H NMR spectrum (data not shown). In the NOESY or TRNOESY experiment, there was no overlap. Therefore, there was no interference caused by the presence of the atropine signal in this structural analysis.

For (*S*)-methacholine, the TRNOE signals were observed starting from the mixing time τ_m = 25 ms. We chose to use τ_m = 50 ms for the measurement because it minimized the appearance of indirect NOEs via spin diffusion and still conferred significant NOE intensities for the distance calculations. The pattern of the cross-peaks in the TRNOESY spectrum was consistent with the conformation with the *gauche* O-C2-C1-N dihedral angle (Fig. 4). The differences in the cross-peak patterns between the NOESY (free) and TRNOESY (receptor-bound) spectra were the ratios of the peak intensities, H2-$^+$NMe$_3$/H2-H$_S$1, and Me2-$^+$NMe$_3$/Me2-H$_S$1. Both of the ratios were lower in the TRNOESY spectrum than in the NOESY spectrum (a/b > a*/b* and c/d > c*/d* in Fig. 4). This indicated the shift in the degree of the O-C2-C1-N dihedral angle of (*S*)-methacholine as it became bound to the M_2 mutant. In addition, the observation that the intensities of the TRNOE cross-peaks, Me2-H$_S$1 (d*) and 2-methyl-H$_R$1 (e*), were consistently equal at various lengths of mixing time (τ_m = 25, 50, and 100 ms) (data not shown), indicated that these distances were approximately equal for the bound ligand. The equal distance between Me2-H$_S$1 and Me2-H$_R$1 indicated that the O-C2-C1-N dihedral angle was approximately +60° and that the binding of the ligand to the receptor led to a 20~30° rotation from the predominant conformation of the free form.

The difference-TRNOE spectrum for (2*S*,4*R*,5*S*)-muscarine was obtained essentially by the same method as for (*S*)-methacholine, except that a longer mixing time, τ_m = 150 ms, was required to detect a sufficiently intense TRNOE. The patterns of the H2-H1 and H3-H1 cross-peaks in the TRNOESY spectrum (receptor-bound form) and the NOESY spectrum (free form) differed from each other (Fig. 5). As found with (*S*)-methacholine, the ratio of the peak intensities, H2-$^+$NMe$_3$/H2-H$_S$1, decreased as (2*S*,4*R*,5*S*)-muscarine became bound to the receptor (a/b > a*/b*), indicating the decrease in the degree of the O-C2-C1-N dihedral angle. In the NOESY spectrum, the order of intensities was H$_R$1-H$_R$3 > H$_S$1-H$_R$3 > H$_S$1-H$_S$3 > H$_R$1-H$_S$3 (d > f > e > c), whereas that in the TRNOESY spectrum was H$_R$1-H$_R$3 > H$_S$1-H$_R$3 > H$_R$1-H$_S$3 > H$_S$1-H$_S$3 (d* > f* > c* > e*). This indicated that H$_S$3 moved away from H$_S$1 whereas H$_R$1 moved closer to H$_s$3 as a result of binding to the receptor (Fig. 5B). This pattern of TRNOE signals, in comparison with that of the NOE signals, indicated that the conformation of the receptor-bound (2*S*,4*R*,5*S*)-muscarine takes the *gauche* O-C2-C1-N dihedral angle at less than +80~90°, most probably around +60°.

Thus, the conformation of receptor-bound (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine is *gauche* with the O-C2-C1-N dihedral angle at approximately +60° but not *trans* with the O-C2-C1-N dihedral angle at +180°, although the *trans* conformation has been suggested to be responsible for the physiological function from the studies with the rigid acetylcholine analogs. In fact, the TRNOESY spectra did not indicate the expected TRNOE signals for the conformer with the *trans* O-C-C-N dihedral angle for (*S*)-methacholine [e.g., strong signals for H2-H$_R$1 (a*) and Me2-$^+$NMe3 (c*) and weak or no signals for H2-H$_S$1 (b*) and Me2-H$_S$1 (d*)) and for (2*S*,4*R*,5*S*)-muscarine (e.g., strong signals for H2-H$_R$1, signals for H$_S$3-$^+$NMe3 and H$_R$3-$^+$NMe3, and weak or no c* and d* signals].

