Characterization of GRK2-Catalyzed Phosphorylation of the Human Substance P Receptor in Sf9 Membranes†

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Received September 15, 1997; Revised Manuscript Received November 10, 1997

ABSTRACT: G protein-coupled receptor kinases (GRKs) phosphorylate agonist-occupied G protein-coupled receptors (GPCRs), resulting in GPCR desensitization. GRK2 is one of the better studied of the six known GRKs and phosphorylates several GPCRs. In a previous study, we documented that GRK2 and GRK3 phosphorylate purified and reconstituted rat substance P receptor (rSPR) [Kwatra et al. (1993) *J. Biol. Chem. 268*, 9161−9164]. Here, we characterize in detail GRK2-catalyzed phosphorylation of human SPR (hSPR) in intact membranes. GRK2 phosphorylates hSPR in urea-washed Sf9 membranes in an agonist-dependent manner with a stoichiometry of 19 ± 1 mol of phosphate/mol of receptor, which increases slightly (1.3-fold increase) in the presence of G$\beta\gamma$. Kinetic analyses indicate that receptor phosphorylation occurs with a K_m of 6.3 ± 0.4 nM and a V_{max} of 1.8 ± 0.1 nmol/min/mg; these kinetic parameters are only slightly affected by G$\beta\gamma$ [K_m = 3.6 ± 1.0 nM and V_{max} = 2.2 ± 0.2 nmol/min/mg]. The lack of a strong stimulatory effect of G$\beta\gamma$ on GRK2-catalyzed phosphorylation of hSPR is surprising since G$\beta\gamma$ potently stimulates GRK2-catalyzed phosphorylation of β_2-adrenergic receptor and rhodopsin. Involvement of G$\beta\gamma$ endogenously present in membranes is ruled out as a source of high levels of hSPR phosphorylation, since receptor phosphorylation was not affected by guanine nucleotides that suppress or enhance the release of endogenous G$\beta\gamma$. The present study determines, for the first time, the kinetics of phosphorylation of a receptor substrate of GRK2 in intact membranes. Further, our results identify hSPR as a unique substrate of GRK2 whose phosphorylation is strong even in the absence of G$\beta\gamma$.

G protein-coupled receptor kinases (GRKs)[1] represent a family of enzymes whose members play a key role in homologous (or agonist-dependent) desensitization of G protein-coupled receptors (GPCRs) (*1*). Studies performed over the last 10 years on GPCRs such as rhodopsin and the β_2-adrenergic receptor (β_2-AR) have shown that homologous desensitization of GPCRs involves two main events: (1) upon agonist binding, the receptor undergoes phosphorylation by a GRK; and (2) the GRK-phosphorylated receptor binds a protein of the arrestin family which disrupts the receptor's coupling to its cognate G protein(s) (*2*−*4*). To date, six different GRKs have been identified: GRK1 (also called rhodopsin kinase), GRK2 (also called β-adrenergic receptor kinase [βARK]), GRK3 (also called βARK2), GRK4, GRK5, and GRK6 (*1*). The hallmark of GRKs is that they all phosphorylate only agonist-occupied or activated GPCRs. Notwithstanding this commonality, GRKs exhibit important differences with respect to tissue distribution and mode of translocation to plasma membranes where their substrates reside. For example, while GRK2, -3, -5, and -6 are widely distributed, GRK1 is localized in the retina and GRK4 is present predominantly in the testis. In addition, GRK1 is anchored to the plasma membrane by a farnesyl lipid present at its C-terminus, whereas GRK2 and GRK3 translocate to membranes by binding G$\beta\gamma$ (*1*). G$\beta\gamma$-subunits also potently activate GRK2- and GRK3-catalyzed phosphorylation of β_2-AR, m_2-muscarinic acetylcholine receptor (m_2-mAChR), and rhodopsin, but these subunits have no effect on GRK5 and GRK6 (*1*, *5*, *6*).

Because GPCRs have a very low natural occurrence, substrate specificity and kinetics of GRKs remain poorly studied. This is because, to detect GPCR phosphorylation by GRKs, GPCRs needed to be purified and reconstituted into phospholipid vesicles (*7*). This difficult approach has identified a few receptor substrates of GRK2, including β_2-AR (*8*), α_2-adrenergic receptor (α_2-AR) (*9*), m_2-mAChR (*10*),

† This work was supported by NIH Grant NS33405. Drs. Kwatra and Schwinn are senior fellows of the Center for the Study of Aging and Human Development, Duke University Medical Center.

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[1] Abbreviations: β_2-AR, β_2-adrenergic receptor; [^{125}I]BHSP, [^{125}I]-Bolton Hunter labeled substance P; DTT, dithiothreitol; G$\beta\gamma$, β,γ-subunits of heterotrimeric G proteins; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(2-thiotriphosphate); GppNHp, 5′-guanylyl-imidodiphosphate; hSPR, human substance P receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mAChR, muscarinic acetylcholine receptor; PBS, phosphate-buffered saline; PEI, poly(ethyleneimine); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda*; SEM, standard error of the mean; SP, substance P.

