Review

Molecular properties of muscarinic acetylcholine receptors

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Abstract: Muscarinic acetylcholine receptors, which comprise five subtypes (M_1-M_5 receptors), are expressed in both the CNS and PNS (particularly the target organs of parasympathetic neurons). M_1-M_5 receptors are integral membrane proteins with seven trans-membrane segments, bind with acetylcholine (ACh) in the extracellular phase, and thereafter interact with and activate GTP-binding regulatory proteins (G proteins) in the intracellular phase: M_1, M_3, and M_5 receptors interact with Gq-type G proteins, and M_2 and M_4 receptors with Gi/Go-type G proteins. Activated G proteins initiate a number of intracellular signal transduction systems. Agonist-bound muscarinic receptors are phosphorylated by G protein-coupled receptor kinases, which initiate their desensitization through uncoupling from G proteins, receptor internalization, and receptor breakdown (down regulation). Recently the crystal structures of M_2 and M_3 receptors were determined and are expected to contribute to the development of drugs targeted to muscarinic receptors. This paper summarizes the molecular properties of muscarinic receptors with reference to the historical background and bias to studies performed in our laboratories.

Keywords: acetylcholine, muscarinic receptor, G protein, G protein-coupled receptor (GPCR), G protein-coupled receptor kinase 2 (GRK2), crystal structure

1. Concept of muscarinic receptors

1.1. Distributions of cholinergic neurons and muscarinic receptors. The concept of muscarinic receptors originated from a report by Dale in 1914,[1] although he did not use the term receptor. He reported that the actions of several kinds of choline esters or other derivatives in various tissues could be divided into muscarinic and nicotinic ones, which are mimicked typically by muscarine and nicotine, respectively. Muscarine is a natural product of certain mushrooms including *Amanita muscaria*, and mimics the actions of parasympathetic neurons, which include vasodilation of vessels, slowing of the heart rate, constriction of bronchioles, elicitation of saliva secretion, constriction of the eye pupils, and so on. The action of muscarine is antagonized by atropine, which is a product of the plant called *Atropa bella-donna* (beautiful lady): the plant shrub has been used for visual appeal, as atropine causes dilation of the pupils (mydriasis).

The endogenous ligand for muscarinic receptors as well as for nicotinic receptors was confirmed to be acetylcholine (ACh) through a series of experiments by Loewi, Dale and others in 1921−1934 (see review ref. 2). ACh is now known to be a neurotransmitter in both the peripheral (PNS) and central (CNS) nervous systems: neurons containing ACh as a neurotransmitter are called cholinergic neurons. Cholinergic neurons in the PNS comprise motor neurons, postganglionic parasympathetic neurons, and preganglionic sympathetic and parasympathetic neurons. Muscarinic receptors are receptors for ACh in postganglionic parasympathetic neurons and are present in the target organs of parasympathetic

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    Abbreviations: ABT: aminobenztropine (muscarinic antagonist used for affinity chromatography); ACh: acetylcholine; cDNA: complementary DNA; DTT: dithiothreitol; E2 loop: the second extracellular loop; I3 loop: the third intracellular loop; M_1 (M_2, M_3, M_4, M_5) receptors: muscarinic acetylcholine receptor M_1 (M_2, M_3, M_4, M_5) subtypes; NMS: N-methylscopolamine (muscarinic antagonist); PrBCM: propylbenzylcholine mustard (irreversible muscarinic antagonist); QNB: quinuclidinyl benzylate (muscarinic antagonist); SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TM1 (2-7): transmembrane segment 1 (2-7).

neurons, which cover almost all organs, whereas nicotinic receptors are receptors for ACh in motor neurons and preganglionic neurons, and are present in skeletal muscles and in post-ganglionic neurons. Muscarinic receptors are also present in preganglionic neurons, where they are involved in regulation of the actions of nicotinic receptors, in sweat glands which are regulated by sympathetic but cholinergic neurons, and in some lymphoma cells.

Cholinergic neurons in the CNS reside in the forebrain including the medial septal nuclei, diagonal band nuclei, Meynert nuclei, and so on: these neurons project to various areas of the cerebrum.[3] Cholinergic neurons are also present in the caudate nucleus as interneurons, which are targeted to GABA-containing neurons competing with dopaminergic neurons. Muscarinic receptors are present in various areas of the brain, which are innervated by cholinergic neurons, both on neurons and glias, and both on postsynaptic and presynaptic membranes. Muscarinic receptors in the CNS are thought to be involved in learning-memory, sleep-waking, attention focusing, motor control, and so on.

1.2. Muscarinic receptors as binding sites for [³H]QNB or [³H]NMS. The term "receptor", including the muscarinic receptor, used to be a functional concept, leaving the molecular entity unknown. Muscarinic receptors should have at least two functions (1) recognition of ACh and (2) initiation of a physiological response. Attempts to define and characterize muscarinic receptors as molecular entities were facilitated through the introduction of radioactive, high-affinity ligands (antagonists) such as tritium-labeled quinuclidinyl benzylate ([³H]QNB) by Yamamura and Snyder[4] (1974), and N-methylscopolamine ([³H]NMS) by Birdsall and Hulme[5] (1976). The binding components of [³H]QNB or [³H]NMS retain the first function of muscarinic receptors at least, and used to be regarded as muscarinic receptors themselves, leaving the second function unknown. [³H]QNB or [³H]NMS binds to muscarinic receptors in membrane preparations with high affinity, the equilibrium dissociation constants being estimated to be 15–80 pM and 50–700 pM, respectively.[6] These binding activities can be easily measured in membrane preparations from brain and other tissues. The affinites of various compounds for muscarinic receptors can be determined by measuring their ability to compete with [³H]QNB or [³H]NMS.

The density of muscarinic receptors in the CNS of rhesus monkey, as assessed as [³H]QNB binding activity, was reported to range from 30 to 1200 pmol/g protein, and to be high in the caudate nucleus, putamen, and cerebral cortex, in accord with expectation during previous physiological experiments.[7] The distribution of muscarinic receptors was also found to be roughly in parallel with that of high affinity choline uptake activity, which was assumed to be a prerequisite step for ACh synthesis and to be present in cholinergic nerve terminals specifically.[8,9] These results support the idea that a major portion of acetylcholine receptors in the brain are muscarinic.[7]

These binding experiments indicated that muscarinic receptors in membrane preparations derived from different tissues exhibit different affinities for specific antagonists depending on the origins of the tissues, although they show similar affinities for atropine, [³H]QNB and [³H]NMS. Two kinds of muscarinic receptors, termed the M_1 and M_2 subtypes, were suggested to be distinguishable based on their different affinities for an antagonist, pirenzepine, and to be dominant in cerebral and atrial tissues, respectively.[10] In addition, a third subtype, M_3, was suggested to be present in smooth muscles, and characterized by low affinity for pirenzepine and high affinity for 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide), for which the M_2 subtype exhibits low affinity.[11]

These receptors may be assumed to represent three distinct entities as the simplest interpretation, but the possibility remained that they might represent three different states of a single entity. The situation was further complicated by the finding that an agonist shows three different affinities for muscarinic receptors in cerebral membranes, although [³H]NMS and atropine each shows a single affinity for them.[12] This result may also be interpreted by means of two different assumptions: (1) a single kind of receptor in three states with the same affinity for an antagonist but different affinities for an agonist, and (2) multiple receptors with the same affinity for an antagonist but different affinities for an agonist.

1.3. Solubilization of muscarinic receptors. Muscarinic receptors, as radioligand binding components, were demonstrated to be solubilizable from membrane preparations of various tissues. Beld and Ariëns[13] (1974) succeeded in solubilizing a stereospecific binding activity with [³H](+)-benzetimide (another radiolabeled ligand for muscarinic receptors) from bovine smooth muscle and caudate nucleus using digitonin, which had been used for

the extraction of visual pigments from suspensions of rod cells. This is the first demonstration that muscarinic receptors may be macromolecules extractable from cell membranes. It is interesting to note that the ligand binding activity is lost on treatment with most common detergents like Triton X-100 or sodium cholate, but not digitonin, and that β adrenergic receptors as well as rhodopsin and muscarinic receptors were also found to be solubilized in their active forms only with digitonin[14] when these three proteins were not thought to belong to the same family.

A problem of using digitonin as a solubilizing agent is that it exists as large micelles, which makes it difficult to characterize solubilized muscarinic receptors in terms of molecular mass: the ligand binding activity in a digitonin extract was found in the fraction corresponding to a mass of around a million.[15] On the other hand, the [³H]QNB-receptor complex, which was solubilized with Lubrol PX from cerebral membranes pretreated with [³H]QNB, was estimated to have a mass of approximately 80 kDa on gel permeation chromatography and sucrose density gradient centrifugation in H_2O and D_2O (Haga, T.,[16] 1980). The mass of 80 kDa was close to the value estimated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of muscarinic receptors covalently labeled with muscarinic ligand [³H]PrBCM (propylbenzilylcholine mustard),[17] suggesting that muscarinic receptors exist as monomers in contrast with nicotinic receptors, which had been indicated to be composed of five subunits.

The bands labeled with either [³H]QNB or [³H]PrBCM were much broader than that expected for a single protein. This suggests one or all of the following possibilities, that (1) muscarinic receptors may be bound covalently with a sugar moiety, (2) muscarinic receptors may consist of multiple subtypes of different sizes, (3) muscarinic receptors may exist in a equilibrium of monomers and dimers, and/or (4) muscarinic receptors may bind with other components in extracts with Lubrol PX or SDS. These possibilities remained to be clarified by further works.
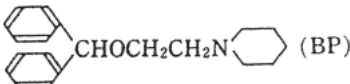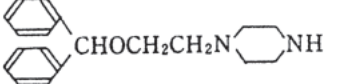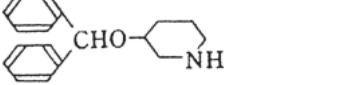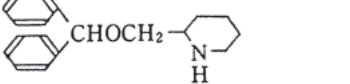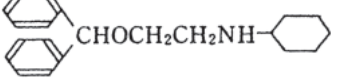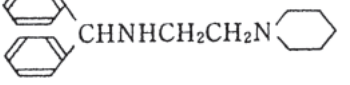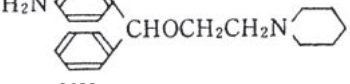
2. Molecular entities of muscarinic receptors

2.1. Development of an affinity chromatography system. In 1981 we started to attempt to purify muscarinic receptors at Burgen's laboratory in the National Institute for Medical Research in London. In order to purify muscarinic receptors it appeared essential to develop an affinity chromatography system with high specificity and high recovery,

because the expression level of muscarinic receptors was known to be very low, an order of pmol/mg protein at most, which requires approximately 10^4-fold purification. Haga, K., who had been engaged in organic synthesis in a laboratory of Ajinomoto Co., started the project with the help of Peck by synthesizing a muscarinic ligand with an amino group: the amino group was expected to bind the ligand with an agarose gel through a linker. The firs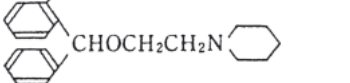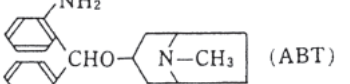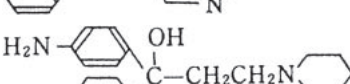t amino compound (APCPP; No. 11 in Table 1) synthesized there exhibited high affinity, with a dissociation constant of nM order for solubilized muscarinic receptors, but was not useful when bound to an agarose gel for unknown reasons. Then, systematic studies for preparation of an affinity gel were restarted in Ichiyama's laboratory in Hamamatsu University School of Medicine in 1982. At first we consulted with Sakakibara in Nagoya City University and obtained advice as to synthetic methods. Then we decided to synthesize various derivatives of a model compound (2-benzhydryloxy-N-ethyl piperazine, BP; No. 1 in Table 1) with an amino group at different positions (No. 2 to 8 in Table 1) in order to determine where the linker between the ligand and the agarose gel should be positioned. The addition of an amino group decreased the affinity for muscarinic receptors by five- to twenty-fold, but the effects of differences in the position of the amino group were relatively small. On the other hand, the effects of the positions became greater when a heptanoic acid moiety (a linker) was coupled to the amino group: the decrease in the affinity for muscarinic receptors was 120-fold on the introduction of the linker to the piperazine group of compound No. 2 in Table 1, whereas the decrease was only 9-fold on introduction of the linker to the phenyl ring of compound No. 8 in Table 1. This indicated that the linker should be placed on the phenyl group. Then, compounds with higher affinity for muscarinic receptors were synthesized by introducing a tropine or quinuclidinyl group instead of a piperazine group (Nos. 9 and 10 in Table 1). Finally ABT (aminobenztropine) was chosen as a ligand for the affinity gel. ABT exhibits a high affinity for solubilized muscarinic receptors, with an equilibrium dissociation constant of 7 nM.