Ligand Docking to the M_2 mAChR Binding Site. The amino acid residues of the M_2 mAChR were successfully fitted into a model derived from the crystal structure of rhodopsin (Palczewski et al., 2000) to form the seven bundles of α helices characteristic of the GPCRs. We took the results obtained by Farrens et al. (1996) and Borhan et al. (2000) into account by moving the transmembrane segments 3 and 6 outward, and rotating the transmembrane segment 6 in a counter-clockwise direction (facing the extracellular surface) at the ligands at the binding site. The *S*-isomer of methacholine was fitted into the putative ligand-binding site of this molecular model, guided by the two intermolecular interactions, between the quaternary amine and Asp 103 and between the acetyl group and Tyr 403. The most stable conformation for (*S*)-methacholine had the *gauche* rather than the

TABLE 1

Kinetic parameters of muscarinic ligands

K_i values were calculated based on the IC_{50} values from the displacement curves of [^{3}H]NMS by the ligands and the K_d value for [^{3}H]NMS reported previously (Furukawa and Haga, 2000). The EC_{50} is the ligand concentration that conferred 50% of the [^{35}S]GTPγS binding maximum in the M_2-G$_{i1}\alpha$. Results are the means ± S.E. of three duplicate experiments. [^{3}H]NMS displacement (Membrane) and [^{35}S]GTPγS binding assays were carried out using membrane fraction of Sf9 cells expressing the M_2 mutant and M_2-G$_{i1}\alpha$, respectively. [^{3}H]NMS displacement (NMR condition) assay was carried out using the purified M_2 mutant after NMR measurement.

Ligands	−log K_I		−log EC_{50}
	Membrane	NMR Condition	
	M		*M*
Acetylcholine	4.71 ± 0.15	4.30 ± 0.23	5.03 ± 0.22
(*S*)-methacholine	4.34 ± 0.11	3.96 ± 0.11	4.62 ± 0.17
(*R*)-methacholine	3.37 ± 0.08	3.01 ± 0.13	2.95 ± 0.03
(2*S*,4*R*,5*S*)-muscarine	4.10 ± 0.04	3.64 ± 0.01	N.D.

N.D., not determined.

trans conformation with the O-C-C-N dihedral angle at +55.5°, as assessed by molecular dynamics with simulated annealing, employing the DISCOVER 3 force field with the freedom to change its conformation (Figs. 6 and 7). The arbitrary fitting of (*S*)-methacholine with the *trans* conformation, followed by the energy optimization, resulted in the conversion to the *gauche* conformation. This confirmed that the *gauche* conformation was preferred over the *trans* for (*S*)-methacholine bound to the M_2 receptor model.

This M_2 mAChR model revealed that several key amino acid residues were located in the proximity of the bound ligand in the transmembrane region (Figs. 6 and 7). These residues included Asp 103, Tyr 104, Thr 190, and Tyr 403. The positively charged quaternary amine group of the (*S*)-methacholine molecule pointed toward the negatively charged Asp 103 residue. The carbonyl oxygen in the acetyl group formed a hydrogen bond with Tyr 403. Furthermore, the carbonyl oxygen and Tyr 403 formed a hydrogen bond network with Tyr 104 and Thr 190 as shown in Fig. 6B. This hydrogen bond network would contribute to the stabilization of the agonist-bound receptor structure. This is consistent with mutational experiments indicating that these residues were important for the agonist binding (Wess et al., 1991; Lu and Hulme, 1999). Recently Lu et al. (2001) proposed a model for the complex of acetylcholine and M_1 mAChR based on the structure of rhodopsin and their mutational studies, where they also assumed that acetylcholine bound to the receptor takes the *gauche* form, adopting our preliminary result (Furukawa et al., 2001). A particular difference between our molecular model and the one by Lu et al. (2001) is that our model includes the rotation of the transmembrane segment 6, and thereby, the disposition of the Tyr 403 residue. This disposition of Tyr 403 brings about a hydrogen bond network among (*S*)-methacholine, Tyr 104, and Thr 190. Without rotation of transmembrane segment 6 and disposition of Tyr 403, the formation of this hydrogen bond network does not seem to be possible.

