Ca^{2+}-Dependent Inhibition of G Protein-Coupled Receptor Kinase 2 by Calmodulin†

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ABSTRACT: Agonist- or light-dependent phosphorylation of muscarinic acetylcholine receptor m2 subtypes (m2 receptors) or rhodopsin by G protein-coupled receptor kinase 2 (GRK2) was found to be inhibited by calmodulin in a Ca^{2+}-dependent manner. The phosphorylation was fully inhibited in the absence of G protein $\beta\gamma$ subunits and partially inhibited in the presence of $\beta\gamma$ subunits. The dose−response curve for stimulation by $\beta\gamma$ subunits of the m2 and rhodopsin phosphorylation was shifted to the higher concentration of $\beta\gamma$ subunits by addition of Ca^{2+}−calmodulin. The phosphorylation by GRK2 of a glutathione *S*-transferase fusion protein containing a peptide corresponding to the central part of the third intracellular loop of m2 receptors (I3−GST) was not affected by Ca^{2+}−calmodulin in the presence or absence of $\beta\gamma$ subunits, but the agonist-dependent stimulation of I3−GST phosphorylation by an I3-deleted m2 receptor mutant in the presence of $\beta\gamma$ subunits was suppressed by Ca^{2+}−calmodulin. These results indicate that Ca^{2+}−calmodulin does not directly interact with the catalytic site of GRK2 but inhibits the kinase activity of GRK2 by interfering with the activation of GRK2 by agonist-bound m2 receptors and G protein $\beta\gamma$ subunits. In agreement with the assumption that GRK2 activity is suppressed by the increase in intracellular Ca^{2+}, the sequestration of m2 receptors expressed in Chinese hamster ovary cells was found to be attenuated by the treatment with a Ca^{2+} ionophore, A23187.

G protein-coupled receptors are known to be phosphorylated in a stimulation-dependent manner by G protein-coupled receptor kinases (GRKs),[1] and the phosphorylation is thought to be involved in desensitization of receptors [see reviews by Palczewski and Benovic (1991), Lefkowitz (1993), and Haga et al. (1994)]. GRK1 and GRK2 are the best characterized among six GRKs cloned up to now. GRK1, which was originally named rhodopsin kinase, phosphorylates rhodopsin in a light-dependent manner. Phosphorylated rhodopsin interacts with arrestin, which upon binding inhibits the activation of G proteins (Gt or transducin) by phosphorylated rhodopsin. GRK2 was originally named β adrenergic receptor kinases or βARK1 but is now known to phosphorylate many different kinds of G protein-coupled receptors, including α1, α2, β1, and β2 adrenergic receptors, m1, m2, and m3 subtypes of muscarinic acetylcholine receptors, substance P receptors, and rhodopsin, in an agonist- or light-dependent manner. A protein named β-arrestin with characteristics similar to those of arrestin has been shown to interact with phosphorylated β adrenergic receptors and thereby inhibit the activation of the G protein Gs by phosphorylated β adrenergic receptors (Lohse et al., 1990). In addition, the phosphorylation of muscarinic acetylcholine receptor m2 subtypes (m2 receptors) by GRK2 has been shown to facilitate the internalization of m2 receptors (Tsuga et al., 1994), although a conflicting result is also reported (Pals-Rylaarsdam et al., 1995). The phosphorylation of β adrenergic receptors by GRK2 had been reported to not be involved in their internalization, but recently, such a linkage was indicated for GRK2-mediated phosphorylation of β adrenergic receptors and their internalization (Ferguson et al., 1995).

Kinase activity of GRK1 and GRK2 is regulated in several ways. First, both GRK1 and GRK2 are activated by their substrates, photoexcited rhodopsin and agonist-bound receptors, respectively. Phosphorylation by GRK1 or GRK2 of peptides corresponding to phosphorylation sites in rhodopsin or β adrenergic receptors was shown to be stimulated by photoexcited rhodopsin (Fowles et al., 1988) or agonist-bound β adrenergic receptors (Chen et al., 1993), respectively. In addition, GRK1 (Palczewski et al., 1991) or GRK2 (Kameyama et al., 1994) was shown to be activated by phosphorylation site-deleted mutants of rhodopsin or m2 receptors in a light- or agonist-dependent manner, respectively. These findings explain at least in part the fact that substrates of GRKs are limited to the activated forms of G protein-coupled receptors and indicate that receptors interact with GRKs at two different sites, phosphorylation sites and activation sites. Second, muscarinic receptor kinase, which is the same as or similar to GRK2, and GRK2, but not GRK1, were shown to be activated by G protein $\beta\gamma$ subunits (Haga & Haga, 1990, 1992; Pitcher et al., 1992; Kameyama et al., 1993). The kinase activity of GRK2 appears to be syner-

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[1] Abbreviations: GRK, G protein-coupled receptor kinase; GRK1, G protein-coupled receptor kinase 1 or rhodopsin kinase; GRK2, G protein-coupled receptor kinase 2 or β adrenergic receptor kinase 1; G protein, guanine nucleotide-binding regulatory protein; m2 receptor, muscarinic acetylcholine receptor m2 subtype; I3, the third intracellular loop; I3−GST, a glutathione *S*-transferase fusion protein containing a peptide corresponding to the central part of the third intracellular loop of m2 receptors; I3−del m2, a m2 receptor mutant that lacks a central part of the third intracellular loop including phosphorylation sites; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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gistically activated by G protein $\beta\gamma$ subunits and agonist-bound receptors (Haga et al., 1994; Kameyama et al., 1994). Recently, the interaction of GRK2 with acidic phospholipids was reported to cause activation of GRK2, although there are some discrepancies among authors (Pitcher et al., 1995; Debburman et al., 1995b; Onorato et al., 1995). Furthermore, the phosphorylation and activation of GRK2 by protein kinase C were also reported (Chuang et al., 1996; Winstel et al., 1996).

