Muscarinic cholinergic receptors (mAChR) transduce their signals by interacting with guanine nucleotide regulatory proteins or G-proteins, as do rhodopsin, β-adrenergic receptors, and other members of the family of seven transmembrane-spanning domain receptors. Activation of mAChR by an agonist has been associated with several biological responses such as: inhibition of adenylylcyclase (1, 2), activation of phospholipases C, A, and D (3–5), stimulation of cGMP accumulation (6), and modulation of ion channels (7–10). Prolonged stimulation of mAChR by agonists results in desensitization of the receptors, that is, a decreased ability of the receptors to transduce signals. Desensitization appears to involve decreases in: the affinity of the receptors for agonist (11–13), the efficiency of receptor/G-protein interactions (4, 14), and/or the number of functional cell surface receptors (15). Activation of protein kinases and phosphorylation of the mAChR seem to be involved in the desensitization process (11–19). Approaches to define the role of receptor phosphorylation in mAChR regulation have been reported in several studies. It has been shown in intact cell studies that agonist activation of chick and porcine heart mAChR leads to phosphorylation of the mAChR on serine and threonine residues to a stoichiometry of 3–5 mol of phosphate/mol of receptor (14, 17, 18). In vitro studies it has been shown that purified mAChR from cardiac tissues, reconstituted into phospholipid vesicles, are excellent substrates for phosphorylation by a β-adrenergic receptor kinase (19) and a similar or related kinase (20). These reactions occur in a strictly agonist-dependent manner. In addition, purified mAChR from chick heart (21) and porcine cerebrum (22) are also excellent substrates for protein kinase C (PKC). Notably, the phosphorylation of the mAChR that is catalyzed in vitro by PKC occurs in an agonist-independent manner (21, 22), which is in marked contrast to the β-adrenergic receptor kinase-catalyzed events (18, 20). The functional effects of mAChR phosphorylation have not been fully defined.

In this work we have studied the functional consequences of phosphorylation of chick heart mAChR by PKC by analyzing the interactions of the receptors with the G-protein Gαs. In addition, we have also determined the ability of Gαs to modify the ability of the receptors to serve as substrates for PKC.

MATERIALS AND METHODS

Purification of mAChR and G-proteins—Muscarinic receptors were purified from chick heart ventricles using the procedure of Haga and Haga (23), as described by Kwatra and Hosey (17), and were reconstituted into lipid vesicles as described previously (18, 24). The specific activity of the purified receptors was ~1 nmol of [3H]quinuclidinyl benzilate (QNB) bound/mg of protein (17). G-proteins were purified from calf brain according to the procedure described by Sternweis and Robishaw (25), which resulted in the purification of a mixture of heterotrimeric Gα and Gβ proteins, in a ratio of 4:1, respectively. Gαs was further purified from the Gα/Gβ mixture by the use of a Mono-Q column (Pharmacia LKB Biotechnology Inc.) as described by Katada et al. (26). Prior to mixing with reconstituted receptors, the amount of Gαs was determined by an [35S]GTPγS binding assay (25). In the text, the amount of Gαs used for experiments refers to the amount assayed before reconstitution.

Purification of Protein Kinase C—Protein kinase C was purified from chick brain according to the procedure of Woodgett and Hunter (27). This procedure results in the isolation of a doublet of 78–80 kDa (27) which probably contains the α, β, and γ isofoms of protein kinase C (28). The PKC preparation was homogeneous as assessed by silver staining after SDS-gel electrophoresis, was highly dependent on calcium, and was insensitive to cyclic nucleotides and calmodulin. Phosphorylation of mAChR by Protein Kinase C—The phosphoryl-
ation of the purified mAChR by protein kinase C was carried out as previously described (21).