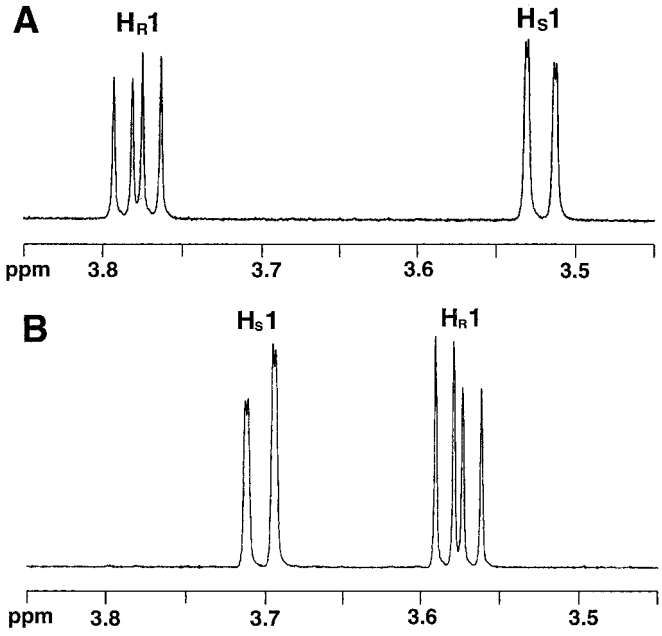

Fig. 3. The ^{1}H-NMR spectra of (*S*)-methacholine (A) and (2*S*,4*R*,5*S*)-muscarine (B) at 800 MHz, focusing on the couplings of H_S1 and H_R1. The coupling constants were, $^3J_{HS1\text{-}H2}$ = 1.3 and $^3J_{HR1\text{-}H2}$ = 9.4 for (*S*)-methacholine, and $^3J_{HS1\text{-}H2}$ = 1.4 and $^3J_{HR1\text{-}H2}$ = 9.4 for (2*S*,4*R*,5*S*)-muscarine. Assuming little or no conformational mixture exists, the degree of the O-C2-C1-N dihedral angle for the predominant conformation can be calculated as +83° and +82° for (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine, respectively, by the equation, $J = 13.89*\cos^{2\phi} - 0.98*\cos\phi + \Sigma\Delta\chi_I\{1.02 - 3.40\cos^2(\xi_i*\phi + 14.9*|\Delta\chi_I|)\}$, where ϕ is the degree of the dihedral angle, $\Delta\chi_I$ is the electronegativity difference between the substituents attached to the H-C-C-H system and the hydrogen, and ξ_I is the correction term (+1 in this case) (Haasnoot et al., 1980).

Discussion

The determination of the ligand conformations that bind mAChRs has been an important issue in the field of pharmacology (Portoghese, 1970). In the present study, we identified the conformations of two muscarinic ligands, (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine, bound to the M_2 mAChR, as having the *gauche* O-C2-C1-N dihedral angle at approximately +60°. This result was further supported by the modeling study of the mAChR-(*S*)-methacholine complex, based on the crystal structure of rhodopsin (Palczewski et al., 2000), which showed that the most energetically preferred conformation of (*S*)-methacholine had a +55.5° O-C2-C1-N dihedral angle. This receptor-bound conformation differs from the one in solution, because (*S*)-methacholine is thought to exist predominantly as a conformer with an +80–85° O-C2-C1-N dihedral angle.

