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The abbreviations used are: mAChRs, muscarinic acetylcholine receptors; m2 receptor, muscarinic acetylcholine receptor m2 subtype; m2LD receptor, m2 receptor mutant that lacks a part of the third intracellular loop (233 to 380 amino acid residues); β–ARs, β–adrenergic receptors; β–ARK, β–adrenergic receptor kinase; G protein, GTP binding regulatory protein; Gs, G protein that stimulates adenylylcyclase; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid; SDS sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid AFDX-116, 11-([2-{(diethylamino)methyl}-1-piperydinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4] benzodiazepine-6-one; β–ARK, β–adrenergic receptor kinase; DTT, dithiothreitol; G protein, guanine nucleotide-binding regulatory protein; GR kinase, G protein-coupled receptor kinase; GTPγS, guanosine 5'-3-O-(thio)triphosphate; I3-GST, GST-fused protein containing a part of m2 receptor third intracellular loop (268 to 324 amino acid residues); NMS, N-methyl scopolamine; PrBCM, propylbenzilcholine mustard; QNB, quinuclidinyl benzilate.

SUMMARY

It has been previously shown that G protein βγ subunits stimulate the agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptors (mAChRs) and rhodopsin by a protein kinase partially purified from porcine brain (mAChR kinase) but not the phosphorylation of rhodopsin by rhodopsin kinase. I report here that the mAChR kinase phosphorylates β–adrenergic receptors (β–ARs) purified from bovine lung in an agonist dependent manner and the phosphorylation is also stimulated by G protein βγ subunits. I also report that recombinant β–adrenergic receptor kinase 1 (β–ARK1) expressed in COS-7 cells phosphorylates mAChRs (human m2 subtype) and rhodopsin in an agonist- or light-dependent manner, respectively, and that this phosphorylation is stimulated by G protein βγ subunits. By contrast, βγ subunits do not stimulate the phosphorylation of mAChRs or rhodopsin by a β–ARK1 mutant lacking a part of the carboxyl-terminal region that is present in β–ARKs but not in rhodopsin kinase. These results indicate that the β–ARK1 is the same as or very similar to the mAChR kinase but is distinguished from the rhodopsin kinase with respect to activation by βγ subunits, and that the extra carboxyl-terminal sequence in β–ARKs is required for the stimulation by βγ subunits. I also make a mutant of the human mAChRs m2 subtype lacking a large part of the third intracellular loop and it was expressed and purified using the baculovirus-insect cell culture system. The mutant was not phosphorylated by β–ARK1, in accordance with the previous assignment of phosphorylation sites to the central part of the third intracellular loop. However, the m2 receptor mutant was capable of stimulating β–ARK1 mediated phosphorylation of a glutathione S-transferase fusion protein containing the m2 phosphorylation sites in an agonist dependent manner. Both mutant and wild type m2 receptors reconstituted with the G protein, Go and Gi2, displayed guanine nucleotide-sensitive high affinity agonist binding, as assessed with displacement by carbamylcholine of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding, and both stimulated [^{35}S]GTPγS binding in the presence of carbamylcholine and GDP. Concentrations of carbamylcholine yielding half-maximal effects on [^{3}H]QNB bindings were indistinguishable for the mutant and wild-type m2 receptors. Moreover, the phosphorylation of the wild type m2 receptor by β–ARK1 did not affect m2 interaction with G proteins as assessed by bindings of [^{3}H]QNB or [^{35}S]GTPγS. These results indicate that the m2 receptor serves both as an activator and as a substrate of β–ARK, and a large part of the third intracellular loop of the m2 receptor does not contribute to interaction with G proteins and its phosphorylation by β–ARK does not uncouple the receptor and G proteins in reconstituted lipid vesicles.

INTRODUCTION

G protein-coupled receptors such as rhodopsin, β–adrenergic receptors and the muscarinic acetylcholine receptors m2 subtype (m2 receptors) are phosphorylated by G protein-coupled receptor kinases (GR kinases) in a light- or agonist-dependent manner (1-4). At least six different GR kinases have been cloned, and they are classified into three subgroups, rhodopsin kinase, β–adrenergic receptor kinases (β–ARK1 and β–ARK2) and others (IT11, GRK5 and GRK6) (5-10).

The agonist-dependent phosphorylation of muscarinic acetylcholine receptors (mAChRs) has been demonstrated by Kwatra et al. using purified β–ARK(11) and by Haga and Haga using a protein kinase (mAChR kinase) that was partially purified from porcine cerebrum (12,13). The β–ARK and mAChR kinase have some common properties, including the inhibitory effects of heparin and salts, the lack of stimulatory effects of calcium or cyclic AMP, the recognition of rhodopsin as a substrate, and similar behaviors during purification (12-15). It is possible, however, that the purified β–ARK preparation as well as the mAChR kinase preparation contain several kinds of kinases belonging to the GR kinase family, and it is not known if β–ARs and mAChRs are phosphorylated by the same kinase or by distinct kinases that have similar properties.

Recently, G protein βγ subunits were found to stimulate the agonist- or light-dependent phosphorylation of mAChRs and rhodopsin by mAChR kinase (13,15). On the other hand, the light dependent phosphorylation of rhodopsin by rhodopsin kinase was not stimulated by βγ subunits (15,16), and it has not been reported if the phosphorylation of β–ARs by the β–ARKs is stimulated by βγ subunits or not. It remains to be shown whether the stimulation by βγ subunits is the exclusive property of a specific kinase phosphorylating mAChRs or whether this property is shared by kinases phosphorylating β–ARs.

The substrate specificity of the β–ARKs is strictly limited to the activated forms of G protein-coupled receptors. In contrast, the phosphorylation sites are not restricted to specific sequences, specific locations in receptors or specific receptors linked to specific G proteins. These findings might be explained by assuming that either the phosphorylation sites are exposed by agonist-induced changes of receptor conformation, or the agonist-bound receptors directly activate β–ARK. The former assumption is unlikely because synthetic peptides containing the phosphorylation sites in β–adrenergic receptors or a glutathione S-transferase fusion protein containing the m2 phosphorylation sites (I3-GST) are poor substrates relative to agonist-bound receptors. Vmax/Km values for these peptides or I3-GST are 1,000-10,000 times lower than those for agonist-bound β–adrenergic receptors or m2 receptors (17,18). On the

other hand, the latter assumption is supported by the findings that light-excited rhodopsin or agonist-bound β–adrenergic receptors stimulate phosphorylation of synthetic peptides (19-21). In addition, I have shown that phosphorylation of I3-GST is synergistically stimulated by G protein βγ subunits and mastoparan or synthetic peptides derived from intracellular domains adjacent to transmembrane segments of the m2 receptor (18). These results suggest that β–ARK activity is synergistically stimulated by βγ subunits and m2 domains adjacent to transmembrane segments exposed by agonists binding. Such putative β–ARK activator domain may be distinct from phosphorylation sites and also from any receptor domains that could interact with βγ subunits. To test this hypothesis, I have used an m2 receptor mutant which lacks a large portion of the third intracellular loop including most of the phosphorylation sites, but retains domains adjacent to transmembrane segments (22). The phosphorylation of I3-GST by β–ARK1 was indeed stimulated by the mutant in an agonist- and βγ subunit- dependent manner although the mutant receptor was not phosphorylated by β–ARK.

Muscarinic receptors as well as dopamine D2 and α2 adrenergic receptors have long third intracellular loops, but their functions have not been elucidated. The third intracellular loop of muscarinic receptor m1 subtype was reported to contribute to down regulation (23) or internalization (or sequestration) of receptors (24). A large part of the third intracellular loop of m1 receptors, however, is unlikely to be involved in the interaction with G proteins because m1 mutants with a large deletion in the loop are still capable of mediating carbamylcholine-stimulated phosphatidylinositol turnover (23,24). In the case of m2 receptors, the third intracellular loop was reported to be involved in the internalization of receptors (22), but it has not been shown whether the loop is involved in G protein interaction.

Present experiments were undertaken to answer these questions, and I present evidence that βγ subunits also stimulate the phosphorylation of β–ARs by the mAChR kinase and the phosphorylation of mAChRs and rhodopsin by recombinant β–ARK1. I also provide evidence that the βγ responsive site is located in the carboxyl-terminal region of the β–ARK1. I also examined whether an m2 mutant with a large deletion in the third intracellular loop can interact with the G proteins, Go and Gi, in a reconstituted system of purified proteins. Further, I examined whether the phosphorylation of wild type m2 receptors by β–ARK may affect their interactions with G proteins.

EXPERIMENTAL PROCEDURES

Materials- [^{3}H]QNB, [^{3}H]NMS, [γ-^{32}P]ATP, and [^{35}S]GTPγS were purchased from Amersham Corp. or DuPont New England Nuclear. Restriction enzymes were from either Bethesda Research Laboratories or Toyobo Corp. *Autographa californica* nuclear polyhedrosis virus and baculovirus transfer vector pVL 1392 and 1393 were donated from Dr. M. D. Summers. Baculovirus transfer vector pBlueBac3 was purchased from Invitrogen. Baculovirus containing human m2 receptor and Sf9 cells were donated from Dr. E. M. Ross. The cDNA of β-ARK1 was donated from Dr. R. J. Lefkowitz. Mammalian expression vector pEF-BOS and COS-7 cells by Dr. S. Nagata and Dr. T. Shimizu. GST fusion protein expression vector pGE-3X was purchased from Pharmacia. Heparin and alprenolol were purchased from Sigma. All other reagents were of analytical grade quality.

Purification of m2 and m2LD receptors, β-ARs, rhodopsin, G proteins, mAChR kinase, and 13-GST- m2 and m2LD receptors expressed in Sf9 cells were purified as described (25,26). Briefly, recombinant virus-transfected Sf9 cells were collected from 3 to 5 l culture medium and homogenized with a Potter-type homogenizer in a solution containing 20 mM Hepes-KOH buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzamidine, 0.5 μg/ml aprotinin, and 2 μg/ml leupeptin (300 to 500 ml). The homogenate was centrifuged at 1,000 x g for 10 min and the supernatant fraction was centrifuged at 100,000 x g for 30 min. The pellet was collected and solubilized with 300 ml of a solution containing 10 mM potassium-phosphate buffer (pH 7.0), 1 mM EDTA, 150 mM NaCl, 1% digitonin and 0.1% sodium cholate. After stirring for 1 hr, the suspension was centrifuged at 100,000 x g for 1 hr. The supernatant was applied to an affinity gel with aminobenztropine (ABT)(400 ml), which had been preequilibrated with buffer A containing 10 mM potassium-phosphate (pH 7.0), 1 mM EDTA, 150 mM NaCl, and 0.1% digitonin. The column was washed with 3 column volumes of buffer A and then connected to a small column of hydroxylapatite (1 ml). Muscarinic receptors were eluted from the ABT agarose by 3 column volumes of buffer A containing 100 μM atropine onto the hydroxylapatite column. The hydroxylapatite column was washed with 100 mM potassium-phosphate buffer containing 0.1% digitonin (pH 7.0), and receptors were eluted with 1 M potassium-phosphate containing 0.1% digitonin (pH 7.0). Ten to twenty per cent of solubilized m2LD receptors were recovered as purified receptors, in contrast to thirty to fifty per cent recovery for wild type m2 receptors. The lower recovery could have resulted from lower affinity of m2LD receptors for hydroxylapatite as compared to wild type m2 receptors.

