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Agonist-Dependent Phosphorylation of Human Muscarinic Receptors in *Spodoptera frugiperda* Insect Cell Membranes by G Protein-Coupled Receptor Kinases

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SUMMARY

Agonist-dependent phosphorylation of G protein-coupled receptors (GPRs) by G protein-coupled receptor kinases (GRKs) is proposed to be a key event initiating homologous receptor desensitization. A technical limitation hindering identification of GPRs as GRK substrates has been the necessity to use purified and reconstituted receptors in GRK assays. Here, the human m2 and human m3 (hm3) muscarinic cholinergic receptors (mAChRs), which couple to attenuation of adenylyl cyclase and stimulation of phospholipase C, respectively, were expressed in *Spodoptera frugiperda* insect cells and an *in vitro* approach to studying GPR phosphorylation by GRKs in crude membranes was developed. The m2 mAChR, a known substrate of certain GRKs, was used to validate the approach. The GRK isoform β-adrenergic receptor kinase (βARK)1 phosphorylated the membrane-bound human m2 mAChRs in an agonist-dependent manner. The results demonstrated that endogenous membrane-bound βγ subunits of G proteins stimulated the phosphorylation of the membrane-bound m2 mAChR. To reveal new GRK substrates, we tested the expressed hm3 mAChRs. The membrane-bound hm3 mAChRs were phosphorylated by βARK1 in an agonist-dependent, $G_{\beta\gamma}$-enhanced manner. This is the first demonstration that hm3 mAChRs can serve as substrates for GRKs. The stoichiometry of receptor phosphorylation was ~2 mol of phosphate/mol of receptors in the absence of $G_{\beta\gamma}$ and ~4 mol of phosphate/mol of receptors upon addition of $G_{\beta\gamma}$. When the specificity of various GRKs towards mAChRs was assessed, βARK2 phosphorylated the agonist-activated hm3 mAChRs as efficiently as did βARK1; however, neither GRK5 nor GRK6 significantly phosphorylated the hm3 mAChRs under similar conditions. The approach of studying GRK-mediated phosphorylation of GPRs in their membrane-bound state identified the hm3 mAChRs as new substrates for GRKs. This approach should be valuable in identifying other new substrates of GRKs and should aid in studies that elucidate GRK/GPR pairing.

Membrane receptors trigger various signal transduction pathways within cells in response to diverse ligands. However, these receptors are rapidly inactivated upon continuous agonist exposure, by a process termed "desensitization." Receptor phosphorylation is considered a key event initiating desensitization for at least one class of cell surface receptors, namely the GPRs. A family of protein kinases thought to mediate GPR phosphorylation are the GRKs (1). Based on studies with rhodopsin and the β_2ARs (2, 3), which are the prototypic models for homologous desensitization, it is proposed that GRKs preferentially phosphorylate only the agonist-occupied form of the GPRs (1-3). The phosphorylated GPRs subsequently bind to "arrestins," which reduce the ability of the GPRs to couple to G proteins (4).

The mAChRs represent a major family of GPRs essential in neuronal function. Five mAChR subtypes (m1-m5) have been identified by molecular cloning (5, 6). Among these, two subfamilies exist; the m1, m3, and m5 mAChRs robustly activate phosphoinositide hydrolysis, whereas the m2 and m4 mAChRs attenuate adenylyl cyclase activity and cAMP production. The present study focuses on one subtype from each

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ABBREVIATIONS: GPR, G protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; β_2AR, β_2-adrenergic receptor; βARK, β-adrenergic receptor kinase; GRK, G protein-coupled receptor kinase; mAb, monoclonal antibody; GST-βARK(466–689aa), glutathione-S-transferase/β-adrenergic receptor kinase amino acids 466–689; QNB, 1-quinuclidinylbenzilate; PrBCM, propylbenzylcholine mustard; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; hm2, human m2; hm3, human m3; Sf9, *Spodoptera frugiperda*; $\beta\gamma_t$, $G_{\beta\gamma}$ subunits of transducin purified from bovine retina; $\beta\gamma_b$, $G_{\beta\gamma}$ subunits purified from rat brain.

subfamily, i.e., the m2 and m3 subtypes of mAChRs. The m2 mAChRs appear to be regulated by a variety of mechanisms, including receptor/G protein uncoupling, receptor sequestration and/or internalization, receptor down-regulation, and gene regulation (7). For rapid homologous receptor desensitization, agonist-dependent phosphorylation of the hm2 mAChRs is postulated to be the primary underlying mechanism (7). In support of this hypothesis, agonists have been shown to induce phosphorylation and desensitization of mAChRs in intact cells (8–11). Additionally, purified chick cardiac mAChRs (predominantly the m2 subtype) were phosphorylated *in vitro* by a GRK, the βARK, in an agonist-dependent manner (12). Furthermore, *in vitro* studies with purified and reconstituted hm2 mAChRs demonstrated that they are excellent substrates for the GRK isoforms βARK1 (13–15) and βARK2 (13), less effective substrates for GRK5 (16), and relatively poor substrates for GRK6 (17). In contrast, much less is known about the role of phosphorylation in the regulation of the m3 mAChRs. However, they have been demonstrated to undergo rapid agonist-dependent phosphorylation (18) and desensitization in intact cells (19). It is possible that GRKs mediate this process, although it is not known whether the m3 mAChRs are substrates for any of the GRKs.

Although hundreds of GPRs are predicted to exist in vertebrates, only six GRKs have so far been identified (20–25). This lack of proportion between the number of GRKs and GPRs suggests that perhaps different GRKs recognize different subsets of GPRs as substrates *in vivo*. The basis and nature of the physiological specificities of GRKs for GPRs remain largely uncharacterized. Until now, GRK-mediated phosphorylation of GPRs has been studied using purified and reconstituted GPRs in *in vitro* GRK assays. Despite extensive cloning of GPRs, few receptors have been successfully purified and reconstituted, thereby limiting the use of current *in vitro* GRK assays. An effective and widely applicable assay to study GRK specificity for GPRs is as yet unavailable. We addressed the question of GRK/GPR specificity for the mAChRs by developing an *in vitro* approach in which GPRs in a membrane environment could be screened as substrates for various GRKs. Because most GPRs are rare membrane proteins, it is difficult to detect their phosphorylation state in membranes over background levels of phosphorylation that are normally observed in membranes due to membrane-associated kinase activities. We used an overexpression system to facilitate detection of GRK phosphorylation of GPRs.

In the present effort, the Sf9 insect cell/recombinant baculovirus system was used to express the hm2 and hm3 mAChRs; the mAChRs in insect cell membranes were then tested as substrates for GRKs, i.e., GRK2 (βARK1), GRK3 (βARK2), GRK5, and GRK6. The following questions were asked. Did GRKs phosphorylate the membrane-bound hm2 mAChRs in a manner similar to that seen with *in vitro* reconstitution assays? Were the hm3 mAChRs substrates for GRKs? Were different GRKs specific for or promiscuous towards the mAChRs? Lastly, because βγ subunits of G proteins have been proposed to act as mediators of receptor phosphorylation by GRKs in reconstitution systems, we asked whether such reactions actually occurred in crude membranes. Several novel insights into the identity of GPR substrates and the roles of G proteins in modulating GRKs were obtained. The approaches developed here should allow for future studies designed to test the functional consequences of the multisite phosphorylation of mAChRs that we have observed.