A negative regulatory mechanism is known for GRK1. Kawamura has found that a protein termed s-modulin inhibits the phosphorylation of photoexcited rhodopsin in frog retina in a Ca^{2+}-dependent manner and that this inhibition of GRK1 plays a major role in light adaptation (Kawamura & Murakami, 1991; Kawamura, 1993). S-Modulin turned out to be a Ca^{2+}-binding protein with three EF-hand domains (Kawamura et al., 1993) that shows homologies to visinin (Yamagata et al., 1990) and recoverin (Langhans-Rajasekaran et al., 1995), which had been identified as Ca^{2+}-binding proteins with three EF-hand domains in chicken cone cells and bovine retina, respectively. Ca^{2+}-binding proteins with three EF-hand domains and homology to s-modulin have also been found in the brain and named as neurocalcin (Terasawa et al., 1992), NVP (neural visinin-like Ca^{2+}-binding protein) (Kuno et al., 1992; Kajimoto et al., 1993), VILIP (visinin-like protein) (Lenz et al., 1992), hippocalcin (Kobayashi et al., 1992), or NCS (neuronal calcium sensors) (De Castro et al., 1995). Recoverin as well as s-modulin are shown to directly inhibit GRK1 by *in vitro* experiments (Kawamura et al., 1993; Klenchin et al., 1995; Chen et al., 1995). On the other hand, recoverin did not affect the GRK2 activity (Chen et al., 1995) . In analogy with GRK1, we may expect that GRK2 and other GRKs are regulated by neurocalcin or other cerebral Ca^{2+} binding proteins. As far as we know, however, inhibition of GRK2 by Ca^{2+} binding proteins has not been reported so far, although NCS-1 and VILIP-1 as well as s-modulin and recoverin are shown to inhibit GRK1 (De Castro et al., 1995).

In the course of attempts to seek the relation between GRK2 and Ca^{2+} binding proteins, we have found that the agonist-dependent phosphorylation of m2 receptors is inhibited by calmodulin. Calmodulin (O'Neil & DeGrado, 1990; James et al., 1995) has four EF-hand domains and is abundant in the brain. In the present paper, we report that calmodulin interferes with the activation of GRK2 by agonist-bound receptors and G protein $\beta\gamma$ subunits in a Ca^{2+}-dependent manner.

EXPERIMENTAL PROCEDURES

Preparation of Calmodulin and Other Proteins. Calmodulin was purified from porcine brain as follows. Frozen brain (200 g) was homogenized in a Tris-HCl buffer solution [40 mM Tris-HCl (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 2.5 μg/mL pepstatin, 0.25 mM PMSF, and 0.5 mM benzamidine; a total volume of 1 L], and the homogenate was centrifuged for 20 min at 30 000 rpm. The supernatant was centrifuged once more after addition of $CaCl_2$ and NaCl to be 4 mM and 0.5 M, respectively. The supernatant was applied to a phenyl-Sepharose CL-4B column (70 mL), which had been equilibrated with a solution containing 40 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1 mM $CaCl_2$. The column was washed and then eluted with a solution containing 40 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 5 mM EGTA. The eluate was dialyzed against solution A [20 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 0.5 mM dithiothreitol] and then applied to a DEAE-Sephacel column (20 mL). The column was eluted with a gradient of solution A and solution A supplemented with 0.3 M NaCl (100 mL each). S100 protein (Isobe & Okuyama, 1978; Schafer & Heizman, 1996) was eluted ahead of calmodulin, and they were separated from each other with rechromatography with a DEAE-Sephacel column (10 mL). Calmodulin showed a single peak in SDS−PAGE with 18% acrylamide and 4.2 M urea. Purified protein was dissolved in 0.1% trifluoroacetic acid and subjected to analysis with matrix-assisted laser desorption ionization/time-of-flight mass spectrometry. Calmodulin preparation gave a single major band of $m/e = 16\,781.0$, which is consistent with the value calculated on the basis of amino acid compositions for porcine calmodulin (16 693).

Human m2 receptors and GRK2 were expressed in and purified from Sf9 cells as described previously (Kameyama et al., 1993), and m2 receptors were reconstituted into lipid vesicles as described (Haga et al., 1996). G protein $\beta\gamma$ subunits and urea-washed rod outer segments containing rhodopsin were purified from bovine brain and retina respectively, as described previously (Haga & Haga, 1992).