β–ARs were partially purified from bovine lung by a method modified from methods used by Caron et al. (27) and Benovic (28). The membrane preparation derived from 1.3 kg lung (350 ml, 4.8 nmol β–AR and 8.8 g protein) was extracted with 0.3% digitonin in a buffer solution (10 mM Tris-HCl (pH 7.4) / 0.1 M NaCl / 5 mM EDTA / 0.5 mM benzamidine / 2.5 μg/ml pepstatin / 0.25 mM PMSF; total volume 1.5 l), and then the pellet was re-extracted with the same buffer solution containing 1% digitonin and 0.13% sodium cholate (total volume 1.6 l). Approximately 60% of β–ARs in membranes were recovered in the second supernatant fraction after centrifugation for 45 min at 40,000 r.p.m. The supernatant fraction was applied to an alprenolol affinity column (500 ml), and after washing the alprenolol column with a buffer solution, a small column of phenyl-Sepharose (10 ml) was connected to the outlet of the alprenolol column. After elution with 0.1 mM alprenolol solution, the phenyl-Sepharose column was separated from the alprenolol column and eluted with a 10 mM Tris-HCl buffer solution containing 1% digitonin and 1 μM alprenolol. The fraction with the [^{3}H]dihydroalprenolol binding activity was applied to a column of DEAE-Sephacel (1 ml), and β–ARs were eluted from the column with a 10 mM Tris-HCl buffer solution containing 0.05% digitonin and 0.5 M NaCl. The recovery of β–ARs was typically 15% (700 pmol).

Rhodopsin-rich membranes were purified from bovine retina as described previously (15).

G protein βγ subunits of Gs (15) and transducin (29) were purified from porcine brain and bovine retina, respectively. βγI and βγII were purified from bovine brain and separated from each other by using Phenyl 5PW chromatography: the βγI were eluted ahead of βγII (30).

Go was purified from porcine brain as described (31), while Gi2 was purified from bovine lung by a similar method as used for purification of brain G proteins (32). Briefly, 2 kg bovine lung were minced and homogenized in 10 mM potassium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzamidine and 2 μg/ml pepstatin A. The homogenate was centrifuged at 1,000 x g for 10 min and the supernatant fraction was centrifuged at 100,000 x g for 30 min. The pellet was collected and solubilized with 2 l of a TED solution (20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol (DTT)) supplemented by 0.5% polyoxyethylene-9-monolaurylether (Lubrol-PX). After stirring for 1 hr, the suspension was centrifuged at 100,000 x g for 1 hr. The supernatant was applied to a DEAE-Sephacel column (1 l) and eluted with a gradient from 0 to 500 mM NaCl in a TED solution. The fractions containing the highest [^{35}S]GTPγS binding activity are collected and applied to Ultrogel AcA 34 column (1 l) followed by elution with a TED solution supplemented by 100

mM NaCl and 0.5% polyoxyethylene-9-monolaurylether. The fractions containing the highest [^{35}S]GTPγS binding activity were collected and applied to the second DEAE-Sephacel column (50 ml) to change the detergent to 1% sodium cholate. The fractions eluted from the second DEAE-Sephacel were diluted 4 times with TED solution then applied to a heptylamine Sepharose column (20 ml). The [^{35}S]GTPγS binding activity was eluted with a linear gradient formed from TED, 200 mM NaCl and 0.3% sodium cholate and TED, 50 mM NaCl and 1.3% sodium cholate. The fractions containing the highest [^{35}S]GTPγS binding activity were collected and checked by SDS-PAGE. Bands at 40 kDa for α subunit and 35 kDa for β subunit were detected, and the 40 kDa bands were reacted with antibody against the decapeptide of the carboxyl-terminus of Gi1 and Gi2 a subunits. From 2 kg tissues, 20 nmol of [^{35}S]GTPγS binding activity were recovered in a 2 mg protein fraction.

mAChR kinase was partially purified from porcine brain as described (15). Approximately 0.4 unit of mAChR kinase was used per assay, where 1 unit was defined as the amount of enzyme that transfers 1 pmol of phosphate/min when assayed in the presence of 20 μM ATP, 30 nM mAChRs, 50 nM βγ subunits, and 1 mM carbamylcholine.

A fusion protein of glutathione S-transferase with a peptide corresponding to a sequence between 268 and 324 of m2 (I3-GST) was expressed in E. coli using an expression vector pGE-3X containing a cDNA corresponding to the above sequence, and purified using glutathione Sepharose according to the procedure recommended by the manufacture, as described previously (18,33).

Construction and expression of mammalian expression vector of β-ARK1 and β-ARK1 mutant- The HindIII fragment of pβ-ARK3A (5) was first inserted into the HindIII site of the plasmid pUC119 (pUC β-ARK1), and the XbaI-SpeI fragment (2.7 kbp) of this plasmid was then transferred to the XbaI site of the expression vector pEF-BOS (34) (pEF-β-ARK1). Mutant β-ARK1 expression vector pEF-β-ARK1 562-632D (deleted amino acid residues of 563 to 632) was prepared by deleting a 213 bp DNA segment between EcoT22I and PstI restriction sites within the carboxyl-terminal segment of the β-ARK1 cDNA as follows. First, a 0.6 kbp PstI restriction fragment encoding the carboxyl-terminus of β-ARK1 cDNA and part of the 3' non-translated region was ligated to EcoT22I-digested β-ARK1 cDNA in pUC119. A 1.7 kbp KpnI-NheI fragment from the resulting plasmid was then isolated and ligated to pEF-β-ARK1 that had been previously digested with KpnI and NheI. Mutants pEF-β-ARK1 563T and pEF-β-ARK1 590T (truncated amino acid residues from 563 or 590 respectively) were constructed by inserting termination codons following the EcoT22I and XhoI sites as follows: oligonucleotides with the sequences 5'-TGACTAGTTGATCAC-3' and 5'-TCGAGTGATCAACTAGTCATGCA-3' were

synthesized, phosphorylated and annealed, and then ligated to the EcoT22I or XhoI site of pUC-β-ARK1.

COS-7 cells were transfected with the resultant vector (pEF-β-ARK1 or mutant expression vectors) (20 μg/dish) or the original vector pEF-BOS using the calcium phosphate method (35). The cells in a 10 cm dish were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 72 hr after transfection and then suspended in 2.5 ml of ice-cold lysis buffer (20 mM Hepes-KOH (pH 8.0) / 5 mM EDTA / 1 mM EGTA / 1 mM phenylmethylsulfonyl fluoride / 1 μg/ml pepstatin / 0.1 mM benzamidine). The suspension was homogenized with a Teflon homogenizer, and then centrifuged for 30 min at 300,000 x g. The supernatant fraction was used as the source of β-ARK1 (2 μl/tube in a typical assay).

Construction of transfer vectors and recombinant baculoviruses of m2LD and β-ARK1- A cDNA for a mutant of human muscarinic receptor m2 subtype lacking a sequence between 235 to 380 (m2LD receptor) was prepared as described (22). The vector containing m2LD cDNA was digested with Eco RI and Bgl II then ligated into Eco RI and Bam HI sites of pVL1392. This plasmid was digested with Bgl II and Hind III and followed by ligation into Bam HI and Hind III sites of pBlueBac3. A cDNA for bovine β-ARK1 was cut from pβ-ARK3A (5) with Eco RI and Nhe I then ligated into Eco RI and Xba I sites of pVL1392. This plasmid was digested with Not I and Bam HI and followed by ligation into Not I and Bgl II sites of pVL1393. This plasmid was digested with Bam HI and Hind III and then ligated into Bam HI and Hind III sites of pBlueBac3. Each transfer vector DNA was cotransfected with Autographa californica nuclear polyhedrosis virus DNA to Sf9 cells by using a calcium-phosphate method and recombinant viruses were selected as described (36,37).

Phosphorylation reaction- Phosphorylation of mAChRs by mAChR kinase or β-ARK1 was carried out as follows. Purified mAChRs (100 pmol in 20 μl) were mixed with crude lipids (0.3 mg) in HEN buffer (20 mM Hepes-KOH buffer (pH 8), 1 mM EDTA, 160 mM NaCl; 0.2 ml) containing 0.09% sodium deoxycholate and 0.02% sodium cholate. The mixture was passed through a column of Sephadex G50 fine (2 ml) preequilibrated with HEN, and the void volume fraction (0.4 ml) was collected and used as a substrate for the phosphorylation reaction. In the standard experiment, an aliquot of the void volume fraction (5 μl) was incubated with a mAChR kinase preparation or a cytosol fraction from COS-7 cells, 1 μM [γ-^{32}P]ATP (5-10 c.p.m./fmol), G protein βγ subunits (2.5 pmol), and 1 mM carbamylcholine in a buffer solution (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM $MgCl_2$, 0.5 mM

EGTA; total volume, 50 μl). In some experiments, the Sephadex procedure was omitted and purified mAChRs (0.2-0.5 pmol) were directly subjected to phosphorylation in the presence of crude lipids (2-3 μg/tube). After incubation for 60 min at 30°C, 25 μl of 5% SDS solution was added to the incubation mixture and an aliquot (45 μl) was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Relative incorporation of ^{32}P into individual bands were estimated by cutting the bands and counting by use of Cerenkov's effect or by using an Image Analyzer (Fuji BAS2000, Fuji Film Corp.).

Phosphorylation of β–ARs by the mAChR kinase was carried out as described for the phosphorylation of mAChRs except that β–ARs reconstituted in crude lipid were precipitated before subjected to phosphorylation. The β–AR preparation was mixed with lipid and then passed through a small Sephadex column. The void volume fraction containing β–ARs (18 pmol) was mixed with a buffer solution HEN containing 0.1 mM isoproterenol and 10 mM dithiothreitol (total volume 400 μl) and then with a 50% polyethylene glycol solution (120 μl). After incubation for 10 min at room temperature, 2 ml of ice-cold HEN solution was added and the mixture was centrifuged for 30 min at 60,000 r.p.m. The pellet was resuspended in a buffer solution (20 mM Hepes-KOH buffer (pH 8.0) and 1 mM EDTA; 50 μl) and an aliquot (1.5 μl/assay) was used as a substrate for the phosphorylation reactions.

Phosphorylation of rhodopsin by mAChR kinase and β–ARK1 was carried out as described (15).

Phosphorylation of I3-GST was carried out as described (18,33), except that 0.1 mM GTP and 10 μM ATP was included in the reaction medium unless mentioned otherwise. The phosphorylation reaction at 30°C was terminated by addition of a 5% SDS solution, and the resulting solution was subjected to SDS-PAGE followed by autoradiography. Incorporation of ^{32}P into individual bands was estimated by cutting the band and counting with the use of Cerenkov's effect or by using an Image Analyzer (Fuji BAS2000, Fuji Film Corp.).

