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Effects of various muscarinic ligands on $M_2AChR-G_{i1\alpha}$ fusion protein expressed in Sf9 insect cells[1]

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KEY WORDS muscarinic receptors; inhibitory G_i G-protein; recombinant fusion proteins; muscarinic agonists; muscarinic antagonists; adenosine triphosphate; guanosine triphosphate; guanosine diphosphate; radioligand assay

ABSTRACT

AIM: To generate $M_2AChR-G_{i1\alpha}$ fusion protein in *baculovirus-Sf9 cells system and detect the effects of* various muscarinic ligands and magnesium ion on the interaction of fused M_2AChR and $G_{i1\alpha}$. **METHODS**: $M_2AChR-G_{i1\alpha}$ fused DNA was generated in a two-step polymerase chain reaction (PCR) and then expressed in Sf9 cells to produce fusion protein. [^{3}H] *L*-quinuclidinyl benzilate (QNB) and [^{35}S] GTPγS binding experiments were performed to study the function of $M_2AChR-G_{i1\alpha}$ fusion protein. **RESULTS**: The expression level of $M_2AChR-G_{i1\alpha}$ was (20.12 ± 0.14) nmol·g^{-1} protein. The affinity of GDP to $G_{i1\alpha}$ partner changed in the presence of different muscarinic ligands. IC_{50} values (95 % confidence limit) of GDP in the presence of acetylcholine (ACh), carbamylcholine, (4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chloro-carbanilate chloride (McN-A-343), pilocarpine, and atropine were 178 (148 - 214) μmol/L, 158 (126 - 199) μmol/L, 66 (56 - 78) μmol/L, 62 (55 - 72) μmol/L, and 5.0 (4.6 - 5.5) μmol/L, respectively, and that in the absence of muscarinic ligand was 15.9 (14.3 - 17.6) μmol/L. Apparent affinity for GDP in the presence of carbamylcholine was markedly decreased with increasing $MgCl_2$ concentrations, although the apparent affinity in the presence of atropine was not affected. **CONCLUSION**: The $M_2AChR-G_{i1\alpha}$ fusion protein has the pharmacological specificity of M_2 receptor and the efficient signaling of the two partners. Affinity of GDP to ligand-bound fusion protein represents the species of muscarinic ligands. Mg^{2+} is necessary for the action of M_2AChR on $G_{i1\alpha}$.

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INTRODUCTION

G protein-coupled receptor (GPCR) initiates vectorial signal transduction cascades via activation of G proteins and the subsequent regulation of effector enzymes. These function to amplify cellular response to extracellular mediators at low concentrations. As part of this process, agonist-occupied GPCR has the capacity to catalytically activate G proteins and induce the exchange of GDP with GTP on G proteins. Then heterotrimeric G_α-$GDP_{\beta\gamma}$ complex dissociates into G_α-GTP and $G_{\beta\gamma}$, which activate effectors respectively[1]. Muscarinic acetylcholine receptors (MAChR) are members of a superfamily of GPCR. Five subtypes of MAChR (m_1 - m_5) have been identified by cloning of cDNA or genes and by their expression in oocytes or cultured cells. The structure of MAChR has the characteristics of GPCR, which are composed of seven transmembrane regions (TM_{1-7}), three extracellular loops (O_{1-3}), three intracellular loops (i_{1-3}), N-terminal outside the cells, and C-terminal inside. The "odd-numbered" muscarinic receptors (M_1, M_3, and M_5) typically couple via the α subunits of the $G_{q/11}$ family, whereas the "even-numbered" members (M_2, M_4) couple via G_i and G_o subunits. This preferential coupling resides at the molecular level mainly in the postulated membrane-proximal regions of the i_2 and i_3 loops of the different receptors, which are notably different between the "odd" and "even" receptor groups, and similar within the two groups. The coupling selectivity at the G-protein level is reflected generally, but not exclusively. Downstream second-messenger pathways activated by the two groups

of muscarinic receptors was observed: phospholipase C_β is activated by the "odd" receptors, whereas adenylyl cyclase is inhibited by the "even" receptors[2,3].