We then attempted to optimize the efficiency of the affinity gel, that is, to increase the amount of bound muscarinic receptors and to decrease non-specific binding of other proteins, according to advice from Nakata and Yamauchi in Asahikawa University School of Medicine and others. Several factors were

Table 1.　Muscarinic ligands with an amino group and their affinity for muscarinic receptors in cerebral membranes and in solubilized form[18]

	Chemical structure	IC$_{50}$ (µM) for inhibition of QNB binding	
		Membrane receptor	Solubilized receptor
1	CHOCH$_2$CH$_2$N⟨⟩ (BP)	0.06	0.6
2	CHOCH$_2$CH$_2$N⟨⟩NH	1	3
3	CHO–⟨⟩NH	0.3	2
4	CHOCH$_2$–⟨⟩ N H	0.4	3
5	CHOCH$_2$CH$_2$NH–⟨⟩	2	10
6	CHNHCH$_2$CH$_2$N⟨⟩	0.8	3
7	H$_2$N⟨⟩ CHOCH$_2$CH$_2$N⟨⟩ (ABP)	0.5	3
8	NH$_2$ CHOCH$_2$CH$_2$N⟨⟩	0.2	2
9	NH$_2$ CHO–⟨N–CH$_3$⟩ (ABT)	0.003	0.05
10	NH$_2$ CHO–⟨N⟩ (ABQ)	0.02	0.25
11	H$_2$N–⟨⟩ OH C–CH$_2$CH$_2$N⟨⟩ (APCPP)	0.004	0.03

A model compound (1, BP), its derivatives with an amino group in different positions (2–8), derivatives of compound 8 with tropin (9) and quinuclidinol (10), and APCPP (11) were synthesized, and their affinities for muscarinic receptors were estimated from their effects on [^{3}H]QNB binding of membrane-bound and solubilized muscarinic receptors.

found to be involved in determination of the efficiency, which included (a) chemical species of the linker, (b) species of the agarose gel, (c) density of bound ligands, (d) concentration of salt in the incubation medium, (e) ratio of gel to applied proteins, and so on. CH-Sepharose gel with a hydrophobic linker such as (CH$_2$)$_5$ showed higher capacity for muscarinic receptors but lower specificity as compared to Epoxy-Sepharose gel (see the structure of ABT agarose in Fig. 1). In addition, it was important to add an appropriate concentration of salt, *e.g.* 0.1–0.2 M NaCl, to the incubation
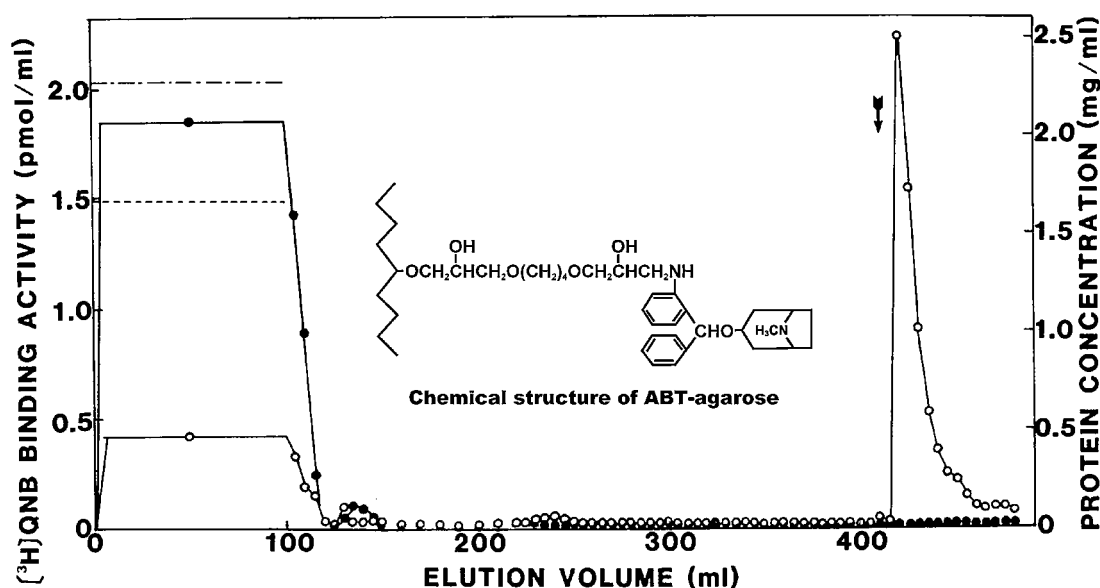
Fig. 1.   Affinity chromatography of muscarinic receptors.[19] A digitonin extract of porcine cerebral membranes (100 ml) was applied to ABT-agarose gel (10 ml). Most proteins (filled circles) were eluted in the flow through fraction, and muscarinic receptors (open circles) were specifically eluted with a muscarinic ligand (arrow). Broken and dotted lines represent the amounts of proteins and muscarinic receptors in the original extract, respectively.

medium, as greater non-specific binding was observed at lower salt concentrations, and less binding of muscarinic receptors at higher salt concentrations.[18] Finally, we prepared an affinity gel comprising agarose gel bound with a muscarinic ligand, amino-benztropine (ABT agarose) (Haga and Haga,[19] 1983). Approximately 1,000-fold purification was attained with a single step of affinity chromatography with ABT agarose.

2.2. Purification of muscarinic receptors. Muscarinic receptors were purified to apparent homogeneity from a digitonin/cholate-extract of porcine brain by ABT agarose and hydroxylapatite chromatography.[20] SDS-PAGE of the purified preparation gave an apparently single band corresponding to approximately 70 kDa. The specific [³H]NMS binding activity of the purified preparation was estimated to be 13 nmol/mg of protein, which is close to the theoretical value of 14 nmol/mg of protein based on the assumption of a single binding site per 70 kDa. Muscarinic receptors were also purified as a [³H]QNB binding component from porcine atria using the ABT agarose and several other chromatography sytems.[21] SDS-PAGE of the purified preparation gave two bands corresponding to apparent molecular weights of 79 kDa and 15 kDa, the 79 kDa component being shown to bind with [³H]PrBCM. These results provide direct evidence

that muscarinic receptors, which are defined as [³H]NMS or [³H]QNB binding components, are protein molecules of 70–80 kDa.

Muscarinic receptors purified from porcine brain and atria had been expected to correspond to the M_1 and M_2 subtypes, which can be discriminated according to the difference in the affinity for pirenzepine: pirenzepine was reported to have a higher affinity for cerebral membrane preparations by a factor of approximately 30 than for atrial ones. The situation, however, was complicated because pirenzepine was indicated to show similar affinity for the two receptor preparations solubilized with digitonin from either rat brain or heart.[22] Thus, purification of muscarinic receptors from both cerebral and atrial membranes was not sufficient to prove that the M_1 and M_2 receptors are distinct molecules. This problem could be solved by cloning cDNA for each receptor, as described later.

As for the affinity of pirenzepine, Nishiyama et al.[23] confirmed that muscarinic receptors purified from either porcine cerebrum or atrium exhibit the same affinity for pirenzepine. This indicates that the affinity for pirenzepine of muscarinic receptors in membrane preparations is not determined solely by their protein moiety but may be affected by other factors such as bound phospholipids. Berstein et al.[24] demonstrated that pirenzepine had a higher affinity

for muscarinic receptors purified from the cerebrum than those purified from the atrium when they were reconstituted into cell membranes pretreated with PrBCM, and that the result was not affected whether cell membranes were prepared from the cerebrum or the atriam. Furthermore, the membrane preparations could be replaced by particular sets of lipid preparations containing cholesteryl hemisuccinate and phosphatidylcholine. Cerebral and atrial muscarinic receptors in membranes appear to be differentially regulated by the same set of cholesterol and phosphatidylcholine to show different affinities for pirenzepine. It is possible that the pirenzepine binding site may involve the lipid moiety or may be affected by interaction with lipids, whereas the binding site for atropine, QNB and NMS does not involve and is not affected by the lipid moiety.

Muscarinic receptors purified from porcine atrium[21),23)] as well as ones purified from porcine cerebrum[20)] showed heterogeneous affinities for a given agonist, although a single kind of muscarinic receptor had been expected to be present in atrium. This indicates that muscarinic receptors may exist in different states with different affinities for agonists. A possible candidate for the different states is the redox state of the cysteine residues: full reduction of muscarinic receptors purified from either cerebra or atria with high concentrations of dithiothreitol (DTT) caused decreases in the affinities for ligands including agonists, and modification by SH reagent dithiobis(2-nitro benzoic acid)(DTNB) caused increases in the affinities for agonists by approximately 30-fold.[23),25)] Treatment of purified muscarinic receptors with these SH reagents did not affect the size of the muscarinic receptors, as judged from sucrose density gradient centrifugation and SDS-PAGE, indicating that intra-molecular, but not inter-molecular, S–S bond(s) is (are) involved in determination of the affinities for ligands.

2.3. Cloning of complementary DNAs for muscarinic receptors. Purification of muscarinic receptors enabled us to determine their partial amino acid sequences, which could be used to clone complementary DNAs (cDNAs) for muscarinic receptors. We collaborated with Numa's and Matsuo's laboratories in Kyoto University and Miyazaki University School of Medicine, respectively. Muscarinic receptors were purified from porcine cerebra in Hamamatsu University School of Medicine and sent to Miyazaki University School of Medicine, where Kangawa *et al.* partially hydrolyzed them and determined amino acid sequences of five peptides. By utilizing the amino acid sequences of these peptides, Kubo *et al.*[26)] (1986) in Kyoto University cloned a cDNA from a library constructed from the mRNAs of porcine cerebrum and confirmed that Xenopus oocytes expressing the cDNA respond to ACh with a specificity expected for muscarinic receptors.[26)] Furthermore, another cDNA was cloned by using a different amino acid sequence from the library constructed from mRNA of porcine atrium.[27)] Difference in the tissue distributions of mRNAs indicated that two cDNAs obtained from libraries of porcine cerebrum and atrium encode the muscarinic receptor M_1 and M_2 subtypes, respectively. A cDNA corresponding to the M_2 subtype was independently cloned by using partial amino acid sequences obtained for muscarinic receptors purified from porcine atria.[28)] These results provide definite evidence that muscarinic receptors are protein molecules, and that the M_1 and M_2 subtypes are two distinct protein molecules with different amino acid sequences. In this paper, hereafter, they are termed M_1 and M_2 receptors respectively.

The amino acid sequences of M_1 and M_2 receptors were deduced from the nucleotide sequences of cDNAs. Three of the five sequences determined for muscarinic receptors purified from porcine brain were found in the sequence of M_1 receptors, and two in that of M_2 receptors. M_1 and M_2 receptors consist of 460 and 466 amino acid residues with calculated molecular weights of 51 and 52 kDa, respectively. The difference between these values and the value of 70 kDa estimated from SDS-PAGE of cerebral muscarinic receptors was supposed to be accounted for by the contribution of the carbohydrate moiety, and was confirmed by endoglycosidase treatment of cerebral and atrial receptors.[29),30)] In fact, the sequences of M_1 and M_2 receptors contain two and three potential N-glycosylation sites with the consensus sequence of NXS/TX in the amino terminal portion.

The amino acid sequences of M_1 and M_2 receptors revealed that they are similar to each other but not to nicotinic receptors, and that they contain seven hydrophobic regions, with a length of 20–24 amino acid residues, which were supposed to constitute transmembrane segments. The aminoterminal portion with glycosylation sites should be in the extracellular phase and the carboxyterminal tail in the intracellular phase. In accord with previous observations on the effects of sulfhydryl reagents, cysteine residues were found in both M_1 and M_2 receptors. Uchiyama *et al.*[31)] suggested that there is

an S–S bond between Cys98 in the first extracellular loop (E1: a loop between transmembrane segment 2 and 3 (TM_2 and TM_3)) and Cys 178 in the second extracellular loop (E2 loop): here cerebral receptors labeled with [^{3}H]PrBCM were analyzed by SDS-PAGE with or without partial hydrolysis, reduction by DTT, endoglycosidase F treatment, and reaction with antibodies against peptides in the specific regions. In the same series of experiments, the binding site of [^{3}H]PrBCM was located between the aminoterminus and the E2 loop. The presence of the S–S bond and localization of [^{3}H]PrBCM binding site to an Asp residue in TM3 were directly demonstrated by peptide map analysis by a group of Hulme.[32]

Screening for cDNAs or genes exhibiting homology to cDNAs of M_1 and M_2 receptors in a cDNA library or genomic library resulted in the identification of other muscarinic receptors, that were termed as M_3, M_4 and M_5 receptors.[33]–[35] The coding regions of these five receptors were found to be in a single exon and to contain no intron, and thus genes with full length coding region could be easily cloned from the genomic library. The presence of M_1 and M_2 receptors, and possibly M_3 receptors, was expected from pharmacological studies, but M_4 and M_5 receptors had not been anticipated before their cDNAs or genes were cloned. The pharmacological properties of these five muscarinic receptors were determined by expressing them initially on Xenopus oocytes, and later on cultured cells such as COS, CHO or HEK293 cells, and are summarized in the refernece.[6]

The presence of five different muscarinic receptors appears to provide an opportunity to develop subtype-specific ligands that may affect only a certain subtype and thus a specific physiological function. In addition, multiple subtypes of muscarinic receptors appear to contribute to subtle regulation by ACh of many physiological functions. For example, Kajimura et al.[36] demonstrated that acid secretion by parietal cells is regulated in a dual way by muscarinic agonists, i.e. stimulation and inhibition with their low and high concentrations, respectively, most probably through different muscarinic receptor subtypes.

2.4. A protein family with seven transmembrane segments.   The presence of seven transmembrane segments in rhodopsin[37] and β adrenergic receptors[38] was suggested by their amino acid sequences, which were determined in 1980 by protein analysis and in 1986 by cDNA screening, respectively. Rhodopsin, β adrenergic receptors, and muscarinic receptors are typical receptors for external stimuli, hormones, and neurotransmitters, respectively. The similarity among these three proteins had been suggested by experiments on their interaction with G proteins, as described in the following section, and was confirmed by cloning of cDNAs for β adrenergic receptors, and M_1 and M_2 muscarinic receptors in 1986, and by identifying the seven putative transmembrane segments. It is interesting to note that the amino acid sequences of M_1 and M_2 receptors are very homologous except the third intracellular loop (I3 loop), but homology among M_1 receptors, rhodopsin and β adrenergic receptors is rather limited in spite of the common property of seven transmembrane segments (Fig. 2). Subsequently, however, the cDNAs for a lot of odorant-, hormone-, and neurotransmitter-receptors have been cloned on the basis of homology to rhodopsin, β adrenergic, and muscarinic receptors. They are called G protein-coupled receptors (GPCRs) or seven-transmembrane (7-TM) receptors.