Experimental Procedures

Materials. Sf9 insect cells were obtained from Invitrogen. Sf-900 II insect cell medium and gentamicin were purchased from GIBCO-BRL. Recombinant baculoviruses encoding the hm2 and hm3 mAChRs were kindly provided by Drs. Elliot Ross and E. M. Parker, University of Texas (Dallas, TX), and Dr. E. G. Peralta, Harvard University (Cambridge, MA), respectively. The m3 mAChRs were epitope-tagged on their carboxyl termini with the KT3 epitope from simian virus 40 large T antigen (26). $G_{\beta\gamma}$, purified from bovine brain was a generous gift from Dr. Pat Casey, Duke University (Durham, NC), and $\beta\gamma_t$ was kindly provided by Dr. Heidi Hamm, University of Illinois (Chicago, IL). Purified GRK6 was kindly provided by Robert Loudon, Thomas Jefferson University (Philadelphia, PA). The hybridoma clonal line producing anti-m2 mAChR mAb (27) was a generous gift from Dr. Mike Schimerlik, Oregon Health Sciences University (Corvallis, OR). The hybridoma clonal line producing anti-KT3 mAb (26) was kindly provided by Dr. G. Walter, University of California, San Diego. [γ-^{32}P]ATP, [*phenyl*-^{3}H]QNB, and Amplify were purchased from Amersham Corp. [2,3-^{3}H]PrBCM was purchased from NEN-DuPont. All other reagents were obtained from previously reported sources (8–13).

Cell culture. Sf9 cells were cultured as described previously (28), either in monolayers or in suspension using Sf-900 II serum-free medium supplemented with gentamicin (50 μg/ml).

Preparation of Sf9 cell membranes. Sf9 cells (at a density of 2 × 10^6 cells/ml for suspension cultures or 1 × 10^7 cells/75-cm^2 T flask) were infected with recombinant baculovirus expressing the hm2 or hm3 mAChRs (or wild-type baculovirus), at a multiplicity of infection of 5. All subsequent operations were performed at 0–4°. At 65–72 hr after infection, the cells were harvested at 4000 rpm for 10 min and resuspended at <10^7 cells/ml in buffer A (20 mM Na-HEPES, pH 7.4, 2 mM EDTA, 1 mM $MgCl_2$, 20 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 20 μg/ml leupeptin, 200 μg/ml iodoacetamide, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A). The cells were homogenized by using a Tri-R Stir-R homogenizer (six or seven strokes at a setting of 7.5.), followed by centrifugation at >100,000 × *g* for 30 min. The resulting pellet was resuspended in buffer A to the original volume by two or three strokes with the homogenizer and the centrifugation step was repeated. The final pellet was resuspended in buffer A at a protein concentration of 2–5 mg/ml. The specific activity of the receptors was estimated by radioligand binding assays with [^{3}H]QNB, as described previously (8).

Affinity labeling of hm2 mAChRs. Affinity labeling of the mAChRs with [^{3}H]PrBCM was performed using the protocol supplied by NEN-DuPont. Estimation of nonspecific binding was determined using a final concentration of 10 μM atropine. Membrane samples labeled with [^{3}H]PrBCM were collected by centrifugation, washed, and electrophoresed on SDS gels containing 8% polyacrylamide. Fluorograms were prepared by soaking the stained gels in Amplify for 30 min; the gels were dried and exposed to Kodak X-Omat film at −70° for 2 weeks.

Purification and reconstitution of hm2 mAChRs. The hm2 mAChRs were purified from Sf9 cell membranes and reconstituted into phospholipid vesicles as described previously (13). The specific activity of the purified receptors was determined by ligand binding assays as described previously (8).

Phosphorylation of membrane-bound mAChRs and purified and reconstituted hm2 mAChRs. The GRKs βARK1, βARK2, GRK5, and GRK6 were overexpressed in Sf9 cells using recombinant baculovirus encoding the various GRKs and were purified as described previously (16, 17, 29). The purified kinases exhibited specific activities (using rhodopsin as substrate) as follows: βARK1, 3017 nmol of P_i/min/mg of protein; βARK2, 2069 nmol of P_i/min/mg of protein; GRK5, 1001 nmol of P_i/min/mg of protein; and GRK6, 51 nmol of P_i/min/mg

of protein (16, 17, 29). The GST-βARK(466–689aa) fusion protein was prepared as reported previously (30). In our assay, the maximal effects of GST-βARK(466–689aa) were observed using 15–18 μM. The reported affinity of GST-βARK(466–689aa) for $G_{\beta\gamma}$ is lower than that of βARK; the K_i for GST-βARK(466–689aa) to prevent $G_{\beta\gamma}$-mediated stimulation of βARK is 7 μM (31), whereas the K_d for βARK binding to $G_{\beta\gamma}$ is 25 nM (31). We routinely used 30–50 nM βARK in our assays. Phosphorylation of the purified and reconstituted hm2 mAChRs (0.1–0.5 pmol) was performed as described previously (13). Phosphorylation of membrane-bound mAChRs by GRKs was carried out in a reaction mixture (100 μl) containing 0.3–1.0 pmol of membrane-bound hm2 mAChRs in 20 mM Tris·HCl, pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$, with or without 1 mM carbachol, with or without 1 mM atropine, and with different GRKs (30–50 nM). The reactions were started by addition of 0.05 mM [γ-^{32}]ATP (500–1000 cpm/pmol) and were incubated at 37° for 15 min or the times indicated. The reactions were stopped by addition of 25 μl of 5× SDS sample buffer (0.0625 M Tris·HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glyceral, 0.001% bromphenol blue) and were electrophoresed on SDS gels containing 6–8% polyacrylamide, using the method of Laemmli (32). Phosphoproteins were visualized and quantified by phosphorimaging, using a BAS 2000 Fuji Bioimage analyzer. Studies with $G_{\beta\gamma}$ subunits ($\beta\gamma_b$, 5–50 nM; $\beta\gamma_t$, 100–200 nM) and GST-βARK(466–689aa) fusion protein (2–18 μM) were performed by the direct addition of these proteins to the *in vitro* assay.

Determination of the stoichiometry of receptor phosphorylation. Basal levels of phosphorylation that were observed with the membrane-bound mAChRs in the presence of the antagonist atropine (or in the absence of the agonist) were normalized to zero in all experiments. No difference in the extent of basal phosphorylation of receptors was observed when atropine was used or no drug was added. This basal phosphorylation varied between 0.1 and 0.5 mol of phosphate/mol of receptors. Agonist-dependent phosphorylation of the receptors was determined as the increase in phosphorylation in the presence of the agonist carbachol. The stoichiometry of phosphorylation of the receptors was expressed as moles of phosphate/mole of receptors.

Immunoprecipitation of membrane-bound hm2 mAChRs. After phosphorylation as described above, the reactions were stopped by addition of NaF to a final concentration of 20 mM. Phosphorylated receptors were solubilized in 1 ml of buffer B (20 mM potassium phosphate, pH 7.4, 5 mM EGTA, 5 mM EDTA, 20 mM NaF) containing 0.8% digitonin and 0.16% cholate. The samples were sonicated on ice for 10 min at 1 pulse/second and then centrifuged at >100,000 × *g* for 1 hr. The resulting supernatants were added to a mixture of Protein G-agarose/anti-m2 mAb that had been preincubated at 4° for 2 hr in buffer B. Solubilized receptors were incubated with the Protein G-agarose/antibody complex for 4 hr at 4°. The beads were collected and washed and receptors were eluted with SDS sample buffer. Immunoprecipitated phosphorylated receptors were visualized by phosphorimaging after SDS-polyacrylamide gel electrophoresis.

Urea treatment of Sf9 cell membranes. Sf9 cell membranes (2–5 mg/ml) containing the expressed hm2 or hm3 mAChRs were treated with varying concentrations of urea (1–5 M) according to procedures modified from those used for stripping rod outer segments of various peripheral proteins, including rhodopsin kinase, with urea (33). The urea-treated membranes were sonicated on ice for 10–15 min, followed by centrifugation at >100,000 × *g* for 30 min. The resulting pellets were rinsed repeatedly to remove excess urea and were finally resuspended in buffer A to the original volume. Recoveries of receptors and total membrane protein were determined by ligand binding and protein assays.