Purification of β–ARK1- β–ARK1 was purified from Sf9 cells by a method modified from that described by Kim et al. (38). Recombinant virus-transfected cells were collected from 500 ml culture media, and homogenized with a Potter-type homogenizer in a solution containing 20 mM HEPES-KOH buffer (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzamidine, 0.5 μg/ml aprotinin, and 2 μg/ml leupeptin (50 ml). The homogenate was centrifuged at 200,000 x g for 30 min. The supernatant was diluted with 10 times volume of a buffer

containing 20 mM HEPES-KOH (pH 7.5) and 5 mM EDTA (buffer B), and applied to a 10 ml SP-Sepharose column followed by elution with a gradient of 0 to 500 mM NaCl in buffer B (100 ml in total). Fractions with the highest m2-phosphorylating activity were diluted with three volumes of buffer B and then applied to a heparin Sepharose column (2 ml) followed by elution with a gradient of 0 to 500 mM NaCl in buffer B (30 ml in total). Fractions with the highest activity were diluted with 10 volume of buffer B and then applied to a 1 ml Mono S column followed by elution with a gradient of 0 to 500 mM NaCl in buffer B (30 ml in total). A single peak of 80 kDa band was detected as β–ARK1 by SDS-PAGE, and from a 500 ml culture, 1 mg of purified β–ARK1 was obtained. Most experiments were carried out with eluates from the heparin column. The specific activity using m2 receptor as substrates was 0.6 nmole phosphate / min / mg protein, assayed in the presence of 100 μM ATP, 400 pM m2 receptor and 1 mM carbamylcholine.

Reconstitution of m2 receptors and G proteins- Purified receptors and G proteins were mixed with crude lipids (0.3 mg) in a solution containing Hepes-KOH buffer (pH 8.0), 1 mM EDTA, 160 mM NaCl, 5 mM DTT, 10 mM $MgCl_2$, 0.09% sodium deoxycholate and 0.02% sodium cholate (total volume 200 μl). The mixture was passed through a column of Sephadex G-50 fine (2 ml) preequilibrated with Hepes-KOH buffer (pH 8.0), 1 mM EDTA, 160 mM NaCl. The void volume fraction (0.4 ml) was collected as reconstituted vesicles and used as a substrate for phosphorylation reactions, and for assay of [^{35}S]GTPγS- and [^{3}H]QNB–binding activity. In some experiments, reconstituted vesicles containing wild type m2 receptors and Go were subjected to phosphorylation by β–ARK1 in the presence or absence of 1 mM ATP (for phosphorylated and non-phosphorylated receptors, respectively) in a solution containing 20 mM Tris-HCl (pH 7.5), 1 mM carbamylcholine, 0.1 mM GTP, 2 mM EDTA, 0.5 mM EGTA, 5 mM DTT and 5 mM $MgCl_2$. After incubation at 37°C for 1 hr, the reaction mixture was reapplied to Sephadex G-50 columns to remove free nucleotides and carbamylcholine. The void volume fractions were used for the analysis of [^{3}H]QNB– or [^{35}S]GTPγS-binding activity.

Binding assay with [^{3}H]NMS, [^{3}H]QNB and [^{35}S]GTPγS- Sf9 membrane preparations (40-50 fmol of [^{3}H]NMS binding sites) were incubated for 60 min at 30°C with 1.5 nM [^{3}H]NMS and various concentrations of antagonists in a buffer solution containing 20 mM Hepes-KOH buffer (pH 8.0), 1 mM EDTA, and 160 mM NaCl (total volume 200 μl). Reconstituted vesicles containing muscarinic receptors and G proteins were incubated with 1.5 nM [^{3}H]QNB and various concentrations of carbamylcholine in the presence or absence of 0.1 mM GTP for 60 min at 30°C or with 50 nM [^{35}S]GTPγS and 1μM GDP in

the presence of 1 mM carbamylcholine or 10 μM atropine for indicated time in a solution containing 20 mM HEPES-KOH (pH 8.0), 160 mM NaCl, 5 mM DTT, 1 mM EDTA and 10 mM $MgCl_2$ (total volume 200 μl). After the incubation, labeled compounds bound to Sf9 membranes or reconstituted vesicles were trapped on glass fiber filter paper (Whatman GF-B) and then analyzed by liquid scintillation counting (31).

[^{3}H]PrBCM labeling of m2 and m2LD receptors- Purified m2 and m2LD receptors were labeled with [^{3}H]PrBCM as described (33). Briefly, purified m2 or m2LD receptors (100 nM) were incubated with 500 nM [^{3}H]PrBCM in the presence or absence of 100 μM atropine in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% digitonin at 30°C for 2 hr (total volume was 100 μl). The reaction was terminated by addition of 10 mM sodium thiosulfate (1 μl) and analyzed by SDS-PAGE followed by autoradiography.

RESULTS AND DISCUSSION

β–ARs were partially purified from bovine lung and subjected to phosphorylation by the mAChR kinase in the presence or absence of isoproterenol and G protein βγ subunits(Fig. 1 A). Several bands were found to be phosphorylated under the present experimental conditions, but only a broad band with a molecular size of approximately 60 kDa was phosphorylated in a manner dependent on the presence of isoproterenol. The purified β–ARs preparation is known to yield a broad band with an apparent molecular size of 64 kDa on SDS-polyacrylamide gel electrophoresis (27,28), supporting the identification of this phosphorylated band as β–ARs. The phosphorylation of β–ARs was markedly increased in the presence of G protein βγ subunits and this effect of βγ subunits was observed only in the presence of isoproterenol but not in its absence. Effects of different concentrations of isoproterenol and alprenolol on the phosphorylation of β–ARs are shown in the Fig 1(b). A half-maximal effect was observed at approximately 1 μM isoproterenol and the stimulation by 10 μM isoproterenol was antagonized by alprenolol with a half-maximal effect at 200 nM. A low concentration of heparin (1 μM) inhibited the phosphorylation of β–ARs (data not shown). These results provide evidence that the mAChR kinase phosphorylates the agonist-bound form of β–ARs and the phosphorylation is stimulated by G protein βγ subunits. The target of βγ subunits, however, remains unknown and may be the kinase or the receptor. If βγ subunits interact with and activate the kinase, the stimulatory effects of βγ subunits should not depend on the species of receptor. I therefore examined the ability of several different species

of βγ subunits to stimulate the phosphorylation of three G protein-coupled receptors, β–ARs, mAChRs and rhodopsin.

Fig. 2 shows the effect of two different βγ subunits purified from bovine brain (30), and transducin βγ subunits from bovine retina (29) on the phosphorylation of β–ARs, mAChRs (m2 subtype), and rhodopsin. The phosphorylation of all three substrates was increased 5- to 10-fold by the increase of concentrations of βγ I and II from 1 to 30 nM, whereas the stimulation by transducin βγ subunits was much less for the phosphorylation of all three substrates compared to βγ I and II. This result is compatible with the assumption that βγ subunits interact with and activate the mAChR kinase, although the possibility remains that each of three different βγ subunits interacts with the three substrates with a similar affinity and that the mAChR kinase phosphorylates the βγ subunits-bound forms of substrates more efficiently than their free forms.

It is important to determine if the phosphorylation of low molecular weight peptide substrates is stimulated by G protein βγ subunits. For example, Palczewski et al. have reported that rhodopsin kinase can phosphorylate peptide C, a peptide derived from the C-terminus of rhodopsin(20). Palczewski et al. have reported that the phosphorylation of peptide C by rhodopsin kinase is greatly stimulated using an illuminated-rhodopsin preparation that lacks C-terminal phosphorylation sites (20). This result suggests that rhodopsin may serve as an activator as well as a substrate for rhodopsin kinase. If the same principle applies to the phosphorylation of mAChRs by the mAChR kinase, phosphorylation may be stimulated by mAChR mutants that lack phosphorylation sites.

Direct evidence for the interaction between βγ subunits and the mAChR kinase has been obtained from experiments showing the effect of βγ subunits on the heat-inactivation of mAChR kinase. The inactivation rate of the mAChR kinase at 45°C was decreased from 0.19 to 0.078 min^{-1} by co-incubating the mAChR kinase with βγ subunits (Fig. 3). Kinetic analysis of the light-dependent phosphorylation of rhodopsin by the mAChR kinase also support the assumption that βγ subunits and rhodopsin bind the mAChR kinase independently and in a random order (15). These results, taken together, suggest that G protein βγ subunits directly activate a kinase(s) that phosphorylate β–ARs and mAChRs, and raise the question whether βγ subunits also stimulate the kinase activities of β–ARK1 and / or β–ARK2.

To answer these questions, I first carried out experiments to determine whether native β–ARK1 kinase produced in COS-7 cells phosphorylates the mAChRs and whether the phosphorylation is stimulated by βγ subunits. The results of these experiments are summarized in Fig. 4 and 5. mAChRs

(m2 subtype) were found to be phosphorylated by extracts of β–ARK1-expressing COS-7 cells but not by extracts of control COS-7 cells (Fig. 4 A). The phosphorylation was dependent on the presence of carbamylcholine and was stimulated 3- to 6-fold by βγ subunits of G proteins (Gs). The concentration of βγ subunits giving a half-maximal effect was approximately 3 nM (Fig. 4 B). The phosphorylation of mAChRs was inhibited by heparin, irrespective of the presence or absence of βγ subunits (Fig. 4 C). The concentration of heparin yielding a half-maximal effect was 10 nM, consistent with the previous results (39). The concentration of carbamylcholine yielding a half-maximal effect was approximately 10 μM and the concentration of atropine that decreased the effect of 300 μM carbamylcholine to 50% was approximately 0.3-1 μM (Fig. 5). Effective concentrations of carbamylcholine and atropine were not changed by the addition of βγ subunits. The stimulatory effect of βγ subunits was not observed in the absence of carbamylcholine or in the presence of atropine. These results provide evidence that β–ARK1 phosphorylates the agonist-bound form of mAChRs and that the phosphorylation is stimulated by G protein βγ subunits, and suggest that β–ARK1 can be distinguished from the rhodopsin kinase with respect to interaction with βγ subunits.

Amino acid sequences of β–ARKs and rhodopsin kinase are reported to be similar to each other except that the β–ARK1 (689 amino acid residues) is 128 amino acid residues longer than the rhodopsin kinase (561 amino acid residues) at the carboxyl-terminus (5-7). I therefore constructed mutants of β–ARK1 lacking all or part of this sequence and expressed them in COS-7 cells. β–ARK1 563T and β–ARK1 590T lack, respectively, the last 127 and 100 amino acid residues of the carboxyl-terminus, and β–ARK1 562-632D contains an internal deletion of 71 amino acid residues (562 to 632) within the carboxyl-terminal end of the protein (Fig. 6 A). I could not detect kinase activity in extracts from cells expressing the mutants β–ARK1 563T and β–ARK1 590T, but could detect the phosphorylating activity of mAChRs in the extract from cells expressing β–ARK1 562-632D. As shown in figure 6 (b) and (c) the kinase activity of mutant β–ARK1 is lower than that of native β–ARK1. The kinase activity of β–ARK1 562-632D in the extract was more labile than that of native β–ARK1: the former activity decreased rapidly in a few days after the extraction but the latter activity was stable at least for a month. The β–ARK1 563T and β–ARK1 590T kinases may be too labile for their activity to be measured under the present experimental conditions.