In 2000, a human genome sequence was reported, and thus the systematic search for GPCRs became possible. We thought that we might find GPCR genes in the human genome because we knew that all GPCRs, which had been identified so far, have the seven hydrophobic region and many of them have no intron in the coding region, and so decided to collaborate with the laboratory of Mitaku in the Tokyo University of Agriculture and Technology, who is a theoretical biophysicist. During the collaboration, Takeda et al.[39] (2002) succeeded in identifying 581 GPCR candidates without an intron in the coding region, 301 of which had not been identified until then. The total number of GPCRs were estimated to be 948, on the assumption that the ratio of intron-less GPCRs is not different between already-registered and not yet-registered GPCRs. The estimated numbers of GPCRs (the proportion of intron-less GPCRs) are as follows: odorant receptors 481 (100%), taste receptors 28 (77%), receptors for endogenous ligands 330 (66%), and ones without homology to already-registered GPCRs 109 (assumed to be 66%).

GPCRs constitute one of the largest superfamilies in the human genome, and function as cell sensors for external stimuli, hormones, and neurotransmitters. GPCRs are believed to have the common properties that they have 7-TM segments and activate GTP-binding regulatory proteins (G proteins). Strictly speaking, however, direct evidence for 7-TM segments has been obtained for limited
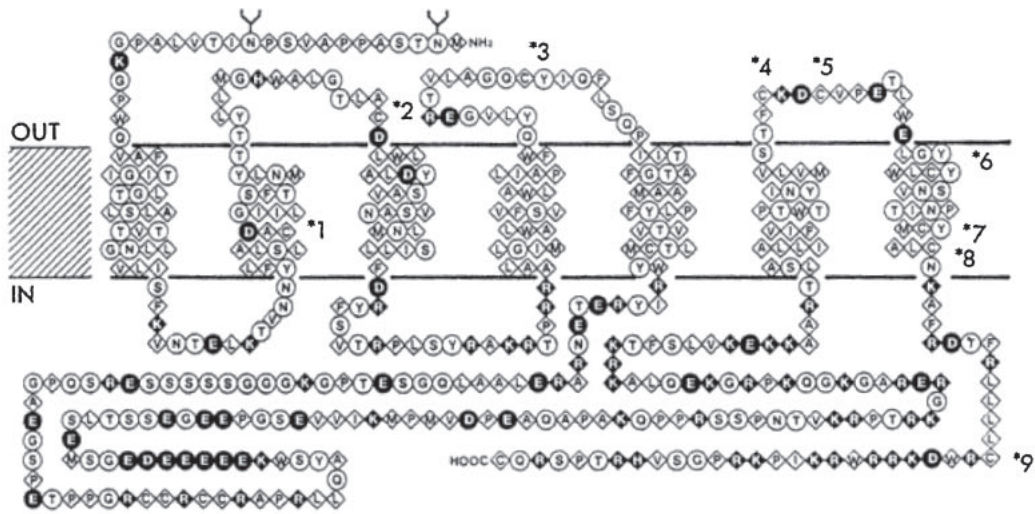
Muscarinic M1 Receptor
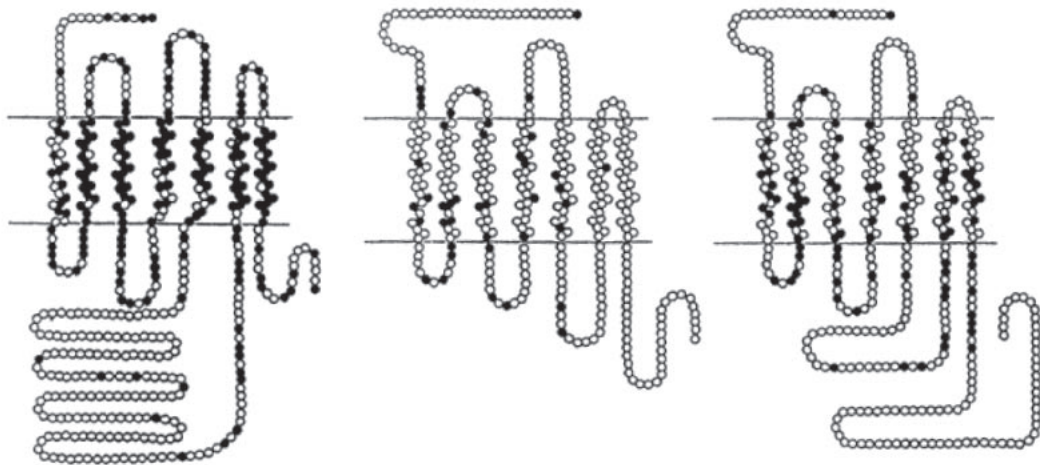
Muscarinic M2 Receptor   **Rhodopsin**   **β Adrenergic Receptor**

Fig. 2.   Topology models of muscarinic M_1,[26)] M_2[27)] receptors, rhodopsin,[37)] and β adrenergic receptors.[38)] In the model of the muscarinic M_1 receptor, ionic residues such as D, E, K, R, H are shown by white letters on the black background, and cysteine residues are numbered. In the models of muscarinic M_2 receptors, rhodopsin and β adrenergic receptors, amino acid residues homologous to those in muscarinic M_1 receptors are shown as black circles.

number of members, including muscarinic receptors, rhodopsin and $\beta2$ adrenergic receptors and several others. Direct evidence for coupling with G proteins is also limited to only several members. On the other hand, GPCRs appear to have other functions besides G protein activation.[40)] M_2 receptors, together with G protein $\beta\gamma$ subunits, has been shown to activate G protein-coupled receptor kinase 2 in an agonist-dependent manner, as described later.

All five muscarinic receptors are distinct from most GPCRs in that they have a long third

intracellular loop (I3 loop, loop between TM5 and TM6) with 160–240 amino acid residues. These long I3 loops are found in $\alpha2$ adrenergic receptors and some 5HT receptors. A long I3 loop is not involved in G protein activation by a receptor but appears to be involved in its regulation.

3. Functions of muscarinic receptors

3.1. β Adrenergic receptor —G protein (Gs)— adenylate cyclase system.   Studies on functions of muscarinic receptors have been built on

the prior work on β adrenergic receptor—G protein (Gs)—adenylate cyclase system, and therefore the historical background of the latter system is introduced here.

Biochemical studies on intracellular events initiated by extracellular hormones originated from the discovery of cyclic AMP (cAMP) by Rall and Sutherland in the late 1950s.[41] They showed that a certain group of hormones, including adrenaline and glucagon, induces increases in cAMP concentration in the cell. Rodbell *et al.* studied glucagon-stimulated cAMP formation in membrane preparations from rat liver, and demonstrated in the early 1970s that guanine nucleotides (GTP or GDP) decrease the affinity for glucagon and that GppNHp (hydrolysis-resistant analogue of GTP) activates the cAMP forming reaction in a pseudo-irreversible manner.[42] Guanine nucleotides have also been shown to decrease the affinity of agonists, but not of antagonists, for β adrenergic receptors in membrane preparations of cultured cells.[43] These results suggested that this signal transduction system requires at least three functional components. It was not known, however, if the hormone receptor, adenylate cyclase, and GTP-recognizing component represent three independent proteins or three functions of a single or two protein(s). In this context, it is interesting to note that the same question was raised for the hormone-dependent increase in cyclic GMP (cGMP), and it turned out later that ANP (atrial natriuretic peptide)-dependent cGMP formation is accomplished by a single protein of ANP receptor/guannyl cyclase, which possesses an extracellular ligand-binding domain, a single transmembrane segment, and an intracellular guanylate cyclase catalytic domain,[44] whereas ACh-dependent cGMP formation needs a series of components, as described later.

Initial biochemical studies indicated that the β adrenergic receptor, which was assessed as the binding component of [^{125}I]IHYP (iodohydroxyben-zylindolol), and the component with GppNHp-activated adenylate cyclase activity could be physically separated as distinct entities.[45] The molecular sizes of the β adrenergic receptor and adenylate cyclase were estimated to be 75,000 and 220,000, respectively.

Molecular identification of a component related with the effect of guanine nucleotides was performed by several groups, particularly Pfeuffer in Germany, Cassel and Selinger in Israel, and Ross and Gilman in USA in the 1970s. Pfeuffer[46] separated the GTP-binding component using a GTP-agarose gel from an extract of pigeon erythrocytes, and demonstrated that the component may confer the adenylate cyclase activity to another component. Cassel and Selinger[47] and Cassel and Pfeuffer[48] showed that β adrenergic agonists enhance the GTPase activity of turkey erythrocyte membranes and proposed the hypothesis that the agonist-bound receptor stimulates the release of GDP from and binding of GTP to the GTP-binding component with GTPase activity, and the component bound with GTP stimulates the adenylate cyclase and is converted to an inactive form bound with GDP by its own GTPase activity. Ross and Gilman used S49 lymphoma cells and their variants (cyc^{-}[49] and unc[50]), and demonstrated through an elegant series of experiments that the cAMP-forming activity in membrane preparations derived from GppNHp-treated S49 lynphoma cells is due to two components: one is labile to high temperature treatment (*e.g.* 50°C for 30 min) and is present in the cyc^{-} mutant, and the other is stable to high temperature treatment and is absent in the cyc^{-} mutant.[51] The former component was shown to be the adenylate cyclase, and the latter was shown to be the component which confers GppNHp-stimulating activity to the former. The latter was named N or G/F and is now named Gs (stimulatory G protein). Subsequently Gs was purified from a Lubrol PX extract of rabbit liver as a component that confers the adenylate cyclase activity on cyc^{-} membranes (see review ref. 52). Gs was found to be composed of three subunits, $\alpha\beta\gamma$, and the α subunit is the GTP-binding component and the substrate of ADP-ribosylation by cholera toxin.

β Adrenergic receptors were purified from a digitonin extract of turkey erythrocytes by an affinity chromatography with a β adrenergic antagonist as a ligand by Strosberg group[53] in France. Interaction between β adrenergic receptors and G protein Gs was studied in details by the groups of Gilman, Lefkowitz, and others, and their direct interaction was confirmed by reconstitution of purified β adrenergic receptors and Gs.[54]

3.2. Receptors —G protein (Gi)— adenylate cyclase inhibition. It appears that muscarinic receptors were generally believed to be linked to cGMP formation or phosphatidylinositol (PI) turnover rather than cAMP in the mid 1970s when the signal transduction system of β receptor-Gs-adenylate cyclase was revealed, although the activation of muscarinic receptors in the heart had been reported to result in inhibition of cAMP formation in the early 1960s.[55] The relation of muscarinic receptors with

cGMP formation or PI turnover was studied in detail in the mid 1990s, as described later.

A series of experiments by Rodbell's group in the 1970s showed that a hormone-dependent decrease in cAMP was also mediated through a mechanism including the action of guanine nucleotides. The responsible protein was indicated to have properties different from Gs,[56] and was termed as Ni and now Gi, inhibitory G protein (see review ref. 57). Katada and Ui[58] demonstrated that the hormone-dependent decrease in cAMP, including the ACh-induced decrease in cAMP,[59] is inhibited by treatment with pertussis toxin, which was originally named IAP (Islet-Activating Protein), and that a protein, which is now known to be the α subunit of Gi, is ADP-ribosylated through the action of the pertussis toxin. Gi was purified from rabbit liver as a substrate of ADP ribosylation by pertussis toxin.[60] Other G protein, which was named as Go, was also purified together with Gi from the brain.[61] Gi and Go are more abundantly present in the brain than Gs, and are estimated to account for 1.5% of membrane protein. Furthermore, light-dependent activation of cGMP phosphodiesterase in the retina was also demonstrated to be mediated by the G protein called transducin and now more often called Gt (see review ref. 62).

All these G proteins, *i.e.* Gs, Gi, Go and Gt, have been demonstrated to be composed of three subunits as an $\alpha_{GDP}\beta\gamma$ trimer in an inactive form, and as α_{GTP} and $\beta\gamma$ in an active form. Agonist-bound receptors catalyze the exchange of GDP and GTP, and the α subunit has GTPase activity to convert GTP to GDP. The dissociated α_{GTP} and $\beta\gamma$ subunit interact with and activate different kinds of effectors. The G proteins are generally classified as to α subunits, *i.e.* whether it activates (Gs) or inhibits (Gi, Go) adenylate cyclase, or activates cGMP-phosphodiesterase (Gt). The α subunit of Gs (Gs-α) is a substrate of ADP-ribosylation by cholera toxin, and the ADP-ribosylation causes inhibition of its GTPase activity and therefore keeps it in an active form (GTP-bound form). The α subunits of Gi and Go are substrates of ADP-ribosylation by pertussis toxin, and the ADP-ribosylation causes uncoupling between receptors and G proteins. Gt-α is the substrate of both cholera toxin and pertussis toxin. The $\beta\gamma$ subunits were also demonstrated to be involved in activation or inhibition of adenylate cyclase (see review ref. 63), and also in other functions such as activation of inward-rectified K^+ channels.[64]

In the early 1980s, the technique to prepare monoclonal antibodies became common. Nukada, who joined in our group in Hamamatsu University School of Medicine in 1981, succeeded in preparing monoclonal antibodies for Gt-α and cloned its cDNA by using the antibody in collaboration with Tanabe *et al.* in Numa's laboratory in Kyoto University.[65] This was the first G protein α subunit, cDNA for which was cloned, and this was followed by cloning of the Gi-α[66] and Gs-α[67] subunits. The amino acid sequences of all these α subunits indicated that they contain a ras domain, which is homologous to the product of an oncogene ras, and another domain, which was later termed the helical domain. The sequences of Gt-α, Gi-α and Go-α, but not Gs-α, contain a cysteine residue as the fourth amino acid from the carboxy-terminus, which is ADP-ribosylated by pertussis toxin. Thereafter, cloning of homologous proteins revealed the presence of three kinds of Gi-α genes, seven kinds of β subunits, and 11 kinds of γ subunits (see review ref. 68). In addition, nine kinds of adenylate cyclase genes were identified, which are differentially regulated by various kinds of G-α and G-$\beta\gamma$ subunits as well as by Ca^{2+}-calmodulin (see reviews refs. 69, 70).