In the periphery, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the CNS, there is evidence that muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation, and memory. Activation of muscarinic receptors in heart preparations produces a reduction in force of contraction and a decrease in the rate of beating. These effects are probably the consequence of inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^+ channels, respectively. Extensive studies with many antagonists have defined this response as being mediated by the M_2 receptor. There is also a large population of M_2 receptors in many smooth muscles and it seems likely that they are involved in antagonizing the relaxant effects of agents that elevate cAMP.

Fusion protein between GPCR and G protein is a novel means to examine the functional interactions of a GPCR with a single G protein. The most salient properties of GPCR-G_α fusion proteins are: (1) the defined 1:1 stoichiometry of the signaling partners; (2) the close physical proximity of the signaling partners; and (3) the tight tethering of G_α to the membrane[4]. The fusion protein techniques have been applied successfully to a number of mammalian GPCR, ie, the β_2-adrenoceptor, α_{2A}-adrenoceptor, adenosine A1 receptor, 5-HT_{1A} receptor, and α-factor receptor (Ste 2) from yeast[5-7]. With respect to G proteins, the short ($G_{s\alpha S}$) and long ($G_{s\alpha L}$) splice variants of $G_{s\alpha}$, the G_i/G_o-proteins $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o1\alpha}$, and the yeast G protein $G_{p\alpha}$ have been fused to, and shown to functionally interact with, GPCR partners.

The aim of our study was to generate a fusion protein of M_2 receptor and $G_{i1\alpha}$ in baculovirus-Sf9 cells expression system and analyze the effects of various muscarinic ligands and magnesium ion on the interaction of fused M_2AChR and $G_{i1\alpha}$.

MATERIALS AND METHODS

Materials The cDNA of human m_2 subtype (PEF-hm_2), bovine $G_{i1\alpha}$ (pG_α28), and pBacPAK9 were generously provided by Prof HAGA Tatsuya (Institute of Biomolecular Science, Gakushuin University, Tokyo, Japan); Sf9 insect cells were kind gifts from Prof CHEN Jian-Guo (Department of Cellular Biology, Institute of Biosciences, Beijing University, Beijing, China); IPL-41 insect cells culture media was from JRH Biosciences Inc; fetal bovine serum, tryptose phosphate broth, pluronic F-68 solution were purchased from Sigma Chemical Co; [^{3}H]QNB (specific activity, 1.8 PBq/mol) and [^{35}S]GTPγS (40.1 PBq/mol) were from Amersham Pharmacia Co; all other reagents were of the highest purity and available from standard suppliers.

Construction of the cDNA encoding fusion protein Two mutations were introduced into the human M_2 receptors used in our experiments. First, putative *N*-glycosylation sites near the amino terminus were eliminated by converting asparagine residues (Asn 2, 3, 6, and 9) in the consensus sequence Asn-X-Ser to aspartic acid residues. Glycosylation is undesirable because structural variability is expected to occur due to heterogenous glycosylation. Second, the central part of the third intracellular loop (235 – 380) of M_2 receptors was deleted. This region is susceptible to proteolysis, and its elimination prevents M_2 receptors from being degraded during the preparation. This domain is involved in phosphorylation by G-protein coupled receptor kinase and the subsequent sequestration of receptors[8]. It is not necessary, however, for activation of G-proteins[9].

M_2AChR-$G_{i1\alpha}$ DNA was generated by a two-step PCR protocol using KOD polymerase. In PCR 1A, the DNA sequence of M_2AChR was amplified with PEF-hm_2 as template using a sense primer 5′ of the newly created *Eco*R Ⅰ site (m_2-*Eco*R Ⅰ) and an antisense primer (m_2-C end) encoding the C-terminus of m_2 receptor. The cDNA of $G_{i1\alpha}$ was amplified in PCR 1B, using pG_α28 as template. The sense primer (m_2-G_{i1}) included the *first 18 bp of the 5′-end of the $G_{i1\alpha}$ and the 18 bp of PEF-*hm_2 in its 3′-extension. The antisense primers (G_{i1}-*Sal* Ⅰ) encoded the five amino acids from C-terminal of $G_{i1\alpha}$ followed by the stop codon and an extra *Sal* Ⅰ site. In PCR 2, the DNA fragments from PCR 1A and PCR 1B were annealed and amplified using the sense primer of PCR 1A and the antisense primer of PCR 1B. The fragments from PCR 2 were digested with *Eco*R Ⅰ and *Sal* Ⅰ and cloned into pBacPAK9 digested with *Eco*R Ⅰ and *Sal* Ⅰ[10]. PCR generated DNA sequences were confirmed by restriction enzyme analysis and enzymatic sequencing.