Results

Agonist-dependent phosphorylation of hm2 mAChRs in Sf9 cell membranes. In this study, we attempted to determine whether GRK-mediated phosphorylation of the mAChRs could be analyzed directly in Sf9 cell membranes. Using the conditions described in Experimental Procedures, with Sf9 cell membranes containing 15–35 pmol of expressed hm2 mAChRs/mg of protein, we first asked whether the hm2 mAChRs in Sf9 cell membranes could be phosphorylated in an agonist-dependent manner with βARK1. The results showed that, in the presence of the agonist carbachol and βARK1, the hm2 mAChRs (at 57–59 kDa) underwent striking agonist-dependent phosphorylation (Fig. 1A). The stoichiometry of agonist-dependent phosphorylation of the hm2 mAChRs averaged 1–2 mol of phosphate/mol of receptors over basal phosphorylation. βARK1-mediated phosphorylation of the hm2 mAChRs was inhibited by heparin, a nonspecific inhibitor of GRKs (34), but not by staurosporine, an inhibitor of protein kinase C and other protein kinases (Fig. 1A). [Heparin is a nonselective inhibitor of GRKs and other proteins; the effect

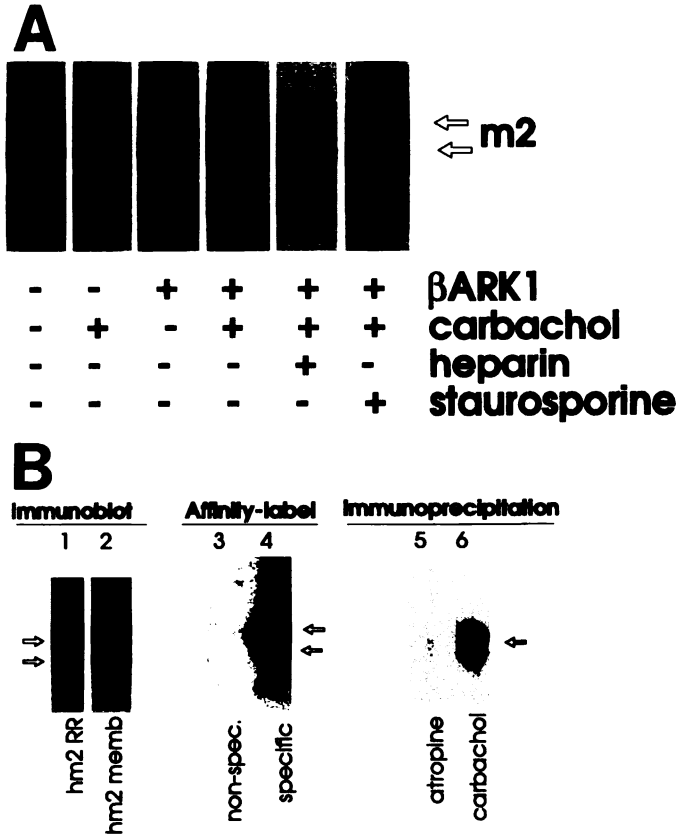

Fig. 1. Agonist-dependent phosphorylation of the hm2 mAChRs in Sf9 insect cell membranes by βARK1. A, Phosphorimage of an SDS gel containing phosphorylated membranes prepared from Sf9 cells expressing hm2 mAChRs. Receptors (0.5 pmol) were phosphorylated with (*lanes 3-6*) or without (*lanes 1* and *2*) βARK1 (45 nM). Reactions contained carbachol (*lanes 2* and *4-6*), heparin (1 μM) (*lane 5*), or staurosporine (100 nM) (*lane 6*) as indicated. Reactions were incubated at 37° for 15 min and processed on an SDS-polyacrylamide (8%) gel as described in Experimental Procedures. B, *Left*, immunoblot identifying the expressed hm2 mAChRs in Sf9 cells. *Lane 1*, hm2 mAChRs (0.5 pmol) purified from Sf9 cells and reconstituted in liposomes; *lane 2*, hm2 mAChRs (0.5 pmol) in Sf9 membranes. Immunoreactive protein was detected with an anti-m2 mAb, using the enhanced chemiluminescence method. *Center*, hm2 mAChRs in Sf9 membranes that were affinity labeled with [^{3}H]PrBCM, an irreversible mAChR antagonist, in the presence (*lane 3*) or absence (*lane 4*) of atropine as described in the text. *Right*, hm2 mAChRs that were phosphorylated in membranes by βARK1 in the absence (*lane 5*) or presence (*lane 6*) of carbachol and [γ-^{32}-P]ATP, then immunoprecipitated with anti-m2 mAb, and visualized as described in Experimental Procedures. *Arrows* indicate position of receptor.

observed here could also have involved an inhibition of receptor/G protein coupling by heparin (35, 36), which could affect the extent of phosphorylation (see below).] No agonist-dependent phosphorylation was observed in the absence or presence of added GRKs in membranes prepared from noninfected Sf9 cells or cells infected with wild-type baculovirus (data not shown). In addition, in most experiments no agonist-dependent phosphorylation of the hm2 mAChRs was observed in the absence of added GRK (Fig. 1A). However, in a few experiments a low level of agonist-dependent phosphorylation was observed in the absence of added GRKs (<0.5 mol of phosphate/mol of receptors). This was likely due to the activity of endogenous insect GRKs. Our laboratory previously suggested that endogenous GRKs in insect cells recognized the hm2 mAChRs in phosphorylation and desensitization assays (11).

To provide additional evidence that the protein phosphorylated in an agonist-dependent manner by βARK1 was indeed the hm2 mAChR, the hm2 mAChRs in Sf9 membranes were identified using three distinct strategies. First, immunoblotting techniques were used to identify the receptors with an anti-m2 mAb. Two receptor species, which co-migrated with the βARK-phosphorylated protein, were identified with the anti-m2 mAb in Sf9 membranes (Fig. 1B). The primary protein identified was a 57–59-kDa band, similar to the hm2 mAChRs purified from Sf9 cells (Fig. 1B) and previously described by our laboratory and others (11, 13, 37, 38). A minor species at 51 kDa was also detected (Fig. 1B). Presumably, the larger form reflects glycosylated receptors (38), whereas the smaller form may represent either nonglycosylated receptors or a breakdown product of the native receptor. In a second series of experiments, we identified the hm2 mAChRs by specific affinity labeling with the mAChR antagonist [^{3}H]PrBCM. The same two species, at approximately 51 kDa and 57–59 kDa, were identified by affinity labeling (Fig. 1B). Finally, immunoprecipitation of the hm2 mAChRs with the anti-m2 mAb was performed after *in vitro* membrane phosphorylation with βARK1 in the presence of carbachol or atropine. The 57–59-kDa species was immunoprecipitated and demonstrated to be phosphorylated only in the presence of agonist (Fig. 1B). These results showed that the membrane protein that became phosphorylated in an agonist-dependent manner by βARK1 was indeed the hm2 mAChR. The detection of more than one species of receptor with the anti-m2 mAb and affinity label correlated well with our observation that sometimes two proteins were phosphorylated in an agonist-dependent manner by βARK1, although the larger species was the dominant phosphorylated form (as seen in Fig. 1B). The other phosphoprotein that migrated slightly faster than the m2 mAChR (Fig. 1A, *lanes 1* and *2*) does not appear to be an m2 receptor, because it was stripped easily by urea treatment (see Fig. 5B) and it was not immunoprecipitated with the m2 mAb. The phosphorylation of this protein was neither increased by carbachol nor affected by atropine. However, as noted above, in some experiments the minor (51-kDa) mAChR species co-migrated with the phosphoprotein described above, making it difficult to clearly differentiate this receptor from the nonreceptor protein.