Phosphorylation Reaction. Phosphorylation of m2 receptors was performed as described previously (Haga et al., 1994). Briefly, a standard reaction medium contained 7−11 nM m2 receptors or urea-washed rod outer segments containing 20−30 nM rhodopsin, 66 nM G protein $\beta\gamma$ subunits, 25 μM calmodulin, 33 nM GRK2, 10 μM [^{32}P]-ATP (1.3 cpm/fmol; 4×10^5 cpm per tube), 20 mM Tris-HCl buffer (pH 7.5), 1 mM carbamylcholine or 10 μM atropine, 2 mM EDTA, 0.5 mM EGTA, 0 or 2 mM $CaCl_2$, and 5 mM $MgCl_2$. The total volume is 30 μL. Incubation was carried out at 30 °C for 60 min, and the reaction was terminated by addition of 20 μL of a 5% sodium dodecyl sulfate (SDS) solution containing medium for SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The mixture was applied to a 12% acrylamide gel for SDS−PAGE analysis, followed by autoradiography. The bands of m2 receptors or rhodopsin were cut out and counted by the use of Cerenkov's effect. In the experiments to see the effect of different concentrations of free Ca^{2+}, the reaction mixture contained 1 mM EDTA, 1 mM EGTA, 0−4 mM $CaCl_2$, and 5−6.5 mM $MgCl_2$. Concentrations of free Ca^{2+} were calculated on the basis of equilibrium binding constants at pH 7.5 for the binding of Ca^{2+} with EGTA ($10^{7.72}$) or EGTA ($10^{7.92}$) and for the binding of Mg^{2+} with EGTA ($10^{2.30}$) or EDTA ($10^{5.91}$). Concentrations of $MgCl_2$ were adjusted so the free Mg^{2+} concentration would be 5 mM. NaCl and NaOH were added so the ionic strength and pH were constant.

Sequestration of m2 Receptors. A stable transfectant expressing porcine m2 receptors was constructed as follows. A *Hin*dIII fragment of pKPM2 (Fukuda et al., 1988) was inserted in the *Xba*I site of pEF-BOS (Mizushima & Nagata, 1990) following the conversion of the end of both fragments to blunt ends (pEF-pm2). CHO-K1 cells (5×10^4 cells) were transfected with 18 μg of expression vectors of pEF-pm2 and 2 μg of pEF-neo according to the calcium phosphate method (Chen & Okayama, 1987). Stable transfectants were selected in the presence of 400 μg/mL geneticin (Life Technologies Inc.) and were subcloned by limiting dilution. Expression of receptors was detected with use of [^{3}H]-quinuclidinyl benzylate ([^{3}H]QNB) binding. The transfec-

tants were cultured in F-12 nutrient mixture (Ham's) (Life Technologies Inc.) supplemented with 10% fetal bovine serum (Cansera International Inc.), 40 units/mL penicillin G (Meiji Seika, Kaisha Ltd.), 40 mg/mL streptomycin sulfate (Meiji Seika, Kaisha Ltd.), and 100 μg/mL geneticin at 37 °C in 95% air and 5% CO_2. The [^{3}H]QNB binding sites of the cells were estimated to be 1.1 pmol/(mg of total homogenate).

Sequestration of m2 receptors was assessed as the loss of [^{3}H]-*N*-metylscopolamine ([^{3}H]NMS, a hydrophilic ligand) binding sites from the cell surface, as described previously (Tsuga et al., 1994). CHO cells (1×10^4 cells/well) were plated onto 12-well culture dishes. Forty to forty-eight hours after plating, the cells were incubated with various concentrations of carbamylcholine with or without 0.4 mM A23187 for 1 h. After the incubation, cells were washed three times with 1 mL of ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 at pH 7.5) per one well and incubated with 1.2−1.6 nM [^{3}H]NMS in Hepes-bufferd saline (25 mM Hepes, 113 mM NaCl, 6 mM glucose, 3 mM $CaCl_2$, 3 mM KCl, 2 mM $MgSO_4$, and 1 mM NaH_2PO_4 at pH 7.4; 0.5 mL per well) at 4 °C for 4 h. After incubation, cells were washed three times with 1 mL of ice-cold PBS per well. Washed cells were dissolved in 0.3 mL of 1% Triton X-100 (w/v), mixed with 4.5 mL of Triton-Toluene cocktail containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis(2-methyl-5-phenyloxazolyl)benzene, and then radioactivity was measured. Quadruplicate samples were assayed for each point.

RESULTS

Human m2 receptors were expressed in and purified from Sf9 cells and subjected to phosphorylation by GRK2, which was also expressed in and purified from Sf9 cells. The agonist-dependent phosphorylation of m2 receptors was found to be inhibited by calmodulin in the presence of Ca^{2+}, as shown in Figure 1a. Phosphorylation of m2 receptors was carried out in the absence of G protein $\beta\gamma$ subunits, and therefore, the inhibitory effect of Ca^{2+}−calmodulin is not due to the suppression of the GRK2-activating effect of $\beta\gamma$ subunits. The phosphorylation was not affected by calmodulin in the absence of Ca^{2+} or by Ca^{2+} alone. The inhibitory effect of Ca^{2+}−calmodulin was also observed for light-dependent phosphorylation of rhodopsin by GRK2 (Figure 1b). The phosphorylation of rhodopsin was fully inhibited by Ca^{2+}−calmodulin, and very little phosphorylation was detected in the presence of Ca^{2+}.

Concentration-dependent effects of calmodulin on phosphorylation of m2 receptors and rhodopsin are shown in Figure 2. Phosphorylation of m2 receptors was fully inhibited by calmodulin at a concentration of less than 25 μM in the absence of G protein $\beta\gamma$ subunits, but the inhibition was partial in the presence of $\beta\gamma$ subunits; EC_{50} values were roughly estimated to be 6 and 17 μM in the absence and presence of $\beta\gamma$ subunits, respectively. On the other hand, phosphorylation of rhodopsin by GRK2 was completely inhibited by calmodulin at a concentration of less than 10 μM with an EC_{50} value of 1−2 μM, irrespective of the presence or absence of G protein $\beta\gamma$ subunits (Figure 2b).