Numbers of studies using rigid acetylcholine analogs have shown that their *trans* isomers induce more muscarinic activities than the *gauche* isomers (Portoghese, 1970; Casy, 1975). Therefore, there has been a general assumption that acetylcholine acts on the mAChRs in a conformation similar to that of the *trans* isomers of the rigid acetylcholine analogs (Taylor and Insel, 1990). However, this assumption had some ambiguity, because the degree of the O-C2-C1-N dihedral angles in the active isomers varies from compound to compound. For example, the dihedral angle of ACTM (Chothia and Pauling, 1970) and 3-dimethylamino-2-hydroxybicyclo[2,2,2]octane (Nelson and Wilson, 1971) were +137° and +120°, respectively, although both of the compounds were originally designed to represent the *trans* conformer of acetylcholine (180°). On the other hand, their inactive *cis* isomers, which were designed to represent the *gauche* conformer of acetylcholine (~60°), were found to have the eclipsed O-C2-C1-N dihedral angle at 0° (Chothia and Pauling, 1970; Nelson and Wilson, 1971). In addition, there were such cases as *cis*- and *trans*-dimethyldiacetoxypiperidium iodide (Lewis et al., 1973) and *d*- and *l*-1-methyl-3-acetoxy-*trans*-decahydroquinolin methiodide (Smissman and Chappell, 1969), where the isomers representing the *gauche* O-C2-C1-N dihedral angle induced more muscarinic activity than those representing the *trans*-O-C2-C1-N. The O-C2-C1-N dihedral angles of the *cis*- and *trans*-dimethyldiacetoxypiperidium iodide isomers were approximately 60° and 180° (Lewis et al., 1973), and those isomers of the *d*- and *l*-1-methyl-3-acetoxy-*trans*-decahydroquinolin methiodide isomers were 74° and 169° (Stephen et al., 1972), respectively. Together, these studies indicate that the rigid acetylcholine analogs with a broad O-C2-C1-N dihedral angles (60 to 137°), rather than exclusively 180°, are able to induce the muscarinic activities (Baker et al., 1971). Thus, these data are not sufficient to determine the precise O-C2-C1-N dihedral angle at which acetylcholine binds to the receptor and induces the pharmacological activity. It should be noted that Partington