Agonist concentration dependence of phosphorylation of hm2 mAChRs by βARK1. We further characterized the agonist-dependent phosphorylation of the membrane-bound hm2 mAChRs by βARK1. A clear dose-dependent effect of agonist to induce phosphorylation of the hm2 mAChRs by βARK1 was observed (Fig. 2). In these experiments, the maximum stoichiometry of receptor phosphorylation averaged 2 mol of phosphate/mol of receptors. The EC_{50} value for carbachol to stimulate βARK-mediated phosphorylation of the hm2 mAChRs was 11.5 μM. This value correlated well with previously published EC_{50} values for carbachol to induce phosphorylation of the mAChRs expressed in intact cardiac cells (9).

Time course and extent of phosphorylation of hm2 mAChRs and role of G proteins. We next determined the time course of receptor phosphorylation by βARK1 over the range of 0–90 min (Fig. 3A). In the presence of atropine, basal receptor phosphorylation of 0.4 mol of phosphate/mol of receptors was observed. When carbachol was added, βARK1 markedly increased receptor phosphorylation by 1.5 mol of phosphate/mol of receptors above that observed in the presence of atropine. Agonist-dependent phosphorylation of the hm2 mAChRs was maximal at 30 min, after which it decreased slightly, perhaps because of membrane-associated phosphatases (Fig. 3A).

The βγ subunits of heterotrimeric G proteins have been shown to physically interact with βARKs (30, 31, 39) and to enhance phosphorylation of receptor substrates in reconstituted systems (13–15, 29). We determined whether the addition of purified $G_{\beta\gamma}$ subunits could potentiate βARK1-mediated phosphorylation of membrane-bound receptors. $\beta\gamma_t$ and $\beta\gamma_b$ stimulated βARK1-mediated phosphorylation of the membrane-bound hm2 mAChRs (Fig. 3A). Both $G_{\beta\gamma}$ subunits markedly increased the initial rates of receptor phosphorylation by βARK1 (Fig. 3A). Furthermore, $\beta\gamma_b$ enhanced the extent of phosphorylation >2-fold and was far more effective than $\beta\gamma_t$ in this regard. These results supported a role for $G_{\beta\gamma}$ subunits

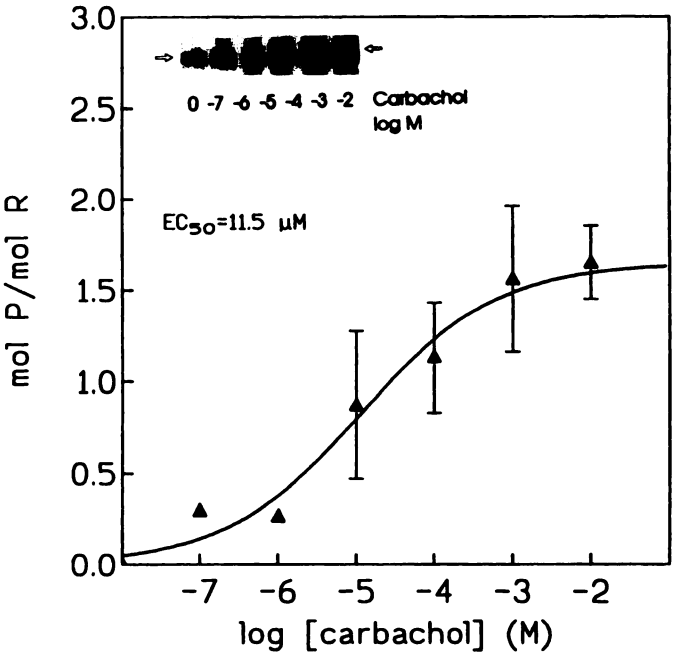

Fig. 2. Agonist concentration dependence of phosphorylation of the hm2 mAChRs with βARK1. The concentration-dependent effect of the agonist carbachol to stimulate βARK-mediated phosphorylation of the hm2 mAChRs is shown. *Inset*, phosphorimage of an SDS gel containing the phosphorylated receptors from a representative experiment. Data presented are the means ± standard errors of three independent experiments. *mol P/mol R*, mol of phosphate/mol of receptor. *Arrows* indicate position of receptor.

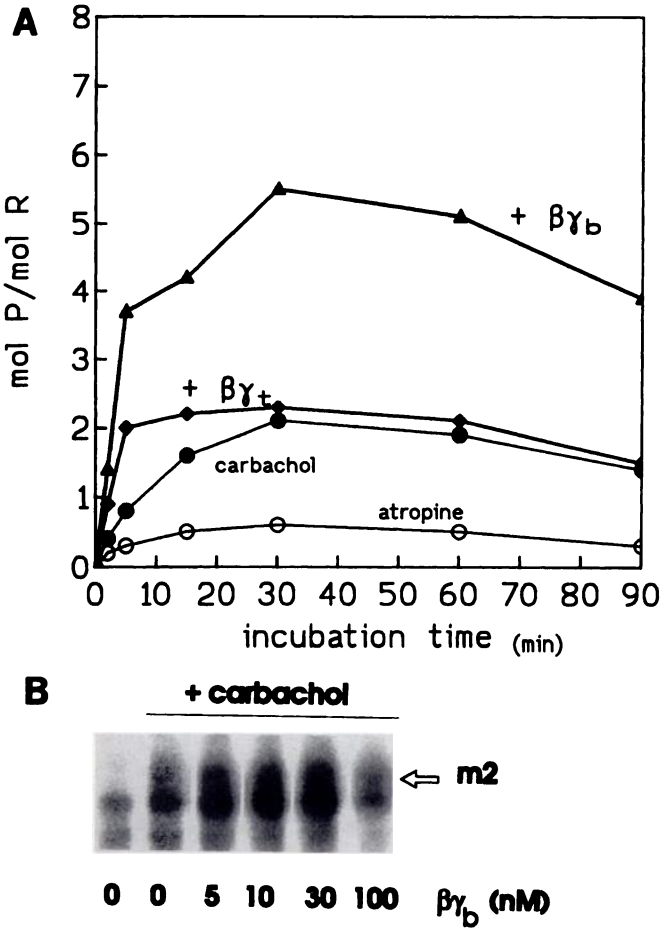

Fig. 3. Time course of receptor phosphorylation by βARKs. A, Representation of the data showing both rate and extent of phosphorylation of the hm2 mAChRs (0.3 pmol) in Sf9 cell membranes by βARK1 (50 nM), over the time course of 0–90 min. Phosphorylation of receptors was carried out in the presence of either atropine or carbachol. Either $\beta\gamma_t$ (100 nM) or $\beta\gamma_b$ (50 nM) was added to reactions containing carbachol, as indicated. *mol P/mol R*, mol of phosphate/mol of receptor. B, Phosphorimage depicting the concentration-dependent effect of $\beta\gamma_b$ (5–100 nM) on βARK1-mediated phosphorylation of the hm2 mAChRs in Sf9 cell membranes.

in enhancing not only the extent but also the rate of membrane-bound receptor phosphorylation by βARK1.

The concentration-dependent stimulation by $\beta\gamma_b$ of βARK1 activity was investigated (Fig. 3B). In the experiment shown, low concentrations of $\beta\gamma_b$ (10–30 nM) maximally enhanced the βARK-mediated phosphorylation of the hm2 mAChRs, whereas higher concentrations of $\beta\gamma_b$ produced a slight inhibition of receptor phosphorylation. In other experiments it was determined that 50–100 nM $\beta\gamma_b$ was required to maximally stimulate βARK1 activity (data not shown). We speculated that a variable amount of endogenous insect $G_{\beta\gamma}$ remained associated with each membrane preparation and that this variable pool of insect $G_{\beta\gamma}$ could actively contribute to receptor phosphorylation by βARKs (see below), modifying the amount of exogenous $\beta\gamma_b$ necessary to maximally potentiate βARK activity. The small inhibition of βARK by $\beta\gamma_b$ that we observed at high concentrations may have been due to the masking of phosphorylation sites on the receptor (13, 15) or βγ acting as a sink for βARK and thus preventing its presentation to substrate. In parallel experiments, results with $\beta\gamma_t$ showed that it was both less efficacious (only up to 40% stimulation of βARK) and less potent (200 nM to achieve maximal effects) than $\beta\gamma_b$ (data not shown). These results are consistent with the observation that $\beta\gamma_t$ was less potent than $\beta\gamma_b$ in the β_2AR system (30).