**Ligand Binding Assays with Reconstituted mAChR and G.**—Reconstitution of phosphorylated and nonphosphorylated mAChR with Gt, was performed as described by Haga et al. (24) using a ratio of 250:1 of Go:receptor. After reconstitution, 40 fmol of mAChR was routinely, as assessed by QNB binding. In the text, the indicated amount of mAChR used refers to the amount of receptors measured by ligand binding after reconstitution, unless otherwise specified. Prior to reconstitution with Gt, the phosphorylated and nonphosphorylated mAChR (which were also subjected to the conditions of the phosphorylation reaction) but without protein kinase C were chromatographed on a Sephadex G-50 column (2 ml) in order to remove the ATP and other reagents of the phosphorylation reactions. Ligand binding to the reconstituted mAChR was carried out with the agonists [H]QNB (Amersham Corp.), and agonist affinity was determined using varying concentrations of the agonist carbachol in competition studies as previously described (24, 29). Briefly, the assay (1 ml) contained 40-50 fmol of reconstituted receptors with or without Gt, 25 mM KPO₄, pH 7.0, 0.8 mM EDTA, 3 mM MgCl₂, 230 mM NaCl, 0.06% bovine serum albumin, 4 mM Hepes, and 0.6-0.7 nM [H]QNB. Reactions were carried out for 75 min at 30 °C and terminated by filtration over GF/F glass fiber filters (Whatman).

**[^35S]GTPyS Binding Assays—**Phosphorylated or nonphosphorylated receptors were reconstituted with Gt, by the direct addition of Gt, to the reconstituted receptors (24) according to the method of Haga et al. (37). The recovery of GTPyS binding activity was 30-40%, similar to that described by Haga et al. (37). For studies of the effect of phosphorylation on the ability of the receptors to stimulate GTPyS binding activity, a proportion of 5 pmol of Gt/pmol of reconstituted receptor was used. Before performing the[^35S]GTPyS binding assays, the above reconstitution mixtures were first incubated 1 h on ice (37) and then incubated for 5 min on ice with GDP (6 μM final concentration), because GDP is known to suppress basal binding of GTPyS but allows stimulation by agonist (30). Reactions were carried out in a final volume of 35-40 μl of a solution containing 25 mM sodium Hepes, pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol, 3 mM MgCl₂, 100 mM NaCl, 0.1% Lubrol (Sigma), and[^35S]GTPyS (Du Pont Co., New England Nuclear) (200,000 cpm/assay tube), with carbachol (0.1 μmol) ± atropine (0.2 μmol) for 0-30 min at 30 °C. The reactions were terminated by adding 2 ml of cold washing buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 25 mM MgCl₂. The bound[^35S]GTPyS was trapped on nitrocellulose filters (type HA, 0.45 μm, Millipore Corp., Bedford, MA) and counted in a liquid scintillation counter.

**GTPase Assays—**The reconstitution of the receptor with Gt, was performed as described above for the GTPyS binding assays. GTPase activity was assayed essentially according to the procedure described by Cerione et al. (31). Briefly, 100 μl of assay volume containing 40 μl of receptor (Go-containing vesicles and 10 μl of Tris-HCl, pH 7.8, 3 mM MgCl₂, 1 mM EDTA, 2 mg/ml bovine serum albumin, 0.5 mM ascorbic acid, 100-200 μM [γ32P]GTP (10,000-40,000 cpm/pmol) (ICN, Irvine, IL), 0.1 mM carbachol ± 0.2 mM atropine, or 1 mM GTP. The reaction mixtures were incubated at 30 °C for 45 min, and were stopped by addition of 10 μl of cold 50% trichloroacetic acid with immediate chilling on ice. The mixtures were centrifuged for 10 min at 2,500 rpm, and then 90 μl of the supernatant was removed and assayed for inorganic[^32P]phosphate as previously described (31).