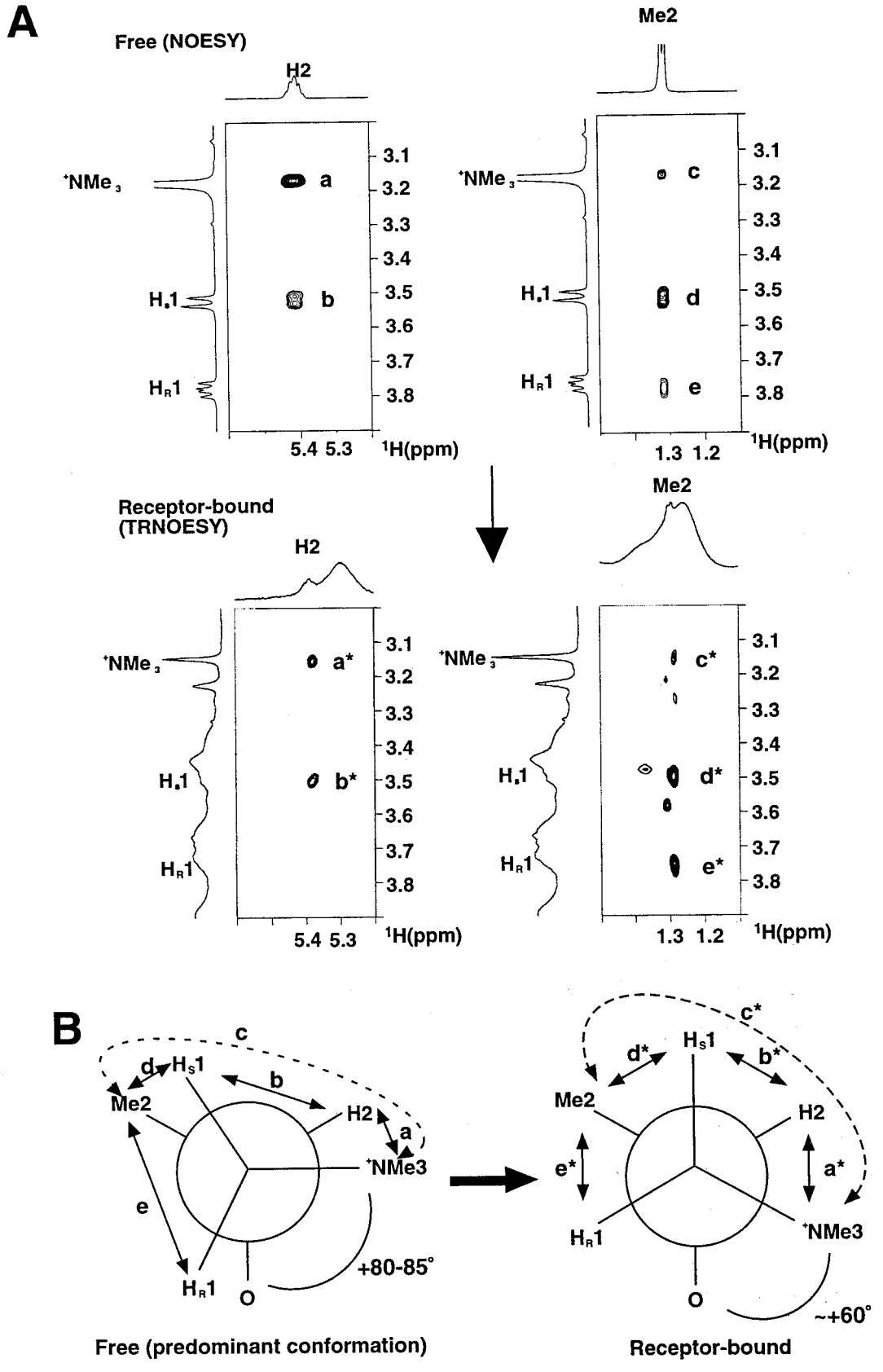

Fig. 4. A, NOESY (top) and TRNOESY (bottom) spectra of (*S*)-methacholine (1.5 mM) in the presence of the purified M_2 mutant (50 μM) at 23°C. The ratios of the cross-peak intensities, H2-$^+NMe_3$/H2-H_S1 and Me2-$^+NMe_3$/Me2-H_S1, decreased (a/b > a*/b* and c/d > c*/d*) as a result of binding to the M_2 mutant. The intensity of the cross-peaks, Me2- H_S1 and Me2-H_R1, was consistently equal (d* = e*) at various mixing time lengths (τ_m = 25, 50, and 100 ms). B, the correlation of the NOE (left) or TRNOE (right) cross-peaks, as explained in the C2-C1 Newman projection. The pattern of TRNOE cross-peaks was consistent with the conformation with the *gauche* O-C2-C1-N dihedral angle at +60°.

et al. (1972) pointed out the absence of simple correlation between the predominant conformations and the potency of the drugs and that Schulman et al. (1983) proposed the significance of the distance between the quaternary amine nitrogen and the ester oxygen of agonists rather than the O-C-C-N dihedral angle for the pharmacological activity.