Role for endogenous membrane-associated G proteins in βARK-mediated phosphorylation of membrane-bound receptors. A critical question concerning the role of $G_{\beta\gamma}$ in GRK activation is whether endogenous membrane-associated G proteins perform the role suggested by studies using model systems and exogenously added $G_{\beta\gamma}$ (14, 15, 30, 31, 39). We asked whether the endogenous insect G proteins played a role in the receptor phosphorylation mediated by βARK1 in the absence of added $G_{\beta\gamma}$.

Firstly, the ability of the guanine nucleotide analogs GTPγS and GDPβS to modulate βARK1-mediated phosphorylation of the hm2 mAChRs in Sf9 membranes was assessed. If endogenous $G_{\beta\gamma}$ indeed contributed to βARK activity in Sf9 membranes, then GTPγS would potentiate and GDPβS would inhibit receptor phosphorylation by βARK1 in this membrane assay, by shifting the equilibrium between the G_α and $G_{\beta\gamma}$ subunits of insect G proteins toward dissociation (GTPγS) or reassociation (GDPβS). The results indicated that GDPβS strongly inhibited and GTPγS stimulated agonist-dependent receptor phosphorylation by βARK1 (Fig. 4A); both analogs acted in a concentration-dependent manner.

Secondly, we evaluated the effects of the GST-βARK(466–689aa) fusion protein, which includes the carboxyl terminus of βARK1 and contains a $G_{\beta\gamma}$ binding domain (30) and which has been shown to inhibit $G_{\beta\gamma}$ effects in reconstituted systems with a K_i of 7 μM (31). When the GST-βARK(466–689aa) fusion protein was added to membranes, βARK1-mediated phosphorylation of the hm2 mAChRs was inhibited significantly (40%) (Fig. 4B). To further confirm that the GST-βARK(466–689aa) protein inhibited $G_{\beta\gamma}$ in membranes, we tested whether it could inhibit the stimulatory effects of added $\beta\gamma_b$ and $\beta\gamma_t$. GST-βARK(466–689aa) effectively inhibited $\beta\gamma_b$ and $\beta\gamma_t$ effects (Fig. 4B). The effects of GST-βARK(466–689aa) occurred in a concentration-dependent manner (data not shown).

The third approach taken to evaluate the role of endogenous $G_{\beta\gamma}$ was to determine the effects of high salt concentrations, which are known to inhibit βARK1 *in vitro* in reconstituted systems (40). However, phosphorylation experiments with rhodopsin and βARK1 showed that $G_{\beta\gamma}$ protected against salt-induced inhibition (39). We observed that the βARK1-mediated phosphorylation of the membrane-bound hm2 mAChRs was insensitive to salt, whereas the βARK1-mediated phosphorylation of the purified and reconstituted hm2 mAChRs was completely inhibited by salt unless $G_{\beta\gamma}$ was added (data not shown). The cumulative results of these different approaches provide consistent evidence that membrane-associated $G_{\beta\gamma}$ subunits participate in the βARK1-mediated phosphorylation of the membrane-bound hm2 mAChRs.

Stripping of hm2 mAChRs in Sf9 cell membranes. Detection of GRK-mediated phosphorylation of the hm2 mAChRs in Sf9 membranes was often hampered by high background levels of phosphorylation of other membrane proteins. We assessed whether we could strip off a significant amount of membrane proteins to reduce this background phosphorylation. The rationale and approach used were based on previous studies with the GPR rhodopsin, in which urea was successfully used to strip peripheral proteins from rod outer segments (33).

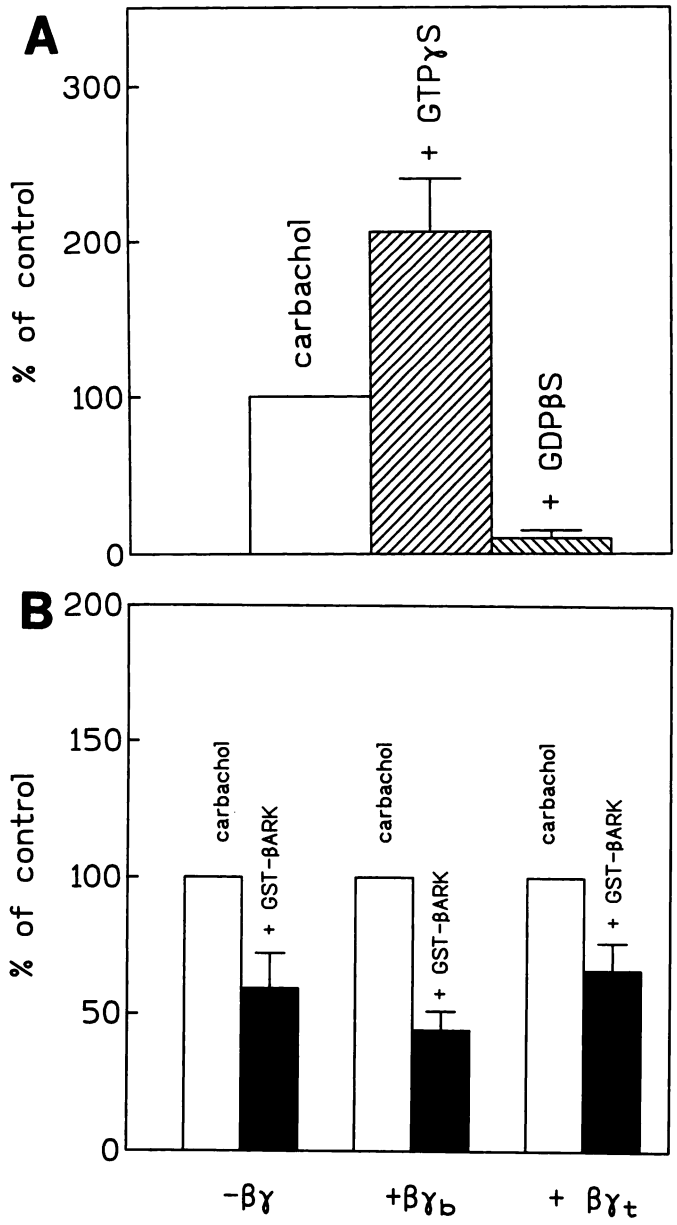

Fig. 4. Role of endogenous G proteins in receptor phosphorylation by βARK1 in Sf9 membranes. A, Effects of guanine nucleotide analogs. The modulation of βARK-mediated phosphorylation of the hm2 mAChRs by guanine nucleotide analogs is shown. Either GTPγS or GDPβS (100 μM each) was added to βARK1 phosphorylation assays containing membrane-bound hm2 mAChRs and carbachol. The stoichiometry of agonist-dependent phosphorylation of the hm2 mAChRs in the presence of carbachol and in the absence of added guanine nucleotide analogs was set to 100% (control), and that observed in the presence of guanine nucleotides was normalized to this value. Data shown are means ± standard errors from three independent sets of experiments. B, Effects of the GST-βARK(466–689aa) fusion protein. The reactions contained carbachol and GST-βARK(466–689aa) (*GST-βARK*) (0–18 μM), with either no added $G_{\beta\gamma}$, $\beta\gamma_b$ (10–30 nM), or $\beta\gamma_t$ (100 nM) as indicated. For each set of reactions, the stoichiometry of agonist-dependent receptor phosphorylation in the presence of carbachol and the absence of added GST-βARK(466–689aa) was normalized to 100%. The data presented are means ± standard errors of three or four independent experiments.

