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An approach to the study of G-protein-coupled receptor kinases: An *in vitro*-purified membrane assay reveals differential receptor specificity and regulation by Gβγ subunits

(phosphorylation assay/receptor kinase specificity)

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ABSTRACT **Phosphorylation of GTP-binding-regulatory (G)-protein-coupled receptors by specific G-protein-coupled receptor kinases (GRKs) is a major mechanism responsible for agonist-mediated desensitization of signal transduction processes. However, to date, studies of the specificity of these enzymes have been hampered by the difficulty of preparing the purified and reconstituted receptor preparations required as substrates. Here we describe an approach that obviates this problem by utilizing highly purified membrane preparations from Sf9 and 293 cells overexpressing G-protein-coupled receptors. We use this technique to demonstrate specificity of several GRKs with respect to both receptor substrates and the enhancing effects of G-protein βγ subunits on phosphorylation. Enriched membrane preparations of the β_2- and α_2-C_2-adrenergic receptors (ARs, where α_2-C_2-AR refers to the AR whose gene is located on human chromosome 2) prepared by sucrose density gradient centrifugation from Sf9 or 293 cells contain the receptor at 100–300 pmol/mg of protein and serve as efficient substrates for agonist-dependent phosphorylation by β-AR kinase 1 (GRK2), β-AR kinase 2 (GRK3), or GRK5. Stoichiometries of agonist-mediated phosphorylation of the receptors by GRK2 (β-AR kinase 1), in the absence and presence of Gβγ, are 1 and 3 mol/mol, respectively. The rate of phosphorylation of the membrane receptors is 3 times faster than that of purified and reconstituted receptors. While phosphorylation of the β_2-AR by GRK2, -3, and -5 is similar, the activity of GRK2 and -3 is enhanced by Gβγ whereas that of GRK5 is not. In contrast, whereas GRK2 and -3 efficiently phosphorylate α_2-C_2-AR, GRK5 is quite weak. The availability of a simple direct phosphorylation assay applicable to any cloned G-protein-coupled receptor should greatly facilitate elucidation of the mechanisms of regulation of these receptors by the expanding family of GRKs.**

Signal transduction through GTP-binding-regulatory (G)-protein-coupled receptors is a dynamically regulated process. Classically, the intensity of the signal wanes over time despite the presence of the agonist. This phenomenon, which is referred to as agonist-mediated desensitization, involves as a key mechanism, the phosphorylation of the receptor by specific kinases (1). Two classes of these enzymes participate. The first is the second messenger-regulated kinases, cAMP-dependent protein kinase A and protein kinase C. The second is a recently appreciated kinase subfamily known as the G-protein-coupled receptor kinases (GRKs) (for review, see ref. 2). Phosphorylation of receptors by GRKs appears to trigger the binding of another protein (arrestin or β-arrestin) thereby uncoupling the receptors from activation of the G-protein/effector systems (2, 3). To date six members of the GRK family have been cloned (GRK1–GRK6) (4–11) including rhodopsin kinase (GRK1) (4) and two forms of β-adrenergic receptor kinase (βARK; βARK1 or GRK2 and βARK2 or GRK3) (5–7). IT-11 (GRK4) was identified by the sequencing of an open reading frame in the vicinity of the Huntington disease locus (8); GRK5 is a recently discovered widely distributed enzyme of unknown function (9, 10); and GRK6 was obtained by low-stringency screening of human heart and lymphocyte cDNA libraries (11, 12). Phosphorylation of receptors by GRKs appears to be strictly dependent on the receptor being in its agonist-activated state. For the β_2-AR, phosphorylation by GRK2 and -3 (13) but not by GRK5 (10) is enhanced by G-protein βγ subunits, which bind to the cytosolic kinases and target them to the membrane bound receptor (13).