Effects of calmodulin on the phosphorylation of m2 receptors and rhodopsin are dependent on the presence of Ca^{2+}, irrespective of the presence or absence of $\beta\gamma$ subunits. Figure 3 shows the effect of increasing concentrations of free Ca^{2+} on the phosphorylation. The phosphorylation of m2 receptors in the presence of calmodulin was gradually inhibited with an increase in free Ca^{2+} concentrations from 10^{-8} to 10^{-3} M, irrespective of the presence or absence of $\beta\gamma$ subunits. On the other hand, the phosphorylation of rhodopsin in the presence of calmodulin was inhibited sharply by an increase in free Ca^{2+} concentrations from 10^{-8} to 10^{-6} M with a−log EC_{50} value of 6.8. The equilibrium

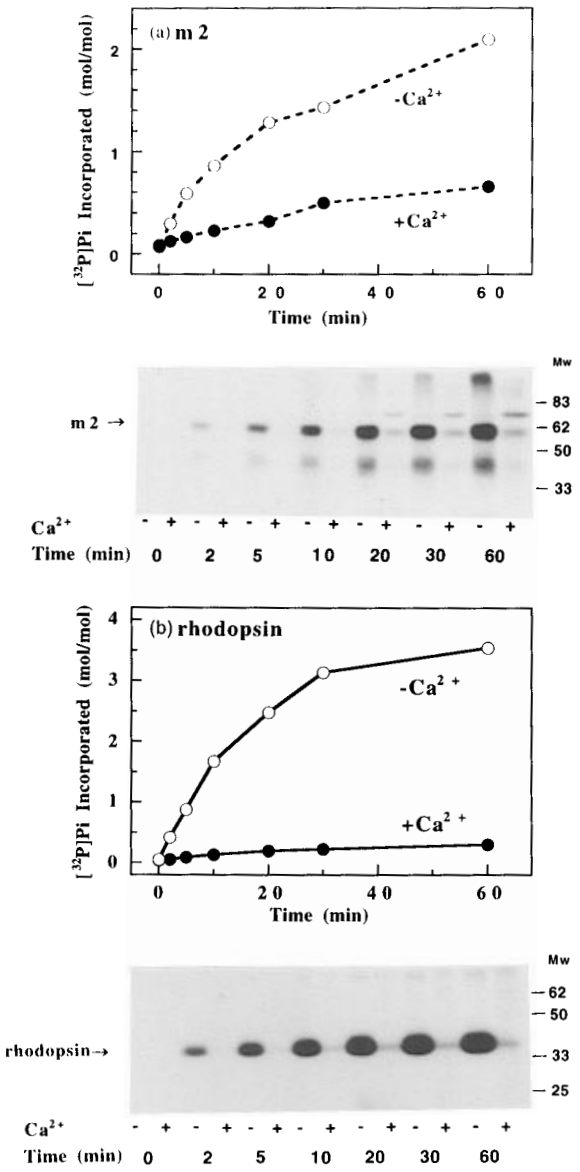

FIGURE 1: Time courses of phosphorylation by GRK2 of muscarinic m2 receptors (a) or rhodopsin (b) in the presence of calmodulin and the presence or absence of Ca^{2+}. Phosphorylation reaction was carried out in the presence of 33 nM GRK2, 25 μM calmodulin, 10 μM [^{32}P]ATP, 2 mM EDTA, 0.5 mM EGTA, plus or minus 2 mM $CaCl_2$, 20 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and (a) 7 nM m2 receptor and 1 mM carbamylcholine or (b) 20 nM rhodopsin and 66 nM $\beta\gamma$ subunits (total volume of 30 μL). G protein $\beta\gamma$ subunits were not included in the reaction mixture for experiment a. After incubation for the indicated times, each incubation mixture was subjected to SDS−PAGE followed by autoradiography and quantification of radioactivity incorporated into m2 receptors or rhodopsin. Virtually no phosphorylation of m2 receptors and rhodopsin was observed in the absence of carbamylcholine or light, respectively, indicating that the results represent agonist- or light-dependent phosphorylation. A minor band with an apparent molecular mass of 39 kDa in part a represents proteolytic fragment of m2 receptors. Another minor band with an apparent molecular mass of 80 kDa has not been identified but may represent autophosphorylation of GRK2.

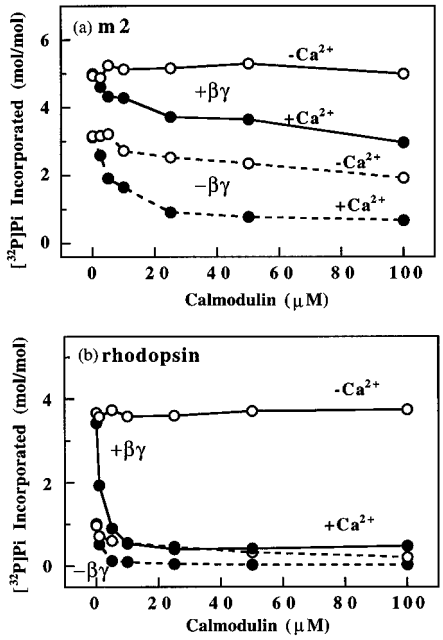

FIGURE 2: Phosphorylation of m2 receptors and rhodopsin in the presence of various concentrations of calmodulin. Experimental conditions are the same as those described in the legend to Figure 1, except that the incubation time was 60 min, 66 nM $\beta\gamma$ subunits were included in some experiments, and different concentrations of calmodulin were added. Experiments were repeated more than three times with essentially the same results, and results of one typical experiment are shown here.