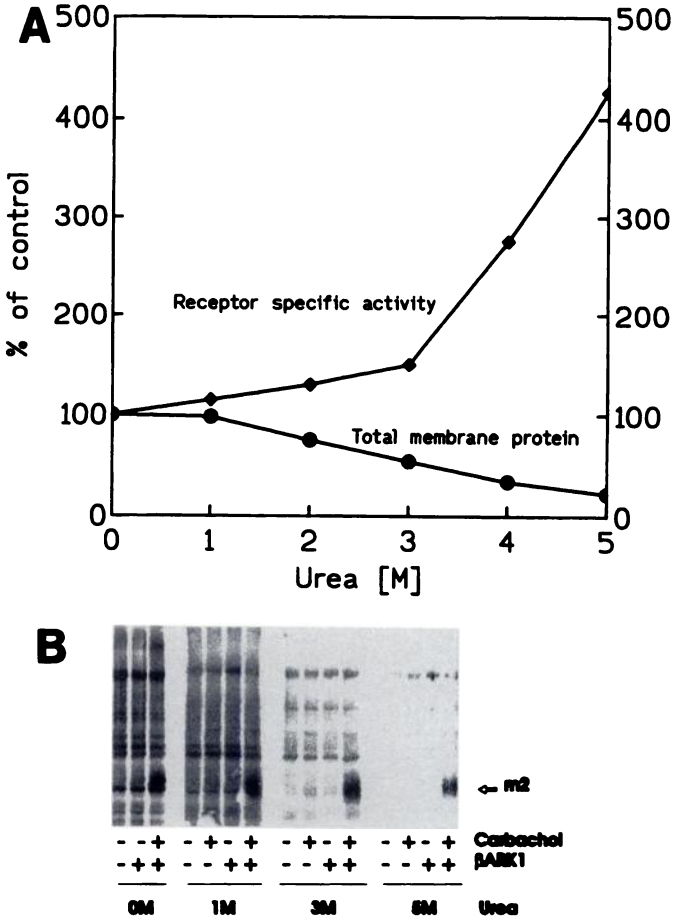

Fig. 5. Effects of urea on density and phosphorylation of the hm2 mAChRs in Sf9 membranes. A, Recovery of total membrane protein and specific activity of the hm2 mAChRs in Sf9 membranes after treatment of membranes with increasing concentrations of urea is shown. Treatments were performed as described in Experimental Procedures. The values for protein content and receptor specific activity, measured as pmol of [^{3}H]QNB binding activity/mg of protein, in the absence of urea (0 urea) were set to 100%. The total content of [^{3}H]QNB binding activity was unaffected by urea treatment, whereas the protein content was decreased by 4-fold. Thus, the specific activity of receptor binding was increased ~4-fold. B, Equal amounts of receptors from membranes treated with 0–5 M urea were phosphorylated by βARK1 in the presence and absence of carbachol. Shown is a phosphorimage of these phosphorylation reactions for membrane samples treated with 0, 1, 3, or 5 M urea. This experiment was repeated three times, with similar results.

Pretreatment of membranes containing the hm2 mAChRs with 0–5 M urea resulted in a concentration-dependent decrease in total membrane protein content of 75% (Fig. 5A), whereas there was no loss of receptor binding activity (data not shown). As a result of the decreased membrane protein, the concentration of the hm2 mAChRs in membranes was enriched 4-fold (Fig. 5A). Agonist-dependent phosphorylation of the urea-treated hm2 mAChRs by βARK1 remained unperturbed when tested with membranes that had been treated with up to 4 M urea and was equivalent to that observed with non-urea-treated receptors (Fig. 5B). Urea, at concentrations of up to 4 M, did not perturb either ligand binding to receptors or the ability of GRKs to recognize the agonist-occupied receptors. At the highest concentration of urea tested (5 M), the amount of agonist-dependent phosphorylation of hm2 mAChRs was slightly reduced (Fig. 5B). The most striking observation was the significant decrease in background membrane phosphorylation with increasing concentrations of urea (Fig. 5B), which markedly facilitated visualization of phosphorylated receptors.

Agonist-dependent phosphorylation of hm3 mAChRs by GRKs in Sf9 cell membranes. An important goal of this

study was to determine whether the hm3 mAChRs could serve as substrates for GRKs. The hm3 mAChRs were expressed in Sf9 cells and membranes were prepared that contained 5–20 pmol of hm3 mAChRs/mg of protein. A significantly high background phosphorylation was observed in membranes from hm3 mAChR-expressing Sf9 cells, and in preliminary studies GRK-phosphorylated receptors could not be detected. To reduce background phosphorylation, membranes containing the hm3 mAChRs were pretreated with 3–4 M urea, and they were then phosphorylated with different GRKs. As shown with membranes containing hm2 mAChRs, urea did not significantly affect ligand binding to the hm3 mAChRs and greatly reduced background phosphorylation (data not shown).

The hm3 mAChRs in urea-stripped membranes were found to be effectively phosphorylated by βARK1 in an agonist-dependent fashion that compared well with phosphorylation of urea-treated hm2 mAChRs (Fig. 6). The phosphorylated hm3 mAChRs migrated as a doublet of 65–70 kDa. As stated earlier, no agonist-dependent phosphorylation was observed in membranes from noninfected cells or cells infected with wild-type virus. Data from several experiments indicated that the stoichiometries of agonist-stimulated phosphorylated hm3 mAChRs averaged 2 mol of phosphate/mol of receptors. Furthermore, $\beta\gamma_b$ markedly enhanced βARK1-mediated phosphorylation of the expressed hm3 mAChRs by 1.5–2-fold, to a total of ~4 mol of phosphate/mol of receptors (Fig. 6). The identity of the 65–70-kDa doublet as the phosphorylated hm3 mAChR was confirmed using the anti-KT3 antibody (data not shown). The protein that exhibited agonist-dependent phosphorylation in the membranes from cells infected with the m3 baculovirus co-migrated with immunoreactive m3 mAChR tagged with the KT3 epitope (data not shown). Because the predicted size of the hm3 mAChRs is 66 kDa, the larger of these two proteins may be a glycosylated species. These results with hm3 mAChRs are the first to demonstrate that these receptors are substrates for a GRK.

Specificity of various GRKs for hm3 mAChRs in native membranes. Another aim in studying the phosphorylation of membrane-bound receptors by GRKs was to test the ability of various GRKs to recognize different mAChRs as substrates and to determine whether they displayed specificity within subtypes of mAChRs. So far, five of the six GRKs cloned are available as purified enzymes, namely rhodopsin kinase, βARK1 (GRK2), βARK2 (GRK3), GRK5, and GRK6. Rhodopsin kinase is expressed primarily in the vertebrate retina and pineal gland and does not phosphorylate purified and reconstituted hm2 mAChRs (14). Therefore, we tested the membrane-bound hm3 mAChRs as substrates for βARK1, βARK2, GRK5, and GRK6, using identical experimental conditions. The hm3 mAChRs were equally well phosphorylated by both βARK isozymes, with stoichiometries of ~1–2 mol of phosphate/mol of receptors (Fig. 7); in contrast to what was observed with βARK1 and βARK2, neither GRK5 nor GRK6 recognized the agonist-occupied hm3 mAChRs as substrates, in the presence or absence of added $G_{\beta\gamma}$ (Fig. 7). Addition of $\beta\gamma_b$ enhanced receptor phosphorylation by both βARK isozymes by 2-fold, up to a stoichiometry of ~4 mol of phosphate/mol of receptors (Fig. 7). A similar profile for GRK specificities was obtained with the membrane-bound hm2 mAChRs (data not shown); these results agreed well with previously published studies with purified and reconstituted hm2 mAChRs, which showed that the m2 mAChRs were excellent substrates *in vitro* for βARK1 and βARK2 and poor substrates for GRK5 and GRK6 (13, 16, 17). A notable difference, though, was that both GRK5 and GRK6 slightly increased hm2 mAChR phosphorylation over basal levels, in an agonist-independent manner, up to 0.5 mol of phosphate/mol of receptors (data not shown). Most of the experiments reported here with βARK1 and hm2 mAChRs were also performed with βARK2, and no significant differences were revealed between the isoforms (data not shown).

