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G Protein-coupled Receptor Kinases of the GRK4 Protein Subfamily Phosphorylate Inactive G Protein-coupled Receptors (GPCRs)*

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Background: GPCR phosphorylation by GRKs initiates arrestin binding and signaling switch to G protein-independent pathways.
Results: GRK5/6 phosphorylate many inactive GPCRs. GRK2/3 and even permanently membrane-tethered GRK2 mutants phosphorylate only active receptors.
Conclusion: Particular receptor-GRK combinations determine the role of GPCR activation in its phosphorylation.
Significance: GRKs can phosphorylate active and inactive GPCRs, promoting arrestin recruitment.

G protein-coupled receptor (GPCR) kinases (GRKs) play a key role in homologous desensitization of GPCRs. It is widely assumed that most GRKs selectively phosphorylate only active GPCRs. Here, we show that although this seems to be the case for the GRK2/3 subfamily, GRK5/6 effectively phosphorylate inactive forms of several GPCRs, including β2-adrenergic and M2 muscarinic receptors, which are commonly used as representative models for GPCRs. Agonist-independent GPCR phosphorylation cannot be explained by constitutive activity of the receptor or membrane association of the GRK, suggesting that it is an inherent ability of GRK5/6. Importantly, phosphorylation of the inactive β_2-adrenergic receptor enhanced its interactions with arrestins. Arrestin-3 was able to discriminate between phosphorylation of the same receptor by GRK2 and GRK5, demonstrating preference for the latter. Arrestin recruitment to inactive phosphorylated GPCRs suggests that not only agonist activation but also the complement of GRKs in the cell regulate formation of the arrestin-receptor complex and thereby G protein-independent signaling.

The strict dependence of rhodopsin phosphorylation on its activation by light was discovered more than 40 years ago (1, 2). The cloning of β_2-adrenergic receptor (β2AR)[2] and demonstration of its homology to rhodopsin (3) established that receptors activating G proteins constitute a structural family, now termed G protein-coupled receptors (GPCRs). Soon thereafter, a kinase that could phosphorylate the β_2-adrenergic receptor (β2AR), termed βARK (now known as GPCR kinase 2 or GRK2), was characterized (4) and cloned (5). GRK2 turned out to be highly specific for the agonist-activated form of the β2AR (4, 6) and was later shown to phosphorylate other GPCRs, including rhodopsin (7) and the α_2-adrenergic receptor (4) in an activation-dependent manner. Rhodopsin kinase (now known as GRK1) was shown to be activated by direct binding to light-activated rhodopsin (8), and GRK2 was also found to be activated by both active β2AR and rhodopsin (9). Active GPCRs thus serve as both substrates and allosteric activators of GRKs, providing a molecular explanation for selectivity of GRKs for the activated form of the receptor (10–13). The dependence on direct interaction with the active receptor is now widely believed to be a general characteristic of all GRKs (14), although it does not explain their activity toward nonreceptor substrates (15). Although constitutive (agonist-independent) activity of GRK4α toward the D1 dopamine receptor (16) and GRK5 toward the β2AR (17, 18) was reported, it was considered to be a rare exception to the rule. Early reports showed "high gain" phosphorylation of rhodopsin in native disc membranes, where hundreds of rhodopsin molecules were phosphorylated for every light-activated rhodopsin (Rho*) (19, 20). However, rhodopsin is incredibly concentrated in discs, occupying about half of the surface (21). Therefore, these findings were interpreted within the framework of the same model; GRK1 was activated by binding to Rho* (8), and due to rapid diffusion in the disc, this complex during its lifetime encountered hundreds of inactive rhodopsin molecules, which were phosphorylated by Rho*-activated GRK1 (22).

GRKs separated into two distinct subfamilies, GRK2/3 and GRK4/5/6, very early in evolution (23, 24). Here, using two representatives of each of these GRK subfamilies and six GPCRs that couple to three different G protein subfamilies, G_s, G_q, and

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[2] The abbreviations used are: β2AR, β_2-adrenergic receptor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; M2R, M2 muscarinic receptor; M3R, M3 muscarinic receptor; D1R, D1 dopamine receptor; D2R, D2 dopamine receptor; 5-HT2C, serotonin receptor 2C; Rho, dark (inactive) rhodopsin; Rho*, light-activated rhodopsin; BRET, bioluminescence resonance energy transfer; PH, pleckstrin homology domain; 5-HT, 5-hydroxytryptamine.