The phosphorylation of mAChRs by β–ARK1 562-632D was dependent on carbamylcholine, but was not stimulated by G protein βγ subunits (Fig. 6 B). This result differs from the phosphorylation by

native β–ARK1 which is dependent upon carbamylcholine and is stimulated by βγ subunits. The light-dependent phosphorylation of rhodopsin by β–ARK1 was also found to be stimulated by G protein βγ subunits, but the phosphorylation of rhodopsin by the β–ARK1 562-632D mutant was not stimulated by G protein βγ subunits (Fig. 6 C). Preliminary data indicate that the affinity of the kinase for rhodopsin was not changed by the mutation. These results suggest that part of the carboxyl-terminal sequence found in β–ARK1 but absent in rhodopsin kinase is for stimulation of the kinase activity by βγ subunits.

The present results indicate that the phosphorylation of β–ARs as well as mAChRs is stimulated by G-protein βγ subunits and that βγ subunits stimulate the activity of the β–ARK1 probably by interacting with the carboxyl-terminal region of the kinase. G protein and βγ subunits are known to influence the activities of several distinct protein kinases by regulating the production of second messengers (40), but have not previously been shown to interact with protein kinases directly. This is the first report to provide evidence that protein kinases may be targets for direct regulation by G protein βγ subunits. Protein kinases can, therefore, be considered to be G protein effectors. G protein βγ subunits have been reported to regulate adenylylcyclase (40,41), phospholipase A_2 (42), and ion channels (43). The present results indicate that βγ subunits play a role in the homologous desensitization of G protein-coupled receptors by activating receptor kinases.

I also attempted to make sure that agonist bound receptors can activate GR kinases, an m2 receptor mutant (m2LD), which lacks a large portion of the third intracellular loop (Fig. 7), was expressed using the baculovirus system. Expression efficiency was similar for wild type m2 and m2LD receptors, as assessed by [^{3}H]QNB binding activity in membrane preparations. Maximal binding capacity was 5 to 10 pmol/mg protein, which correspond to roughly 5-10 x 10^6 [^{3}H]QNB binding sites/cell (26). Affinities of muscarinic ligands were estimated from displacement of [^{3}H]NMS binding. Both m2 and m2LD receptors had a higher affinity for AFDX-116 (11-([2-{(diethylamino)methyl}-1-piperydinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one) than for pirenzepine, in contrast to m1 receptors (Fig. 8). IC_{50} values for displacement by pirenzepine of [^{3}H]NMS binding were estimated to be 0.13 ± 0.03, 2.9 ± 0.4, and 3.7 ± 0.8 μM for m1, m2 and m2LD receptors, respectively, and the values for displacement by AFDX-116 were 9.4 ± 1.4, 0.33 ± 0.05, and 0.30 ± 0.09 μM for m1, m2 and m2LD receptors, respectively, (means ± S. E. n=3) (Fig. 8). These results indicate that m2LD receptors retain the same ligand binding capacity and selectivity as wild type m2 receptors.

Purified m2LD receptors were detected as a major band of 35 kDa in SDS-PAGE, in contrast to a major band of 59 kDa and a minor band of 39 kDa for wild type receptors (Fig. 9 A). The 39 kDa component is a proteolytic fragment of m2 receptors (33). The difference in apparent sizes between 59 and 35 kDa was larger than that expected from the number of deleted amino acid residues of 146, but the cause of this discrepancy is unknown. These bands were labeled by an irreversible muscarinic ligand [^{3}H]PrBCM, and the labeling was blocked by atropine, providing evidence that these bands correspond to muscarinic receptors (Fig. 9 B).

Purified m2 and m2LD receptors were reconstituted with the purified G proteins, Go, in crude lipid vesicles and were subjected to phosphorylation by β–ARK1 in the presence of GTPγS. Intact m2 receptors of 59 kDa and the proteolytic fragment of 39 kDa were phosphorylated in an agonist-dependent manner (Fig. 9 C). In contrast, no phosphorylation was detected in the 35 kDa band for m2LD under the same experimental conditions (Fig. 9 C). This result is consistent with previous assignment of phosphorylation sites in m2 receptor (33), i. e. , two of four serine residues (residues 232, 234, 250, 251) in the sequence between 232 and 251, and 5-6 serine and 3-4 threonine residues in the sequence between 271-311 (15). The m2LD receptor lacks the sequence between 233 and 380, which includes most of the putative phosphorylation sites except serine residue of 232. Serine residue 232 might not be phosphorylated even in the wild type m2 receptor or they are no longer phosphorylation sites as a result of structural changes caused by deletion of the sequence between 233 and 380.

A glutathione S-transferase fusion protein containing the sequence between 268 and 324 in the third intracellular loop of the m2 receptor (I3-GST) was subjected to phosphorylation by β–ARK1 in the presence of purified m2LD receptors reconstituted with G protein βγ subunits in lipid vesicles. Phosphorylation of I3-GST was stimulated in the presence of carbamylcholine (Fig. 10 A). The phosphorylation was dependent on the presence of G protein βγ subunits, and virtually no phosphorylation was detected in their absence (data not shown). Fig. 10 B shows the phosphorylation of I3-GST as a function of I3-GST concentration. The apparent Km for I3-GST was estimated to be 0.3 μM. This value is lower than the Km value of 7.3 μM for phosphorylation in the presence of only βγ subunits, and is comparable to the Km value of 0.17 μM for phosphorylation in the presence of 100 μM mastoparan as an activator (18). The effect of carbamylcholine was inhibited by atropine in a dose-dependent manner (Fig. 10 C). Concentration of atropine yielding half maximal effects in the presence of 1 mM carbamylcholine was 0.5 μM. This value is identical to that estimated for inhibition of phosphorylation of m2 receptors in the presence of 1 mM carbamylcholine (13). In the absence of m2LD

receptor, phosphorylation of I3-GST is not affected by either carbamylcholine or atropine (data not shown). These results indicate that the effect of carbamylcholine is mediated through its binding to m2LD receptors and that the carbamylcholine-bound m2LD receptors interact with and activate β–ARK.

Purified m2 and m2LD receptors were reconstituted with the purified G proteins, Go or Gi2, in lipid vesicles. Fig. 11 A shows displacement curves by carbamylcholine of [^{3}H]QNB binding to m2 or m2LD receptors reconstituted with Go in the presence or absence of 0.1 mM GTP. Displacement curves shifted to the right in the presence of 0.1 mM GTP for both m2 and m2LD receptors. Displacement curves in the absence of GTP were shallower than those expected from the mass action at a single site and were therefore fitted to a two-sites model. Proportions of the high affinity sites and IC_{50} values of carbamylcholine for displacement of [^{3}H]QNB binding to the high- and low-affinity sites were estimated to be 64 ± 6%, 1.4 ± 0.2 μM and 370 ± 90 μM for m2 receptors, and 64 ± 12%, 3.0 ± 0.8 μM and 770 ± 110 μM for m2LD receptors, respectively, (means ± S.E., n=3). Displacement curves in the presence of GTP fitted to those expected from a single site and concentrations of carbamylcholine giving a half maximal effect were estimated to be 770 ± 240 and 1630 ± 480 μM for m2 and m2LD receptors, respectively, (means ± S.E., n=3). Similar results were obtained with Gi from bovine lung, which consist mainly of Gi2 (data not shown). These results indicate that both the m2LD receptors and m2 receptors can interact with Go and Gi2 and undergo similar conformational changes in the ligand binding domain by interaction with G proteins.

Fig. 11 B shows the time course of [^{35}S]GTPγS binding to Go reconstituted with m2 or m2LD receptors in lipid vesicles. The binding of [^{35}S]GTPγS (50 nM) was carried out in the presence of 1 μM GDP (44-46). Stimulation by carbamylcholine was observed for both the m2 or m2LD receptors to the same extent. Similar results were obtained with a Gi preparation from bovine lung (data not shown). These results indicate that both the m2LD and m2 receptors can activate Go and Gi2.

m2 receptors (5 nM) were reconstituted with Go (10-15 times molar excess over m2 receptors) in crude lipid vesicles and then subjected to phosphorylation in the presence of 1 mM ATP, 0.1 mM GTP, 1 mM carbamylcholine and excess β–ARK1 (up to 50 nM) for 60 min at 30°C. Control samples were treated in the same way except for omission of ATP or β–ARK1. Phosphorylated or control samples were passed through a small column of Sephadex G-50 to remove muscarinic ligands and nucleotides. Fig. 12 A and 12 B show the displacement curves by carbamylcholine of [^{3}H]QNB binding in the presence or absence of GTP and the time course of [^{35}S]GTPγS binding in the presence of carbamylcholine or atropine, respectively. Under these experimental conditions, I did not find any significant differences

between phosphorylated and non-phosphorylated receptors as for the GTP shift of carbamylcholine displacement curves of [^{3}H]QNB binding or stimulation by carbamylcholine of [^{35}S]GTPγS binding. I also measured the potency of carbamylcholine to stimulate [^{35}S]GTPγS binding in the presence of different concentrations of $MgCl_2$. Fig. 12 C shows the effect of carbamylcholine concentrations on [^{35}S]GTPγS binding in the presence of 15 mM $MgCl_2$. Carbamylcholine concentrations yielding a half maximal effect on [^{35}S]GTPγS binding were estimated to be 4.1 ± 1.0 and 5.8 ± 1.1 μM (mean ± S.E. n= 5) in the presence of 1.5 mM $MgCl_2$ for non-phosphorylated and phosphorylate receptors, respectively, and 2.0 ± 1.0 and 3.0 ± 1.0 μM (mean ± S.E. n= 5) in the presence of 15 mM $MgCl_2$ for non-phosphorylated and phosphorylated receptors, respectively. Phosphorylated receptors tend to have lower sensitivity to carbamylcholine, but the differences in EC_{50} values between phosphorylated and non-phosphorylated receptors were not significant. Phosphorylation of m2 receptors during the preincubation of m2 receptors with β–ARK1 in the presence of 1 mM ATP was confirmed by adding [γ-^{32}P]ATP to the preincubation medium or by labeling m2 receptors with [γ-^{32}P]ATP after the preincubation.

In the present studies we show that m2LD receptors, which lack a large portion of the third intracellular loop, can bind muscarinic ligands with the same specificity as wild type m2 receptors, and that agonist-bound m2LD receptors can interact with and activate G proteins, Go and Gi2. These results indicate that most of the third intracellular loop of m2 is not necessary for the ligand binding and interaction with G proteins. This conclusion is consistent with the previous findings that the ligand binding sites of muscarinic receptors are located in the transmembrane domain and the sites for interaction with G proteins are located to the second intracellular loop, amino- and carboxyl-terminal segments of the third intracellular loop and the carboxyl-terminal tail (47,48). This conclusion is also consistent with the fact that the third intracellular loops are short for many G protein-coupled receptors including receptors that are known to interact with pertussis toxin-sensitive G proteins (Gi and Go), such as adenosine A1 and opioid receptors.