Phosphorylation of G-protein-coupled receptors by GRKs is an important mechanism by which the intensity and duration of agonist stimulation are regulated. These mechanisms regulate light perception in mammals and flies as well as the biological activity of hormones and neurotransmitters in various mammalian systems (1, 2, 14). Recent evidence suggests that these mechanisms may also be important in olfactory perception (15, 16). However, direct biochemical evidence for the phosphorylation of G-protein-coupled receptors exists for only a handful of receptors including rhodopsin, β_2-AR, α_2-C_{10}-AR (the AR whose gene is located on human chromosome 10), the muscarinic (M_2) receptors, and substance P receptors (17–21). Moreover, almost nothing is known of the receptor specificity of the GRKs and the determinants that govern specific receptor–kinase interactions. This relative lack of information is largely due to the lack of a simple reliable biochemical assay for receptor phosphorylation and the difficulties in preparing suitable quantities of purified reconstituted G-protein-coupled receptors for such studies. In the past, GRK-catalyzed phosphorylation of G-protein-coupled receptors had to be accomplished either in whole cells with subsequent purification of receptor or with purified reconstituted receptors. In this report, we describe the development of a direct biochemical assay for receptor phosphorylation in which purified membranes overexpressing the receptors are used as substrates. We utilize this approach to demonstrate specificity for three GRKs both with respect to receptor substrates and the enhancement of activity by G-protein βγ subunits.

EXPERIMENTAL PROCEDURES

Expression of β_2-AR and α_2-C_2-AR. Recombinant human β_2-AR and α_2-C_2-AR (the AR whose gene is located on human

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Abbreviations: AR, adrenergic receptor; βARK, β-adrenergic receptor kinase; G protein, GTP binding regulatory protein; GRK, G-protein-coupled receptor kinase.

chromosome 2) were expressed in *Spodoptera frugiperda* (Sf9) cells using baculovirus expression technology as recommended by PharMingen. Human β_2-AR was also transiently transfected in human embryonic kidney (293) cells by a modified calcium phosphate method (22). Expression levels of β_2-AR and α_2-C_2-AR were determined by radioligand binding using [^{125}I]iodocyanopindolol (NEN) and [^{3}H]rauwolscine (NEN), respectively, as described (23, 24).

Sucrose Gradient Centrifugation. Cells were harvested 48 h after infection (Sf9) or 60 h after transfection (293), washed in ice-cold phosphate-buffered saline, and lysed in a hypotonic buffer (20 mM Tris·HCl/2 mM EDTA, pH 7.4) containing leupeptin (10 μg/ml), benzamidine (10 μg/ml), aprotinin (10 μg/ml), pepstatin A (5 μg/ml), and 0.2 mM phenylmethylsulfonyl fluoride (buffer A). Crude membranes were collected by centrifugation at 40,000 × *g* for 30 min (4°C), resuspended, and homogenized with a Polytron (Brinkman Instruments) on ice for 15 s in buffer A. Crude membranes were fractionated on a sucrose gradient as described (25). Briefly, crude membranes (2–15 mg of proteins) were layered on top of stepwise gradient consisting of 3.5 ml of 60% (wt/vol) sucrose and 4 ml of 35% sucrose prepared in buffer A and centrifuged at 115,000 × *g* for 90 min (4°C). At the end of centrifugation, the 0–35% sucrose interface containing a light membrane fraction was collected, washed in buffer A containing 250 mM NaCl, and centrifuged at 40,000 × *g* for 30 min (4°C). Pellets were resuspended once again in buffer A containing 250 mM NaCl and centrifuged at 40,000 × *g* for 30 min (4°C). Finally, membrane pellets were washed in buffer A alone, centrifuged, resuspended in buffer A, and stored at −70°C.