**RESULTS**

**Effect of Protein Kinase C Phosphorylation of Chick Heart mAChR on the Ability of the Receptors to Stimulate GTPyS Binding to Gt—**Chick heart muscarinic receptors were purified, reconstituted into lipid vesicles, and phosphorylated by PKC as previously described (21). Phosphorylation occurred to an extent of 4-5 mol of P/mol of receptor and was unaffected by the presence of agonist (Ref. 21 and see below). To determine the effect of phosphorylation of mAChR by PKC on the interaction of the receptors with Gt, we first analyzed agonist-stimulated binding of GTPyS to Gt. Phosphorylated and nonphosphorylated mAChR were reconstituted with Gt, and the binding of GTPyS to Gt was monitored as a function of time in the presence of the agonist carbachol, plus and minus the antagonist atropine. As shown in Fig. 1A, when nonphosphorylated receptors were characterized, carbachol stimulated the binding of the nucleotide to Gt, ~70% of the total GTPyS binding after 30 min of reaction was due to agonist-stimulated binding. This effect was prevented by the presence of atropine in the reaction mixture. In contrast, the phosphorylated receptors (Fig. 1B) exhibited a diminished capacity for agonist-stimulated binding of GTPyS to Gt. With the phosphorylated receptors a 35-40% decrease of the carbachol-stimulated GTPyS binding to Gt, was observed relative to the nonphosphorylated receptors. These results suggest that the phosphorylation of the mAChR by PKC may impede the interaction of the receptor with Gt, which may result in a decrease in agonist-stimulated binding of GTPyS to the G-protein.

**Effect of Phosphorylation of the mAChR by Protein Kinase C on the Ability of the Receptors to Modulate the GTPase Activity of Gt—**To further determine the effect of PKC phosphorylation of mAChR on mAChR/Gt, interactions, we studied the ability of the receptors to stimulate the GTPase activity of Gt, in an agonist-dependent manner. Nonphospho-
phosphorylated receptors were used as controls. As shown in Fig. 2, the presence of mAChR plus carbachol in the reaction mixtures stimulated the catalytic activity of Go by ~13-fold. This effect was prevented by atropine. In contrast, the phosphorylated receptors stimulated GTPase activity to a significantly greater degree. Only ~45–50% of the P, released in the control was observed in the presence of carbachol and the phosphorylated mAChR. These results are consistent with those obtained with the GTPyS binding assays and indicate that phosphorylation of the receptors by PKC affects mAChR coupling to Go, and perturbs the capacity of the agonist-activated mAChR to stimulate GTPyS binding to Go, and the GTPase activity of Go.

Effect of Protein Kinase C Phosphorylation of mAChR on the Ability of Go to Induce High Affinity Agonist Binding to mAChR—The capacity of Go to induce high affinity agonist binding to the phosphorylated and nonphosphorylated mAChR was also studied. Phosphorylated and nonphosphorylated mAChR were reconstituted with Go using a proportion of 1 pmol of receptor/250 pmol of G-protein (24). Agonist affinity was determined in competition studies as described previously (21–29). As shown in Fig. 3, in the absence of Go, both the phosphorylated and the nonphosphorylated receptors exhibited a single low affinity state for the agonist carbachol with a \( K_d \) of ~110 \( \mu \)M. In the presence of Go, both the nonphosphorylated and the phosphorylated receptors also exhibited a high affinity state with a \( K_d \) of ~30–40 nM for carbachol and the dose-response curves were best fit with a two-state model. The percentage of mAChR exhibiting high affinity for carbachol was not different between the phosphorylated and the nonphosphorylated receptors under the conditions tested (Fig. 3). These results indicate that the phosphorylation of mAChR by PKC may not affect the ability of Go to induce high affinity agonist binding to the receptor.