Characterization of hm3 mAChR phosphorylation. In time course experiments, in the presence of $G_{\beta\gamma}$, we observed that receptor phosphorylation by βARKs was rapid and the $t_{1/2}$ was <10 min (data not shown). We characterized the concentration dependence of agonists to induce phosphorylation of the hm3 mAChRs by βARK1 and βARK2 (Fig. 8, *inset*). The agonist carbachol induced phosphorylation of the hm3

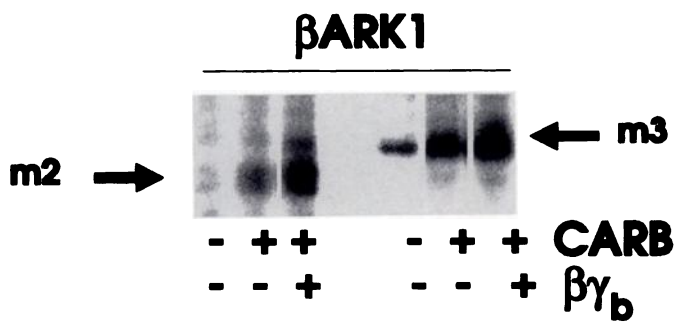

Fig. 6. Agonist-dependent phosphorylation of the hm3 mAChRs by GRKs in Sf9 membranes. Depicted is a phosphorimage comparing agonist-dependent phosphorylation of the hm2 and hm3 mAChRs by βARK1 in Sf9 membranes. Urea-treated Sf9 membranes containing the hm2 mAChRs (0.2 pmol) (*left*) or the hm3 mAChRs (0.2 pmol) (*right*) were phosphorylated with βARK1 (50 nM) under the indicated conditions. *CARB*, carbachol.

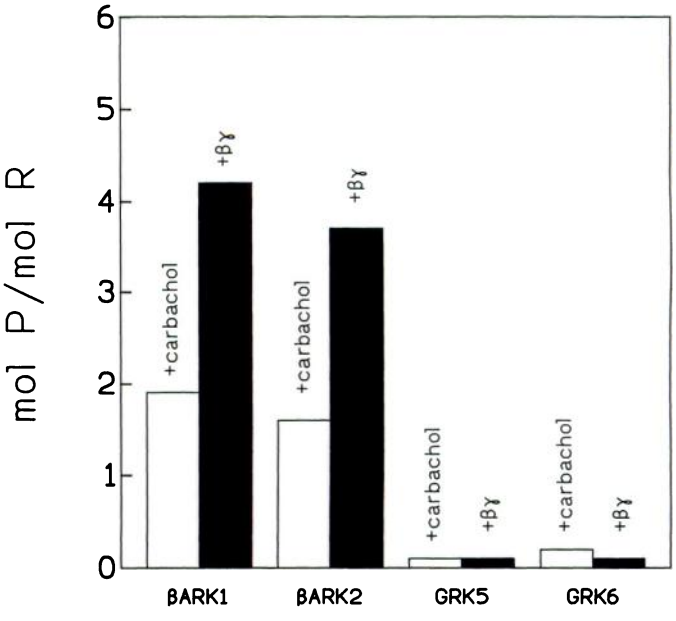

Fig. 7. GRK specificity for the hm3 mAChRs in Sf9 membranes. The ability of βARK1, βARK2, GRK5, and GRK6 to mediate agonist-dependent phosphorylation of the hm3 mAChRs in urea-treated membranes in the absence (□) and presence (■) of $\beta\gamma_b$ (10–50 nM) is shown. Receptor phosphorylation in the presence of atropine was taken as zero and the stoichiometry of agonist-dependent receptor phosphorylation is shown in mol of phosphate/mol of receptors (*mol P/mol R*). The data shown are means from two independent experiments.

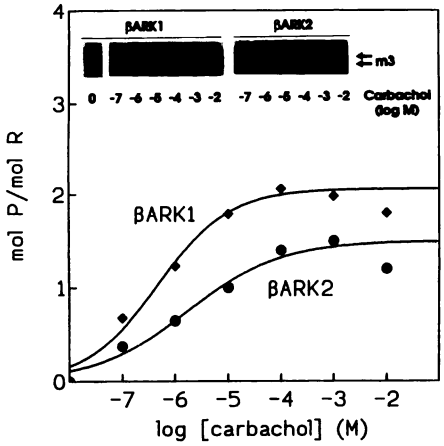

Fig. 8. Agonist concentration dependence of phosphorylation of hm3 mAChRs by βARKs. The concentration-dependent effect of carbachol to induce phosphorylation of the hm3 mAChRs by βARK1 and βARK2 is shown. Agonist-dependent phosphorylation of receptors in the presence of atropine was taken as zero. *mol P/mol R*, mol of phosphate/mol of receptor. *Inset*, phosphorimage of this experiment.

mAChRs by βARK1, in a concentration-dependent manner, up to a stoichiometry of ~2 mol of phosphate/mol of receptors (Fig. 8). The EC_{50} for the βARK1-mediated phosphorylation was 0.8 μM. These experiments were performed in the absence of $G_{\beta\gamma}$. Similar results were obtained with βARK2. The concentration dependence of carbachol effects on βARK2-mediated phosphorylation of the hm3 receptor showed that the EC_{50} was 1.3 μM. The maximal stoichiometry of phosphorylation catalyzed by βARK2 was ~1.5 mol of phosphate/mol of receptors in the absence of $G_{\beta\gamma}$ (Fig. 8). These results demonstrated that βARK2 phosphorylated the membrane-bound hm3 mAChRs almost as effectively as did βARK1.

Discussion

In this report, an *in vitro* approach to examine GRK-mediated phosphorylation of mAChRs in membranes was used to reveal novel substrates for GRKs, address GRK/GPR pairing among mAChRs, and assess the role of G proteins in GRK-mediated phosphorylation of membrane-bound mAChRs. Our results showed that βARK1 and βARK2 equivalently phosphorylated the hm2 mAChRs in Sf9 membranes, in an agonist-dependent manner. Both endogenous insect $G_{\beta\gamma}$ and purified exogenous $G_{\beta\gamma}$ stimulated receptor phosphorylation by βARKs in native membranes, and these $G_{\beta\gamma}$ effects were inhibited by a GST-βARK(466–689aa) fusion protein. A membrane-stripping approach was used to significantly decrease background phosphorylation in membranes and enhanced our ability to visualize agonist-dependent GPR phosphorylation without affecting receptor function. The applicability of this membrane assay for screening other GPRs for GRK specificities was successfully tested with the hm3 mAChRs, a novel GRK substrate.

Several important new findings have emerged from the present study. This report is the first to demonstrate that the hm3 mAChRs are substrates for the GRKs. A previous study demonstrated that the hm3 mAChRs were rapidly phosphorylated in intact CHO cells in an agonist-dependent manner (18). The kinases mediating phosphorylation of the hm3 mAChRs in these cells were not identified; however, a recent study showed that a heparin-insensitive, membrane-associated kinase was capable of mediating agonist-dependent phosphorylation of the hm3 mAChRs in CHO cell membranes (41). Whether this membrane-associated kinase is a GRK is unknown. The present study demonstrated that the hm3 mAChRs are substrates *in vitro* for βARK1 and βARK2. The stoichiometry of phosphorylation of the hm3 mAChR was ~2 mol of phosphate/mol of receptors in the absence of $G_{\beta\gamma}$ and ~4 mol of phosphate/mol of receptors in the presence of $G_{\beta\gamma}$. The time course of receptor phosphorylation was rapid (in the presence of added $G_{\beta\gamma}$). We also observed that, in the absence of added GRK, the hm3 receptor was phosphorylated (0.3 mol of phosphate/mol of receptors). These present observations are in agreement with previous *in vivo* labeling studies with hm3 mAChR-expressing CHO cells (18). Furthermore, our laboratory has determined by reverse transcriptase-polymerase chain reaction analysis that CHO cells express both βARK isoforms.[1] Taken together, these results suggest that βARKs may be involved in the regulation of hm3 mAChRs *in vivo*.