S0006-2960(97)02302-7 CCC: $15.00
Published on Web 01/15/1998

rat substance P receptor (rSPR) (*11*), and m_1-mAChR (*12*). However, phosphorylation of even this small group of GPCRs by GRK2 has not been well characterized, and K_m and V_{max} data are available only for the phosphorylation of β_2-AR (*13*). In addition, since these data were obtained using purified β_2-AR reconstituted into phospholipid vesicles, they may not adequately represent how the receptor behaves in intact membranes. To date, the kinetics of GRK2-catalyzed phosphorylation of a membrane-bound GPCR has not been reported. Recently, however, methods have been developed to examine GRK2-catalyzed phosphorylation of adrenergic and muscarinic receptors in Sf9 membranes (*14*, *15*). In the present study, we have characterized GRK2-catalyzed phosphorylation of human SPR (hSPR) in Sf9 membranes. GRK2 phosphorylates membrane-bound hSPR to a high stoichiometry, and the phosphorylation occurs with a nanomolar value of K_m in the absence of G$\beta\gamma$. Interestingly, addition of G$\beta\gamma$ does not have a marked effect on GRK2-catalyzed phosphorylation of hSPR.

EXPERIMENTAL PROCEDURES

Materials. Aprotinin, bacitracin, chymostatin, leupeptin, phenylmethanesulfonyl fluoride, soybean trypsin inhibitor, guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), guanosine 5′-*O*-(2-thiotriphosphate) (GTPγS), and SP were from Sigma (St. Louis, MO); 5′-guanylyl-imidodiphosphate (GppNHp) was from Boehringer-Mannheim (Indianapolis, IN); [^{125}I]-Bolton Hunter labeled SP ([^{125}I]BHSP) (2200 Ci/mmol), [^{3}H]-SR140333 (27.8 Ci/mmol), and [^{32}P]-γ-ATP (3000 Ci/mmol) were from New England Nuclear (Boston, MA). Grace's insect cell media, gentamicin, and fetal bovine serum were from GIBCO (Gaithersburg, MD); Sf9 cells were from Invitrogen (San Diego, CA); DEAE-Sephacel and heparin-Sepharose were from Pharmacia (Piscataway, NJ); and bovine retinas were from Pel-Freez (Rogers, AK). The nonpeptide SPR antagonist CP99994-1 was a gift from Dr. Saul Kadin, Pfizer Inc. (Groton, CT). G$\beta\gamma$ purified from bovine brain and purified β_2-AR reconstituted into phospholipid vesicles were kindly provided by Dr. R. J. Lefkowitz, Duke University Medical Center (Durham, NC). An antibody against the 15 amino acids from the C-terminus of hSPR (KTMTESFSFSSNVLS) was raised in rabbits by Southern Biotechnology Associates Inc. (Birmingham, AL). This hSPR antibody is suitable for immunoblotting as well as immunoprecipitating the receptor.

Expression of the hSPR in Sf9 Cells. A recombinant baculovirus containing the cDNA of hSPR was prepared as previously described (*16*). Sf9 cells were grown in spinner flasks to a density of 2×10^6 in Grace's insect cell culture media supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/mL gentamicin. The cells were infected with hSPR-containing baculovirus at a multiplicity of infection of 5. At 60−72 h postinfection, infected cells were harvested by centrifugation at 2000 rpm for 10 min; the cell pellet was washed twice with phosphate-buffered saline (PBS) and stored at −70 °C.

Preparation of Sf9 Cell Membranes. PBS-washed cells from a 250 mL culture were resuspended in 15−20 mL of buffer A (0.25 M sucrose, 10 mM EDTA, 20 mM Tris-HCl pH 7.4) supplemented with several protease inhibitors including leupeptin (10 μg/mL), phenylmethanesulfonyl fluoride (0.1 mM), aprotinin (5 μg/mL), and soybean trypsin inhibitor (10 μg/mL). Unless otherwise stated, these protease inhibitors were present in all buffers used for membrane isolation. The cells were homogenized with a polytron (PT 3000, Brinkman) at maximum speed for 30 s, and the homogenate was centrifuged at 500*g* for 10 min. The supernatant was saved and the pellet extracted three times with 5−10 mL of buffer A. Supernatants from low-speed centrifugations were combined and centrifuged at 45000*g* for 25 min. The pellet was resuspended in 20 mL of buffer B (20 mM Tris-HCl, pH 7.4, 2 mM EDTA) and centrifuged at 45000*g* for 25 min; the resulting pellet was resuspended in buffer B at a protein concentration of 4−6 mg/mL and stored at −70 °C.

Washing of hSPR-Containing Sf9 Membranes with Urea. hSPR-containing Sf9 membranes were resuspended in 5 M urea in buffer B, incubated on ice for 15 min, and then centrifuged at 45000*g* for 25 min. The resulting pellet was resuspended in buffer B and centrifuged; this washing step was repeated three times. The final pellet was resuspended in buffer B at a protein concentration of 2−3 mg/mL and stored at −70 °C. Treatment with urea decreased protein concentration by 70%, while more than doubling the specific activity of hSPR.