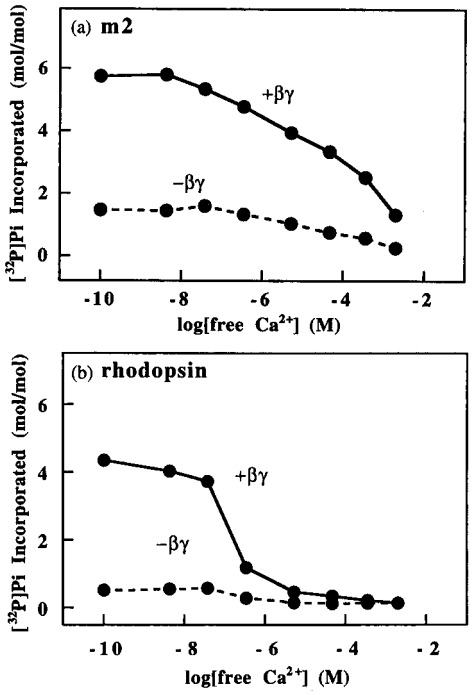

FIGURE 3: Effect of various concentrations of free Ca^{2+} on phosphorylation of m2 receptors and rhodopsin. Experimental conditions are the same as those described in the legend to Figure 2, except that concentrations of calmodulin, EGTA, EDTA, and free Mg^{2+} are 25 μM and 1, 1, and 5 mM, respectively, and concentrations of free Ca^{2+} are adjusted as described in Experimental Procedures.

dissociation constants for Ca^{2+} binding are reported to be approximately 10^{-7} and 10^{-6} M for the carboxy-terminal and amino-terminal domains of calmodulin, respectively (James et al., 1995; Schafer & Heizman, 1996). The finding that the phosphorylation of rhodopsin in the presence of calmodulin is inhibited more than 90% in the presence of 10^{-6} M Ca^{2+} indicates that the binding of 2 mol of Ca^{2+} to the carboxy-terminal domain of calmodulin is sufficient to exert its inhibitory effect. On the other hand, the phosphorylation of m2 receptors may be partially inhibited by $(Ca^{2+})_2$−calmodulin and fully inhibited by $(Ca^{2+})_4$−calmodulin.

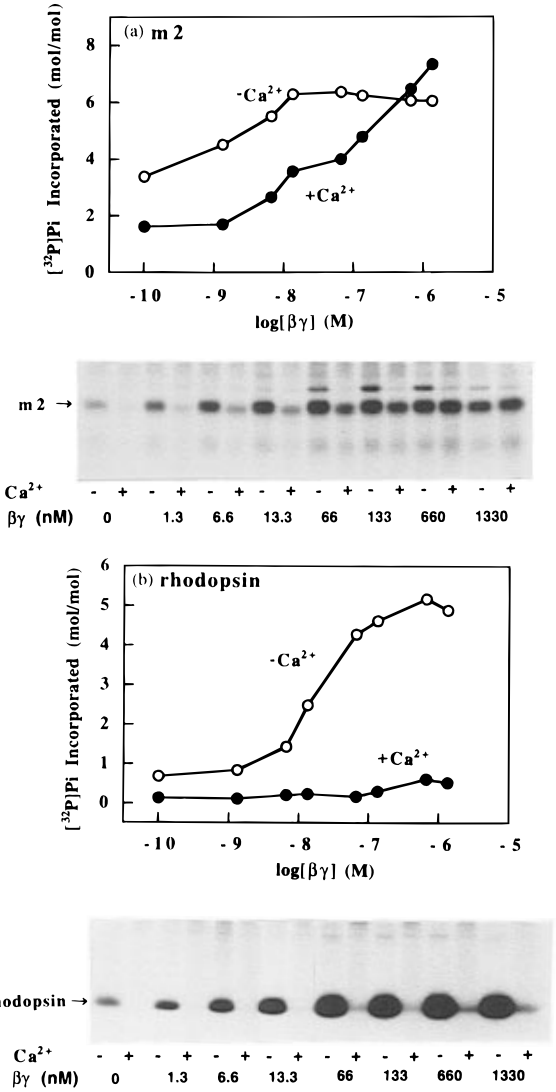

FIGURE 4: Effects of various concentrations of $\beta\gamma$ subunits on phosphorylation of m2 receptors and rhodopsin. Experimental conditions are the same as those described in the legend to Figure 2, except that concentrations of calmodulin are 25 μM and different concentrations of $\beta\gamma$ subunits are used.

Figure 4 shows the effect of increasing $\beta\gamma$ concentrations on the phosphorylation of m2 receptors and rhodopsin in the presence of calmodulin and in the presence or absence of Ca^{2+}. Phosphorylation of m2 receptors in the absence of Ca^{2+} increased with an increase of $\beta\gamma$ subunits from 10^{-10} to 10^{-8} M, irrespective of the presence of calmodulin, and EC_{50} values were estimated to be 1.7 nM. The dose−response curves shifted to the right in the presence of Ca^{2+} and calmodulin, and the EC_{50} value was estimated to be 74 nM (Figure 4a). This result suggests that the stimulatory effect of $\beta\gamma$ subunits is suppressed by Ca^{2+}−calmodulin in a competitive manner. Phosphorylation of rhodopsin in the absence of Ca^{2+} increased with an increase of $\beta\gamma$ subunits from 5×10^{-9} to 5×10^{-7} M, and the EC_{50} value was estimated to be 14 nM. In contrast with the phosphorylation of m2 receptors, the phosphorylation of rhodopsin in the presence of Ca^{2+} and calmodulin was only slightly stimulated by $\beta\gamma$ subunits at concentrations of up to 1 μM (Figure 4b).