3.3. Interaction of muscarinic receptors with Gi-type G proteins. Indirect evidence for the interaction between muscarinic receptors and G proteins is based on the observation that agonist but not antagonist binding to muscarinic receptors in membranes of rat heart is affected by GTP and GDP,[71] which is similar to the original effect of guanine nucleotides on the agonist binding to β adrenergic receptors.[43] These results indicated that muscarinic receptors as well as other receptors such as $\alpha 2$ adrenergic receptors interact with guanine nucleotide-related protein(s). The guanine nucleotide-sensitive high affinity agonist binding of muscarinic receptors in membrane preparations derived from porcine caudate nucleus was found to be abolished by treatment with a sulfhydryl reagent, N-ethylmaleimide (NEM),[72] although guanine nucleotide-sensitive agonist binding to β adrenergic receptors was hardly affected by NEM treatment. These results suggest that the putative G protein involved in agonist binding of muscarinic receptors is different from Gs-type G proteins.

To obtain direct evidence for the interaction between muscarinic receptors and G proteins, we brought muscarinic receptors purified from porcine brain to Ui's laboratory in Hokkaido University, where Gi had been purified. We, together with

Katada and Kurose, examined their interaction as muscarinic agonist-enhanced GTPase activity in the reconstituted system. Thus, muscarinic receptors were shown to interact with Gi in a pertussis toxin-sensitive manner.[73] This is the first direct demonstration that at least one of the functions of muscarinic receptors is to interact with and activate Gi-type G proteins. Furthermore, quantitative reconstitution of muscarinic receptors and G proteins Gi and Go indicated that approximately 50% of muscarinic receptors reconstituted with one of either Gi or Go showed GppNHp-sensitive high affinity for ACh (Fig. 3) or other agonists. The proportion of high affinity sites did not increase further on the addition of both Gi and Go. GDP or GTP as well as GppNHp abolished the high-affinity agonist binding in the same concentration ranges, respectively, and no conversion between GTP and GDP was detected under the experimental conditions used[74] (Fig. 3). The simplest interpretation of these results is that (1) 50% of muscarinic receptors interact with either Gi or Go in the absence of GTP and GDP, (2) the muscarinic receptor-Gi/Go complex shows high affinity for agonists, and (3) the complex bound with GTP or GDP dissociates into Gi/Go and muscarinic receptors with low affinity for agonists. These interpretations infer that the muscarinic receptor-guanine nucleotide-free Gi/Go complex exhibits high affinity for agonists and may exist in a stable form at least *in vitro*, although the muscarinic receptor-Gi/Go-GTP (GDP) complex does not exhibit high affinity for agonists or such a complex is not formed in a stable form.

Muscarinic receptors purified from porcine brain were found to interact also with Gn, which was purified from porcine brain as a novel [^{35}S]GTPγS binding component. Gn is a substrate of ADP-ribosylation by pertussis toxin but different from Gi or Go. The gene species of α subunits for Gi and Gn are thought to correspond to Gi$_1\alpha$ and Gi$_2\alpha$, respectively, but the gene species of β and γ subunits for Gi and Gn have not been determined yet. In the reconstitution system, muscarinic agonists were found to stimulate the release of [^{3}H]GDP bound to Gi, Go or Gn, and the amount of released [^{3}H]GDP was 5–10 times the amount of muscarinic receptors. These results indicate that a single muscarinic receptor molecule interacts with multiple G proteins and catalytically stimulates the release of [^{3}H]GDP bound to them.[75),76)]

When the above reconstitution experiments were carried out, it was not known which subtypes
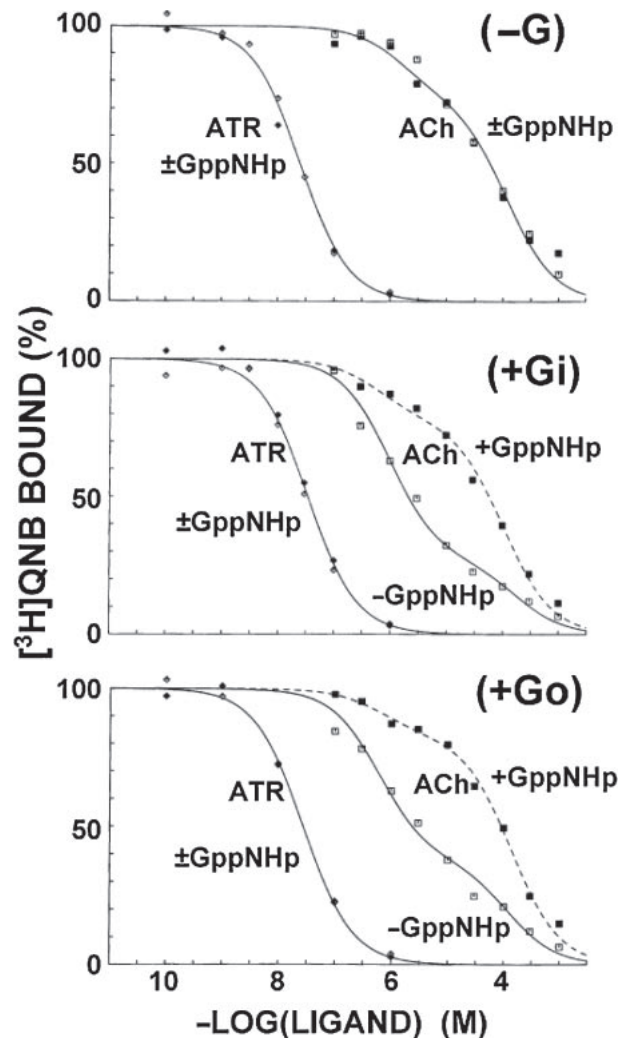

Fig. 3. Interaction of muscarinic receptors with G protein Gi or Go.[74] Muscarinic receptors purified from porcine cerebrum were reconstituted in lipid vesicles without or with G protein Gi or Go, which were purified from the same tissue, and were incubated with [^{3}H]QNB in the presence of various concentrations of ACh or atropine (ATR) and in the presence or absence of GppNHp (GTP analogue). The interaction of muscarinic receptors with Gi or Go was demonstrated as GppNHp-sensitive, high-affinity agonist binding. The displacement curves by ATR of [^{3}H]QNB binding were not affected by the presence or absence of Gi, Go or GppNHp. On the other hand, the displacement curves by ACh in the presence of Gi or Go, but not in their absence, were shifted to the right in the presence of GppNHp. Quantitative analysis indicated that approximately 50% of the muscarinic receptors showed a high affinity for ACh in the presence of Gi or Go and the absence of GppNHp. Interpretation of these results is given in the text.

of muscarinic receptors are present in cerebral tissues and thus it was not clear which subtypes interacted with Gi, Go or Gn. Based on the present knowledge

that all five subtypes are present in the brain, and that M_2 and M_4 receptors interact with Gi-type G proteins, it is reasonable to suppose that M_2 and M_4 receptors interact with Gi, Go and Gn. More specifically, Ikegaya *et al.*[77] showed that muscarinic receptors purified from porcine atria (M_2 receptors) interact with three kinds of Gi-type G proteins, Gi, Go and Gn.

Shiozaki and Haga[78] (1992) examined the interaction between atrial muscarinic receptors and Gi in more detail in the reconstitution system and demonstrated that (1) M_2 receptors show three different affinities for agonists depending on the presence or absence of Gi and Mg^{2+} ions, (2) agonists cause a decrease in the affinity for GDP, (3) the presence of Mg^{2+} ions is necessary for the agonist-dependent decrease in affinity for GDP, and (4) the effect of an agonist was more prominent on the affinity for GDP rather than on that for GTP. Studies using M_2-$Gi1\alpha$ fusion proteins confirmed these observations, and also indicated that muscarinic partial agonists such as pilocarpine and McN343 caused a partial decrease in the affinity for GDP that was intermediate between those in the presence and absence of a full agonist[79] (Fig. 4). These results indicate that the M_2-Gi fusion protein may be used as a ligand-screening system, and also suggest that the M_4-Gi fusion protein could be used for the screening of M_4-specific agonists or positive allosteric modulators, which may be useful for the treatment of schizophrenia. GPCR-$G\alpha$ fusion proteins could be used to screen endogenous ligands for orphan receptors[80] and would be useful for searching for agonists for Gi/Go-coupled receptors (see a review ref. 81).

Based on these results, a simplified model was proposed for the interaction between M_2 and Gi.[76] In the model, the ACh-M_2-Gi complex is assumed to be formed as the transition state for the GDP/GTP exchange reaction, which contributes to reduction of the barrier for transition from Gi-GDP to Gi-GTP through Gi. This action of ACh-M_2 on Gi was noticed to be similar to the action of actin on myosin, where actin facilitates the exchange of ATP/ADP in myosin by forming an actin-ATP/ADP-free myosin complex. In the simplest form of the model, approximately 100-fold difference in the affinity for ACh with or without Gi was thought to correspond to a reduction of the barrier by 3 kcal/mol and to an increase in GDP dissociation rate by 100-fold. Whether the model is accepted or not, it would be safe to conclude that the function of ACh-bound M_2 is to accelerate the dissociation of GDP from Gi.
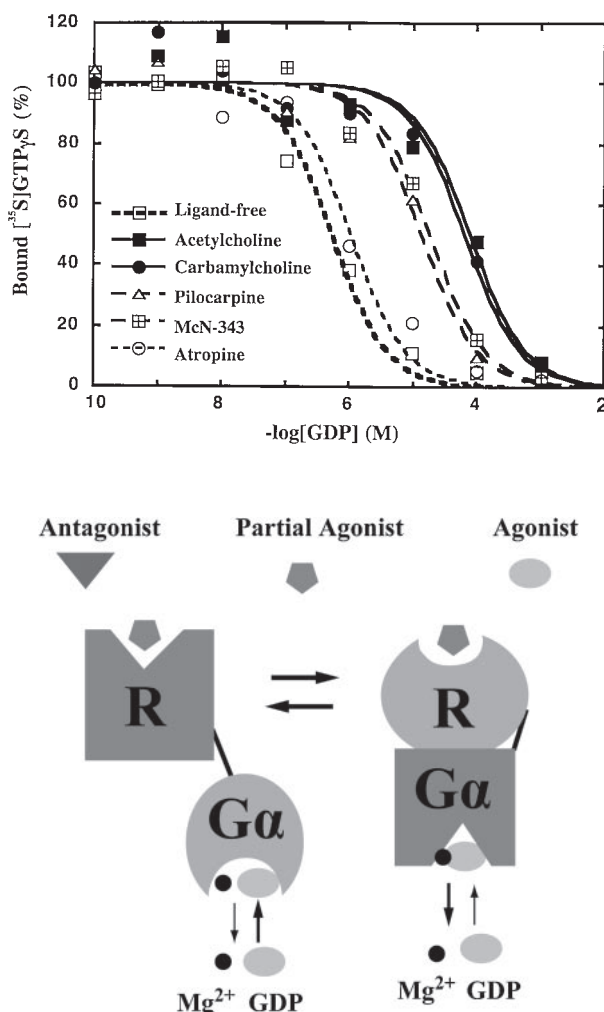

Fig. 4.   Displacement by GDP of [^{35}S]GTPγS binding to an M_2-$Gi\alpha$ fusion protein in the absence and presence of antagonists, partial agonists or agonists.[79] Insect cell (Sf9) membranes expressing M_2-$Gi\alpha$ fusion proteins were incubated with 50 nM [^{35}S]GTPγS and various concentrations of GDP in the presence of full agonists (ACh, carbmaylcholine), partial agonists (pilocarpine, McN-343), or antagonist (atropine), and [^{35}S]GTPγS bound to the fusion proteins was trapped and counted. The displacement curves by GDP of [^{35}S]GTPγS binding shifted to the right in the presence of partial agonists and full agonist, indicating that the affinity for GDP decreased in the presence of agonist, partial agonist, and antagonist in this order. In the model, M_2-$Gi\alpha$ fusion proteins (R-$G\alpha$) are assumed to take two conformations: one conformation with a high affinity for GDP is dominant in the absence of ligand or the presence of antagonist, the other conformation with a low affinity for GDP is dominant in the presence of full agonist, and the two conformations are assumed to be in equilibrium in the presence of partial agonists.

3.4. Muscarinic receptors —G protein (Gq)—phospholipase C.
Hokin and Hokin[82] showed in the 1950s that stimulation by ACh of pancreas and

brain cortex slices causes incorporation of [^{32}P]phosphate into phospholipids, particularly phosphatidyl inositol. After a long series of studies by various groups for almost thirty years, a mechanism was proposed by which the stimulation of muscarinic and other receptors leads to activation of phospholipase C, which catalyzes the formation of diacylglycerol and inositol trisphosphate (IP3) from phosphatidylinositol 4,5 bisphosphate (PIP2), and that IP3 acts on intracellular organelles causing the release of Ca^{2+} ions into the cytoplasm (see review ref. 83). This signal transduction system, which is called the PI response, was indicated to involve a lot of receptors and to be ubiquitous in various tissues. M_1, M_3 and M_5 receptors were shown to be coupled with PI turnover, whereas M_2 and M_4 are only weakly coupled with PI turnover (see reviews refs. 34, 35).

Receptors and phospholipase C were suggested to be linked through G proteins, and the relevant G proteins were putatively termed Gp, but it took a time to identify the molecular entity of Gp, which is now called Gq-type G proteins. Retrospectively, the difficulty in identifying Gq proteins appears to be due to that (1) a good assay system like cyc$^-$ cells for the identification of Gs was not available in the case of Gq, (2) Gq-type G proteins do not bind [^{35}S]GTPγS without activation by agonist-bound receptors, in contrast with Gi and Go proteins, which could be purified as [^{35}S]GTPγS binding components, (3) toxins like cholera and pertussis toxins were not available for Gq-type G proteins, and (4) there was some confusion as to the sensitivity to pertussis toxin as there are two kinds of phospholipase Cβ, which are activated by Gq- and Gi-type G proteins in pertussis toxin-resistant and -sensitive manners, respectively.