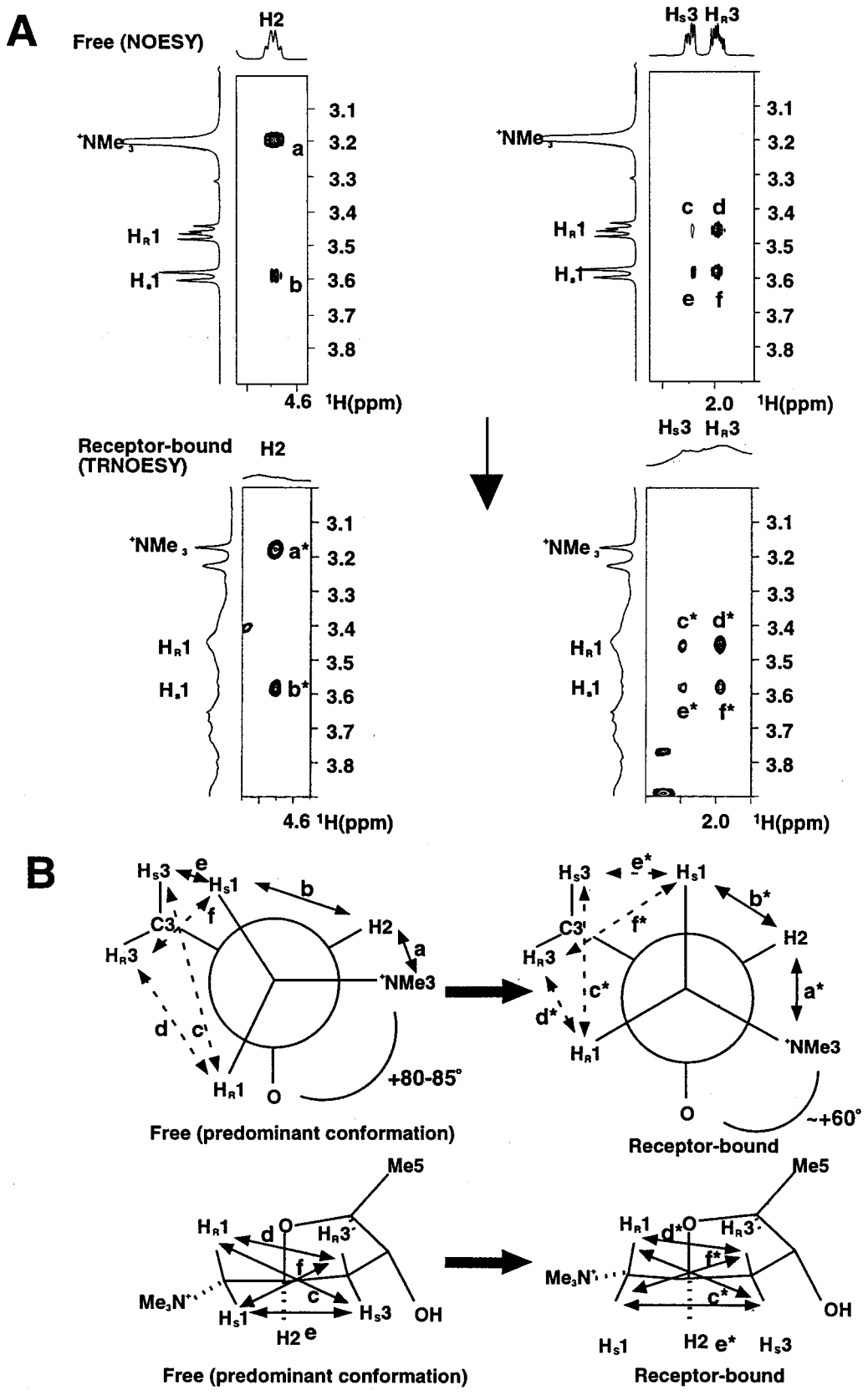

Fig. 5. A, NOESY (top) and TRNOESY (bottom) spectra of (2*S*,4*R*,5*S*)-muscarine (1.5 mM) in the presence of the purified M_2 mutant (50 μM) at 23°C. The ratio of the cross-peak intensity, H2-$^{+}$NMe3/H2-H_R1 decreased (a/b > a*/b*) as a result of binding to the M_2 mutant. The order of cross-peak intensities in the free form was H_R1-H_R3 (d) > H_S1-H_R3 (f) > H_S1-H_S3 (e) > H_R1-H_S3 (c), whereas that in the receptor-bound form was H_R1-H_R3 (d*) > H_S1-H_R3 (f*) > H_R1-H_S3 (c*) > H_S1-H_S3 (e*). B, the correlation of the NOE (left) or TRNOE (right) cross-peaks, as explained in the C2-C1 Newman projection and the side view of (2*S*,4*R*,5*S*)-muscarine. The pattern of TRNOE cross-peaks was consistent with the conformation with the *gauche* O-C2-C1-N dihedral angle at approximately +60°.