Generation of recombinant baculovirus, cell culture, and membrane preparation Recombinant

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baculovirus was generated in Sf9 cells using the BaculoGOLD transfection kit (Pharmingen) according to the instructions of the manufacturer. After initial transfection, recombinant baculovirus was isolated by plaque purification according to the protocol provided by Pharmingen. High-titer virus stocks were generated by three sequential amplifications of plaque-purified virus colonies. The isolated M_2AChR cDNA was introduced into baculovirus as above. The recombinant viruses were used for the large scale of production of isolated M_2 receptor and M_2AChR-$G_{i1\alpha}$ fusion protein according to the methods of BacPAKTH baculovirus expression system manual. Sf9 cells were homogenized and centrifuged as previous described[11]. Pellets were resuspended in 20 mmol/L HEPES-KOH buffer (pH 8.0) containing edetic acid 1 mmol/L, $MgCl_2$ 1 mmol/L, and a mixture of protease inhibitors (PMSF 0.5 mmol/L, leupeptin 5 mg/L, pepstatin 5 mg/L, and benzamidine 5 mmol/L), and stocked in aliquots at −80 ℃ until use.

[^{3}H]QNB and [^{35}S]GTPγS binding assays Before experiments, membranes were resuspended in HEN buffer containing HEPES-KOH 20 mmol/L (pH 8.0), edetic acid 1 mmol/L, NaCl 160 mmol/L. Sf9 membranes expressed M_2 receptor or M_2AChR-$G_{i1\alpha}$ fusion protein (about 50 μg protein per tube) were suspended in 100 μL HEN buffer supplemented with [^{3}H]QNB (10 nmol/L). Non-specific binding was determined with atropine 10 μmol/L. In the standard assay for [^{35}S]GTPγS binding, the membrane preparations were incubated with [^{35}S]GTPγS 0.3 nmol/L and different concentrations of GDP, GTP, ATP, or $MgCl_2$ in the presence of various muscarinic ligands. Incubations were performed for 60 min at 30 ℃. The reactions were terminated with 2.5 mL cold Na^+-K^+ buffer (Na_2HPO_4 0.05 mol/L, KH_2PO_4 0.05 mol/L, pH 7.2). Bound [^{3}H]QNB or [^{35}S]GTPγS was trapped on glass fiber filter paper, washed three times with 2.5 mL cold Na^+-K^+ buffer, and then counted with a liquid scintillation counter.

Statistical analysis Protein concentrations were determined by phenol agent protein assay. Data were analyzed by nonlinear regression, using the SPSS (8.0 version) program.

RESULTS

Expression of isolated M_2AChR protein and M_2AChR-$G_{i1\alpha}$ fusion protein in Sf9 insect cells The 3′ end of the M_2AChR coding region was fused in phase with the 5′ end of that of the $G_{i1\alpha}$ subunit (Fig 1). Sf9 insect cells, less of endogenous $G_{i1\alpha}$-like G proteins were transfected with this construct.

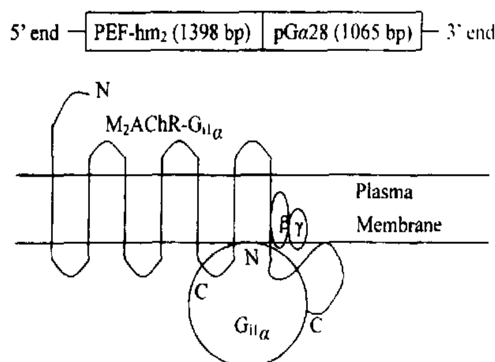

Fig 1. Expression of the M_2AChR-$G_{i1\alpha}$ fusion gene in Sf9 insect cells. The 3′ end of the coding region for M_2AChR was fused in phase with the initiation code of that of $G_{i1\alpha}$. This resulted in the fusion of the N-terminus of $G_{i1\alpha}$ to the C-terminus of the M_2 muscarinic receptor.