Receptor Phosphorylation by GRKs. Recombinant GRK2 (βARK1), GRK3 (βARK2), and GRK5 expressed in Sf9 cells and G-protein βγ subunits from bovine brain were purified to near homogeneity (>90%) as reported (5, 7, 10, 26). Phosphorylation of purified and reconstituted β_2-AR (>75% purity) (27, 28), β_2-AR membranes, and α_2-C_2-AR membranes by purified GRK2, GRK3, or GRK5 was performed as described (5, 7, 10) for purified receptor preparations. Briefly, the reaction mixtures (25 μl) consisted of ≈1 pmol of receptor, 10–60 nM kinase, ±300–600 nM G-protein βγ subunits, and ±100 μM (−)-isoproterenol (for β_2-AR) or ±100 μM epinephrine (for α_2-C_2-AR) in 20 mM Tris·HCl, pH 8.0/2 mM EDTA/10 mM $MgCl_2$/1 mM dithiothreitol. The reactions were started by adding 100 μM ATP (3000–6000 cpm/pmol; NEN) and incubated at 30°C. At the times indicated, the phosphorylation was stopped with 25 μl of SDS-sample-loading buffer [8% (wt/vol) SDS/25 mM Tris·HCl, pH 6.5/10% (vol/vol) glycerol/1% mercaptoethanol/0.005% bromophenol blue] and electrophoresed (SDS/PAGE) on SDS/10% polyacrylamide gels. Phosphorylation stoichiometries of receptor were determined either by excising the receptor bands and measuring radioactivity or by using a PhosphoImager (Molecular Dynamics) as described (29).

RESULTS

Membrane Fractionation and *in Vitro* Phosphorylation of β_2-AR and α_2-C_2-AR by GRK2. Infection of Sf9 cells with β_2-AR or α_2-C_2-AR or transfection of 293 cells with β_2-AR led to the expression of functional receptors at a level of ≈15 pmol/mg of crude membrane protein. Further purification of these crude membrane preparations on a discontinuous sucrose gradient by centrifugation followed by a high salt wash yielded an ≈20-fold enrichment of receptor specific binding activity for Sf9 cells and a 7- to 10-fold enrichment for 293 cells. Thus, final specific activity of receptor binding in the purified membrane preparations was 100–300 pmol/mg of protein.

As shown in Fig. 1, these purified membrane preparations could be used in an *in vitro* assay to phosphorylate G-protein-coupled receptors with GRKs. The β_2-ARs either from Sf9 membranes (Fig. 1*B*) or 293 membranes (Fig. 1*C*) could be phosphorylated by βARK1 (GRK2) in an agonist-dependent fashion and appeared as a major band on SDS/PAGE gels centered at ≈50 kDa. For comparison, Fig. 1*A* shows the pattern of phosphorylation obtained with β_2-AR purified from Sf9 cells and reconstituted into phospholipid vesicles. Furthermore, GRK2-catalyzed phosphorylation of β_2-AR in membranes was significantly enhanced by G-protein βγ subunits, a phenomenon previously demonstrated in the phosphorylation of the purified and reconstituted β_2-AR. Under the same conditions, α_2-C_2-AR in purified Sf9 membranes could also be phosphorylated by GRK2 in an agonist-dependent fashion that was enhanced by G-protein βγ subunits (Fig. 1*D*). For both β_2-AR and α_2-C_2-AR either in Sf9 membranes or 293 membranes, the stoichiometry of receptor phosphorylation was ≈1 mol of phosphate per mol of receptor in the presence of maximal concentrations of agonist and reached ≈3 mol of P_i per mol of receptor in the presence of G-protein βγ subunits. As documented earlier (13), using purified and reconstituted β_2-AR, the stoichiometry of phosphorylation is 1–2 mol of P_i per mol of receptor in the presence of agonist alone and rises to 7–10 mol of P_i per mol of receptor upon addition of agonist and G-protein βγ subunits. The phosphorylated band at ≈80 kDa, which is apparent in Fig. 1, presumably represents autophosphorylation of GRK2 and it, too, appears to be enhanced in the presence of βγ subunits.