We started characterizing the PI turnover initiated by activation of M_1 receptors with Nukada at Tokyo University in 1989. Nakamura et al.[84] (1991) identified two new G protein α subunits, which were termed $G_{L1}\alpha$ and $G_{L2}\alpha$, and are not substrates of pertussis toxin, by cloning of the corresponding cDNAs with the use of hybridization with cDNA for $Gi_1\alpha$. $G_{L1}\alpha$ and $G_{L2}\alpha$ were found to be the same as the α subunits of Gq-type G proteins $G_{14}\alpha$ and $G_{11}\alpha$ respectively, which were independently cloned by Strathmann and Simon.[85] We collaborated with Takenawa in Tokyo University and Kikkawa in Kobe University, and constructed a reconstitution system for M_1 receptors, Gq-type G proteins, and phospholipase C-β1, and could confirm that the function of M_1 receptors is to interact with and activate Gq-type G proteins, which activate

phospholipase C β1: we expressed M_1 receptor and Gq-type G proteins ($G\beta_1\gamma_2$ + one of $G_{L1}\alpha$ ($G_{14}\alpha$), $G_{L2}\alpha$ ($G_{11}\alpha$) or Gqα) in Sf9 cells using baculovirus, purified them and reconstituted them with purified phospholipase C-β in phospholipid vesicles, and demonstrated that M_1 receptors can activate all three Gq-type G proteins, but not Go, and that these Gq-type G proteins activate phospholipase C-β1.[86] In these studies, the baculovirus-Sf9 system was very useful for preparing large amounts of proteins, particularly membrane proteins such as muscarinic receptors, which are expressed only in a limited amount in mammalian cultured cells or tissues. The baculovirus-Sf9 system was established in our laboratory by Kameyama, who had learned it at the laboratory of Ross in Texas University. It is interesting to note that guanine nucleotide-sensitive high affinity agonist binding was not detected in the M_1-Gq system so clearly as compared with in the M_2-Gi system, suggesting that the mechanism of activation by receptors of G proteins might be different between the two systems.

The relation of muscarinic receptors with the formation of cGMP appears to involve many steps of intra- and inter-cellular signal transduction, in contrast with the relation of muscarinic receptors with the inhibition of cAMP formation. In the case of relaxation of arterial smooth muscle, (1) muscarinic M_3 receptors in endothelial cells respond to ACh and initiate the signal transduction leading to formation of NO (nitric oxide) through many steps including Gq, phospholipase Cβ1, IP3 receptor, Ca^{2+}/calmodulin, and NO synthase, (2) NO diffuses into smooth muscle and there activates NO receptor with guanylate cyclase activity, and (3) cGMP activates protein kinase G which leads to relaxation of the muscle and then vasodilation (see a review ref. 87).

Recently muscarinic receptors, particularly M_1 and M_3 receptors, were shown to be linked to activation of $G_{12/13}$-type G proteins, which leads to activation of small GTP-binding proteins Rho through activation of RhoGEF (RhoGTPase nucleotide exchange factor) (see a review ref. 88).

4. Regulation of muscarinic receptors

4.1. Synergistic activation of G protein-coupled receptor kinase 2 (GRK2) by agonist-bound muscarinic receptors and G protein $\beta\gamma$ subunits. In the early 1990s we started studies on the regulatory systems of muscarinic receptors. At first, we examined the phosphorylation by protein kinase C of muscarinic receptors. Muscarinic recep-

tors purified from porcine brain were found to be phosphorylated at their carboxyterminal tail, but the phosphorylation did not affect their ability to interact with muscarinic agonists and Gi/Go.[89),90)] In addition, the reconstituted system of M_1 receptors, Gq-type G proteins, and phospholipase C-β was also shown to be not affected by treatment with protein kinase C.[86)] The phosphorylation by protein kinase C of muscarinic receptors was not dependent on the presence of a muscarinic agonist, in contrast with the phosphorylation by GRK2 described hereafter.

When we started studies on the phosphorylation of muscarinic receptors, rhodopsin and β adrenergic receptors were known to be phosphorylated in a light- or agonist-dependent manner by rhodopsin kinase (GRK1 in the present terminology[91)]) and β adrenergic receptor kinase (βARK, GRK2 in the present terminology[92)]), respectively, and these phosphorylations were reported to affect their function (see review ref. 93). Kwatra and Hosey[94)] (1986) reported that muscarinic receptors in chick heart may be phosphorylated in an agonist-dependent manner by an endogenous, unidentified kinase. We then started to search for this kinase that specifically phosphorylates muscarinic receptors in an agonist-dependent manner. We found that a preparation partially purified from a brain extract may phosphorylate both cerebral and atrial muscarinic receptors in an agonist-dependent manner.[95)] Unexpectedly, the activity was found to be affected by G protein Go in a dual manner that is stimulation at lower concentrations of it and inhibition at higher concentrations of it, and the stimulatory and inhibitory effects were reproduced by the Go-$\beta\gamma$ subunits and Go-$\alpha\beta\gamma$ trimer, respectively.[96)] The inhibition by the Go-$\alpha\beta\gamma$ trimer was abolished on the addition of either GTP or GDP,[95)] which is consistent with the model that M_2 receptors form a complex with guanine nucleotide-free Go, but not with GTP or GDP-bound Go. Further experiments indicated that the kinase preparation may also phosphorylate rhodopsin in light- and G$\beta\gamma$-dependent manners, whereas the phosphorylation of rhodopsin by rhodopsin kinase was dependent on light but not affected by the presence of $\beta\gamma$ subunits.[97)] Furthermore, any $\beta\gamma$ subunits derived from Gs, Gi, or Go stimulated the agonist- or light-dependent phosphorylation of muscarinic receptors and rhodopsin. These results suggested that the stimulatory effect of $\beta\gamma$ subunits would be due to the specific property of the kinase preparation, and not due to the species of substrates or $\beta\gamma$ subunits.

The partially purified kinase was supposed to be similar to or the same as GRK2, because of the similar purification procedure, and similar effects of salts and heparin, but the stimulatory effect by $\beta\gamma$ subunits on phosphorylation of rhodopsin[98)] and muscarinic receptors[99)] by GRK2 had not been reported. Kameyama et al.[100)] examined the effect of $\beta\gamma$ subunits on GRK2, the cDNA of which was supplied by Lefkowitz in Duke University, and showed that GRK2 may phosphorylate muscarinic receptors as well as β adrenergic receptors in agonist- and G$\beta\gamma$-dependent manners. In addition, rhodopsin was also phosphorylated by GRK2 in light- and G$\beta\gamma$-dependent manners. GRK2 is homologous to rhodopsin kinase except that GRK2 has an extra C-terminal domain of approximately 130 amino acid residues. We assumed that the C-terminal tail may be involved in the interaction with G$\beta\gamma$. In accord with this assumption, a mutant with a deletion in the C-terminal tail was shown to phosphorylate muscarinic receptors in an agonist-dependent but G$\beta\gamma$-independent manner.[100)] In 2003, this assumption was directly confirmed by the crystal structure of the GRK2-G$\beta\gamma$ complex, where the C-terminal tail of GRK2 is bound with the G$\beta\gamma$ subunit.[101)]

Phosphorylation sites for rhodopsin kinase or GRK2 in rhodopsin or β adrenergic receptors were shown to be in the C-terminal tail, but those in muscarinic receptors had not been determined yet. Nakata et al.[102)] located the phosphorylation sites in the central part of the third intracellular loop (I3) but not in the C-terminal tail. Furthermore, the amino acid sequences of putative phosphorylation sites in rhodopsin, β adrenergic receptors, and M_2 receptors were shown not to be similar to each other. This raises the question why GRK2 specifically phosphorylates only agonist-bound GPCRs. One of the simplest explanations is to assume that the phosphorylation sites are hidden inside GPCRs in their inactive state and are exposed in their active state induced by agonist binding. To test this assumption, we compared the phosphorylation of M_2 receptors, and a fusion protein of glutathione S-transferase (GST) and a peptide (268–324) containing the phosphorylation sites (I3-GST). If the assumption is the case, I3-GST would be as good a substrate as agonist-bound M_2 receptors.

Against the assumption, I3-GST turned out to be a poor substrate for the kinase. The phosphorylation of I3-GST, however, was found to be synergistically enhanced by G protein $\beta\gamma$ subunits and mastoparan, which is known to mimic agonist-bound

receptors.[103] The stimulatory effect of mastoparan could be reproduced by synthetic peptides corresponding to the sequences of intracellular segments adjacent to the transmembrane helices of muscarinic receptors in I2, I3 and the carboxyterminal tail. Furthermore, I3-GST was found to be phosphorylated by GRK2 in an agonist-dependent manner in the presence of G protein $\beta\gamma$ subunits and I3-deleted M_2 receptors, which lack the central part of the I3 loop including phosphorylation sites[104] (Fig. 5 (a)). These results indicate that M_2 receptors interact with GRK2 at two different sites: (1) the phosphorylation sites in the central part of I3 that serves as the substrate and (2) intracellular segments that may serve as a GRK2 activator. It will be reasonable to assume that agonist binding induces a conformational change of M_2 receptors at sites adjacent to transmembrane segments, which interact with and activate GRK2 together with G protein $\beta\gamma$ subunits (see review ref. 93). Furthermore, $G\alpha\beta\gamma$ trimer might compete with GRK2 on these sites, which would explain the finding[95] that the agonist-dependent phosphorylation of muscarinic receptors is inhibited by a guanine nucleotide-free $G\alpha\beta\gamma$ trimer.

4.2. Phosphorylation-dependent internalization of muscarinic M_2 receptors. Muscarinic receptors as well as β adrenergic receptors and other GPCRs are subject to agonist-induced desensitization, and agonist-dependent phosphorylation of muscarinic receptors is supposed to be involved in the desensitization. Particularly we have examined the agonist-induced internalization of M_2 and M_4 receptors with Kameyama in Tokyo University in the 1990s, and with Yoshida, Ichiyama, and Hashimoto in Gakushuin University in the 2000s. The internalization of muscarinic receptors could be easily determined as the decrease in [3H]NMS binding activity of cultured cells as [3H]NMS is a quaternary ion and does not penetrate the cell membrane. On the other hand, down regulation, that represents agonist-induced loss of muscarinic receptors from the cell, was determined as the agonist-induced decrease in the binding activity of [3H]QNB, which is a tertiary amine, can penetrate the cell membrane, and binds with M_2 receptors both on the cell surface and in intracellular organelles. Actually, cultured cells expressing M_2 receptors were treated with an agonist for 0 to 16 hours at 37°C, washed with cold buffer, and incubated with [3H]NMS or [3H]QNB for 4 hours at 4°C, and then bound [3H]NMS or [3H]QNB was determined: here it is presumed that at 4°C M_2 receptors may bind with [3H]NMS or [3H]QNB but
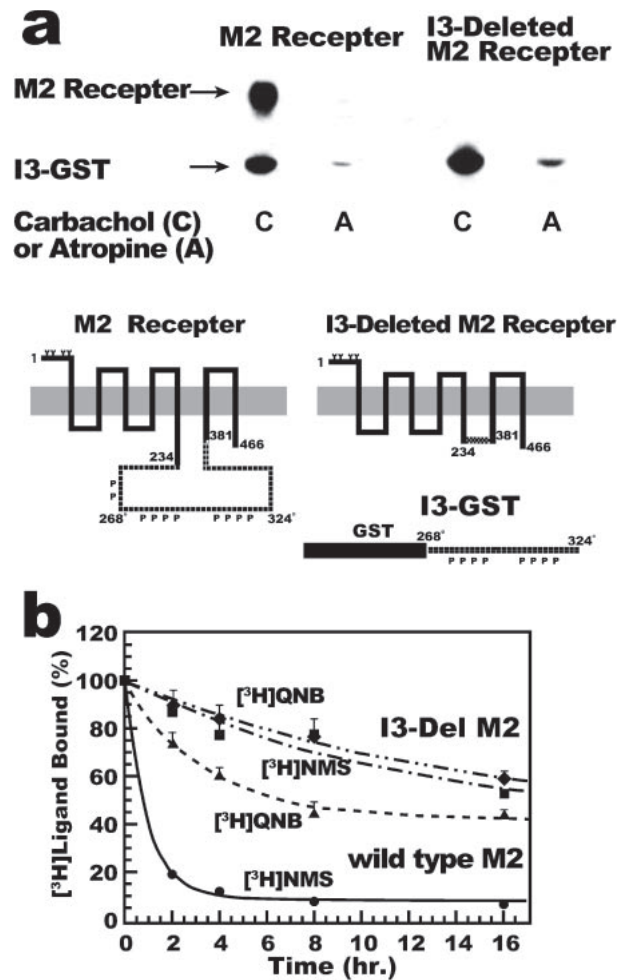

Fig. 5.   (a) Agonist-dependent phosphorylation of I3-GST in the presence of M_2 or I3-deleted M_2 receptors[104] and (b) agonist-induced internalization and down-regulation of M_2 and I3-deleted M_2 receptors.[106] (a) I3-GST was subjected to phosphorylation by GRK2 in the presence of M_2 or I3-deleted M_2 receptors with an agonist (carbachol) or antagonist (atropine), followed by SDS-PAGE of the reaction mixture and detection of [32P]labeled bands through autoradiography. (b) CHO cells expressing M_2 or I3-deleted M_2 receptors were incubated at 37°C with an agonist carbamylcholine for indicated time, and then subjected to [3H]NMS or [3H]QNB binding at 4°C. Agonist-induced internalization and down-regulation of M_2 and I3-deleted M_2 receptors were measured as the decrease in the [3H]NMS and [3H]QNB binding activity, which represent the amount of M_2 receptors in the cell surface and in the whole cell, respectively.

may not be internalized from the cell surface nor be recycled back there.