The finding that m2LD receptors are not phosphorylated by β–ARK1 is consistent with our previous assignment of phosphorylation sites by protein-chemical methods (33). This result, together with the finding that agonist-bound m2LD receptors activate β–ARK1, indicates that GR kinases interact with m2 receptors in at least two segregated regions, i. e. , regions serving as substrates and regions serving as activators. A similar conclusion has been reported for the interaction between rhodopsin and rhodopsin kinase (20). Phosphorylation sites have been assigned but receptor domains serving as activators have not been assigned for both rhodopsin and m2 receptors. Any β–ARK activator domains are expected to overlap with the sites for interaction with G protein α subunits, because phosphorylation of m2 receptors

are known to be inhibited by G protein $\alpha\beta\gamma$ trimers but are activated by $\beta\gamma$ subunits(4,13). Further, phosphorylation of rhodopsin is known to be inhibited by G protein $\alpha\beta\gamma$ trimers (16,49). β-ARK activator domains in m2 receptors are likely to include domains adjacent to transmembrane segments because (1) these domains are retained in m2LD receptors, (2) peptide fragments derived from these domains are known to activate β-ARK1 (18), (3) several of these domains are considered to be sites for interaction with G proteins. One can postulate that the agonist binding to m2 receptors induces a conformational change in these regions, which enables m2 receptors to interact with and activate either G proteins or β-ARK1. Thus, the agonist-bound receptor is proposed to activate β-ARK1 synergistically with G protein $\beta\gamma$ subunits.

The finding that a large part of the third intracellular loop in m2 receptors is not necessary for G protein interaction is consistent with the result that phosphorylation of the central part of the third intracellular loop in m2 receptors by β-ARK1 does not affect the interaction of m2 receptors with G proteins. This latter finding, however, differs from results obtained by Richardson et al. (50). These authors reported that the stimulation of [^{35}S]GTPγS binding by carbamylcholine is lower for G proteins (Go) reconstituted with phosphorylated m2 receptors than for Go reconstituted with non-phosphorylated m2 receptors and that the difference is more apparent when m2 receptors were phosphorylated in the presence of excess $\beta\gamma$ subunits. It remains to be seen whether the phosphorylation of m2 receptors in vivo results in uncoupling between receptors and G proteins or not.

The physiological functions of m2 receptor phosphorylation by GR kinases remain to be clarified. The possibility that the phosphorylation sites of m2 receptors are directly involved in the interaction with ligand or G proteins was excluded by the present experiments. The present results also argue against an indirect effect of phosphorylation on the interaction with G proteins, although this needs to be confirmed in the intact tissue. It is also possible that the interaction with G proteins of phosphorylated, but not of non-phosphorylated, receptors is impaired by yet another protein. Phosphorylation of rhodopsin and β-adrenergic receptors by rhodopsin kinase and β-ARK, respectively, has marginal effects on their interaction with G proteins but the interaction of phosphorylated rhodopsin and phosphorylated β-adrenergic receptors with G proteins is impaired in the presence of arrestin and β-arrestin, respectively (51-53). Recently β-arrestin has been reported to interact with m2 receptors in a phosphorylation-dependent manner (54). It remains to be determined whether the binding of β-arrestin to m2 receptors

affect the interaction of m2 receptors with G proteins. Lastly, the phosphorylation by GR kinases may be related to internalization (or sequestration) of m2 receptors.

m2LD receptors were originally constructed in order to examine possible effects of the third intracellular loop on internalization of m2 receptors. When m2 and m2LD receptors were expressed in U293 cells, the degree of internalization was lower with m2LD than with m2 receptors. Loss of [^{3}H]NMS binding activity upon carbamylcholine exposure for 2 h was estimated to be 58% and 14% for m2 and m2LD receptors, respectively (22). Furthermore, substitution of serine and threonine by alanine residues in a sequence from 307 to 311 resulted in partial inhibition of internalization. These serine and threonine residues are candidates for phosphorylation sites by GR kinases (33). These results are consistent with, though do not prove, the assumption that phosphorylation by GR kinase is involved in internalization of m2 receptors.

In these studies, I have presented evidence that an m2 receptor mutant that lacks a large portion of the third intracellular loop can activate both G proteins and β–ARK1, implying that a large part of the third intracellular loop containing phosphorylation sites by GR kinases is not necessary for the interaction between m2 receptors and G proteins and that m2 receptors interact with GR kinases in two segregated regions serving as substrates and activators. We speculate that possible functions of phosphorylation of m2 receptors by GR kinases could be initiation of internalization of receptors and/or stimulation of binding of additive proteins like β–arrestin.

In conclusion, I would like to show the hypothesized scheme of mAChRs(Fig. 13). The function of mAChRs, as well as other G protein coupled receptors, is to activate G proteins, that facilitate the conversion of G proteins from the trimer form ($\alpha GDP\beta\gamma$) to the dissociation form ($\alpha GTP + \beta\gamma$). The activation of G proteins is followed by activation of different kinds of effectors by αGTP. βγ subunits are also acting on effectors such as inward rectifying potassium channel(43), facilitation or suppression of activation by αsGTP of adenylylcyclase depending on the type of the enzyme(41), and activation of phospholipase Cβ2 subtype. The present result suggest that βγ subunits as well as agonist bound receptors function as activators of G protein coupled receptor kinases and thereby facilitate homologous desensitization.

Agonist bound mAChRs facilitate the dissociation of GDP from G proteins (G, $\alpha GDP\beta\gamma$) and thereby a ternary complex (aRG or $aR\alpha\beta\gamma$) of agonist (a), mAChR (R) and guanine nucleotide free G proteins is formed. Addition of GTP or GTPγS results in the dissociation of the αGTP or αGTPγS from the ternary complex. It is not known if βγ subunits directly interact with mAChRs or indirectly through

α subunits, and if the βγ subunits is also dissociated from aR on addition of GTP or GTPγS. The direct interaction between βγ subunits and rhodopsin has been shown. It is tempting to speculate that the βγ subunits remains to bound to aR and G protein coupled receptor kinase bind to the aRβγ complex and thereafter phosphorylates the receptor. It is proposed a working hypothesis that (1) the kinase and α subunits compete for βγ subunits and the sites of mAChRs which were exposed by binding of agonist, and (2) in a putative complex of aR, βγ subunits and the kinase, kinase interacts with and is activated by both βγ subunits and the exposed sites of mAChRs.

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Fig. 1 Phosphorylation of β–ARs by the mAChR kinase. Fig. 1 A Effects of isoproterenol and G protein βγ subunits. β–ARs were partially purified from bovine lung and subjected to phosphorylation by mAChR kinase partially purified from porcine brain. A broad band with an apparent molecular size of 60 kDa was identified as β–ARs. Concentrations of β–ARs, G-protein βγ subunits, and isoproterenol were 14 nM, 25 nM and 10 μM, respectively.

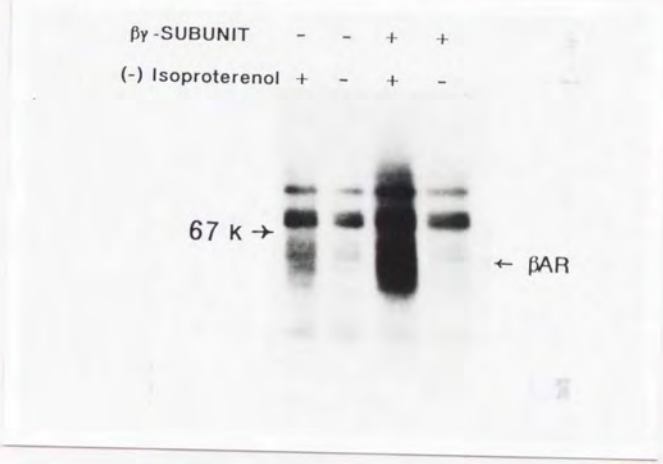


Fig. 1 B Effects of different concentrations of isoproterenol and alprenolol on the phosphorylation of β–ARs. Concentrations of β–ARs and G protein βγ subunits were 14 nM and 25 nM, respectively.

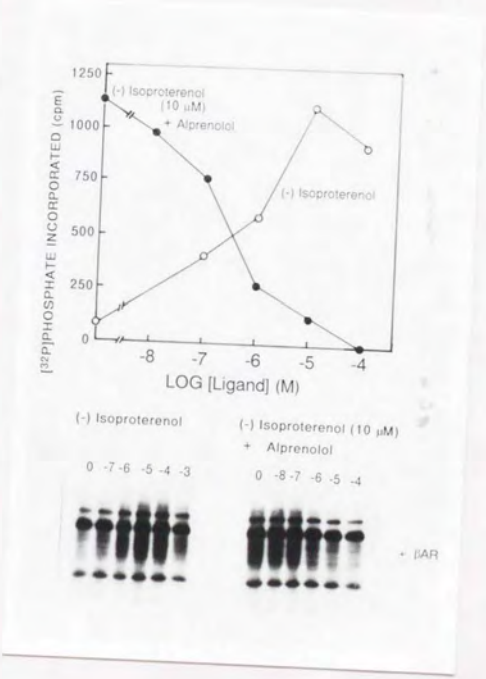


Fig. 2 Effects of different species of βγ subunits on the phosphorylation of mAChRs, β–ARs and rhodopsin by the mAChR kinase. βγ I and II were purified from bovine brain and transducin βγ subunits from bovine retina. Phosphorylation of mAChRs (25 nM) and β–ARs (14 nM) was carried out in the presence of 1 mM carbamylcholine and 10 μM isoproterenol, respectively, as described in the EXPERIMENTAL PROCEDURE and the phosphorylation of rhodopsin (150 nM) under fluorescent light as described previously (15).

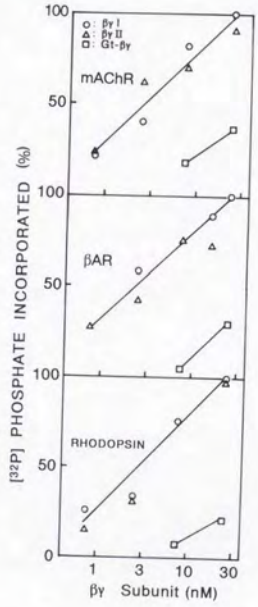


Fig. 3 Effects of G protein βγ subunits on the heat-inactivation of the mAChR kinase. The mAChR kinase (0.54 unit, 20 μl) in a 20 mM Tris-HCl and 1 mM EDTA solution was mixed with G protein βγ subunits in a 10 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol and 0.7% CHAPS solution (2 pmol, 2 μl; closed circle) or the buffer solution (2 μl; open circle), incubated for indicated time at 45°C, then cooled and mixed with the buffer or βγ subunits, respectively, and then used for the phosphorylation of mAChRs (1 pmol) as described in the EXPERIMENTAL PROCEDURES. After the incubation for 30 min, the incorporation of [^{32}P]phosphate into mAChR bands was measured.