Both M_2AChR protein and M_2AChR-$G_{i1\alpha}$ fusion protein were expressed stably in Sf9 cells. And the expression level was (4.53±0.07) and (20.12±0.14) nmol/g protein respectively, assessed by saturation analysis of specific binding of the muscarinic receptor antagonist [^{3}H]QNB. Non-specific binding, as defined in the presence of atropine 10 μmol/L, was less than 10 % of the measured total binding when [^{3}H]QNB 10 nmol/L was used.

[^{35}S]GTPγS binding assays Ligand-regulated GTPγS binding to G proteins is a sensitive method for studying GPCR-G protein coupling. Addition of the muscarinic receptor agonist ACh to membranes expressed M_2AChR did not result in activation of endogenous G_i proteins as measured by the extent of [^{35}S]GTPγS binding, and no significant difference was detected among ACh, McN-A-343, and atropine (Fig 2). However, ACh activated M_2AChR-$G_{i1\alpha}$ fusion protein which induced significant elevation of bound [^{35}S]GTPγS. Moreover, these three kinds of muscarinic ligands led to different amount of [^{35}S]GTPγS binding on the M_2AChR-$G_{i1\alpha}$ fusion protein.

Effects of full agonists, partial agonists, or antagonist on displacement by GDP, GTP, or ATP of [^{35}S]GTPγS binding The GTPγS binding

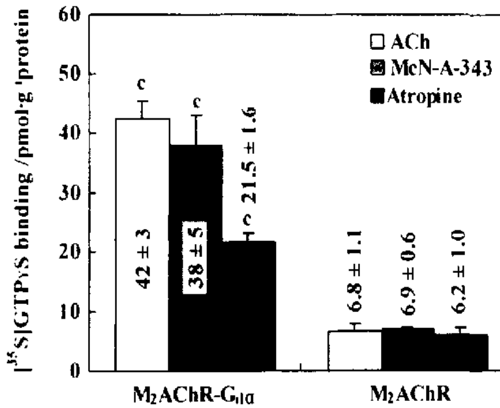

Fig 2. Ligand-mediated stimulation of $[^{35}S]GTP\gamma S$ binding to M_2AChR-$G_{i1\alpha}$ and M_2AChR exposed to ACh 100 μmol/L, McN-A-343 100 μmol/L, or atropine 10 μmol/L. $n=6$. $\bar{x} \pm s$. $^cP<0.01$ vs M_2AChR.

assay has already been successfully employed in Sf9 membranes to monitor coupling between various types of receptors and G-proteins[12,13]. In agreement with other systems, GDP reduced basal $[^{35}S]GTP\gamma S$ binding in Sf9 membranes expressing M_2AChR-$G_{i1\alpha}$ fusion protein (Fig 3). The affinity of GDP to $G_{i1\alpha}$ protein partner changed in the presence of different muscarinic ligands. IC_{50} values (95 % confidence limit) of GDP in the presence of ACh, carbamylcholine, McN-A-343, pilocarpine, and atropine were 178 (148 - 214) μmol/L, 158 (126 - 199) μmol/L, 66 (56 - 78) μmol/L, 62 (55 - 72) μmol/L, and 5.0 (4.6 - 5.5) μmol/L, respectively, and that in the absence of muscarinic ligand was 15.9

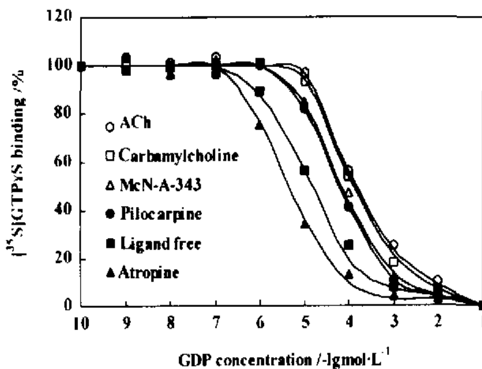

Fig 3. Displacement by GDP of $[^{35}S]GTP\gamma S$ binding to M_2AChR-$G_{i1\alpha}$ fusion protein in the presence of ACh 100 μmol/L, carbamylcholine 100 μmol/L, McN-A-343 100 μmol/L, pilocarpine 100 μmol/L, and atropine 10 μmol/L or in the absence of muscarinic ligand. The values were the means of six incubations.