Radioligand Binding Assays. hSPR expression in Sf9 membranes was determined by using the antagonist radioligand [^{3}H]SR140333, which binds to hSPR in Sf9 membranes with a K_d of 0.8 nM (*17*). The assays were performed with saturating levels of [^{3}H]SR140333 (3−5 nM) and 1−2 μg of membrane protein in buffer C (50 mM Tris-HCl, pH 7.4, 1−2 mM $MgCl_2$, 5 mg/mL bovine serum albumin (BSA), 40 μg/mL bacitracin, 20 μg/mL chymostatin, and 4 μg/mL leupeptin); incubation time was 60 min at room temperature. Receptor-bound radioligand was separated from free radioligand by vacuum filtration over GF/C filters presoaked in 0.25% poly(ethylenimine) (PEI). Filters were washed (4 × 2 mL) with ice-cold 10 mM Tris-HCl, pH 7.4, dried in air, mixed with 5 mL of Lefko-Fluor (Research Products International Corp., Mount Prospect, IL), and counted in a scintillation counter. Nonspecific binding was determined with 1 μM CP99994-1. Binding assays using [^{125}I]BHSP were performed as described previously (*11*).

Purification of GRK2. Bovine GRK2 was expressed in Sf9 cells and purified as described previously with some modifications (*11*). Briefly, 250 mL of Sf9 cells infected with GRK2 baculovirus were centrifuged at low speed, and the pellet was washed with PBS and then homogenized in a Teflon glass homogenizer in 10 mL of buffer D (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA with protease inhibitors). The homogenate was centrifuged, and NaCl was added to the supernatant to a final concentration of 10 mM. The supernatant was then incubated with 2 mL of DEAE-Sephacel for 30 min at 4 °C with mixing. The suspension was poured into a column, and the flow-through (plus a 5-mL wash with buffer) was collected and, after 3-fold dilution with buffer D (20 mM HEPES, pH 7.4, 5 mM EDTA, 0.02% Triton X-100), was applied to a 5-mL heparin-Sepharose column at a rate of 1 mL/min. The column was washed with 50 mL of buffer D containing 100 mM NaCl and GRK2 eluted with a 100-mL gradient from 100 mM NaCl to 700 mM NaCl in buffer D. Fractions of 2 mL were collected and assayed for phosphorylation activity using rhodopsin as

the substrate. The fractions with GRK2 activity were pooled, concentrated in Centricon-30, diluted with glycerol to a final concentration of 50%, and stored at −20 °C. The purified GRK2 preparation exhibited one major band with an apparent molecular mass of 80 kDa when analyzed by SDS−PAGE and Coomassie staining. We estimate purity at >70%.

In Vitro Phosphorylation of hSPR with GRK2. The phosphorylation assays were performed at 30 °C in a total volume of 50 μL containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, 20 nM purified GRK2, the desired amount of Sf9 membrane proteins to give hSPR concentration varying from 1 to 60 nM, and other reagents as noted in the figure legends. G$\beta\gamma$ was used at either 50 or 100 nM since it maximally stimulates GRK2-catalyzed phosphorylation of hSPR at approximately 50 nM; this value is similar to the K_d of 32 nM reported for the binding of G$\beta\gamma$ to GRK2 (*13*). Reactions were started by adding 5 μL of [^{32}P]-γ-ATP (1.0−1.3 mM; 2−3 cpm/fmol) and stopped by adding 25 μL of SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 10% freshly added 2-mercaptoethanol). Receptor phosphorylation was visualized by subjecting the samples to SDS−PAGE on 10% acrylamide gels followed by autoradiography. For determining the stoichiometry of receptor phosphorylation, the phosphorylation reactions were performed with 1 nM hSPR (50 fmol/50 μL reaction) since phosphorylation at higher concentrations of hSPR gives lower stoichiometries. To calculate phosphorylation stoichiometry, the phosphorylated receptor bands were excised from the dried gels and counted in a scintillation counter. After basal receptor phosphorylation was subtracted, the extent of SP-induced receptor phosphorylation was calculated as moles of phosphate/moles of receptor. For kinetic studies, the concentration of membrane-bound hSPR varied from 1 to 60 nM and initial rates were determined by stopping the reactions after 3 min; the reaction rate was linear up to 6 min at the lowest receptor concentration (1 nM). K_m and V_{max} values were obtained by a nonlinear fit of the data to the Michaelis−Menten equation using the computer program KaleidaGraph (Synergy Software, Reading, PA).

Preparation of Urea-Treated Rod Outer Segment Membranes. Rod outer segment membranes were prepared from bovine retinas (Pel-Freez, Rogers, AK) as described (*18*) and then incubated for 15 min at room temperature with 5 M urea in 10 mM HEPES, pH 7.4. Urea-washed membranes were pelleted by centrifugation at 100000*g* for 45 min. The pellet was washed three times with 10 mM HEPES, 100 mM NaCl, pH 7.4, then resuspended in 10 mM HEPES, pH 7.4 at a concentration of 1−3 mg/mL and stored at −70 °C. Phosphorylation reactions using bovine rhodopsin as the substrate were performed as described above for hSPR.

RESULTS

GRK2-Catalyzed Phosphorylation of hSPR in Sf9 Membranes. We tested the ability of purified GRK2 to phosphorylate hSPR in Sf9 membranes (Figure 1). In the presence of SP, there is a striking increase in the phosphorylation of a 43 kDa protein (Figure 1, lane 6). This phosphorylated protein is identified as hSPR because it is recognized by an hSPR-specific antibody through both immunoblotting and immunoprecipitation (data not shown).