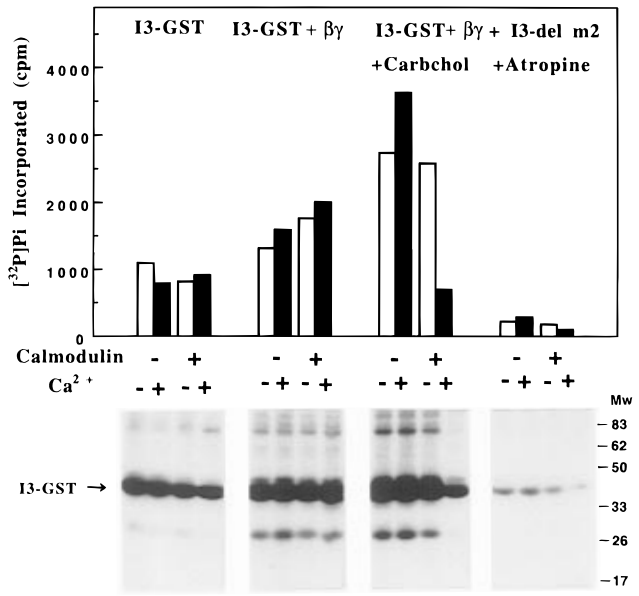

FIGURE 5: Phosphorylation by GRK2 of a glutathione *S*-transferase fusion protein containing a peptide corresponding to the central part of the third intracellular loop of m2 receptor (I3−GST) in the presence or absence of Ca^{2+}, calmodulin, $\beta\gamma$ subunits, a m2 receptor mutant lacking the central part of the third intracellular loop including phosphorylation sites (I3−del m2), 1 mM carbamylcholine, or 10 μM atropine. Experimental conditions are the same as those described in the legend to Figure 2, except that m2 receptors were replaced by 8 μM I3−GST with or without 16 nM I3−del m2 receptors.

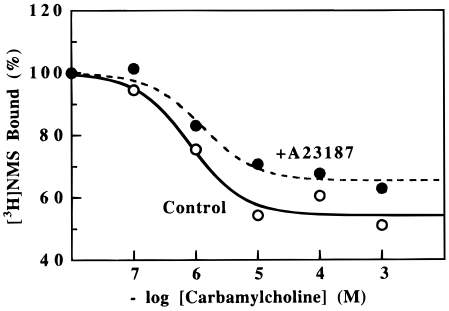

FIGURE 6: Effect of A23187 on sequestration of [^{3}H]NMS binding sites on CHO cells. CHO cells expressing porcine m2 receptors were incubated with indicated concentrations of carbamylcholine for 60 min with or without 0.4 mM A23187 and then subjected to the [^{3}H]NMS binding assay at 4 °C for 4 h. Experiments were repeated three times with essentially the same results, and results of one typical experiment are shown. The curves were fitted to the equation $R_{max}EC_{50}/(EC_{50} + [\text{carbamylcholine}]) + (100 - R_{max})$. The values of R_{max} were 46 and 35% in the absence and presence of A23187, respectively, and the values of EC_{50} were 0.8×10^{-6} and 1.3×10^{-6} in the absence and presence of A23187, respectively.

Differences in effective concentrations of $\beta\gamma$ subunits and Ca^{2+}−calmodulin between phosphorylation of m2 receptor and rhodopsin indicate that the m2 receptor−GRK2 system has a higher affinity for $\beta\gamma$ subunits and a lower affinity for Ca^{2+}−calmodulin compared with the rhodopsin−GRK2 system. These results suggest that both $\beta\gamma$ subunits and Ca^{2+}−calmodulin directly interact with m2 receptors and rhodopsin.

The question of whether the inhibition by Ca^{2+}−calmodulin is caused by their interaction with and inhibition of GRK2 or their interaction with m2 receptors or rhodopsin followed by concealing of their phosphorylation sites or by suppression of their GRK2-activating effects arises. To address this issue, we examined the effect of calmodulin on phosphorylation by GRK2 of a glutathione *S*-transferase fusion protein containing a peptide corresponding to the central part of the third intracellular loop of m2 receptors (I3−GST), which includes GRK2 phosphorylation sites. As shown in Figure 5, the phosphorylation of I3−GST by GRK2 was not affected by Ca^{2+} and calmodulin, irrespective of the presence or absence of $\beta\gamma$ subunits. The phosphorylation of I3−GST is stimulated by carbamylcholine in the presence of $\beta\gamma$ subunits and a m2 receptor mutant that lacks a central part of the third intracellular loop including phosphorylation sites (I3−del m2). The carbamylcholine-dependent phosphorylation of I3−GST by GRK2 in the presence of I3−del m2 was found to be markedly suppressed by calmodulin in the presence of Ca^{2+}. This result indicates that Ca^{2+}−calmodulin does not inhibit the catalytic activity of GRK2 or conceal the phosphorylation sites but suppresses the agonist-dependent, GRK2-activating effect of I3−del m2 receptors.