The βARK isoforms are almost ubiquitous in their tissue distribution and, so far, few differences in their specificities have been determined (1). To date only two exceptions, one with olfactory receptors (42–44) and the other with thrombin receptors (45), have been reported. With olfactory receptors, βARK2 was shown to exhibit a marked specificity over βARK1 as a result of its preferential expression in olfactory receptor neurons (42–44), whereas βARK2 was specific for thrombin receptors even when both isoforms were overexpressed in the same cells (45). Therefore, although it is not entirely surprising that both βARK isozymes recognized the hm3 mAChRs in membranes, it will be important to determine whether βARK1 or βARK2 is responsible for the phosphorylation of the hm3 mAChRs *in vivo*. Along with the substance P (46) and thrombin (45) receptors, the hm3 mAChR becomes the third type of phosphoinositide-linked GPR that has been found to be recognized by GRKs.

A second notable finding was that GRK5 and GRK6 did not recognize either the membrane-bound hm3 or hm2 mAChRs in an agonist-dependent manner. Little is known about the substrate specificity of GRK5 and GRK6. Both GRK5 and GRK6 phosphorylate the m2 mAChR, rhodopsin, and the β_2AR; however, these receptors are all relatively poor substrates for these GRKs, compared with βARK (16, 17). For example, the stoichiometries of phosphorylation of the m2 mAChR were 4–10 mol of phosphate/mol of receptors for the βARK isoforms (13) and ~1 and 0.6 mol of phosphate/mol of receptors for GRK5 and GRK6, respectively (16, 17). Although the present studies further suggest that hm3 and hm2 mAChRs may not be substrates for GRK5 and GRK6 *in vivo*, it is possible that unidentified components essential for GRK5 and GRK6 activity may be missing from the membrane system developed here.

A third conclusion from the present work is that endogenous membrane-associated $G_{\beta\gamma}$ participates in modulating βARK activity toward ligand-activated GPRs, lending critical support to the current proposal that $G_{\beta\gamma}$ activates GRKs (1). Guanine nucleotide analogs regulate the availability of dissociated (free) $G_{\beta\gamma}$, and the GST-βARK(466–689aa) fusion protein binds only to dissociated $G_{\beta\gamma}$ *in vitro* (30). The results obtained with these modulators strongly suggested that the level of membrane-bound $G_{\beta\gamma}$ was important in determining how efficiently βARKs phosphorylated their substrates. The effects of activation of

[1] R. P. Rylaarsdam and M. M. Hosey, unpublished observations.

endogenous or exogenous G proteins by receptors might have led to activation of other signaling pathways that could have contributed to the phosphorylation observed. However, the agonist-dependent phosphorylation was not inhibited by staurosporine, which inhibits protein kinase C and other kinases (Fig. 1). The GST-βARK(466–689aa) fusion protein only partially inhibited βARK-mediated phosphorylation of the hm2 mAChRs. In this regard, many studies showed that both heterotrimeric G protein- and ligand-activated GPRs stimulated GRK function *in vitro* (13, 39, 47–49). In the present study, holo-G proteins could have contributed to βARK-mediated phosphorylation of the membrane-bound mAChRs.

In this study, we characterized in detail the phosphorylation of the hm2 mAChRs as model GPRs in the membrane system, because agonist-dependent phosphorylation of the hm2 mAChRs was previously investigated both in intact tissues and in *in vitro* reconstitution systems, offering a basis for comparative evaluation (8–15). Upon comparison of the results from different systems, one significant difference was evident, concerning the stoichiometry of agonist-dependent phosphorylation of the hm2 mAChRs determined by these different approaches. In the present studies, the hm2 mAChRs were phosphorylated to only 1–2 mol of phosphate/mol of receptors without addition of exogenous $G_{\beta\gamma}$, whereas addition of $G_{\beta\gamma}$ increased receptor phosphorylation by βARKs to 4–5 mol of phosphate/mol of receptors. These latter stoichiometries are similar to those obtained in *in vivo* studies of intact cells, in which m2 mAChRs were phosphorylated in an agonist-dependent manner to an extent of 4–5 mol of phosphate/mol of receptors (8–11). The stoichiometries of receptor phosphorylation obtained in *in vivo* studies should include any potential contributions of $G_{\beta\gamma}$. In view of such an argument, the present results suggest that phosphorylation of the hm2 mAChRs in membranes more closely resembled the *in vivo* environment in intact cells than did phosphorylation of purified hm2 mAChRs reconstituted in phospholipid vesicles, which were phosphorylated with up to 9–10 mol of phosphate/mol of receptors by βARKs in the presence of $G_{\beta\gamma}$ (13–15). The reasons for higher stoichiometries obtained with reconstituted receptors are poorly understood. Recent studies with rhodopsin showed that the regulatory protein arrestin regulates the number of sites phosphorylated on rhodopsin and that phosphorylation of 1–2 sites/receptor is enough to allow binding of arrestins to phosphorylated rhodopsin (50). In studies described here, it is possible that membrane-associated insect arrestins regulate the extent of mAChR phosphorylation achieved by βARKs in Sf9 membranes.

With the m2 mAChRs, the putative GRK phosphorylation sites are thought to be in the third intracellular loop (7, 38). However, the identities of these sites are not yet known. Thus, we are unable to determine whether the sites phosphorylated by βARK *in vitro* are similar to or different from those phosphorylated in intact cells (8–11). Future experiments will determine the identity of the phosphorylation sites in the hm2 and hm3 mAChRs. Furthermore, although we have not shown the functional consequences of receptor phosphorylation in the present study, future studies will assess the effects of βARKs on receptor/G protein uncoupling in membranes, in a manner similar to that used for the reconstituted m2 mAChRs (13).

Current *in vitro* approaches used for testing the specificity of GRKs for GPRs require that receptors be purified and reconstituted. To date, only a handful of GPRs have been successfully purified to homogeneity and demonstrated to be GRK substrates (1). The only GPR that has been routinely studied as a GRK substrate in membranes *in vitro* is rhodopsin. High expression of rhodopsin in rod outer segments (90% of total membrane protein) may explain the feasibility of using the membrane-localized protein in phosphorylation assays. However, most GPRs are rare membrane proteins expressed at much lower levels in native membranes, and attempts to study phosphorylation by GRKs in these circumstances have failed. The results in the present study support our initial hypothesis that GPRs must be expressed at high levels for GRK phosphorylation of GPRs to be detected. Higher receptor expression levels appear necessary to afford easier visualization of GRK-phosphorylated GPRs over the background phosphorylation often seen in membranes. In this regard, stripping of membranes containing hm3 mAChRs was necessary to reduce background phosphorylation and allow detection of the phosphorylated GPRs. Similarly, while this manuscript was being prepared, a study was reported in which sucrose gradient purification of Sf9 cell membranes containing expressed adrenergic receptors resulted in enriched receptor density and reduced background phosphorylation, allowing successful detection of phosphorylated GPRs (51).

In summary, we have used an *in vitro* approach to study phosphorylation of GPRs in membranes, and we report novel findings about the specificities of the GRKs for the hm2 and hm3 mAChRs and the regulation of GRKs by G proteins. This approach may expedite the study of other GPRs and may reveal potentially novel mechanisms with regard to their regulation by GRKs.

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