Tsuga et al.[105] showed that coexpression of GRK2 together with M_2 receptors in COS7 cells causes dramatic increases in both the phosphorylation and internalization of M_2 receptors in an agonist-
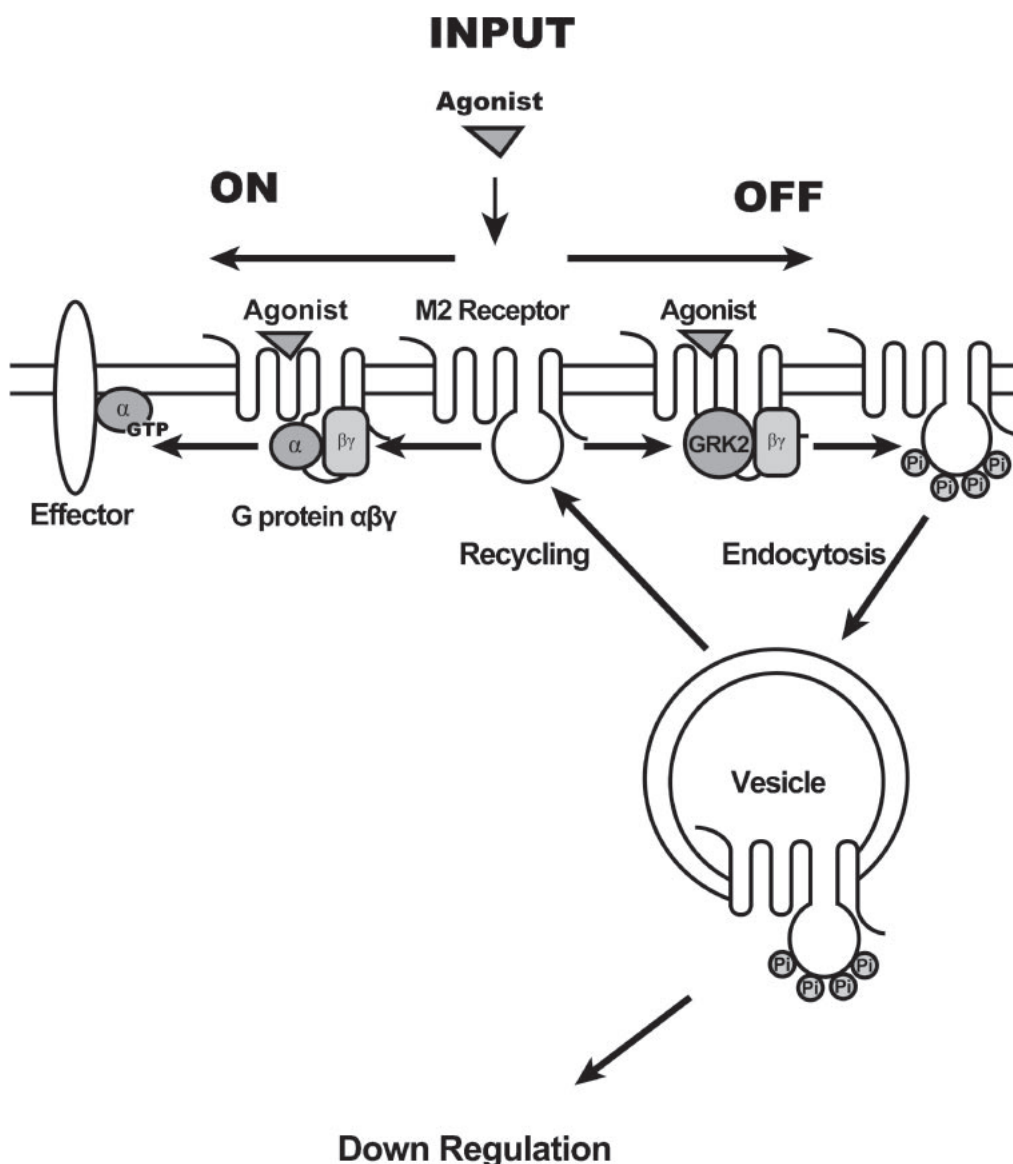
Fig. 6. A model of agonist-induced activation of G protein ("on" reaction) and agonist-induced phosphorylation and internalization of M_2 receptors ("off" reaction).

dependent manner. Furthermore, both internalization and down regulation of M_2 receptors were shown to be dependent on the presence of the I3 loop: when CHO cells expressing M_2 receptors or I3-deleted M_2 receptors are exposed to an agonist continuously, approximately 90% of the M_2 receptors were internalized with a half life of 10 min as was shown by decline of [³H]NMS binding and 60% of them were down-regulated with a half life of 2.3 hours as was shown by decline of [³H]QNB binding, whereas 60% of I3-deleted M_2 receptors were internalized and down-regulated with a half life of 8–10 hours[106]

(Fig. 5(b)). These results indicate that internalization and down regulation of muscarinic receptors are governed by the I3 loop, and that the agonist-induced phosphorylation of the I3 loop by GRK2 is the initial and essential step (Fig. 6).

Agonist-dependent internalization is also observed for muscarinic receptors other than M_2 receptors. The extent and rate of internalization of muscarinic M_1-M_5 receptors expressed in COS-7 and BHK-21 cells differed from one subtype to another.[107] In addition, the effect of co-expression of GRK2, dominant negative GRK2, GRK4, GRK5,

GRK6, dynamin and dominant-negative dynamin was also different depending on the subtype species. These subtype-specific properties are assumed to be due to the different I3 loop sequences among the five subtypes. To test this assumption, we examined the internalization and recycling of the M_2-M_4 chimera with replacement of the I3 loop by another subtype such as M_2-M_4(I3)-M_2 and M_4-M_2(I3)-M_4, which were expressed in HEK293 cells. The agonist-dependent internalization of the M_2-M_4(I3)-M_2 chimera was shown to be greatly reduced on coexpression of the dominant negative dynamin as was the case for M_4 receptors, whereas the agonist-dependent internalization of the M_4-M_2(I3)-M_4 chimera was hardly affected by coexpression of the dominant negative dynamin as was the case for M_2 receptors. In addition, M_2-M_4(I3)-M_2 chimera and M_4 receptors were shown to be recycled back to the cell surface after removal of the agonist, whereas no recycling was observed for the M_4-M_2(I3)-M_4 chimera and M_2 receptors.[*1] These results demonstrate the major role of the I3 loop in the agonist-dependent internalization/recycling of muscarinic receptors. In contrast with these observations, the possibility remains that other parts of receptors might also be involved in the agonist-dependent internalization of M_4 receptors, because the internalization of I3-deleted M_4 receptors lacking major phosphorylation sites was found to be enhanced by coexpression of GRK2.[*2] It remains to be determined if GRK2 phosphorylates other sites of M_4 receptors besides the I3 loop, phosphorylates other proteins, or exerts its effect independently of the kinase activity.

We assumed that all five I3 loops have different conformations, each of which recognizes a different protein. To test this assumption, Ichiyama et al.[108] (2006) have expressed a fusion protein of M_2-I3 (208–388) with glutathione S-transferase (GST-I3) in E. coli and purified it. The I3 part of GST-I3 was shown to have no secondary structure by circular dichroism (CD) and nuclear magnetic resonance (NMR) measurements. Furthermore, differential CD between wild type M_2 and I3-deleted M_2 receptors indicated that the I3 loop does not have any secondary structure even when it is in M_2 receptors. This finding is rather unexpected but is consistent with the finding that purified M_2 receptors are very susceptible to proteolysis at the I3 loop during

storage at 4°C. This finding raises the question of how the I3 loop may exert its function.

Hashimoto et al.[109] (2008) attempted to localize the part that is involved in agonist-dependent internalization and recycling of M_4 receptors. Experiments involving the deletion of various portions of the M_4-I3 loop indicated that a segment of 21 amino acid residues is necessary for the internalization and recycling of M_4 receptors. Furthermore, I3-deleted M_2 receptors, which could hardly be internalized in response to an agonist, were found to be internalized and then be recycled back to the cell surface in an agonist-dependent manner through insertion of the 21 segment of M_4 receptors. These results suggest that this segment is recognized by other proteins, and that these hypothetical proteins function to initiate internalization and recycling of receptors. A search for such proteins that specifically interact with the different I3 loops of the five muscarinic subtypes should be fruitful for molecular studies on regulation of muscarinic receptors.

4.3. Other aspects of muscarinic receptor regulations. In a series of experiments on the phosphorylation of muscarinic receptors by GRK2, GRK2 was found to be inhibited by calmodulin in a Ca^{2+} ion-dependent manner.[110] In addition, GRK2 was found to bind with and phosphorylate tubulin,[111] although GRKs had been known to phosphorylate only activated forms of GPCRs. Yoshida et al.[112] (2003) identified the phosphorylation sites as Ser and Thr residues in the C-terminal domain of β-tubulin, which is located on the outermost surface of microtubules. Microtubule formation from tubulin in vitro was shown to be affected by approximately 10^{-5} M Ca^{2+} ions,[113] which was later shown to be mediated through calmodulin. These preliminary results regarding the interactions among GRK2, Ca^{2+}-calmodulin, and tubulin might indicate the possible involvement of GRK2 in intracellular signaling or cytoskeleton formation. It is interesting to note that a diverse function of GRK2 and other GRKs, besides their role in receptor desensitization, has been indicated recently.[114]

Muscarinic receptors may be modified after their translation. Muscarinic receptors were supposed to be glycosylated based on the observation that they are bound to lectin gels,[21] affected by endoglycosidase treatment,[29] and so on. Ohara et al.[30] (1990) showed that endoglycosydase F treatment of muscarinic receptors purified from porcine cerebrum caused a decrease in their molecular size from 70 to 50 kDa, as assessed as the mobility of [^{3}H]PrBCM-labeled

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[*1] Jojima, E., Saito, H., Yoshida, N. and Haga, T. unpublished results.
[*2] Morisawa, K., Saito, K., Kakegai, R., Hashimoto, Y. and Haga, T. unpublished results.

band on SDS-PAGE. The endoglycosidase treatment, however, did not affect their ligand binding activity nor their ability to interact with G proteins, indicating that the glycosylation moiety is not involved in the primary functions of muscarinic receptors, although it may be involved in the intracellular translocation of muscarinic receptors.

Hayashi and Haga[115] expressed muscarinic M_2 receptors in Sf9 cells using baculovirus and have shown that they are labeled with [³H]palmitic acid added to the incubation medium. The labeling rate was greatly accelerated by the presence of an agonist. Such labeling was not observed for a Cys458Ala mutant, indicating that Cys 458 in the C-terminal tail of M_2 receptors is palmitoylated. The Cys458Ala mutant, which was expressed in Sf9 cells and purified from them, may interact with and activate Gi2- and Go-type G proteins, although the rate of the agonist-dependent increase in [³⁵S]GTPγS binding was slower as compared to the corresponding rate for wild-type M_2 receptors. These results indicate that the palmitoylation of Cys458 in M_2 receptors is not necessary for their function but facilitates their interaction with G proteins.

Mieda,[116),117] together with Saffen, studied the regulation of M_4 gene expression and identified regulatory functions of the 5′-flanking region in the M_4 receptor gene: the proximal 435-bp sequence contains a constitutive promoter and the 635-bp sequence a cell-type-specific silencer element. Furthermore, the neuron-restrictive silencer/repressor element (NRSE/RE1) upstream of the M_4 gene was shown to be necessary and sufficient to repress M_4 promoter activity in non-neuronal cells. The activity of neuronal-restrictive silencer factor/RE1-silencing transcription factor (NRSF/REST) was suggested to depend upon the species of promoter to which it is linked and upon the protein species that bind to this promoter.

5. Structure of muscarinic receptors

5.1. A large scale preparation of purified QNB-M_2 complex. In the late 1990s, we started to attempt to crystalize M_2 receptors and to determine their tertiary structures. We chose a M_2 mutant with a deletion in the central part of I3 because the I3 loop had been shown to be very susceptible to proteolysis and to be broken down during the purification procedure. In addition, asparagine residues in three N-glycosylation consensus sequences in the N-terminal portion were mutated to aspartic acid residues in order to avoid glycosylation, which might

cause heterogeneity of M_2 receptors. We expressed the M_2 mutant with N2, 3, 6, 9D and a deletion of 235–380 in the I3 loop in cultured insect cells (Sf9) by using the baculovirus system,[118] and purified it by using ABT-agarose, and demonstrated that it has the ability to interact with and activate Gi in agonist-dependent manners. For approximately 10 years since the late 1990s, we have continued large-scale expression of the M_2 mutant, which will be called here as M_2 or the M_2 receptor in this text. The M_2 receptor was expressed in Sf9 cells, with a scale of 20–40 l of cultured solution per month, which express 1–2 mg of M_2 receptors per 1 liter of medium, in Wakenyaku Co. and Toyobo Co. initially, and then in Gakushuin University, where Okada, Hayashi, Matsuyama, Watanabe and others contributed. Thus, we expressed 20–80 mg of M_2 receptors per month and several grams of them in total. Almost all of these M_2 receptors were used for attempts at crystallization.

In attempt to crystalize M_2 receptors, one of the most critical problems was to find an appropriate detergent. As described earlier, muscarinic receptors had been known to be solubilized in their active form by digitonin, but the purified M_2 receptors were found to be bound with more than 10 times as much digitonin as M_2 receptor proteins. M_2 receptors lose their ligand-binding activity in most detergents other than digitonin, although some detergents including lysophosphatidyl choline,[119] and dodecyl maltoside[120] were reported to be used for the solubilization of muscarinic receptors with their ligand binding activity. Rinken *et al.*[121] showed that atrial muscarinic receptors could be solubilized by sucrose monolaurate and partially purified in the detergent. We also tried to solubilize M_2 receptors in dodecyl maltoside and other alkyl sugars, and to purify them in these detergents, but found that the ligand binding activity was gradually lost during the purification procedure, probably because of the instability of M_2 receptors in these detergents.