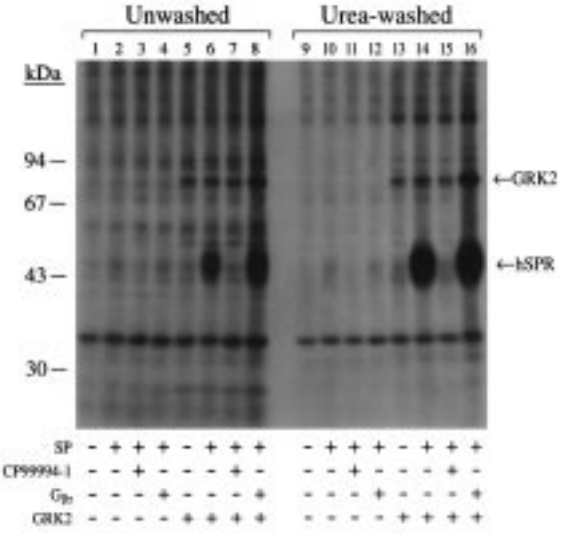

FIGURE 1: GRK2-catalyzed phosphorylation of hSPR in unwashed and urea-washed Sf9 membranes. Phosphorylation reactions were performed in a total volume of 50 μL containing 50 fmol of hSPR either in unwashed Sf9 membranes with 40 pmol of hSPR/mg of protein or in urea-washed Sf9 membranes with 90 pmol of hSPR/mg protein, 20 nM GRK2, 0.1 mM [^{32}P]-γ-ATP (2−3 cpm/fmol), with or without 1 μM SP, with or without 1 mM hSPR antagonist CP99994-1 (a 1000-fold excess of the antagonist over SP was used to completely block the stimulation by SP), with or without 50 nM G$\beta\gamma$, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 10 mM $MgCl_2$. The reaction mixtures were incubated at 30 °C for 60 min, the reactions stopped by adding 25 μL of SDS sample buffer, and the reaction mixtures analyzed by SDS−PAGE/autoradiography as described in Experimental Procedures. The experiment was repeated twice with similar results.

Only activated hSPR served as a GRK2 substrate because no phosphorylation of the receptor is seen in the absence of SP (Figure 1, lane 5) or when stimulation by SP is blocked by hSPR antagonist CP99994-1 (Figure 1, lane 7).

We next determined whether GRK2-catalyzed phosphorylation of membrane-bound hSPR becomes more pronounced by washing the membranes with urea; this treatment removes loosely attached proteins and was initially used on rod outer segment membranes to eliminate endogenous rhodopsin kinase activity (*19*). It was found that 5 M urea gives optimal results in terms of increased specific activity of hSPR (>2-fold) and reduced background phosphorylation (Figure 1, lanes 9−16). As can be seen, the GRK2-catalyzed phosphorylation of hSPR in urea-washed membranes (lanes 13−16) is similar to that seen in unwashed membranes (lanes 5−8) except that the background phosphorylation is low and the extent of receptor phosphorylation is higher (cf. lanes 5−8 and 13−16). Therefore, urea-washed membranes were used in all the subsequent experiments. As discussed below, GRK2-activator G$\beta\gamma$ causes only a slight increase in SP-induced hSPR phosphorylation (cf. lanes 14 and 16). However, G$\beta\gamma$ causes a significant increase in the autophosphorylation of GRK2 (cf. GRK2 band in lanes 14 and 16).

Interestingly, a low level of an endogenous GRK-like activity was detected both in unwashed (Figure 1, lanes 1−4) and in urea-washed membranes (Figure 1, lanes 9-12). This endogenous GRK activity phosphorylates hSPR in a stimulus-dependent manner (Figure 1, lanes 2 and 10); no phosphorylation of the receptor is seen in the absence of SP (Figure 1, lanes 1 and 9) or when SP stimulation is blocked by the SPR antagonist CP99994-1 (Figure 1, lanes 3 and 11). The