In order to see if the GRK2 activity is affected by Ca^{2+}, we have examined the effect of a Ca^{2+} ionophore, A23187, on the sequestration of m2 receptors expressed in CHO cells. The sequestration of m2 receptors is a specific and sensitive sensor of GRK2 activity because it is facilitated by coexpression of GRK2 and attenuated by coexpression of a dominant negative form of GRK2 (Tsuga et al., 1944). As shown in Figure 6, the sequestration of m2 receptors, which was assessed as the loss of [^{3}H]NMS binding sites from the cell surface, was found to be attenuated in the presence of A23187 as was the case in the coexpression of dominant negative GRK2. This result is consistent with, although it does not prove, the idea that the GRK2 activity is suppressed by the increase in the Ca^{2+} concentration.

DISCUSSION

In the present paper, we have shown that Ca^{2+}-bound calmodulin inhibits the light- or agonist-dependent phosphorylation of rhodopsin or m2 receptors by GRK2. Possible targets of Ca^{2+}−calmodulin include GRK2, m2 receptors (or rhodopsin), and $\beta\gamma$ subunits. Ca^{2+}−calmodulin is reported to interact with G protein $\beta\gamma$ subunits (Asano et al., 1986; Katada et al., 1987). However, the interaction of calmodulin with $\beta\gamma$ subunits does not explain the inhibition observed in the absence of $\beta\gamma$ subunits. Ca^{2+}−calmodulin is thought to interact with GRK2, because the inhibitory effect of Ca^{2+}−calmodulin was apparent on the phosphorylation of rhodopsin by GRK2 but was much less apparent on the phosphorylation of rhodopsin by GRK1 [unpublished data and De Castro et al., (1995)]. Furthermore, Ca^{2+}−calmodulin may also interact with m2 receptors (or rhodopsin), because effective concentrations of calmodulin and free Ca^{2+} are apparently higher for the phosphorylation of m2 receptors than for the phosphorylation of rhodopsin. It is theoretically possible that GRK2 undergoes conformational changes by binding with m2 receptors or rhodopsin and that GRK2 bound with rhodopsin has a higher affinity for Ca^{2+}−calmodulin than GRK2 bound with m2 receptors. However, it is more likely, though not proven, that Ca^{2+}−calmodulin interacts with both GRK2 and m2 receptors (or rhodopsin) and has a higher affinity for the GRK2−rhodopsin complex than for the GRK2−m2 complex.

In a previous experiment, G protein $\beta\gamma$ subunits have been reported to stimulate the phosphorylation of m2 receptors and rhodopsin at similar concentrations (Kameyama et al., 1993). In the present experiments using a wide range of $\beta\gamma$

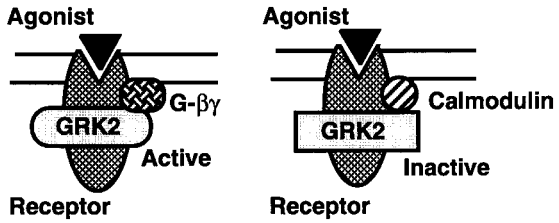

FIGURE 7: Schema for the interaction among agonist-bound m2 receptor, GRK2, G protein $\beta\gamma$ subunits, and Ca^{2+}-bound calmodulin. It is assumed that GRK2 is synergistically activated by agonist-bound receptors and $\beta\gamma$ subunits, and calmodulin interferes with the activation of GRK2 by agonist-bound receptors and $\beta\gamma$ subunits.

concentrations, it became apparent that higher concentrations of $\beta\gamma$ subunits were required for stimulation of rhodopsin phosphorylation than for stimulation of m2 receptor phosphorylation. This result is consistent with the assumption that $\beta\gamma$ subunits interact with m2 receptors and rhodopsin. There are some reports indicating the direct interaction between $\beta\gamma$ subunits and rhodopsin (Kelleher & Johnson, 1988; Phillips & Cerione, 1992), although no direct evidence is available for interaction between $\beta\gamma$ subunits and m2 receptors. The direct interaction between $\beta\gamma$ subunits and GRK2 has been shown by different lines of evidence such as activation of phosphorylation of synthetic substrates by $\beta\gamma$ subunits (Haga et al., 1994) and interaction of $\beta\gamma$ subunits with partial peptides of GRK2 (Koch et al., 1993) or GRK2 itself (Kim et al., 1993). Thus it is most likely that $\beta\gamma$ subunits interact with both GRK2 and rhodopsin (or m2 receptor).

The inhibition by Ca^{2+}−calmodulin of phosphorylation of m2 receptors was suppressed in the presence of excess $\beta\gamma$ subunits, and the dose−response curves for $\beta\gamma$ subunits were shifted to the right by addition of Ca^{2+}−calmodulin. The simplest explanation for these findings is to assume that there are two kinds of ternary complexes, GRK2−m2 receptor−$\beta\gamma$ subunits and GRK2−m2 receptor−Ca^{2+}−calmodulin, and that GRK2 is active in the former complex and not active in the latter complex (Figure 7). It is assumed here that Ca^{2+}−calmodulin and $\beta\gamma$ subunits each interact with both GRK2 and m2 receptor and that the two ternary complexes are mutually exclusive. This does not necessarily mean that Ca^{2+}−calmodulin and $\beta\gamma$ subunits bind to the same domains in m2 receptors and GRK2. It is possible that the binding of Ca^{2+}−calmodulin and $\beta\gamma$ subunits excludes the binding of the other by steric hindrance.