We also noticed that muscarinic receptors could be solubilized in their active forms when porcine cerebral membranes were pre-treated with a muscarinic ligand such as carbamylcholine or atropine, and then solubilized with sodium cholate in the presence of this ligand.[122] [³H]QNB, which was pre-bound to muscarinic receptors in membrane preparations, appeared to remain bound to receptors after solubilization in different kinds of detergents. Based on these preliminary results, we adopted the following strategy to prepare muscarinic receptors that could

be used for their crystallization. (1) M_2 receptors are solubilized in the absence of a muscarinic ligand with digitonin, (2) solubilized M_2 receptors are bound to an ABT-agarose column in digitonin and eluted from the column with atropine, (3) eluted M_2 receptors are bound to a small column of hydroxyapatite (HA), (4) the HA column are washed and incubated with QNB in digitonin, and washed with a Na cholate solution and then with any chosen detergent, (5) the QNB-M_2 complex is eluted with a high salt solution in a given detergent, concentrated, and then dialyzed against any chosen medium, and (6) the concentrated QNB-M_2 complex is subjected to crystallization. Here it was essential to have M_2 receptors bound with muscarinic ligands such as ABT, atropine, and QNB in digitonin, and then to let digitonin dissociate from M_2 receptors by washing the HA column with Na cholate before the detergent changes to an alkyl sugar. Digitonin is bound with receptors so tightly that it can not be replaced by detergents like alkyl sugars.

In a typical large scale purification, M_2 receptors were solubilized with 1% digitonin/0.2% Na cholate from Sf9 cell pellets derived from 20 l of cultured solution expressing 800 nmol (30 mg) of M_2 receptors, the extract was applied to two columns of ABT-agarose (500 ml each), the eluate with atropine from the ABT columns was led to a HA column (30 ml), and then the QNB-M_2 receptors were eluted with decyl maltoside from the HA column. The recovery of purified QNB-M_2 receptors was estimated to be approximately 50% of solubilized receptors by using [^{3}H]QNB instead of non-labeled QNB. We also observed that [^{3}H]QNB was very tightly bound to M_2 receptors and dissociation of [^{3}H]QNB was hardly observed at 4°C, indicating that the QNB-M_2 complex may be stable at 4°C.

5.2. Crystallization of QNB-M_2 complex. In 1999, we established a system for large scale purification of M_2 receptors and started attempt to crystallize them. We consulted X ray crstallography specialists such as Nakasako in Keio University, Yoshikawa and Shinzawa-Itoh in Hyogo Prefectural University, Tsukihara in Osaka University, Harada in Tokyo University and others. We adopted a crystal screen kit (Hampton Research) and attempted to crystallize several kinds of M_2 preparations purified in different detergents. In 2000, the crystal structure of rhodopsin was reported,[123] which was the first structure determined for a member of the GPCRs. Rhodopsin was shown to have seven-transmembrane segments, as was expected from amino acid sequences, although no direct evidence had been obtained until then. We consulted with and obtained advice from Okada a major contributor to determination of the rhodopsin structure, and tried to crystalize M_2 receptors under the same or similar conditions as those used for rhodopsin crystallization, that involves ammonium sulfate as a precipitant. However, we could not detect any sign of crystallization of M_2 receptors under such conditions.

Then we returned to the Hampton kits and examined 500 conditions of the kits for several preparations (more than 2000 conditions in total) by means of the vapor diffusion method. Most attempts were carried out in the cold room around 4°C. In 2001, we could obtain the first needle-shaped crystal with a few diffraction points, although the resolution was approximately 32 Å. The resolution of diffraction points was improved to 18 Å in a few months. One year later, we could obtain a rod-shaped crystal with an orthorhombic-type unit cell ($111 \times 69 \times 109$ Å) by using decylmaltoside as detergent and low molecular weight polyethyleneglycol as the precipitant. The resolution of the diffraction points was 9 Å (Fig. 7). The improvement in one year had been so great that we expected to obtain crystals good enough to be used for structure determination in a few years. However, no improvement in the quality of crystals was attained in the next five years, although persistent endeavors were made using a lot of M_2 receptor preparations and various kinds of crystallization conditions, which included the use of different kinds of alkyl sugars, polyethyleneglycols, the addition or no addition of lipids, various kinds and concentrations of salts and buffers, and various incubation temperatures.

In 2007, Kobilka *et al.* reported the structure of β_2 adrenergic receptors.[124] They used two unique techniques to facilitate the crystallization of β_2 adrenergic receptors: (1) introduction of lysozyme of bacteriophage T4 (T4L) into the I3 loop of β_2 adrenergic receptors and (2) adoption of the lipidic cubic phase method. T4L had been known to be a well-folded protein and was introduced to increase the hydrophilic regions, which was expected to facilitate inter-molecular interaction. The lipidic cubic phase method was developed by Rosenbusch and Landau,[125] and was used to crystallize bacteriorhodopsin. With the lipidic cubic phase method, membrane proteins are designed to be crystallized in the lipidic phase consisting of oleic acid instead of the aqueous phase.[126] We had also considered adopting the lipidic cubic phase method, and visited and

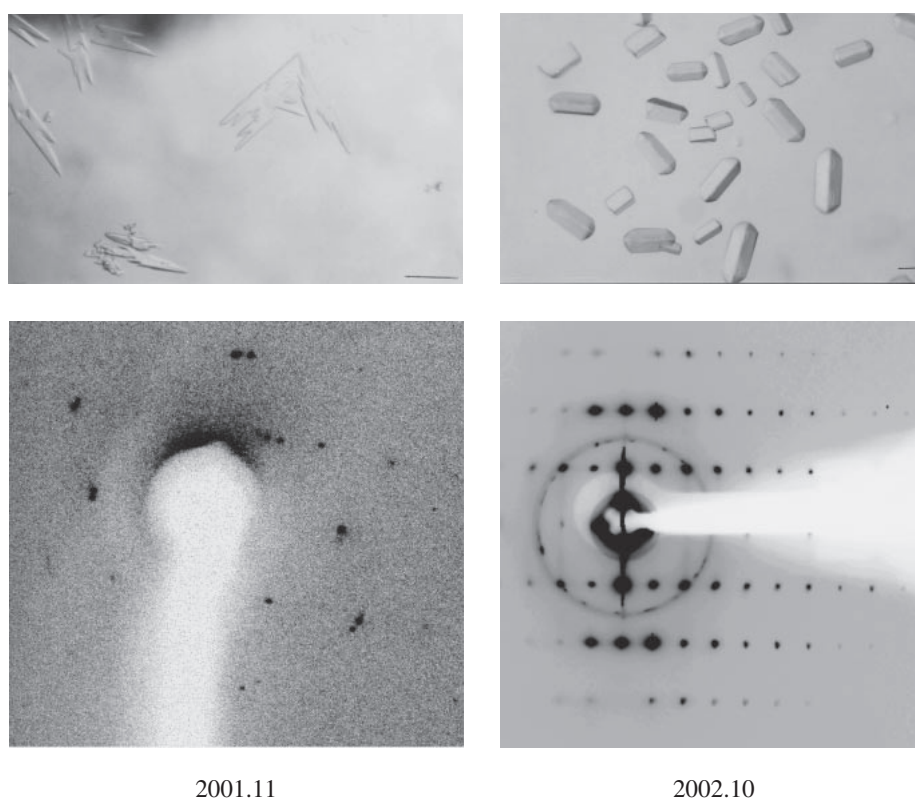2001.11   2002.10

Fig. 7.   Crystals and diffraction patterns of M_2 receptors. I3-deleted M_2 receptors were crystalized by the vapor-diffusion method. The resolution of diffraction points was 9 Å.

consulted with Rosenbusch in Basel in the late 1990s. However, we did not adopt the method because we could obtain crystals in 2001–2002 with the usual vapor diffusion method and decided to improve the quality of the crystals by means of the same vapor diffusion method.

We had started collaboration with the group of Iwata in Kyoto University, when the structure of β_2 adrenergic receptors was reported. We decided to introduce T4L in the I3 loop of M_2 receptors following the success for β_2 adrenergic receptors. Kobayashi in Iwata's laboratory constructed the cDNA of M_2-T4L, and we expressed M_2-T4L using the Sf9-baculovirus system and tried to crystalize it at Gakushuin University. Soon we could obtain crystals of QNB-M_2-T4L with the vapor diffusion method, but the resolution was not good enough to be used for structure determination. We continued to attempt to improve the quality of the crystals for a few more years but were not successful. Then, we reconsidered to adopting the lipidic cubic phase method, discussed this with Kobayashi and Kobilka by e-mail, and decided to send the purified QNB-M_2-

T4l protein to the laboratory of Kobilka in Stanford University for trial crystallization. We had expected a long series of experiments after the initial survey in Kobilka's laboratory when we sent the sample of purified QNB-M_2-T4L in January 2011. To our surprise, a month later we were informed that Kruse succeeded in crystallizing M_2-T4L in the first preliminary trial and that the quality of the crystals was so good that the structure would be determined soon. Actually in February 2011, we could see the 3 Å structure of the QNB-M_2-T4L complex.[127] The combination of T4L and the lipidic cubic phase has been shown to be useful for determination of the structures of many GPCRs including muscarinic M_3 receptors,[128] opioid receptors,[129] and others.

5.3. The overall structure of M_2 receptor. As was expected from the amino acid sequence, the structure of M_2 receptors was demonstrated to include seven transmembrane segments[127] (Fig. 8). Another α helix (Helix 8) was also detected in the carboxyterminal tail. The overall transmembrane structure of M_2 receptors including Helix 8 is very similar to those of rhodopsin[123] and β_2 adrenergic
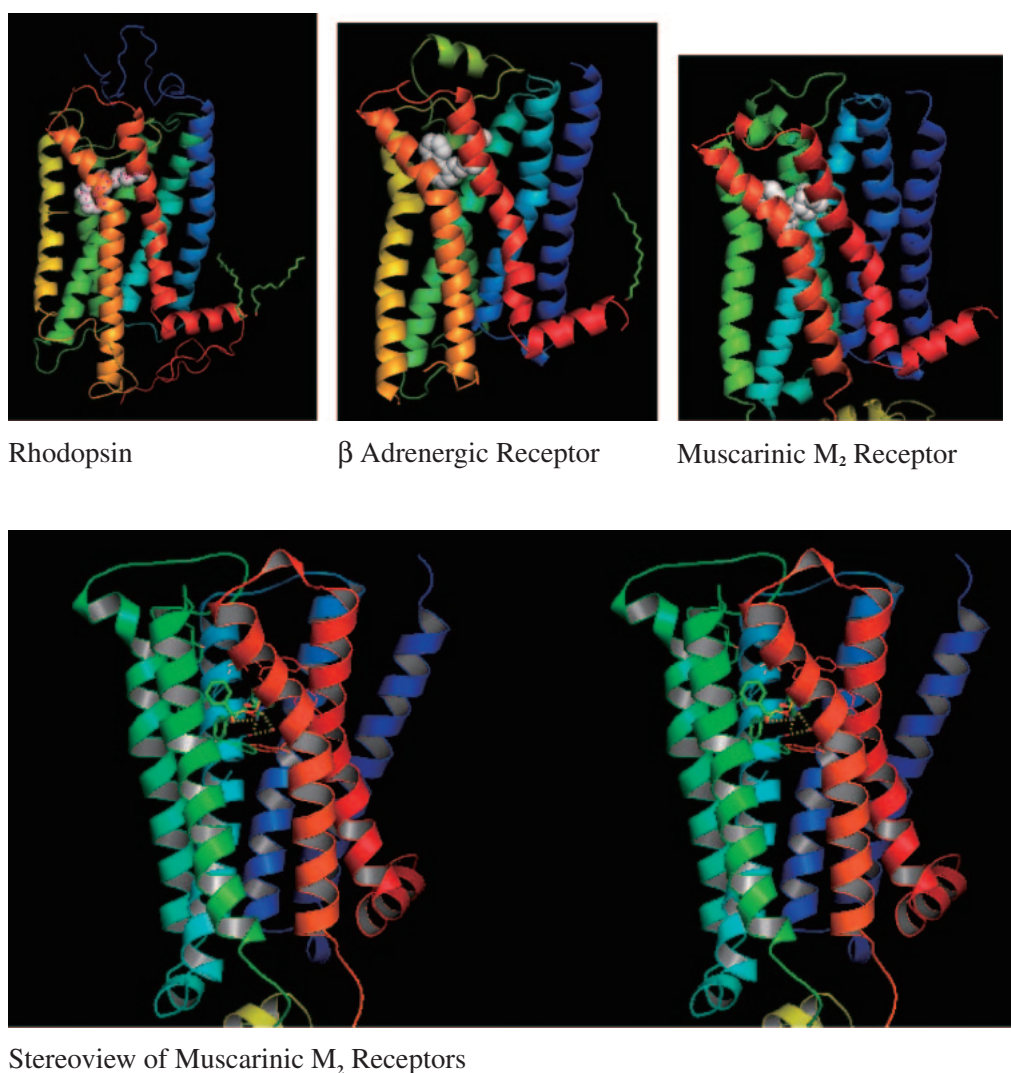
Rhodopsin   β Adrenergic Receptor   Muscarinic M₂ Receptor

Stereoview of Muscarinic M₂ Receptors

Fig. 8.   Crystal structures of rhodopsin,[123] β adrenergic receptors[124] and muscarinic M_2 receptors.[127] Rhodopin, β adrenergic receptors, and muscarinic M_2 receptors bound with retinol, carazolol (β antagonist), and QNB, respectively, all of which are colored white and found at similar positions. The upper side is intracellular phase, and the bottom site is extracellular phase. Amino terminus is in the intracellular phase, and carboxy terminus in the extracellular phase. Transmembrane segments 1 to 7 in M_2 receptors are colored with dark blue (TM1), blue (TM2), light blue (TM3), blue-green (TM4), green (TM5), orange (TM6) and red (TM7), respectively.

receptors[124] (Fig. 8). As was expected from bio-chemical studies,[31],[32] a conserved S–S bond between Cys 96 and Cys 173 was detected between the N terminus of TM3 and extracellular loop 2 (E2). In addition, another S–S bond between Cys413 and Cys416 was detected in E3 loop. Palmitoylation in the C-terminal tail had been expected to be present from biochemical studies[115] but the palmitoyl residue was not detected in the present structure probably because of the low resolution. In contrast with transmembrane segments, the extracellular and intracellular loops appear to have a specific structure distinct from those of rhodopsin and β_2 adrenergic receptors. QNB bound to M_2 receptors was found to reside approximately one third-inside from the extracellular phase, to be surrounded by transmem-brane segments 2 to 7 (TM2 to TM7), and not to be exposed to either the extracellular or intracellular phase. The position of QNB is very similar to that of retinal in rhosopsin and of carazolol (β adrenergic antagonist) in β_2 adrenergic receptors (Fig. 8, see atomic models of ligands colored white).