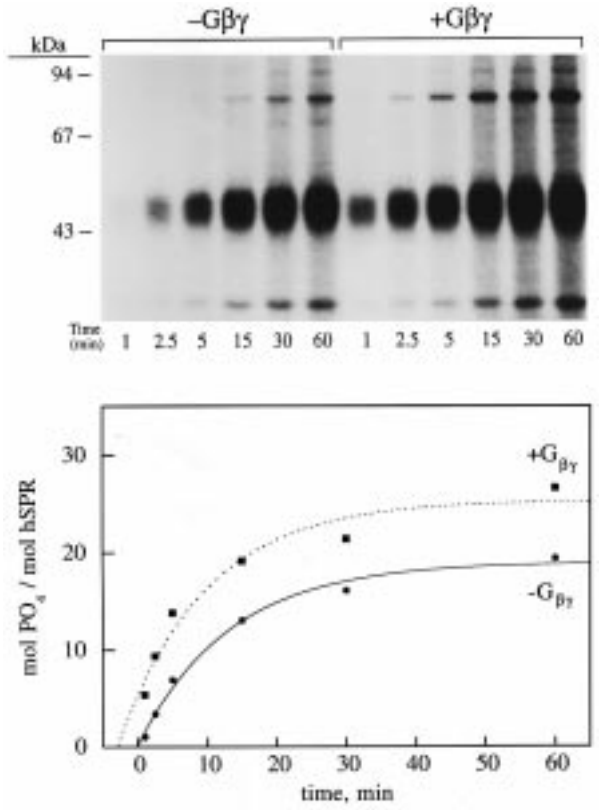

FIGURE 2: Time course of hSPR phosphorylation by GRK2 in Sf9 membranes in the absence and presence of G$\beta\gamma$. Phosphorylation reactions were performed in a total volume of 50 μL and contained 50 fmol of hSPR in urea-washed membranes with 90 pmol of hSPR/mg of protein, 20 nM GRK2, 50 nM G$\beta\gamma$, 1 μM SP, 0.1 mM [^{32}P]-γ-ATP (2−3 cpm/fmol), 20 mM Tris-HCl, pH 7. 4, 2 mM EDTA, and 10 mM $MgCl_2$. The reaction mixtures were incubated at 30 °C for the indicated times, the reactions stopped by adding 25 μL of SDS sample buffer, and the reaction mixtures analyzed by SDS−PAGE/autoradiography as described in Experimental Procedures. After autoradiography, receptor bands were excised and counted, and the stoichiometry of phosphorylation was determined. The experiment was repeated twice with similar results.

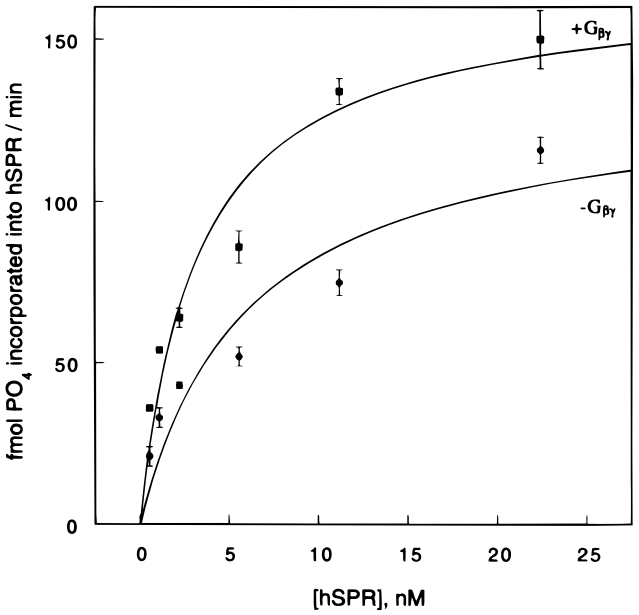

FIGURE 3: Kinetic analysis of GRK2-catalyzed phosphorylation of agonist-occupied hSPR in Sf9 membranes in the presence and absence of G$\beta\gamma$. Phosphorylation reactions were performed in a total volume of 50 μL containing 1−60 nM hSPR in urea-washed Sf9 membranes (70−90 pmol of hSPR/mg of membrane protein), 20 nM GRK2, 50 nM G$\beta\gamma$, 1 μM SP, 0.1 mM [^{32}P]-γ-ATP (2−3 cpm/fmol), 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 10 mM $MgCl_2$. The reaction mixtures were incubated for 3 min at 30 °C, and the reactions stopped by the addition of 25 μL of SDS sample buffer. Phosphorylation of the receptor was analyzed by SDS−PAGE/autoradiography, and initial rates were determined by excising the receptor band and counting for radioactivity as described in Experimental Procedures. Data points are the mean of three separate experiments each performed in duplicate. Data were fitted to the Michaelis−Menten equation using the computer program KaleidaGraph (Synergy Software, Reading, PA).

presence of an endogenous GRK-like activity in Sf9 membranes has also been noted in experiments using m_2-mAChR as the substrate (*14*). It should be noted that the phosphorylation of hSPR by endogenous GRK2-like activity is only a small fraction (<5%) of the phosphorylation observed with added GRK2 (cf. lanes 10 and 14), and it is not enhanced by G$\beta\gamma$ (Figure 1, lanes 4 and 12).

Another characteristic of the GRK2-catalyzed phosphorylation of hSPR is that G$\beta\gamma$, which only slightly stimulates the phosphorylation of activated-hSPR (Figure 1, lanes 14 and 16; Figure 2), increases the basal phosphorylation of hSPR to a stoichiometry of about 6 mol of phosphate/mol of receptor (e.g., see Figure 5, lane 11). This increase in the basal phosphorylation of hSPR is not blocked by the hSPR antagonist CP99994-1 (data not shown). A similar effect of G$\beta\gamma$ on GRK2-catalyzed basal phosphorylation of reconstituted β_2-AR has been reported (*6*).