In the schema depicted in Figure 7, it is assumed that GRK2 is synergistically activated by $\beta\gamma$ subunits and agonist-bound receptors and that GRK2 interacts with agonist-bound receptors at two different sites, a phosphorylation site and an activation site. Phosphorylation sites of m2 receptors are located in the central part of the third intracellular loop (Nakata et al., 1994), and activation sites are thought to be in the region adjacent to transmembrane segments (Haga et al., 1994). Ca^{2+}−calmodulin does not inhibit the phosphorylation of I3−GST by GRK2 but suppresses the stimulation by the agonist-bound, I3-deleted m2 receptor mutant of the phosphorylation of I3−GST. This indicates that Ca^{2+}−calmodulin does not affect the interaction of GRK2 with the phosphorylation site but interferes with the interaction of GRK2 with the activation site of agonist-bound m2 receptors. Calmodulin binding sites are comprised of a basic amphiphilic α-helix with hydrophobic residues in one face and basic amino acids in the other face (James et al., 1995). Mastoparan, a bee venom, is an amphiphilic peptide and binds with calmodulin. Mastoparan is known to mimic agonist-bound receptors and activate GRK2 (Haga et al., 1994; Murga et al., 1996) as well as G proteins (Higashijima et al., 1988). Thus, it will be reasonable to assume that Ca^{2+}−calmodulin binds with agonist-bound receptors at the region adjacent to transmembrane segments and thereby interferes with the activation of GRK2 by agonist-bound receptors. Consistent with this assumption, GRK2 is known to be activated by peptides corresponding to the second intracellular loop, the carboxy-terminal part of the third intracellular loop, and the amino-terminal segments of the carboxy-terminal tail (Haga et al., 1994), and the regions adjacent to transmembrane segments of G protein-coupled receptors including muscarinic receptors are shown to form basic amphiphilic α-helices (Strader et al., 1989; Duerson et al., 1993; Blin et al., 1995).

Inhibition by recoverin of rhodopsin phosphorylation by GRK1 has been examined in detail (Klenchin et al., 1995; Chen et al., 1995). The free Ca^{2+} concentration giving a half-maximal effect (EC_{50}) was estimated to be 1−3 μM, and EC_{50} values for recoverin in the presence of excess free Ca^{2+} were estimated to be 3.4 μM (Klenchin et al., 1995) or 8 and 0.8 μM for nonacylated and myristoylated recoverin, respectively (Chen et al., 1995). As shown in the Results, the phosphorylation of m2 receptors in the presence of calmodulin was gradually inhibited with increases in free Ca^{2+} concentrations from 10^{-8} to 10^{-3} M. The EC_{50} value for calmodulin was estimated to be 6 μM in the absence of $\beta\gamma$ subunits. This is in contrast with values obtained for the phosphorylation of rhodopsin by GRK2; the phosphorylation was inhibited with the increase of free Ca^{2+} from 10^{-8} to 10^{-6} M with EC_{50} values of 0.1 μM for free Ca^{2+} and 1−3 μM for calmodulin. This may reflect the facts that rhodopsin and m2 receptors are embedded in different membranes, urea-stripped photoreceptor and Sf9 membranes, respectively, and the two membranes must contain different compositions of phospholipids, which are known to affect GRK2 activity (Pitcher et al., 1995; Debburman et al., 1995b; Onorato et al., 1995). Alternatively, it is possible to speculate that Ca^{2+}−calmodulin may have better target receptors other than m2 receptors and that the phosphorylation of m2 receptors by GRK2 may be inhibited by Ca^{2+}-binding proteins other than calmodulin. It should be noted that a number of different kinds of G protein-coupled receptors have been indicated to be substrates of GRK2 (Diviani et al., 1996; Kurose & Lefkowitz, 1994; Freedman et al., 1996; Haga et al., 1996; Debburman et al., 1995a; Kwatra et al., 1993; Tiberi et al., 1996; Prossnitz et al., 1995) and that different kinds of Ca^{2+}-binding proteins with homology to s-modulin have been identified in the brain recently (Terasawa et al., 1992; Kuno et al., 1992; Kobayashi et al., 1992; De Castro et al., 1995).

The present studies on the sequestration of m2 receptors suggest that the activity of GRK2 to phosphorylate m2 receptors in CHO cells is suppressed by the increase in intracellular Ca^{2+}. The evidence, however, is not direct, and it is theoretically possible that the increase in Ca^{2+} causes the decrease in the sequestration without affecting the GRK2 activity, although such an result has not been reported. The possibility that the phosphorylation by GRKs of m2 and other G protein-coupled receptors is regulated by calmodulin or other Ca^{2+}-binding proteins under physiological conditions still remains a challenging question. We may speculate about a possible physiological meaning of the Ca^{2+}-dependent inhibition of GRK2 by the analogy with light adaption of

the rhodopsin system. Activation of m2 or other Gi/Go-coupled receptors mediates the inhibition of the Ca^{2+} channel and by inference is expected to cause the decrease in intracellular Ca^{2+}, which will enhance the GRK2 activity and then cause the greater desensitization of these receptors.

In conclusion, we have presented evidence that the agonist-dependent phosphorylation of muscarinic m2 receptors by GRK2 is inhibited by calmodulin in a Ca^{2+}-dependent manner and have suggested the possibility that the phosphorylation and desensitization of G protein-coupled receptors by GRK2 may be modified by the increase in the intracellular Ca^{2+} concentration through action of calmodulin or other Ca^{2+}-binding proteins.

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