$G_{i/o}$, we systematically tested the agonist dependence of receptor phosphorylation by different GRKs in intact cells. We found that although phosphorylation of all GPCRs by GRK2/3 is strictly agonist-dependent, GRK5/6 demonstrate significant activation-independent phosphorylation of most GPCRs tested, with the level of phosphorylation in the absence of agonist reaching that achieved in its presence with some receptors. We show that inverse agonists do not reduce β2AR phosphorylation by GRK5, demonstrating that this is indeed an activation-independent process. Moreover, we found that the phosphorylation of inactive β2AR by GRK5 in intact cells facilitates the binding of both nonvisual arrestins and that constitutive phosphorylation of β2AR with the V2 C terminus leads to constitutive arrestin recruitment to this receptor. Thus, the ability to phosphorylate inactive GPCRs appears to be a common characteristic of the GRK4 subfamily. This activation-independent phosphorylation leads to constitutive arrestin binding, suggesting that GRKs of this subfamily can stimulate basal receptor cycling and/or arrestin-mediated signaling in the absence of agonists.

EXPERIMENTAL PROCEDURES

Materials—All restriction and DNA-modifying enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech (Manassas, VA) or Invitrogen. All other reagents were from Sigma.

GRK Purification—All GRKs were purified from baculovirus-infected High-5 cells. Bovine GRK2 was expressed as the S670A mutant, which eliminates a C-terminal MAPK site (25) and contains a C-terminal hexahistidine tag. Bovine GRK1 and GRK5 were expressed as truncated forms ending at residues 535 and 561, respectively, followed by the exogenous amino acids VDHHHHHH to facilitate purification by Ni^{2+}-nitrilotriacetic acid chromatography. All GRKs were purified essentially as described previously for bovine $GRK1_{535}$-H_6 (12).

Rhodopsin Purification and in Vitro Phosphorylation—Rhodopsin (Rho) in native disk membranes was purified from bovine retina as described previously (26). Opsin was generated from rhodopsin, as described previously (27). Rhodopsin was phosphorylated by purified GRKs *in vitro* in kinase buffer (KB) (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM ATP) in the presence of [γ-^{32}P]ATP to yield final specific activity of 3,000–4,500 cpm/pmol. Purified bovine Rho (0.5 μg) or opsin (0.5 μg) was phosphorylated by purified GRKs (0.5 μg) in 12-μl assays for 20 min at room temperature under bright light or in the dark. Reactions were stopped by the addition of an equal volume of SDS sample buffer and subjected to disc electrophoresis on 4% stacking and 10% running gels. The gels were stained with Coomassie (GelCode Blue, Pierce) to visualize protein bands, destained with water, dried, and exposed to x-ray film. Receptor bands were excised, and the radioactivity was quantified by liquid scintillation counting, whereupon the stoichiometry of the phosphorylation was calculated.

Receptor Purification, Reconstitution into HDL Particles, and in Vitro Phosphorylation—The β2AR was expressed in Sf9 cells and purified by FLAG affinity chromatography, followed by alprenolol-Sepharose affinity chromatography, as described previously (28). Purified receptors were reconstituted into HDL particles, as described previously (29). Briefly, a 3:2 mol/mol ratio of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol was dissolved in a cholate buffer (50 mM sodium cholate, 100 mM NaCl, 20 mM HEPES, pH 7.5, 1 mM EDTA), then mixed with receptor at a ratio of 40:1 mol/mol lipids/receptor. MSP1 protein was then added to a final concentration of 3 mg/ml, and the reaction was incubated on ice for 1 h. 10 mg of Bio-Beads SM-2 adsorbent was then added, and the sample was rotated for 4 h at 4 °C.

Receptor phosphorylation was performed in KB in the presence of [γ-^{32}P]ATP to yield a final specific activity of 800–1,800 cpm/pmol. Purified human β2AR (3.3 μM) in the presence of an agonist BI-167107 (0.2 μM) (30) or inverse agonist carazolol (0.2 μM) (31) was phosphorylated by purified GRK2 or GRK5 (1.7 μM) for 20 min at 22 °C in 10-μl assays. Preliminary experiments showed that the phosphorylation is nearly linear within this period. Reactions were stopped by the addition of an equal volume of SDS sample buffer and subjected to disc electrophoresis on 4% stacking and 10% running gel. The gels were stained with Coomassie (GelCode Blue, Pierce) to visualize protein bands, destained with water, dried, and exposed to x-ray film. Receptor bands were excised, and the radioactivity was quantified by liquid scintillation counting, whereupon the stoichiometry of the phosphorylation was calculated.