Characterization of GRK2-Catalyzed Phosphorylation of Agonist-Occupied hSPR in Urea-Washed Sf9 Membranes. Agonist-induced phosphorylation of hSPR by GRK2 is dependent on the concentration of SP and occurs with an EC_{50} of 30 ± 9 nM (n = 3) (data not shown). This EC_{50} reflects the binding of SP to the receptor in the low-affinity state (*11*). Further, this value is similar to the EC_{50} of 100 nM that we reported earlier for SP to stimulate GRK2-catalyzed phosphorylation of reconstituted rSPR (*11*).

The time course of hSPR phosphorylation in the absence and presence of G$\beta\gamma$ is shown in Figure 2. As can be seen, there is substantial hSPR phosphorylation in the absence of G$\beta\gamma$. Although G$\beta\gamma$ increases both the initial rates and the extent of hSPR phosphorylation, the effect is modest. We calculate that GRK2 phosphorylates the agonist-occupied hSPR to a stoichiometry of 19 ± 1 (SEM, n = 3) mol of phosphate/mol of receptor, and it increases to 24 ± 1 (SEM, n = 3) mol of phosphate/mol of receptor in the presence of G$\beta\gamma$, representing a 1.3-fold increase.

Kinetics of GRK2-Catalyzed Phosphorylation of Agonist-Occupied hSPR in Urea-Washed Sf9 Membranes. Figure 3 shows initial rates of GRK2-catalyzed phosphorylation of agonist-occupied hSPR in the presence and absence of G$\beta\gamma$ as a function of increasing concentrations of membrane-bound hSPR. These curves were fitted to the Michaelis−Menten equation, and the Michaelis constant (K_m) and the maximum velocity (V_{max}) were calculated. In the absence of G$\beta\gamma$, GRK2 phosphorylates agonist-occupied hSPR with a K_m value of 6.3 ± 0.4 nM and V_{max} value of 1.8 ± 0.1 nmol/min/mg (SEM; n = 3); in the presence of G$\beta\gamma$, the phosphorylation occurs with K_m = 3.6 ± 1.0 nM and V_{max} = 2.2 ± 0.2 nmol/min/mg (SEM; n = 3). As a control, we determined the K_m value for GRK2-catalyzed phosphoryla-

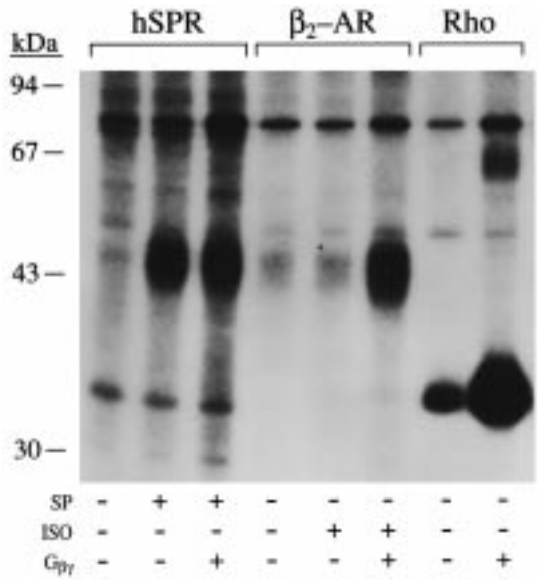

FIGURE 4: Effect of G$\beta\gamma$ on GRK2-catalyzed phosphorylation of hSPR, β_2-AR, and rhodopsin (Rho). Phosphorylation reactions were performed in a total volume of 50 μL containing the indicated receptor (either 50 fmol of hSPR in urea-washed Sf9 membranes with 90 pmol hSPR/mg protein, 500 fmol of purified β2AR reconstituted into phospholipid vesicles, or 0.35 μg of urea-washed rod outer segment membranes), 20 nM GRK2, 0.1 mM [^{32}P]-γ-ATP (2−3 cpm/fmol), 50 nM G$\beta\gamma$, 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 10 mM $MgCl_2$. Concentrations of SP and isoproterenol (ISO) were 1 and 100 μM, respectively. Reaction mixtures were incubated at 30 °C for 60 min, the reactions stopped by adding 25 μL of SDS sample buffer, and the reaction mixtures analyzed by SDS−PAGE/autoradiography as described in Experimental Procedures. The experiment was repeated three times with similar results.

tion of activated rhodopsin in urea-washed rod outer segment membranes and found it to be 7 μM. This value is similar to the K_m value of 4−6 μM reported for GRK2-catalyzed phosphorylation of rhodopsin by other investigators (*20*, *21*) and attests to the reliability of our K_m and V_{max} data on GRK2-catalyzed phosphorylation of hSPR.

Effect of G$\beta\gamma$ on GRK2-Catalyzed Phosphorylation of Membrane-Bound hSPR. It is well-established that G$\beta\gamma$ increases both initial rates (>10-fold) and extent of phosphorylation (3−6-fold) of several GRK2 substrates including β_2-AR (*6*, *13*), m_2-mAChR (*22*), and rhodopsin (*6*, *22*). To ensure that these results could be achieved using our experimental conditions, we examined the effect of G$\beta\gamma$ on GRK2-catalyzed phosphorylation of hSPR in Sf9 membranes along with purified β_2-AR reconstituted into phospholipid vesicles, and rhodopsin in rod outer segment membranes (Figure 4). As can be seen, G$\beta\gamma$ potently activates the phosphorylation of β_2-AR and rhodopsin, but the effect on hSPR phosphorylation is little. It should be mentioned that, although we have used reconstituted β_2-AR in the experiment shown in Figure 4, a potent effect of G$\beta\gamma$ on GRK2-catalyzed phosphorylation of β_2-AR in Sf9 membranes has also been reported (*14*). Similarly, G$\beta\gamma$ increases (by 2-fold) GRK2-catalyzed phosphorylation of m_2-mAChR in Sf9 membranes (*15*).