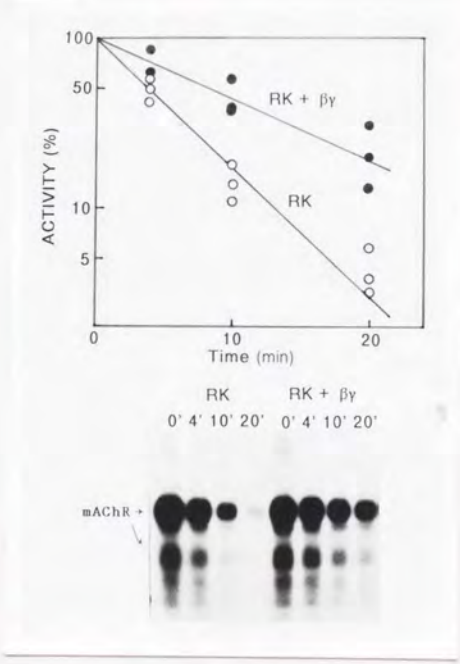


Fig. 4 Phosphorylation of mAChRs by β–ARK1 and its activation by G protein βγ subunits. Fig. 4 A mAChRs (m2 subtype) were subjected to phosphorylation by extracts prepared from COS-7 cells transfected with pEF-BOS (lane 1,2) or with pEF-BOS containing cDNA encoding β–ARK1 (pEF-β–ARK) (lane 3,4). Concentrations of mAChRs, carbamylcholine and atropine in the reaction mixture were 33 nM, 1 mM and 10 μM, respectively. G protein βγ subunits were not added to the reaction mixture.

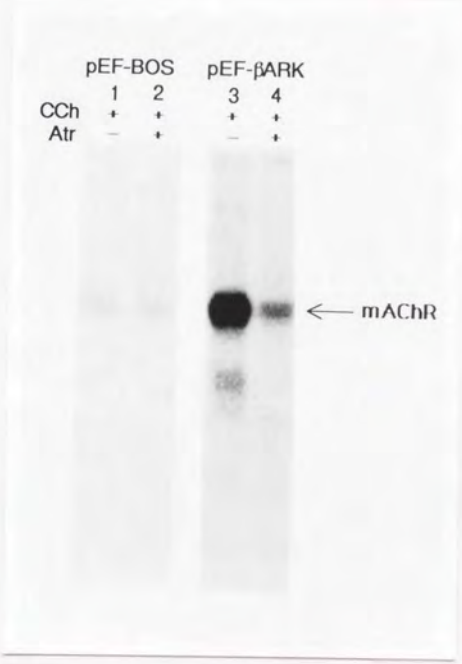


Fig. 4 B Effect of G protein βγ subunits (Gs). Concentrations of mAChRs and carbamylcholine were 10 nM and 1 mM, respectively. Inset shows the autoradiogram of phosphorylated mAChRs: the concentrations of added βγ subunits were 0 (lane 1), 0.5 pM (lane 2), 5 pM (lane 3), 50 pM (lane 4), 500 pM (lane 5), 5 nM (lane 6), 50 nM (lane 7), 500 nM (lane 8) respectively.

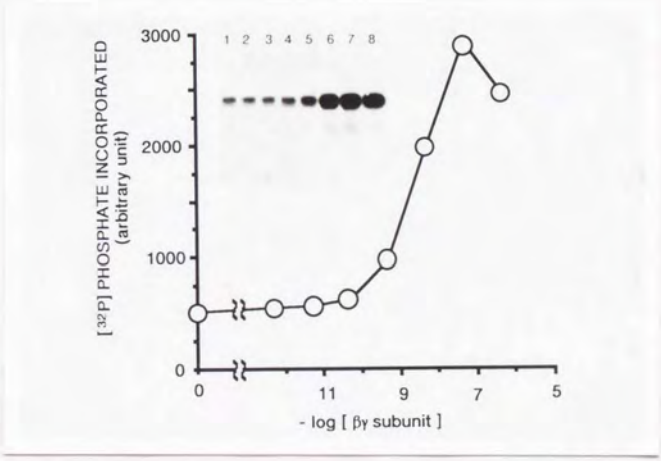


Fig. 4 C Inhibition by heparin of the phosphorylation of mAChRs by β–ARK1. Concentrations of mAChRs and carbamylcholine were 10 nM and 1 mM, respectively.

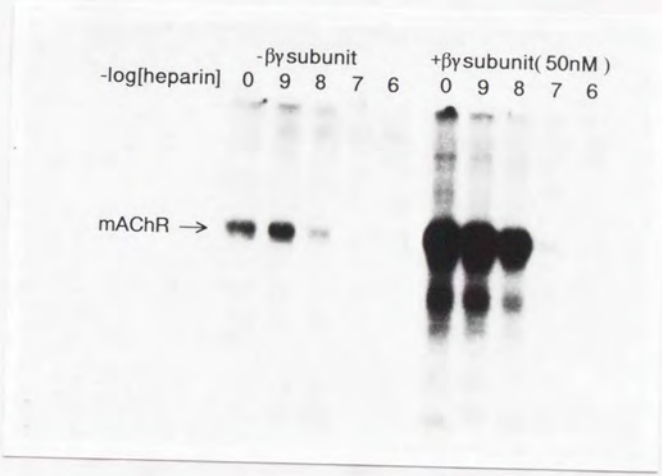


Fig. 5 Effects of different concentrations of carbamylcholine and atropine on the phosphorylation of mAChRs by the β-ARK1. mAChRs were phosphorylated by β-ARK1 as described in the legend to Fig. 4 except that various concentration of carbamylcholine and atropine were used as indicated.

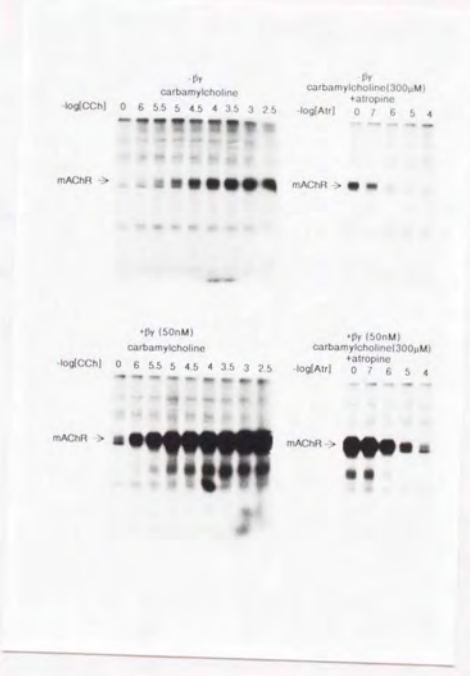


Fig. 6 A Structures of rhodopsin kinase, native β–ARK1, and mutant β–ARK1 562-632D.

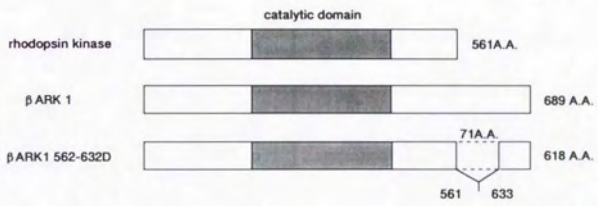


Fig. 6 B Phosphorylation of mAChRs by β–ARK1 and the mutant β–ARK1 562-632D. mAChRs were subjected to phosphorylation by extracts prepared from COS-7 cells transfected with pEF-BOS containing cDNA encoding β–ARK1 (pEF-β–ARK, lane 1-4) or pEF-BOS containing cDNA encoding mutant β–ARK1 lacking amino acid residues of 562 to 632 (pEF-β–ARK1 562-632D, lane 5-8). Experimental conditions were the same as described in the legend to Fig. 4 B.

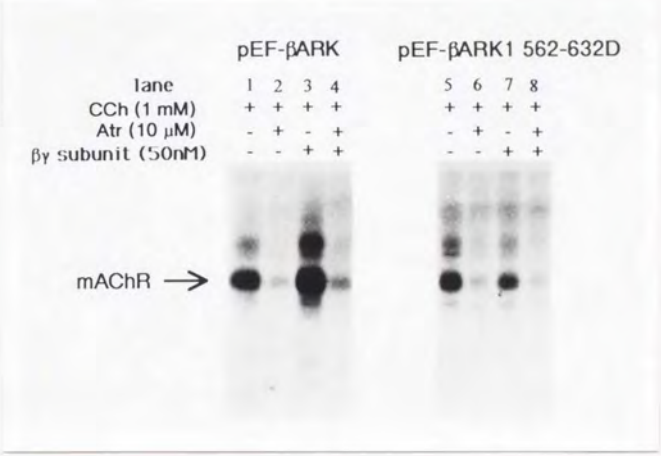


Fig. 6 C Phosphorylation of rhodopsin by pEF-β–ARK (lane 1-4) or pEF-β–ARK1 562-632D (lane 5-8). The phosphorylation was carried out as described in the legend to Fig. 2, except that COS-7-expressed kinases were used as described in the legend to Fig. 4.

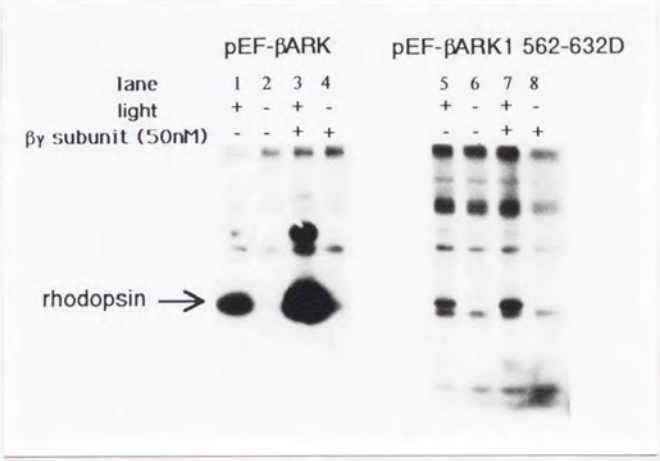


Fig. 7 Schematics of the m2 receptor. The m2LD receptors lack the sequence between 233 and 380, and β3-GST contains the sequence between 268 and 324. P indicates candidates of phosphorylation sites by GR kinase(33).