5.4. Orthosteric binding site of M_2 receptors. QNB is surrounded by 18 amino acid residues, at
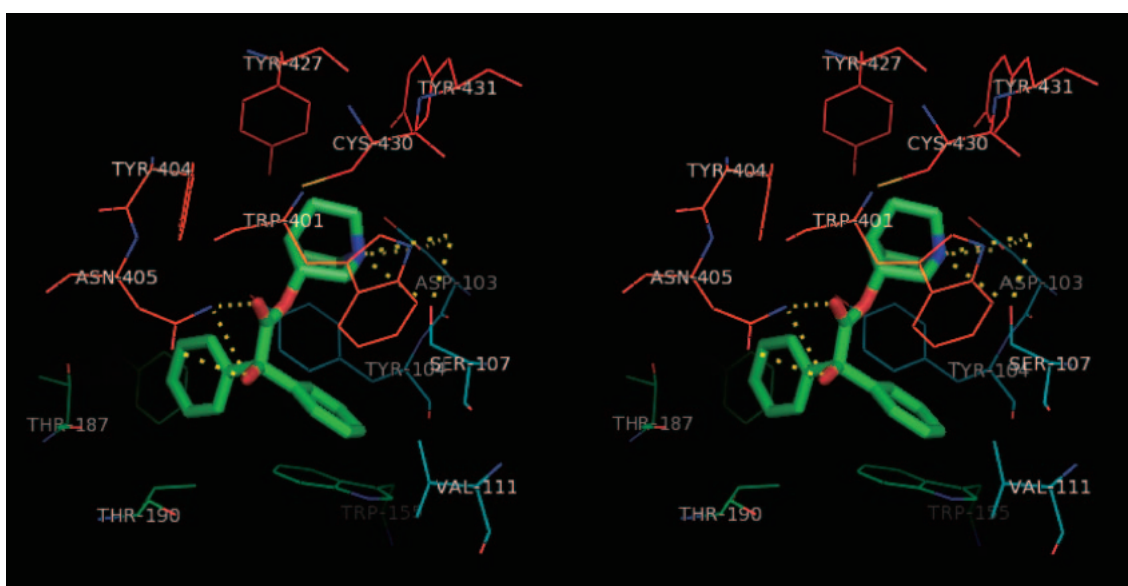
Fig. 9.   Interaction of muscarinic M_2 receptors and QNB (orthosteric antagonist)[127] (stereo view). QNB (green) is bound with Asn405 in TM6 through hydrogen bonds, interacts with Asp103 in TM3 electrostatically, and is bound with a lot of hydrophobic amino acids through van-del-Waals interaction. Hydrogen bonds and electrostatic interaction are demonstrated with dotted lines of yellow.

least one atom of which is within 4 Å from one atom in QNB (Fig. 9). Among them, QNB interacts with Asp103 in TM3 electrostatically, with Asp405 in TM6 through hydrogen bonds, and with many Tyr, Trp and Phe residues through van-der-Waals interactions. These multiple interactions explain the extremely high affinity for QNB of M_2 receptors. Among these 18 residues surrounding QNB, 17 are common to all M_1 to M_5 receptors, which is consistent with the previous finding that five muscarinic receptors have similar affinities for QNB. QNB is supposed to bind to the orthosteric site of muscarinic receptors, that is defined as the ACh binding site. Thus the present finding is consistent with and explains the previous results that most orthosteric ligands show similar affinities for the five muscarinic receptors[6] and that it is difficult to develop subtype-specific orthosteric ligands.

Most amino acid residues surrounding QNB were suggested to be involved in the ligand binding by site-directed mutagenesis studies, which had been carried out on M_1,[130),131)] M_2[132)] and M_3[133)] receptors. This consistence appears to give credit to that the determined structure would represent the active structure of M_2 receptors, at least as for antagonist binding, and that mutagenesis studies would provide useful information on the ligand binding structure. One of merit of structure determination is that it provides direct information on the role of each

residue. For example, the mutation of Asp103 in M_2 receptors into Ala caused a decrease in the affinity for QNB of more than 100-fold, whereas the mutation of Asp103 into Glu hardly affected the affinity for QNB.[132)] This finding apparently indicates the involvement of an electrostatic interaction between Asp103 and QNB. Actually, two oxygen atoms on the carboxyl group of Asp103 were found to be located 3.1 and 3.9 Å from the nitrogen atom of the quaternary amine of QNB in the crystal structure of the QNB-M_2 complex.[127)] Furthermore, the mutation of Asn405 to Ala in M_2 receptors was shown to decrease the affinity for QNB more than 100-fold,[132)] but it remained unknown whether this effect is due to the loss of direct interaction between Asn405 and QNB or to a conformational change of M_2 receptors induced by the mutation. In the crystal structure, Asn405 was shown to form hydrogen bonds with QNB, which is supposed to contribute to the high affinity of QNB.[127)]

5.5. Allosteric binding sites of M_2 receptors. Some muscarinic ligands have been reported to interact with muscarinic receptors but not to compete with an orthosteric ligand such as [3H]NMS or ACh. Gallamine[134)] and alcuronium[135)] are original ligands with such properties: gallamine suppresses the [3H]NMS binding of cardiac muscarinic receptors (M_2 receptors) without competing with [3H]NMS, whereas alcuronium enhances the [3H]NMS

binding. They are presumed to interact with sites distinct from the orthosteric site and are called allosteric ligands, and their binding sites are called allosteric sites. Allosteric ligands, which enhance or suppress the binding of orthosteric ligands positive- or negative-cooperatively, are now called as positive allosteric modulators or negative allosteric modulators respectively. Guo et al.[136] found that lapachone as well as alcuronium acts on M_2 receptors subtype-specifically and enhances the [^{3}H]NMS binding. Another type of allosteric ligand is the allosteric activator, which activates the receptor without being affected by orthosteric antagonist. An allosteric activator TBPB (1-(1′-2-methylbenzyl)-1,4′-bipi-peridin-4-yl)-1H-benzo[d]imidazole-2(3H)-one) is reported to be M_1-specific and not to affect M_2-M_5 receptors.[137] It is reasonable to assume that allosteric modulators and allosteric activators are subtype-specific because allosteric sites are not the sites of physiological ligands and thus are not conserved, in contrast with the orthosteric site.

Mutagenesis studies of M_2 receptors indicated that the allosteric sites include amino acid residues in the E2 loop and the aminoterminal end of TM7.[138]–[140] The allosteric sites were located between the QNB binding site and the extracellular phase in the structure of M_2 receptors.[127] The amino acid sequences of the allosteric sites are relatively different among the five muscarinic receptors as compared with their orthosteric sites. It is unexpected that the putative allosteric sites of M_3 receptors have a similar overall structure as those of M_2 receptors in spite of sequence differences.[128] The same structure of the E2 loop might be required for maintenance of the transmembrane structure. In any cases, the structures of allosteric sites may provide information important for developing ligands acting on the allosteric sites.

6. Future problems

6.1. Interaction of ACh and muscarinic receptors. ACh is known to take on two conformations with a trans or gauche Cα–Cβ bond. Dose-response analysis involving diasteromers of conformationally rigid ACh analogues has indicated that the trans form of ACh is the active form for muscarinic receptors.[141] Then, we assumed that ACh bound to M_2 receptors would take on the trans form and attempted to confirm this when sufficient amounts of purified receptors became available. Furukawa et al.[142] prepared purified M_2 receptors bound to methacholine (α-methyl acetylcholine), and

determined the Cα–Cβ bond of methacholine bound to M_2 receptors by measuring the transferred nuclear Overhauser effect (TRNOE). Unexpectedly, the angle was estimated to be 60°, which indicates the gauche form rather than the trans one. We interpreted these results as indicating that ACh binds with M_2 receptors in its gauche form and undergoes a conformational change from the gauche to the trans form when ACh-bound M_2 is activated and bound with G proteins (Fig. 10 (a)). M_2 receptors are known to show high affinity for agonists when reconstituted with G proteins in the absence of guanine nucleotides, whereas they show low affinity for agonists in the absence of G proteins[74),77]] (Fig. 3). Thus, we assume that the low and high affinity ACh binding of M_2 receptors represent the ACh (gauche) − M_2 and ACh (trans) − M_2-Gi interactions, respectively. It should be noted that the ACh-M_2-Gi complex represents the transition state for the reaction of M_2 receptors and Gi. This assumption, however, has not been proved yet, but it has not been disproved either. It remains to be determined by TRNOE measurement if the Cα–Cβ bond angle of ACh bound to M_2-Gi complex is trans or gauche.

We tentatively put ACh instead of QNB in the crystal structure of the QNB-M_2 complex, finding that the space occupied by QNB is too large to be occupied by ACh, and we could not determine which form of ACh, trans or gauche, fits the M_2 structure (Fig. 10 (b)). The conformational change in muscarinic receptors during their activation has been discussed,[143] based on the crystal structure of agonist-β_2 adrenergic receptor-Gs complex,[144] but no information nor discussion has been presented so far regarding a possible conformational change of muscarinic or adrenergic agonists during activation of their receptors. Determination of the crystal structure of the ACh-M_2-Gi complex could give a crucial answer for or against the assumption that the conformational change of M_2 receptors is accompanied by the conformational change of ACh.

6.2. Positive allosteric modulators as possible therapeutic agents. Muscarinic receptors are assumed to be targets of drugs for various diseases including Alzheimer disease, schizophrenia, Parkinson disease, and peptic ulcer.[145] These drugs are expected to be subtype-specific in order to avoid side effects due to their actions on other subtypes. Several subtype-specific orthosteric ligands have been developed, but their specificity for one subtype is limited mostly because of the conservation of related amino acids among the five receptors.[6] On the other
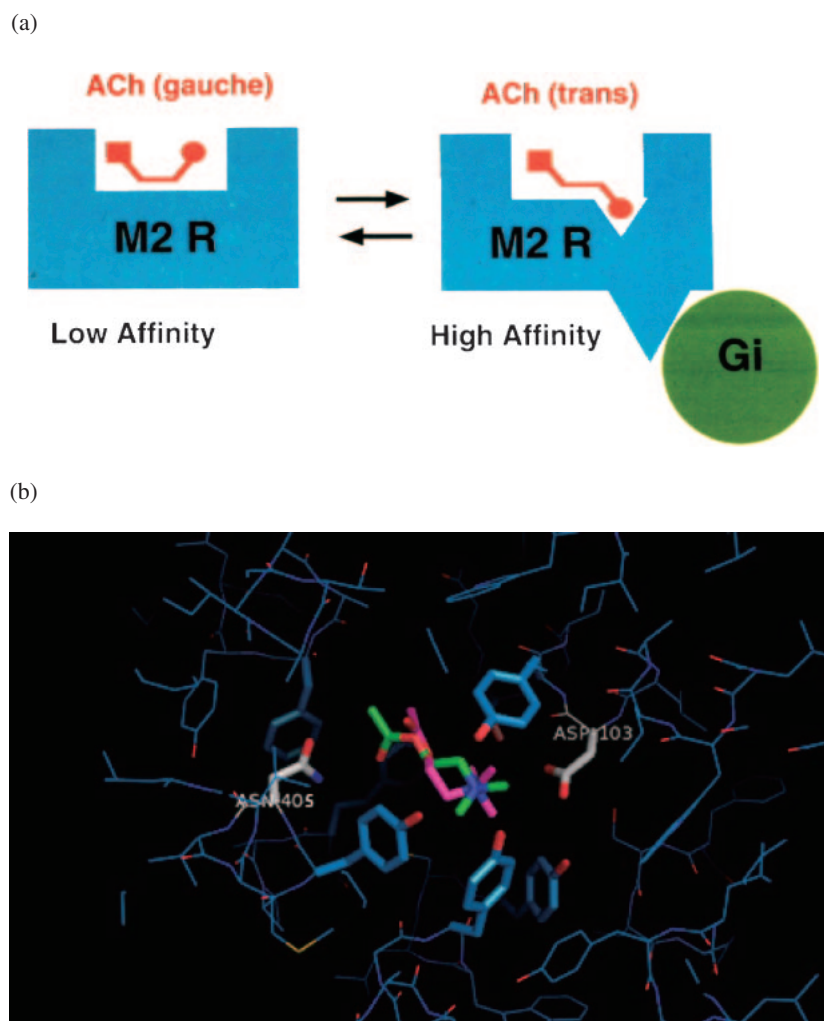
(a)

(b)

Fig. 10.   Gauche and trans forms of ACh in muscarinic M_2 receptors.[127],[142] (a) A model for the conformational change of ACh in the transition of M_2 receptors from the inactive, low affinity, free state to the active, high-affinity, Gi-bound state. (b) ACh with a gauche (pink) or trans (green) form of $C\alpha$–$C\beta$ bond was inserted at the position of QNB in the M_2-QNB crystal structure.

hand, subtype-specific allosteric ligands have been developed (see recent reviews refs. 146–148). Particularly, M_1- and M_4-specific positive allosteric modulators have been extensively developed for treatment of Alzheimer's disease and schizophrenia, respectively. These ligands are expected to enhance the activity of endogenous ACh by increasing its potency for a specific muscarinic receptor subtype. The merits of these allosteric modulators as compared with orthosteric agonists are that (1) their effects are subtype-specific and (2) they enhance only the receptors bound with ACh. Thus, they are expected to enhance specific muscarinic receptors when and where they are activated by ACh released under physiological conditions.

The structures of allosteric sites have been reported for M_2 and M_3 receptors, and are expected to be reported for M_1, M_4 and M_5 receptors in the near future. We look forward to developing positive allosteric modulators for Alzheimer's disease, schizophrenia, and other diseases based on these structures.

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Profile

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