Time Course of GRK2 Phosphorylation of β_2-AR. The results shown in Fig. 2 compare the time courses of GRK2-mediated phosphorylation of the β_2-AR in membranes with that of the purified reconstituted receptors. The phosphorylation rate for the membranes is ≈3 times faster than that of the purified reconstituted receptors ($t_{1/2}$, ≈5 min vs. ≈15 min).

Specificity of Phosphorylation of Membrane-Bound AR by Various GRKs. GRK2, -3, and -5 effectively phosphorylated the β_2-AR in Sf9 membranes in the presence of the agonist isoproterenol (100 μM) (Fig. 3). Interestingly, while GRK2 and -3 phosphorylate β_2-AR to ≈1 mol of P_i per mol of receptors, the extent of phosphorylation with GRK5 in the presence of agonist is twice as high (≈2 mol of P_i per mol of receptor) (Fig. 3). However, the GRK2 or GRK3 phosphorylation of β_2-AR membranes was greatly enhanced by G-protein βγ subunits, whereas the GRK5 phosphorylation was independent of Gβγ subunits. Thus, in the presence of Gβγ both GRK2 and GRK3 are more effective than GRK5 in phosphorylating membrane-bound β_2-AR, in agreement with

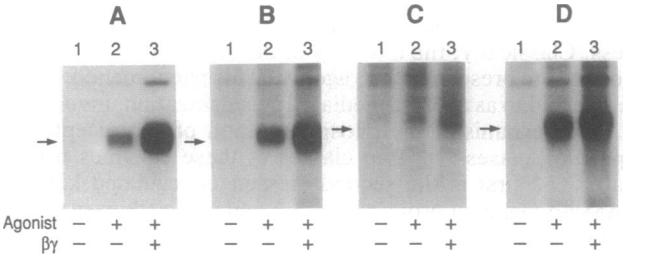

FIG. 1. Phosphorylation of β_2-AR and α_2-C_2-AR by GRK2. β_2-AR purified and reconstituted into phospholipid vesicles (*A*), β_2-AR in membranes from Sf9 cells (*B*), β_2-AR in membranes from 293 cells (*C*), and α_2-C_2-AR in membranes from Sf9 cells (*D*) were phosphorylated by GRK2 at 30°C for 20 min in the absence (lanes 1) or in the presence of isoproterenol (100 μM) for β_2-AR or epinephrine (100 μM) for α_2-C_2-AR (lanes 2 and 3) or plus G-protein βγ subunits (lanes 3). The experiments were replicated four to seven times with similar results.

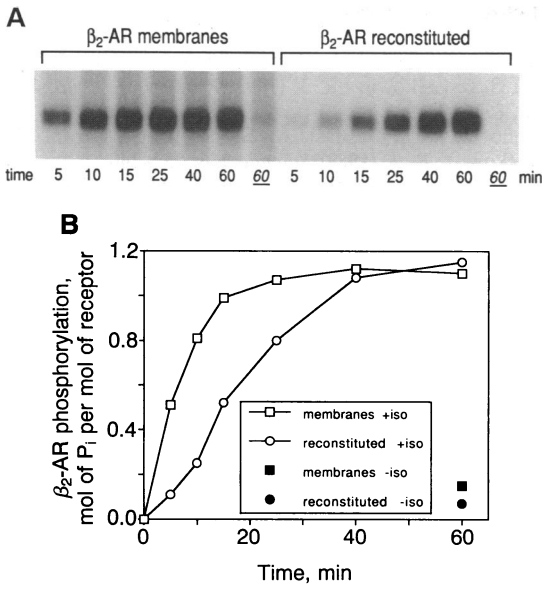

FIG. 2. Time course of GRK2 phosphorylation of β_2-AR in membranes and reconstituted phospholipid vesicles. β_2-AR in membranes and purified β_2-AR reconstituted into phospholipid vesicles were phosphorylated at 30°C for the times indicated in minutes in the presence of isoproterenol (iso, 100 μM). A 60-min point without isoproterenol is also shown as 60. The stoichiometries of P_i incorporation in autoradiograms of SDS/PAGE gels (*A*) were quantitated and plotted in *B*. The experiments were repeated three times with similar results.

earlier results obtained with purified reconstituted β_2-AR (10).