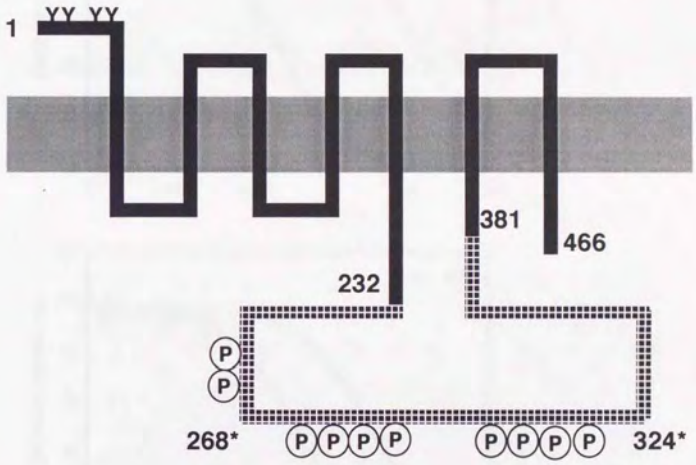


Fig. 8 Displacement by pirenzepine (-○-) or AFDX-116 (-△-) of [^{3}H] NMS binding to m1, m2 and m2LD receptors. [^{3}H]NMS binding assay was carried out using SF9 membrane preparations containing m1 (A), m2 (B) or m2LD (C) receptors, as described under EXPERIMENTAL PROCEDURES. Data shown are taken from an experiment representative of three repeated experiments that yielded similar results.

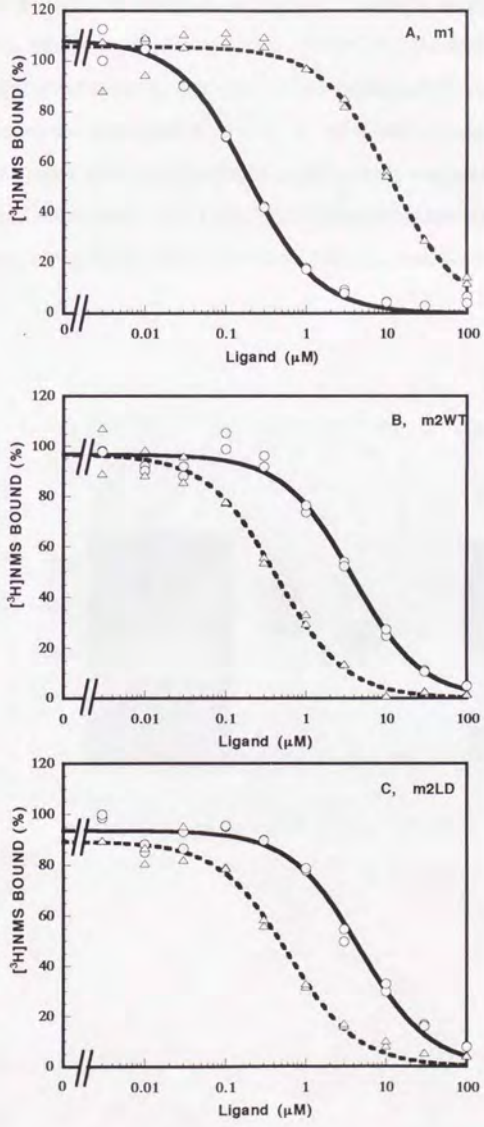


Fig. 9 Characterization of purified m2 and m2LD receptors: (A) Silver staining, (B) Labeling with [^{3}H]PrBCM, (C) Phosphorylation by β–ARK1. m2 and m2LD receptors were purified from Sf9 cell membranes and then subjected to SDS-PAGE followed by silver staining (A), to labeling with [^{3}H]PrBCM followed by SDS-PAGE and autoradiography (B), or to phosphorylation by β–ARK1 followed by SDS-PAGE and autoradiography (C). A: lane 1, molecular weight markers; lane 2, m2 receptors; lane 3, m2LD. Each lane contains 10 pmol of [^{3}H]QNB binding sites. B: lane 1, m2 receptors labeled in the absence of atropine; lane 2, m2 receptors labeled in the presence of 100 μM atropine; lane 3, m2LD receptors labeled in the absence of atropine; lane 4, m2LD receptors labeled in the presence of 100 μM atropine. C: lane 1 and 2, m2 receptors phosphorylated in the presence of 1 mM carbamylcholine or 100 μM atropine, respectively; lane 3 and 4, m2LD receptors phosphorylated in the presence of 1 mM carbamylcholine or 100 μM atropine, respectively. Each lane contains 100 fmol of [^{3}H]QNB binding sites.

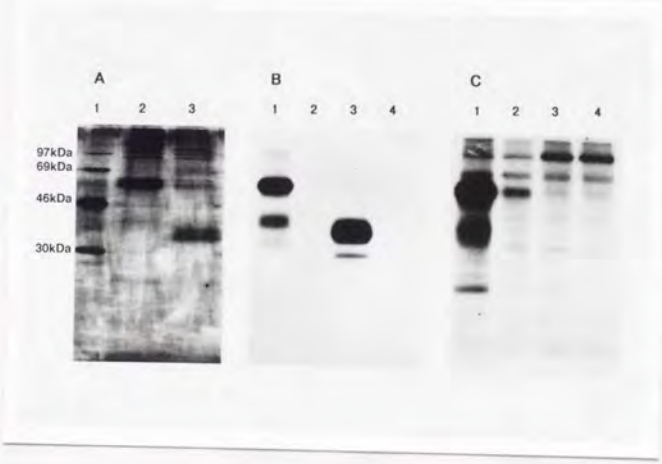


Fig. 10 Effect of carbamylcholine on phosphorylation of I3-GST in the presence of m2LD receptors. Fig. 10 A: I3-GST (8 μM) was incubated with 10 μM [γ-^{32}P]ATP in the presence of m2LD receptors (17 nM), G protein $\beta\gamma$ subunits (66 nM), β-ARK1 (0.15 μg/tube), and 1 mM carbamylcholine (—○—) or 10 μM atropine (- -●- -) for indicated periods. A portion of the reaction mixture was subjected to SDS-PAGE followed by autoradiography and then counting of the radioactivity in the I3-GST band. The same results were obtained in duplicate experiments. Inset shows the autoradiography of phosphorylated bands of I3-GST (arrow) for indicated time incubated with carbamylcholine (CCh) or atropine (Atr).

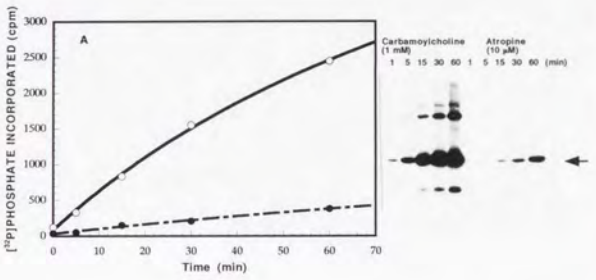


Fig. 10 B: Effects of concentrations of I3-GST in the presence of 1 mM carbamylcholine (-●-) or 10 μM atropine (-○-). The other conditions are the same as described in A. Inset shows the autoradiography of phosphorylated bands of I3-GST (arrow) for indicated concentrations of I3-GST incubated with carbamylcholine (CCh) or atropine (Atr) for 60 min.

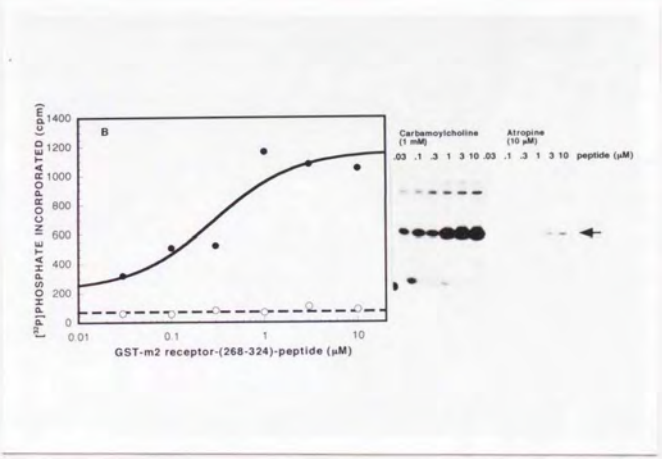


Fig. 10 C: Effects of concentrations of atropine on phosphorylation of I3-GST in the presence of 1 mM carbamylcholine. The other conditions are the same as described in A.

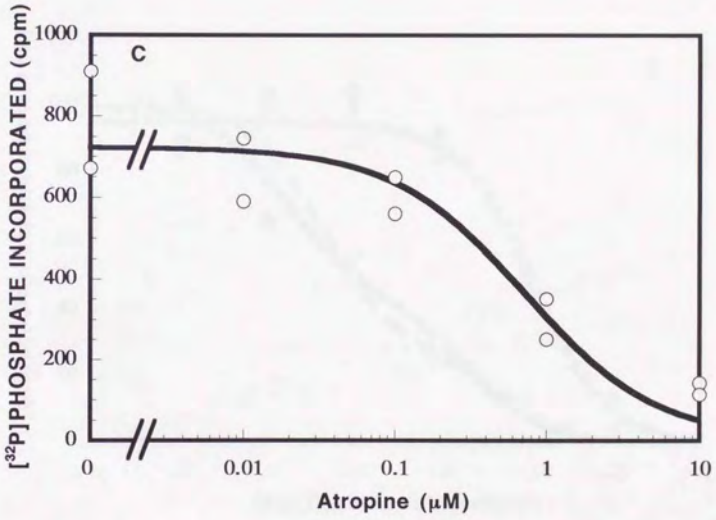


Fig. 11 Interaction of m2 and m2LD receptors with Go. Fig. 11 A: Displacement by carbamylcholine of [^{3}H]QNB binding to m2 (circles) or m2LD (triangles) receptors reconstituted with Go in the presence (filled symbols) or absence (open symbols) of 0.1 mM GTP.

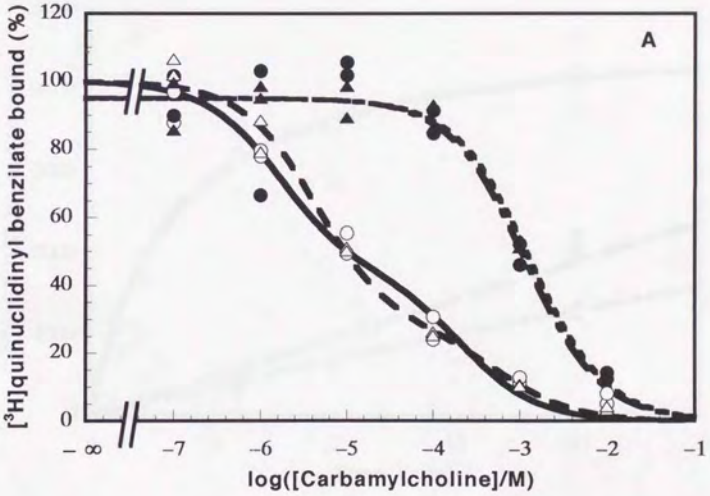


Fig. 11 B: Binding of [^{35}S]GTPγS to Go (400-600 fmol/tube) reconstituted with m2 (circles) or m2LD (triangles) receptors (40 fmol/tube) in the presence of 1 mM carbamylcholine (open symbols) or 10 μM atropine (filled symbols). Concentrations of [^{35}S]GTPγS and GDP in the reaction mixture are 50 nM and 1 μM, respectively. Data shown are taken from an experiment representative of three repeated experiments that yielded similar results.