(14.3 - 17.6) μmol/L. Compared with the system no ligand existed, the IC_{50} values of GDP in the presence of full agonists ACh and carbamylcholine were about 11 times higher than the value of that condition, and that value in the presence of antagonist atropine was almost 3 times lower.

Similar change was also observed for $[^{35}S]GTP\gamma S$ displacement by GTP, although the extent was less than that by GDP (Fig 4A). The IC_{50} values of GTP in the presence of ACh, McN-A-343, and atropine were 17.8 (15.8 - 20.0) μmol/L, 10.2 (9.3 - 11.2) μmol/L, and 4.47 (4.17 - 4.78) μmol/L, respectively. With ATP instead of GTP repeating the above experiments, no $[^{35}S]GTP\gamma S$ displacement was detected by the increasing concentrations of ATP (Fig 4B).

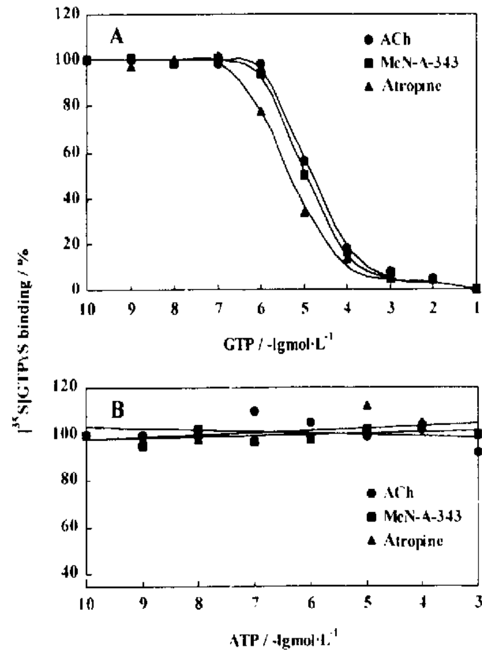

Fig 4. Displacement by GTP (A) or ATP (B) of $[^{35}S]GTP\gamma S$ binding to M_2AChR-$G_{i1\alpha}$ fusion protein in the presence of ACh 100 μmol/L, McN-A-343 100 μmol/L, or atropine 10 μmol/L. The values were the means of five or six incubations.

Effects of $MgCl_2$ on apparent affinity of M_2AChR-$G_{i1\alpha}$ for GDP At the core of every G protein is a guanine nucleotide-binding domain where GTP- and Mg^{2+}-binding sites are tightly coupled. So we performed displacement experiments to analyze the effects of Mg^{2+} on the interaction of M_2AChR and $G_{i1\alpha}$. When carbamylcholine existed, the affinity of GDP to

M_2AChR-$G_{i1\alpha}$ decreased with an increase in Mg^{2+} concentrations. The IC_{50} values of GDP increased from 3.16 μmol/L (95 % confidence limit: 2.88 – 3.47 μmol/L) in the absence of $MgCl_2$ to 6.01 (5.50 – 6.60) μmol/L, 12.6 (11.2 – 14.1) μmol/L, and 79 (69 – 91) μmol/L in the presence of $MgCl_2$ 0.1, 1.0, and 10 mmol/L, respectively (Fig 5A). However, the displacement curves in the presence of atropine were not affected by various concentrations of $MgCl_2$ and IC_{50} values of GDP were estimated to be 3.06 (2.82 – 3.39 μmol/L) – 6.31 (5.62 – 7.08) μmol/L (Fig 5B).