As shown in Fig. 4, the α_2-C_2-AR in membranes was comparable to the β_2-AR in serving as a substrate for either GRK2 or GRK3. In contrast, however, the extent of phosphorylation of the α_2-C_2-AR by GRK5 was only ≈20% of that of the β_2-AR and phosphorylation was independent of G-protein $\beta\gamma$ subunits. In other words, GRK5 was a much less effective kinase for modifying α_2-C_2-AR than either GRK2 or GRK3, especially in the presence of G-protein $\beta\gamma$ subunits (6- to 8-fold difference). These results clearly demonstrate that biological specificity does exist in GRK phosphorylation of G-protein-coupled receptors.

DISCUSSION

In this paper, we report the development of an *in vitro* biochemical approach that facilitates the assessment of the properties of various GRKs in terms of their abilities to phosphorylate G-protein-coupled receptors and to be regulated by G-protein $\beta\gamma$ subunits. Specifically, we show that GRK2, -3, and -5 effectively phosphorylate the β_2-AR in an agonist-dependent fashion. G-protein $\beta\gamma$ subunits, which have been shown to enhance receptor phosphorylation in purified reconstituted β_2-AR preparations (13), enhance phosphorylation in the *in vitro* membrane assay also. GRK2 and -3 are also efficient enzymes for α_2-C_2-AR phosphorylation in contrast to GRK5, which is markedly less effective at phosphorylating the α_2-C_2-AR. In addition, in contrast to GRK2 and -3, no effects of $\beta\gamma$ subunits are observed on the activity of GRK5 for phosphorylating either the β_2- or α_2-C_2-AR.

The hallmark of GRKs is that they can only phosphorylate receptors in their agonist-activated state and this conformation requires the receptor to be embedded in its native environment of a bilayer. In the past, the only G-protein-coupled receptor system that has been amenable to direct biochemical studies in plasma membranes has been the visual

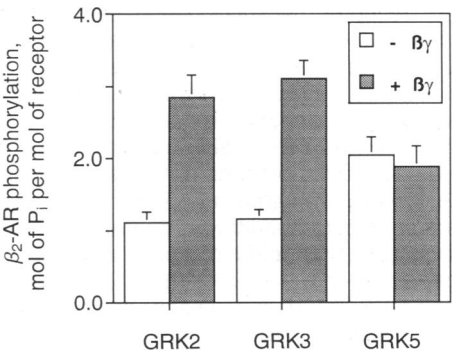

FIG. 3. Comparison of β_2-AR phosphorylation by GRKs. β_2-AR in membranes from Sf9 cells was phosphorylated in the presence of isoproterenol (100 μM) at 30°C for 20 min by GRK2, GRK3, or GRK5, with (stippled bars) or without (open bars) G-protein $\beta\gamma$ subunits. The maximum stoichiometries of P_i incorporation were determined from at least three experiments. Error bars represent SEM.

transduction system in which rod outer segments containing in excess of 80% rhodopsin are used. For other G-protein-coupled receptors, however, despite repeated attempts, over several years, it has never been possible to phosphorylate receptors with GRKs by using plasma membrane preparations (J. L. Benovic, M. M. Kwatra, M.G.C., and R.J.L., unpublished observations). Previous experiments with ARs and GRKs have been performed in whole cells with subsequent receptor purification or with purified receptors reconstituted in phospholipid vesicles (1). This has severely limited exploration of these mechanisms in detail. The approach described here, which uses an enriched membrane preparation from insect or mammalian cells expressing high levels of receptors should be readily applicable to any G-protein-coupled receptor that can be expressed in such heterologous systems. This development thus represents a major advance to elucidate these mechanisms.