Within the simplest assumption that the receptor-bound conformation of a ligand represents the pharmacologically active form, the present results indicate that the conformation with O-C2-C1-N at +60° induces pharmacological activity. This interpretation is compatible with the studies of *cis*-dimethyldiacetoxypiperidinium iodide and *d*-1-methyl-3-acetoxy-*trans*-decahydroquinolin methiodide, in which the *cis*- or *d*-isomers (O-C2-C1-N dihedral angle at 60° and 74°)

are preferred to the *trans*- or *l*-isomers (O-C2-C1-N dihedral angle at 180° and 169°) in inducing pharmacological activities (Smissman and Chappell, 1969; Lewis et al., 1973). However, it cannot explain the pharmacological activities induced by many other rigid analogs with an O-C2-C1-N dihedral angle such as 120°.

On the other hand, there is the possibility that the receptor-bound ligand assumes multiple conformations, depending on the state of the mAChR. It is known that the M_2 mutant, or M_2 mAChR, as well as other GPCRs, binds agonists with high and low affinity, depending on the presence and absence of G proteins and guanine nucleotides (Hulme et al., 1983; Haga et al., 1986). In this study, the NMR measurements were carried out for the purified M_2 mutant in the absence of G protein. Under these conditions, the M_2 mAChR and the M_2 mutant are known to bind to the agonist with low affinity (Hulme et al., 1983; Haga et al., 1986). This low-affinity state represents the initial binding of the agonists to the receptor, before the association with G proteins. The receptors show high-affinity agonist binding when they associate with G proteins in the absence of guanine nucleotides. This high-affinity state corresponds to the transition state for the GTP/GDP exchange in the receptor-G protein complex, which leads to the pharmacological activity (Gilman, 1987; Haga and Haga, 1987). The high-affinity state has ≤1000-fold higher affinity for the agonist than the low-affinity state (Shiozaki and Haga, 1992). It will be interesting to elucidate whether such a change in the state of the receptor accompanies the conformational shift of the ligand, from the initially bound conformation to another one with distinct O-C2-C1-N dihedral angles.

In summary, we determined the receptor-bound conformation of (*S*)-methacholine and (2*S*,4*R*,5*S*)-muscarine, in the absence of G protein, to have the *gauche* O-C2-C1-N dihedral angle at approximately +60°. The mAChR recognizes the conformer with an O-C2-C1-N dihedral angle at +60°, which differs from the predominant conformer in solution with an O-C2-C1-N dihedral angle at +80–85°.