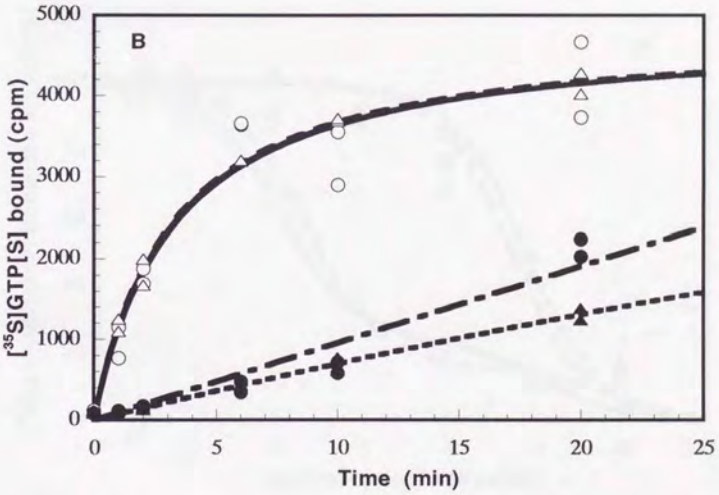


Fig. 12 Interactions of phosphorylated or non-phosphorylated m2 receptors with Go. Fig. 12 A: Displacement by carbamylcholine of [^{3}H]QNB binding to non-phosphorylated (circles) or phosphorylated (triangles) m2 receptors reconstituted with Go in the presence (filled symbols) or absence (open symbols) of 0.1 mM GTP. Proportions of the high affinity sites and IC_{50} values of carbamylcholine for displacing [^{3}H]QNB binding to the high- and low-affinity sites were estimated to be 78 ± 2%, 1.2 ± 0.1 μM and 1000 ± 200 μM for non-phosphorylated receptors, and 83 ± 1%, 1.9 ± 0.3 μM and 3000 ± 2000 μM for phosphorylated receptors, respectively (means ± S. D. , n = 3). Displacement curves in the presence of GTP fitted to those expected from a single site and IC_{50} values of carbamylcholine for [^{3}H]QNB binding were estimated to be 650 ± 200 μM and 800 ± 40 μM for non-phosphorylated and phosphorylated receptors, respectively (means ± S. E. , n = 3).

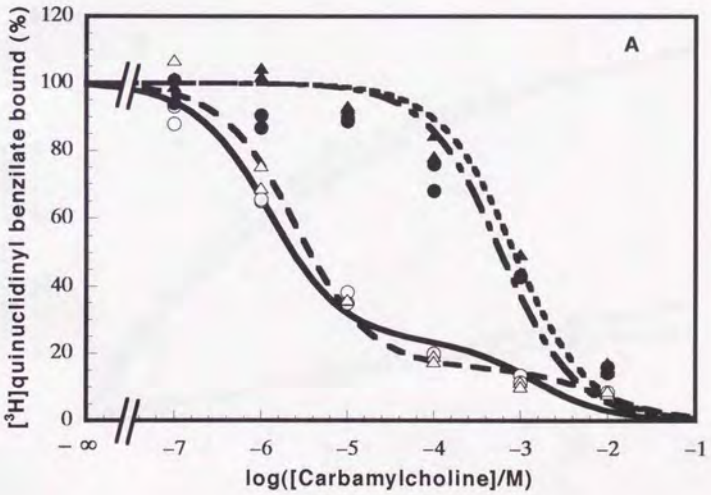


Fig. 12 B: Binding of [^{35}S]GTPγS to Go (400-600 fmol/tube) reconstituted with non-phosphorylated (circles) or phosphorylated (triangles) m2 receptors (40 fmol/tube) in the presence of 1 mM carbamylcholine (open symbols) or 10μM atropine (filled symbols). Concentrations of [^{35}S]GTPγS and GDP in the reaction mixture are 50 nM and 1 μM, respectively. Data shown are taken from an experiment representative of three independent experiments that yielded similar results. The other conditions are the same as described in legend to Fig. 11 B

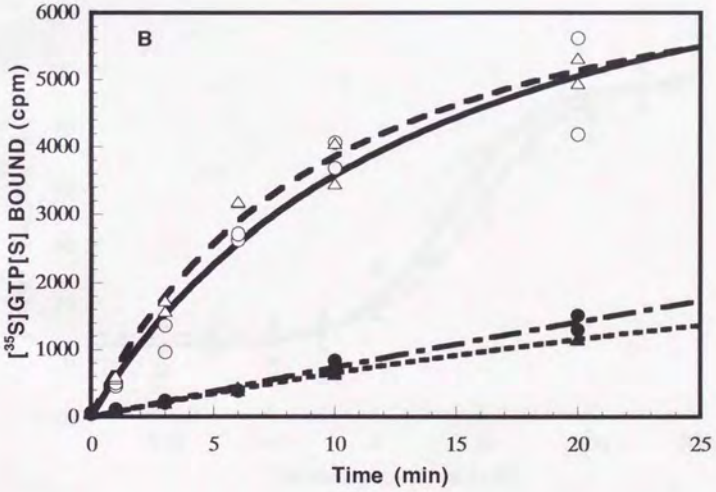


Fig. 12 C: Effect of carbamylcholine concentrations on [^{35}S]GTPγS binding to Go reconstituted with non-phosphorylated (circles) or phosphorylated (triangles) m2 receptors after incubation for 15 min at 30°C. Other experimental conditions were the same as in B except for the presence of 15 mM Mg2+. The counts of [^{35}S]GTPγS binding in the presence and absence of 1 mM carbamylcholine were normalized to 100% and 0%, respectively. Data shown are taken from an experiment representative of five independent experiments that yielded similar results.

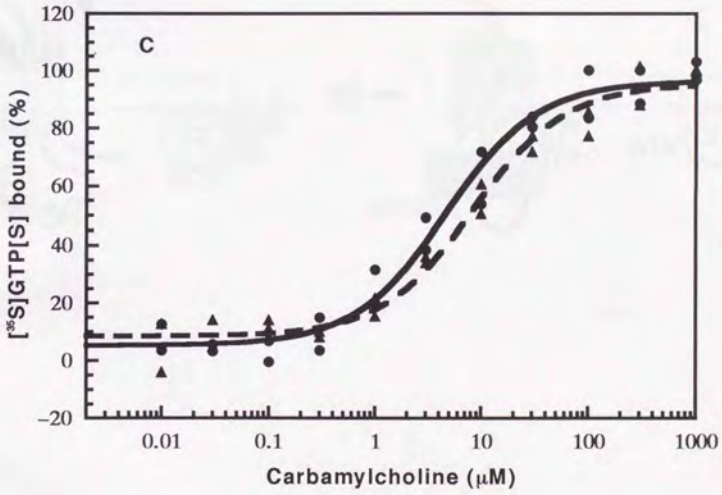
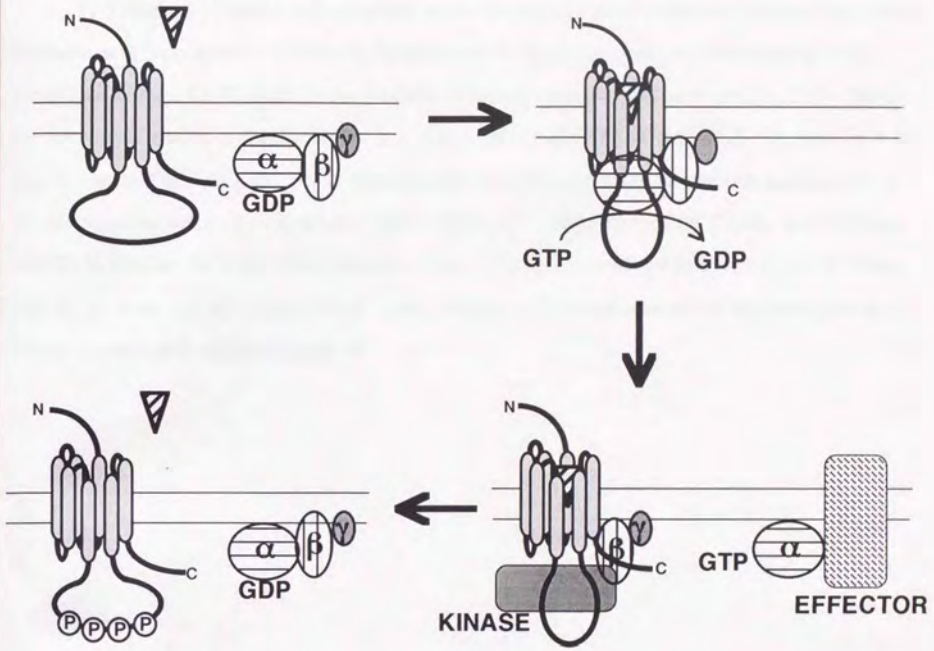


Fig. 13 Scheme of receptor phosphorylation by G protein coupled receptor kinase.



ACKNOWLEDGMENTS

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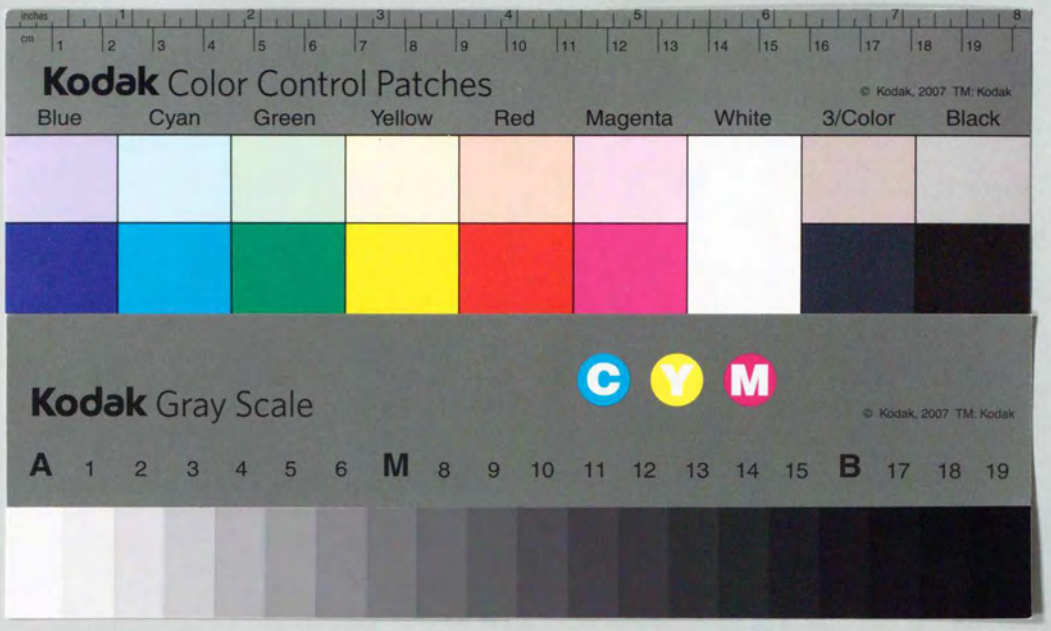
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