The reason for the lack of a pronounced effect of G$\beta\gamma$ on GRK2-catalyzed phosphorylation of hSPR is not clear. However, since the phosphorylation of hSPR occurs to a high stoichiometry without added G$\beta\gamma$, one possibility is that

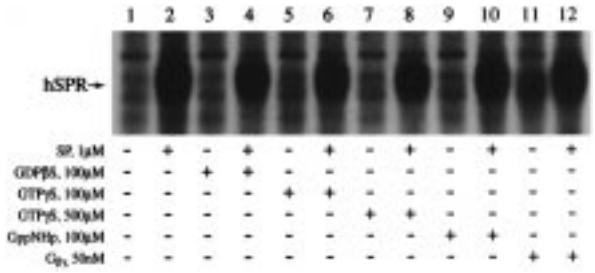

FIGURE 5: Effect of guanine nucleotides on GRK2-catalyzed phosphorylation of hSPR in urea-washed Sf9 membranes. Phosphorylation reactions were performed in a total volume of 50 μL containing 50 fmol of hSPR in urea-washed Sf9 membranes with 60 pmol of hSPR/mg of protein, 20 nM GRK2, 0.1 mM [^{32}P]-γ-ATP (2−3 cpm/fmol), 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM $MgCl_2$, and other reagents as shown. The reaction mixtures were incubated for 60 min at 30 °C, the reactions stopped by adding 25 μL SDS sample buffer, and the reaction mixtures analyzed by SDS−PAGE/autoradiography as described in Experimental Procedures. The experiment was repeated twice with similar results.

GRK2 may be activated by G$\beta\gamma$ endogenously present in Sf9 membranes. This is a plausible hypothesis since we have previously shown that hSPR in Sf9 membranes couples to three endogenous G proteins, $G\alpha_o$, $G\alpha_{q/11}$, and $G\alpha_s$ (*16*, *23*), and hSPR activation should release G$\beta\gamma$. The receptor remains coupled to endogenous G proteins in urea-washed Sf9 membranes as binding of agonist radioligand [^{125}I]BHSP to hSPR is sensitive to guanine nucleotides (data not shown). Therefore, we examined the role of endogenous G$\beta\gamma$ of Sf9 membranes in hSPR phosphorylation by performing the phosphorylation reactions with either GDPβS (which reduces free G$\beta\gamma$) or GTPγS/GppNHp (which increases free G$\beta\gamma$). This approach has recently been used to examine the role of endogenous G$\beta\gamma$ in GRK2-catalyzed phosphorylation of muscarinic receptors in Sf9 membranes (*15*). As shown in Figure 5, neither GDPβS nor GTPγS/GppNHp caused any significant change in GRK2-catalyzed phosphorylation of agonist-occupied hSPR. Of note, GTPγS/GppNHp did not increase basal phosphorylation of hSPR (Figure 5, lanes 5, 7, and 9) whereas exogenously added 50 nM G$\beta\gamma$ increases basal phosphorylation of hSPR (Figure 5, lane 11). These data indicate that either the released G$\beta\gamma$ was not available to interact with GRK2 or the amount of released G$\beta\gamma$ was not sufficient to activate GRK2. The latter possibility is probably the case since our phosphorylation reactions were performed using small amounts (~0.5 μg) of Sf9 membrane proteins, which may not provide the nanomolar concentrations of G$\beta\gamma$ needed for GRK2 activation. These results suggest that endogenous G$\beta\gamma$ of Sf9 membranes does not contribute to high hSPR phosphorylation observed in the absence of exogenously added G$\beta\gamma$. Therefore, the higher level of hSPR phosphorylation by GRK2 observed in the absence of G$\beta\gamma$ appears to be an inherent property of hSPR.

DISCUSSION

A main finding of the present study is that GRK2 phosphorylates membrane-bound hSPR with high stoichiometry (19 ± 1 mol of phosphate/mol of receptor without G$\beta\gamma$ and 24 ± 1 mol of phosphate/mol of receptor with G$\beta\gamma$) and a low K_m value. The high stoichiometry of hSPR phosphorylation is not surprising because hSPR contains 26 serine/threonine residues on its carboxyl tail and 12 serine/