Effect of Go on the Phosphorylation of mAChR by Protein Kinase C—In view of the effects of PKC on mAChR/Go,

![Fig. 2. Effect of protein kinase C phosphorylation of chick heart mAChR on the ability of the receptors to modulate agonist-stimulated GTPase activity of Go. Control (nonphosphorylated, left) and phosphorylated (right) mAChR were reconstituted with Go as indicated in Fig. 1. The assays of GTPase activity were performed as described under “Materials and Methods.” The data shown are representative of two experiments performed in duplicate. Maximum P, released is defined as the amount of P, obtained with the control (nonphosphorylated) receptors after 45 min of reaction in the presence of carbachol. Each assay contained 0.5 pmol of reconstituted receptors. The values presented are corrected for basal activities, which were 0.10 and 0.12 mol of P, released/mol of Go for control and phosphorylated receptors, respectively. The corrected activities were: control receptors with carbachol, 3.29 mol of P, released/mol of Go, and with atropine 0.03 mol of P, released/mol of Go; phosphorylated receptors with carbachol, 0.16 mol of P, released/mol of Go, and with atropine 0.025 mol of P, released/mol of Go. Slashed bars represent experiments performed in the presence of carbachol, and cross-hatched bars experiments performed in the presence of carbachol plus atropine.

FIG. 3. Competitive inhibition by carbachol of \(^{[3]H}\)NQNB binding to control (nonphosphorylated) or phosphorylated mAChR reconstituted in the presence or absence of purified Go. Ligand binding assays were performed as described under “Materials and Methods.” Data from control (nonphosphorylated) mAChR are shown in panel A and from phosphorylated mAChR in panel B. Receptors with no Go added (○); receptors reconstituted with Go (●), using 1 pmol of reconstituted receptor per 250 pmol of Go. The assays contained 40–60 fmol of reconstituted receptors with or without Go. Curve fitting was performed with the LIGAND program, and the resulting parameters are: for control receptors, without Go, \( K_I \) = 110 ± 22 \( \mu \)M, \( R_L \) = 100%; and with Go, \( K_I \) = 0.027 ± 0.009 \( \mu \)M, \( R_L \) = 53 ± 11%, \( K_I \) = 93 ± 18 \( \mu \)M, \( R_L \) = 47 ± 9%; for phosphorylated receptors, without Go, \( K_I \) = 127 ± 41 \( \mu \)M, \( R_L \) = 100%; and with Go, \( K_I \) = 0.042 ± 0.013 \( \mu \)M, \( R_L \) = 48 ± 5%, \( K_I \) = 81 ± 12 \( \mu \)M, \( R_L \) = 52 ± 9%. The results shown are from three separate experiments performed in duplicate. \( K_I \) and \( K_R \) refer to \( K_I \) values for high and low affinity states, respectively. \( R_L \) and \( R_L \) refer to the percentage of receptors exhibiting the respective affinity.

interactions, it was also of interest to determine if Go could modify the ability of mAChR to serve as substrates for PKC. Purified and reconstituted mAChR were subjected to phosphorylation by protein kinase C in the absence and the presence of Go, plus and minus the agonist carbachol. As shown in the autoradiogram of Fig. 4A, phosphorylation of the mAChR in the absence of Go (lanes 1 and 2) results in a major phosphorylated band at ~80 kDa which corresponds to the chick heart mAChR. The stoichiometry of phosphorylation was ~5 mol of phosphate/mol of receptor (21). The phosphorylation reaction was unaffected by agonist (lane 2) as reported previously (21). In contrast, the presence of Go (5 pmol of Go/1 pmol of mAChR) in the reaction mixture markedly inhibited the ability of protein kinase C to phosphorylate the reconstituted mAChR (lane 5). The stoichiometry of phosphorylation was reduced to ~1.2 mol of P/mol of receptors. This inhibition of mAChR phosphorylation by Go, occurred in the absence or presence of agonist (lane 6). Go was also subjected to phosphorylation by protein kinase C (lanes 3 and 4), but no significant phosphorylation of Go was observed under these conditions. Only a faint band at ~78 kDa was observed which corresponds to autophosphorylated PKC.

We further studied the effects of various concentrations of Go on the PKC-mediated phosphorylation of mAChR. Pur-
with or without Go (1 pmol of reconstituted receptor/5 pmol of Go).

Purified Go and chick heart were inserted into phospholipid vesicles and reconstituted. Phosphorylation by PKC was carried out for 60 min at 30°C in the presence or absence of carbachol. Reaction mixtures containing reconstituted receptors (0.7 pmol) and purified Gα (lanes 3 and 4) were electrophoresed on SDS gels and visualized by autoradiography.