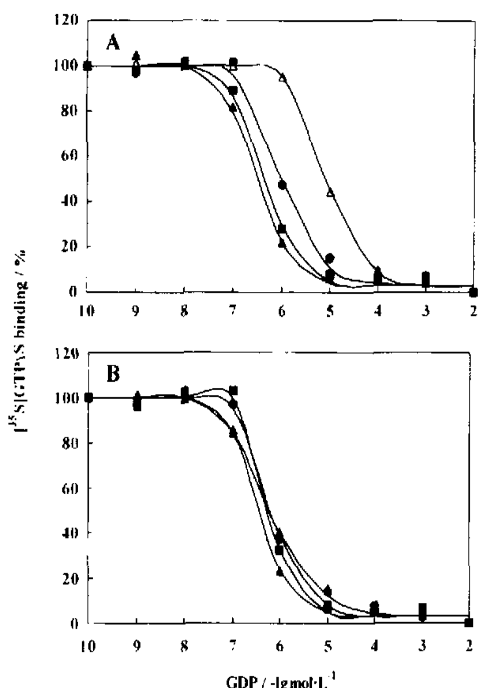

Fig 5. Effects of Mg^{2+} on the displacement by GDP of the [^{35}S]GTPγS binding to $G_{i1\alpha}$ fused with M_2AChR in the presence of carbamylcholine 100 μmol/L (A) or atropine 10 μmol/L (B). Various concentrations of Mg^{2+} [(△) 10 mmol/L, (●) 1.0 mmol/L, (■) 0.1 mmol/L, and (▲) 0 mmol/L] were used in the experiments. The values were the means of six incubations.

DISCUSSION

Several observation in our studies demonstrate that M_2AChR-$G_{i1\alpha}$ fusion protein expressed in Sf9 insect cells is functional. (1) [^{3}H] QNB binding experiments showed that the number of M_2AChR binding sites was (20.12 ± 0.14) nmol/g protein and the pharmacological specificity of the receptor moiety for agonists and antagonists was maintained (Fig 2). (2) The fusion protein was capable of efficient signaling as shown by the ACh and McN-A-343-induced [^{35}S]GTPγS binding (Fig 2). (3) Coupling of the isolated M_2AChR to endogenous $G_{i\alpha}$-like G proteins of insect cells was poor (Fig 2). Therefore, Sf9 cells provide an excellent background for studying M_2AChR-$G_{i1\alpha}$ fusion protein without the need to introduce mutations in the C-terminus of $G_{i1\alpha}$.

The stimulus for our present study was observations regarding the importance of the GDP affinity to $G_{i1\alpha}$ protein for M_2AChR and $G_{i1\alpha}$ coupling. The M_2AChR-$G_{i1\alpha}$ fusion protein had the lowest affinity for GDP when bound to full agonist, the medium affinity bound to partial agonist, and the highest affinity to antagonist. So the affinity for GDP on the M_2AChR-$G_{i1\alpha}$ fusion protein depends on and stands for the species of muscarinic ligands. Analyzing the GDP affinity of M_2AChR-$G_{i1\alpha}$ in the presence of various ligands will be helpful to find out full agonists, partial agonists, and antagonists specific to M_2AChR. And the fusion protein techniques can be used for other muscarinic receptor subtypes to solve the problem that lack of agonists and antagonists with high selectivity for any single receptor subtype, which is dogging the pharmacological characterization of muscarinic receptor subtypes[2]. These selective agents also has potential therapeutic application in area such as Alzheimer's disease, Parkinson's disease, asthma, analgesia, and disorders of intestinal motility, cardiac, and urinary bladder function.

The finding that difference was observed in the presence or absence of atropine indicates that the M_2AChR-$G_{i1\alpha}$ fusion protein have constitutive activity. Atropine inhibited this constitutive activity as an inverse agonist. An explanation for these findings could be that the lower the GDP affinity of G_α is, the lower is the M_2AChR activation energy required to catalyze GDP release from a $G_{i1\alpha}$ protein. M_2AChR exist either in an inactive "R" state or an active "R*" state. M_2AChR isomerize from R to R* spontaneously, and this process is referred to as constitutive activity. Receptor agonists stabilize the "R*" state and increase basal G protein activity, whereas inverse agonists stabilize the "R" state and reduce basal G protein activity.

Compared with GDP and GTP, ATP had no capacity of inhibiting the [^{35}S]GTPγS binding to M_2AChR-$G_{i1\alpha}$ (Fig 4). This finding suggests that $G_{i1\alpha}$ are selective for guanine nucleotides because they discriminate against

adenine nucleotides. The relative lack of affinity for adenine nucleotides is due not only to loss of a favorable interaction (with Asp-119 of $G_{i1\alpha}$) at the 1 position of the adenine ring, but also to the unfavorable juxtaposition of two hydrogen-bond donors, the exocyclic NH_2 at position 6 of the adenine ring with the amido group of Asn-116.