Several factors may contribute to the phosphorylation of G-protein-coupled receptors by GRKs in the enriched membrane preparations: (*i*) the 10- to 20-fold enrichment in specific activity of receptors might be sufficient to provide enough receptor in a single assay to observe the phosphorylated protein, (*ii*) a reduction of background phosphorylation due to the removal of other kinases by purification or salt wash of membranes may enhance the detection of GRK-phosphorylated receptors, and (*iii*) the possible removal of putative GRK inhibitors present in crude preparations may also contribute to the success of this approach. As shown in

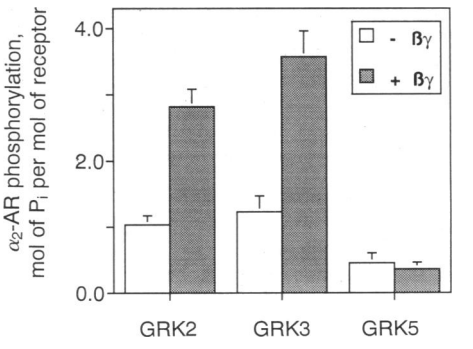

FIG. 4. Comparison of α_2-C_2-AR phosphorylation by GRKs. α_2-C_2-AR in membranes from Sf9 cell membranes was phosphorylated in the presence of epinephrine (100 μM) at 30°C for 20 min by GRK2, GRK3, or GRK5, with (stippled bars) or without (open bars) G-protein $\beta\gamma$ subunits. The maximum stoichiometries of P_i incorporation were determined from at least three experiments.

this study, receptor phosphorylation can be observed in purified membranes from more than one cell type, Sf9 and 293 cells. However, the success of this approach is likely to depend on more complex factors than simply overexpression and membrane enrichment since membranes from COS-7 cells, which express comparable amounts of receptors, do not provide suitable substrates in this assay. Interestingly, background phosphorylation in COS-7 cell membranes is usually much higher. In addition, photoaffinity-labeling experiments suggest that unlike receptors in Sf9 and 293 cells, β_2-ARs expressed in COS-7 cells migrate as multiple diffuse bands upon SDS/PAGE presumably due to heterogeneous glycosylation (data not shown). This may further impair the ability to concentrate the [^{32}P]receptor species in a narrow band within the gel.

Phosphorylation of G-protein-coupled receptors in the native plasma membrane environment provides a versatile approach to the study of these regulatory mechanisms that, to our knowledge, has not previously been available. In addition, from the data presented here, it appears that the membrane assay more closely reflects the whole cell environment than purified receptors in reconstituted phospholipid vesicles. Thus, GRK2 phosphorylation of β_2-AR was much faster than that of reconstituted receptors, narrowing the gap in the kinetics of receptor phosphorylation between *in vivo* and *in vitro* studies (19, 30, 31). In addition, the maximum stoichiometry of β_2-AR phosphorylation in membranes in the presence of agonist and $\beta\gamma$ subunits is much closer to what is routinely observed in whole-cell phosphorylation experiments (3–4 mol of P_i per mol of receptor) as opposed to the higher stoichiometry (7–10 mol of P_i per mol of receptor) often demonstrated with reconstituted receptors (13, 17). The factors responsible for these differences are currently unknown.

We also demonstrate here, using the *in vitro* assay, that marked specificity exists in the ability of various GRKs to phosphorylate G-protein-coupled receptors. Whereas previous studies had indicated that specificity existed between the ability of rhodopsin kinase and βARK to phosphorylate rhodopsin and β_2-AR (7), little or no difference was found in the ability of GRK2 and -3 to phosphorylate other receptors. Here we clearly show that the α_2-C_2-AR receptor that is an excellent substrate for GRK2 and -3 is an extremely poor substrate for GRK5. These results demonstrate how the availability of a direct biochemical approach to study G-protein-coupled receptor regulation has the potential to markedly facilitate acquisition of information concerning the specificity and mechanism of the GRKs.

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