Phosphorylation by PKC was concentration-dependent. The extent of inhibition was quantified by determining the stoichiometries of phosphorylation (Fig. 5, lower panel). When mACHR were reconstituted with Gα in a ratio of 10 pmol of Gα/pmol of mACHR, only ~0.8 mol of phosphate/mol of receptor was incorporated. This effect of Gα was prevented by the presence of GTPyS (100 μM) in the reaction mixture (Fig. 5, lane 5 in upper panel and asterisk in lower panel). This latter result was predicted if the inhibitory effect of Gα was due to an interaction of mACHR with Gα.

**DISCUSSION**

In previous work we reported that mACHR purified from chick heart and reconstituted into phospholipid vesicles can serve as effective substrates for PKC (21). In contrast to results obtained in in vitro phosphorylation studies using β-adrenergic receptor kinase (19), phosphorylation of the chick heart mACHR by PKC was unaffected by agonist occupancy. Similar results were obtained by Haga et al. (22) using mACHR purified from porcine brain. The functional consequences of mACHR phosphorylation by PKC in mACHR signaling were not fully elucidated in our previous study; however, several studies have reported that PKC may play an important role in the regulation of mACHR function (reviewed in Ref. 32). It has been shown that the exposure of neuroblastoma cells to phorbol esters, well known activators of PKC, results in a decrease in the number of mACHR on the cell surface (15, 32, 33) and attenuation in receptor-mediated stimulation of phosphatidylinositol hydrolysis (4, 34) and cGMP production (35). The inhibition of these stimulatory effects has been proposed to be associated with a feedback regulation of mACHR by PKC phosphorylation (36), but direct evidence for this has not been demonstrated. In this work we present evidence that phosphorylation of mACHR by PKC may directly affect the coupling of the receptors to Gα, which results in a decrease in mACHR-mediated stimulation of GTPyS binding and the catalytic activity of the G-protein.

The interaction of mACHR with Gα has been analyzed in several studies (24, 30, 37). It has been shown that agonist interaction with mACHR effectively stimulates the binding of GTPyS to Gα as well as the hydrolysis of GTP by Gα in reconstituted vesicles containing purified mACHR and Gα (30, 37). Similar results were obtained in this work under control conditions using nonphosphorylated mACHR. When these functions were monitored in reconstituted vesicles containing mACHR phosphorylated by PKC, a ~35–50% inhibition was observed compared with the nonphosphorylated receptors. These results suggest that the phosphorylation of mACHR by PKC can perturb receptor/Gα coupling and diminish the transducing capacity of the receptor. It has not yet been demonstrated if similar events occur in intact cells. However, mACHR-mediated stimulation of phosphoinositide turnover may result in an increase in the intracellular concentration of diacylglycerol and Ca2+ and a consequent activation of PKC (36). Thus PKC may regulate mACHR function by phosphorylating the receptors, resulting in altered receptor/G-protein coupling.