Apparent affinity for GDP in the presence of carbamylcholine was markedly decreased with increasing $MgCl_2$ concentrations, although the apparent affinity in the presence of atropine was not affected (Fig 5). These results indicate that M_2AChR-$G_{i1\alpha}$ fusion protein has a high affinity for GDP in the presence of muscarinic antagonist irrespective of the presence or absence of $MgCl_2$, where $G_{i1\alpha}$ is supposed to be independent of interaction with M_2 receptors. $G_{i1\alpha}$ has a low affinity for GDP in the presence of both carbamylcholine and $MgCl_2$, where $G_{i1\alpha}$ is assumed to be associated with M_2 receptors. These findings suggest that Mg^{2+} is necessary for the action of M_2AChR on $G_{i1\alpha}$ and a complex of agonist, M_2AChR, $G_{i1\alpha}$, and Mg^{2+} has a lower affinity for GDP, that is, the complex without bound nucleotides is the transition complex for GDP-GTP exchange reaction catalyzed by agonist bound receptors.

In conclusion, the present experiments suggest that the M_2AChR-$G_{i1\alpha}$ fusion protein expressed with baculovirus-Sf9 cells system has the pharmacological specificity for M_2 receptor and the capability of efficient signaling of two partners. Apparent affinity of GDP to ligand-bound fusion protein represents the species of muscarinic ligands. Mg^{2+} is necessary for the action of M_2AChR on $G_{i1\alpha}$ protein, and the agonist-M_2AChR-G protein complex formed in the presence of $MgCl_2$ is an intermediary for the action of M_2AChR on G protein.

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不同的毒蕈碱配体对在 Sf9 昆虫细胞内表达的 M_2AChR-$G_{i1\alpha}$融合蛋白的作用[1]

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关键词 毒蕈碱受体；抑制的 G_iG 蛋白；重组融合蛋白质类；毒蕈碱激动剂；毒蕈碱拮抗剂；腺苷三磷酸；鸟苷三磷酸；鸟苷二磷酸；放射配位体测定

目的：表达 M_2AChR-$G_{i1\alpha}$融合蛋白，检测不同配体或镁离子对 M_2AChR 与 $G_{i1\alpha}$间相互作用的影响. **方法**：两步 PCR 建立 M_2AChR-$G_{i1\alpha}$融合 DNA，并在 Sf9 细胞内表达. [^{3}H]QNB 和[^{35}S]GTPγS 结合实验检测 M_2AChR-$G_{i1\alpha}$融合蛋白功能. **结果**：M_2AChR-$G_{i1\alpha}$的表达水平为(20.12 ± 0.14) nmol·g^{-1} protein. 不同配体的存在使融合蛋白中 $G_{i1\alpha}$与 GDP 的亲和力

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发生变化. 乙酰胆碱，卡巴胆碱，McN-A-343，毛果芸香碱和阿托品存在以及无配体存在时，GDP的IC_{50}值(95 %可信区间)分别是178 (148-214) μmol/L，158 (126-199) μmol/L，66 (56-78) μmol/L，62 (55-72) μmol/L，5.0 (4.6-5.5) μmol/L 和 15.9 (14.3-17.6) μmol/L. 在卡巴胆碱的作用下，$G_{i1\alpha}$随Mg^{2+}浓度增加而减小对GDP的亲和力，而阿托品使$G_{i1\alpha}$不受Mg^{2+}浓度的影响，始终保持对GDP的高亲和性. **结论**：杆状病毒-Sf9细胞系统表达的M_2AChR-$G_{i1\alpha}$具备M受体配体结合特性和组分间的偶联功能. M_2AChR-$G_{i1\alpha}$对GDP的亲和性决定于M_2受体配体的性质，分析GDP亲和力的大小有利于筛选和鉴别M_2亚型特异性药物. Mg^{2+}离子是M_2AChR-$G_{i1\alpha}$与低亲和性GDP结合必需的阳离子.

(责任编辑　朱倩蓉)

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