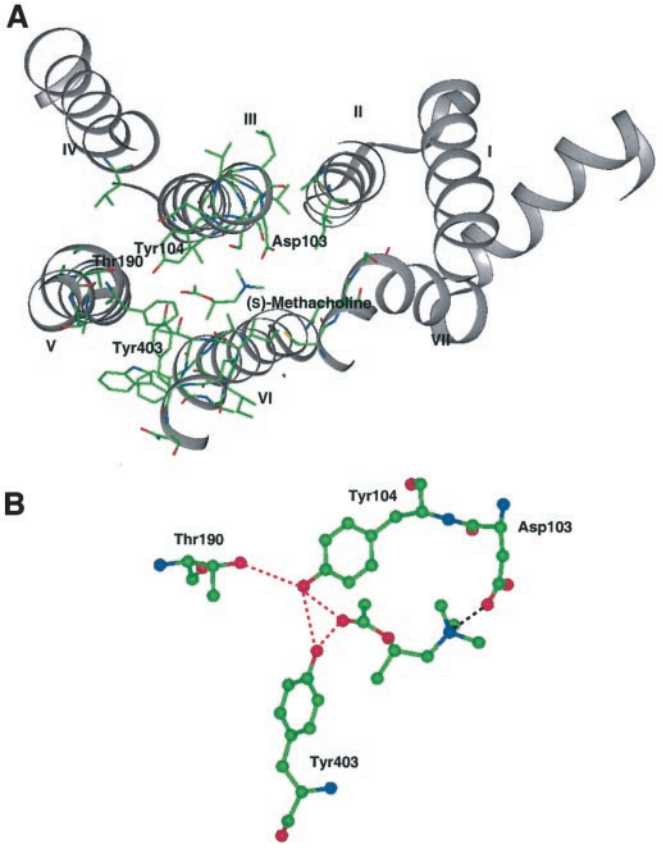

Fig. 6. A, the molecular model of the M_2 mAChR photoactive rhodopsin model, based on the crystal structure of rhodopsin, as seen from the extracellular side of the M_2 receptor. The helices (in ribbons) are numbered in order from TM1–7 from the N terminus. The conformation with the *gauche* O-C2-C1-N dihedral angle (+55.5°) for (*S*)-methacholine fit well with this molecular model, both energetically and spatially. Residues within 9 Å from the ligand are shown. Loops and hydrogen atoms are omitted for clarity. B, the putative hydrogen bond network at the ligand-binding site. The red broken lines indicate hydrogen bonds (~2.9 Å) between the ligand and the residues. The black broken line indicates the ionic interaction between the quaternary amine and the carboxylate oxygen of Asp103. Carbon, nitrogen, oxygen, and sulfur atoms are colored green, blue, red, and yellow, respectively. Illustrations of hydrogen atoms are omitted for clarity.

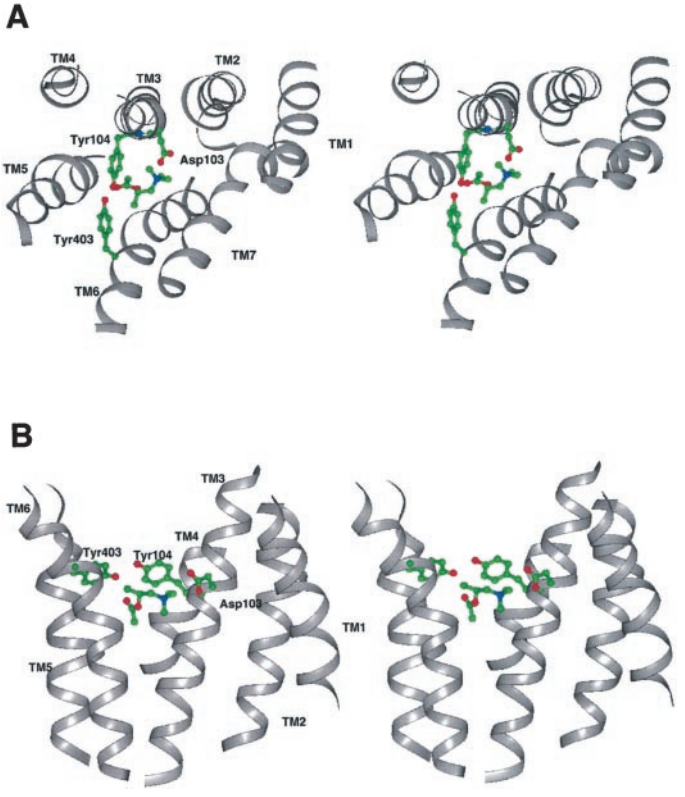

Fig. 7. The stereoview of the molecular model of the M_2 mAChR as seen from the extracellular side (A) and from the side of the transmembrane regions with the extracellular space at the top and the intracellular space at the bottom (B). The helices (in ribbons) are numbered in the order from the N terminus as TM1–7. The conformation with the *gauche* O-C2-C1-N dihedral angle for (*S*)-methacholine fit well with this molecular model both energetically and spatially. The key residues suggested to take roles in the binding of agonists from the mutagenesis studies, such as Asp 103, Tyr 104, and Tyr 403, are near the quaternary amine and acetyl groups of (*S*)-methacholine and are likely to make contacts.

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