It has been shown that prolonged exposure of neuroblastoma cells to phorbol ester also results in a decrease in the affinity of mACHR for agonist (38). In order to investigate
the possibility that this might be due to the phosphorylation of mAChR by PKC, we studied the ability of Gs to induce high affinity agonist binding to the phosphorylated and nonphosphorylated receptors. A ratio of 1 pmol of mAChR/250 pmol of Gs was used in the reconstitution process in order to facilitate detection of the effects of the G-protein (37). Surprisingly, both the phosphorylated and the nonphosphorylated receptors showed a similar percentage of high affinity binding (48 and 53%, respectively) and a similar affinity for the agonist carbachol (Kd of ~30–40 nM). These apparently different effects of phosphorylation on receptor/G-protein interactions may be explained in several ways. First, the phosphorylation of mAChR by PKC may affect receptor-mediated activation of Gs without affecting the ability of Gs to induce high affinity agonist binding to the receptors. This possibility seems plausible in light of studies with the β-adrenergic receptors which suggest that different domains are involved in receptor-mediated activation of Gs and effects of Gs on receptor affinity (51, 52). A second interpretation of the differential effects of mAChR phosphorylation in receptor/Gs interaction is that the presence of a third component like arrestin or β-arrestin may be necessary to observe certain effects of phosphorylation (39, 53). Third, the possibility that the relatively high proportion of Gs receptor used for the ligand binding assays (versus the lower proportion used in GTPase activity and GTP-γ-S binding assay) could have masked an effect of the phosphorylation of mAChR by PKC to modify Gs-induced high affinity agonist binding. On the other hand, the results we obtained are consistent with those reported by Haga et al. (22) who assessed effects of PKC phosphorylation on mAChR purified from porcine brain. In contrast, previous results from our group (21), from studies in which we used a mixture of Gs/Gαs showed a positive effect of the PKC phosphorylation of mAChR on the G-protein induced high affinity state of the receptors. This may have been due to the mixture of G-proteins used or some differences in the conditions of the reactions. The mixture of Gs/Gαs used in our previous study (21) was only ~70% pure as assessed by silver staining after SDS-gel electrophoresis and we cannot discard the possibility that impurities in the mixture may have contributed to the difference in results. Furthermore the previous study of the effects of the mixture of Gs/Gαs on high affinity agonist binding was performed in the presence of 0.1% Lubrol plus 0.1% cholate, whereas the present studies were performed in the absence of cholate. We have not yet tested whether these factors contributed to the results, nor do we know how phosphorylation of the receptor affects interactions with purified isoforms of Gs. We plan to address these issues in future studies.

In view of the ability of PKC to modify mAChR/Gs interactions, it is interesting that Gs can inhibit the phosphorylation of the receptors by PKC. This inhibition caused by Gs was reversed by the presence of GTP-γ-S in the reaction mixture but was unaffected by the presence of agonist. Agonists are thought to stabilize receptor/G-protein interactions, whereas the binding of GTP-γ-S to Gs should result in an uncoupling of the receptors from the G-protein and a dissociation of the G-protein trimer into its α and βγ subunits. The G-protein-mediated inhibition may be due to the interaction of the third cytoplasmic loop of the receptor with the G-protein, where the potential sites for PKC phosphorylation are located. This inhibitory effect of Gs on mAChR phosphorylation has important implications for the regulation of the receptors by PKC in intact cells, as it suggests that only receptors dissociated from G-proteins may serve as substrates for PKC.

In summary, the present results indicate that the phosphorylation of chick heart mAChR by PKC interferes with the capacity of the receptors to stimulate GTP-γ-S binding and GTPase activity of the G-protein Gs, without affecting the ability of Gs to induce high affinity agonist binding state of the receptors. The presence of Gs in the reaction mixtures results in an inhibition of the phosphorylation of mAChR by PKC. The inhibitory effect of Gs was completely suppressed by the nucleotide GTP-γ-S, indicating that it was specifically due to the presence of the G-protein in the reconstituted system.

A question that remains to be clarified is: how many subtypes of mAChR(s) undergo phosphorylation by PKC? Molecular cloning studies have demonstrated that there are at least five distinct subtypes of mAChR (m1–m5) (40–47). Studies with several neuronal cells suggest that m1, m3, and m4 subtypes may be regulated by PKC. It has been shown that mammalian m2 mAChR purified from cardiac tissue are apparently not substrates for PKC (22, 50). For the present studies we have used purified chick heart mAChR. So far, two subtypes have been reported to be present in chick heart, m2 and m4 (48, 49), with the m2 receptor being expressed at much higher levels than the m4 receptor (49). If the protein levels of the two receptors reflect the amount of mRNA expressed, then the chick heart contains predominantly m2 receptors. Using different expression systems which will allow us to obtain significant amounts of expressed subtypes of mAChR with relative ease, we expect to address the question of which subtypes of mAChR are regulated by PKC in the near future.

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