threonine residues on its intracellular loops (*24*). Previously, we reported that GRK2 phosphorylates partially purified and reconstituted rat SPR with a stoichiometry of 9 mol of phosphate/mol of receptor (*11*). It is possible that hSPR was phosphorylated with high stoichiometry here because it was phosphorylated in the more physiological milieu of intact membranes. While this appears to be an attractive possibility, recent data on GRK2-catalyzed phosphorylation of adrenergic and muscarinic receptors in Sf9 membranes indicate that these receptors are phosphorylated to a lower extent than when they are reconstituted into phospholipid vesicles. It has been reported that α_2-ARs and β_2-ARs in Sf9 membranes are phosphorylated with a stoichiometry of 1 mol of phosphate/mol of receptor without G$\beta\gamma$ and 3 mol of phosphate/mol of receptor with G$\beta\gamma$ (*14*). In contrast, phosphorylation of β_2-AR in phospholipid vesicles occurs with a stoichiometry of 4 mol phosphate/mol of receptor and increases to 11 mol of phosphate/mol of receptor in the presence of G$\beta\gamma$ (*13*). Similarly, GRK2 phosphorylates m_2- and m_3-mAChRs in Sf9 membranes with a stoichiometry of 2 mol of phosphate/mol of receptor without G$\beta\gamma$ and 4 mol of phosphate/mol of receptor in the presence of G$\beta\gamma$ (*15*), whereas reconstituted m_2-mAChR is phosphorylated to a stoichiometry of 4−5 mol of phosphate/mol receptor, which increases 2−3-fold in the presence of G$\beta\gamma$ (*25*). Clearly, while both adrenergic and muscarinic receptors can be phosphorylated to high stoichiometries in reconstituted systems, their phosphorylation in membranes is generally low. Thus, hSPR is the first substrate of GRK2 which exhibits extensive phosphorylation in intact membranes. It should be pointed out that a high stoichiometry of hSPR phosphorylation is observed only when the phosphorylation reaction is performed at limiting concentrations of hSPR (1 nM). Since limiting concentrations of membrane-bound adrenergic and muscarinic receptors were not used in the above-mentioned studies (*14*, *15*), it is possible that their reported stoichiometries of phosphorylation may be lower than true values.

We find that GRK2-catalyzed phosphorylation of hSPR occurs with a low K_m value (4−6 nM). A comparison of these data with other receptor substrates of GRK2 must wait until similar data on GRK2-catalyzed phosphorylation of other receptors in membranes are available. Currently, the only kinetic data available are on GRK2-catalyzed phosphorylation of purified and reconstituted β_2-AR. It is reported that recombinant GRK2 purified from Sf9 cells (a preparation of GRK2 comparable to that used in the present study) phosphorylates reconstituted β_2-AR with a K_m of 194 nM, which decreases to 49 nM in the presence of G$\beta\gamma$ (*13*). Thus, in the absence of G$\beta\gamma$, GRK2 has a 40-fold-higher affinity for membrane-bound hSPR than for reconstituted β_2-AR.

Our data also allows a direct comparison between the K_m of GRK2-catalyzed phosphorylation of membrane-bound hSPR and the K_m of GRK1-catalyzed phosphorylation of membrane-bound rhodopsin, a physiological substrate of GRK1. It is reported that GRK1 phosphorylates membrane-bound rhodopsin with a K_m of 4 μM (*26*), and this value is 1000-fold higher than the K_m of 4−6 nM with which GRK2 phosphorylates membrane-bound hSPR. It is possible that GRK2 phosphorylates hSPR with a relatively low K_m, presumably to compensate for the low natural occurrence of hSPR, whereas GRK1 phosphorylates rhodopsin with a relatively high K_m since the natural occurrence of rhodospin in rod outer segment membranes is very high (*18*).

The finding that hSPR phosphorylation occurs optimally in the absence of the GRK2 activator G$\beta\gamma$ is novel, suggesting that hSPR by itself can maximally stimulate GRK2. While it is known that GRK2 is activated by its receptor substrates (e.g., β_2-AR and m_2-mAChR) (*26*−*28*), receptor-mediated activation of GRK2 is greatly enhanced in the presence of G$\beta\gamma$ due to synergistic interactions between receptor and G$\beta\gamma$ (*29*). Thus, an apparent lack of substantial stimulation by G$\beta\gamma$ of GRK2-catalyzed phosphorylation of hSPR raises the question whether hSPR is also causing the stimulation of activity normally brought about by G$\beta\gamma$. In this context, it is interesting to point out that examination of the amino acid sequences of hSPR and Gβ_1 indicates that a set of five amino acids (SFSFS; 398−402) on the carboxyl tail of hSPR is 80% (4 out of 5) identical with a set of five amino acids (SVSFS; 275−278) present toward the carboxyl end of Gβ_1 (*30*). Whether this similarity between the segments of hSPR and Gβ_1 is the cause of the high hSPR phosphorylation in the absence of G$\beta\gamma$ remains to be tested. It should also be mentioned that a recent study shows that distinct combinations of G$\beta\gamma$ are needed to stimulate GRK2 activity against specific GPCRs (*31*). Therefore it is also possible that the stimulation of GRK2-catalyzed phosphorylation of hSPR by G$\beta\gamma$ requires a unique combination of Gβ and Gγ which is not present in G$\beta\gamma$ purified from bovine brain.

In conclusion, we demonstrate that hSPR is an excellent substrate of GRK2 and, unlike other receptor substrates of GRK2, it exhibits substantial phosphorylation in the absence of G$\beta\gamma$.

ACKNOWLEDGMENT

We would like to thank William Barry for technical assistance and David Kellogg and Susan Tumey for editorial assistance.

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BI972302S