Receptor and GRK Constructs—Expression constructs for wild type (WT) β2AR, β2AR with vasopressin V2 receptor C terminus (32–34), M2 muscarinic receptor (M2R), D1 dopamine receptor (D1R), and D2 dopamine receptor (D2R) N-terminally tagged with triple HA were described previously (35, 36). Similar construct for M3 muscarinic receptor (M3R) purchased from Addgene was used. cDNA of the unedited (5-HT2C-INI) and fully edited (5-HT2C-VGV) 5-HT2C receptor was kindly provided by Dr. Ronald B. Emeson (Vanderbilt University). Both receptors were N-terminally tagged with triple HA tag and subcloned into pcDNA3, with sequence confirmed by dideoxy-sequencing. Full-length WT untagged bovine GRK2 and GRK3 and human GRK5 and GRK6 (kindly provided by Dr. J. L. Benovic, Thomas Jefferson University) in pcDNA3 were used in most experiments. The membrane-tethered GRK2 mutants were constructed by attaching the amphipathic helix plus palmitoylation motif from GRK6A or the amphipathic helix from GRK5, in both cases followed by the geranylgeranylation motif ACVLL at the GRK2 C terminus, yielding H6Ger and H5Ger membrane-tethered mutants, respectively. The mutations were introduced by PCR and confirmed by dideoxy-sequencing. All GRKs were subcloned into pcDNA3.

Cell Culture and Transfection—HEK293A and HEK293FT cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37 °C and 5% CO_2. Lipofectamine2000 (Invitrogen) (1:2.5 DNA/lipid) in Opti-MEM was used to transfect cells. DNA amounts in each transfection were kept constant by the addition of empty vector, where necessary. All experiments were conducted 48 h post-transfection.

Antibodies—Anti-HA antibody for immunoprecipitation was from Roche Diagnostics; anti-phosphothreonine antibodies were from Cell Signaling Technology (Beverly, MA), and anti-phosphoserine was from Invitrogen. The antibody against

phospho-Ser$^{355/356}$ of β2AR, which was specifically phosphorylated by GRKs (18), was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Stimulation and Immunoprecipitation—HEK292FT cells co-transfected with the indicated receptor and GRK clones were stimulated with appropriate agonists for 10 min at 37 °C (control cells were exposed to vehicle for the same time), scraped off plates, collected by centrifugation in phosphate-buffered saline, and resuspended in the immunoprecipitation buffer (IPB) containing 50 mM Tris-HCl, 2 mM EDTA, 250 mM NaCl, 10% (v/v) glycerol, 0.5% Nonidet P-40, 20 mM NaF, 1 mM sodium orthovanadate, and 10 mM *N*-ethylmaleimide. Benzamidine (2 mM final concentration) and phenylmethylsulfonyl fluoride (1 mM) were added immediately before use. Cells were lysed at 4 °C for 1 h and centrifuged to remove the debris. The supernatant was pre-cleared by incubating with 25–30 μl of protein G-agarose for 1 h at 4 °C. Receptors were then immunoprecipitated by incubating the supernatant overnight at 4 °C with anti-HA antibody (1–2 μg per 60-mm dish) and 20–25 μl of protein G-agarose. Beads were washed three times with IPB, and bound proteins were eluted by boiling in Laemmli SDS buffer for 5 min.

Western Blotting—The proteins were analyzed by reducing SDS-PAGE and Western blotting onto Immobilon-P (Millipore, Bedford, MA) membrane. The membrane was blocked with 5% nonfat dry milk in TBS at room temperature for 1 h and then incubated in TBS supplemented with 0.1% Triton X-100 and 1% BSA and appropriate primary antibody overnight at 4 °C. Blots were incubated with secondary antibodies coupled with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature, and bands were visualized by SuperSignal enhanced chemiluminescence reagent (Pierce). The bands on the film were quantified using VersaDoc 4 (Bio-Rad). GRKs used as standards were expressed in High Five cells and purified to homogeneity as described previously, as follows: human GRK2$_{S670A}$-H$_6$ (12, 37), bovine GRK5-H$_6$ (38), human palmitoylation-deficient GRK6 (pal$^-$) (13), and bovine GRK3 (39).

BRET Assay—BRET assays were used to measure the GRK-dependent binding of Venus-arrestin to β2AR-RLuc8, as described recently (35, 36, 40). HEK293FT cells were co-transfected with β2AR-RLuc8, Venus-arrestin-2 or -3, and GRK2, GRK5, or equal amounts of empty vector using Lipofectamine2000 according to the manufacturer's instructions. Isoproterenol (10 μM) or vehicle was added to the cells 5 min before the addition of 5 mM coelenterazine-h. The BRET ratio was calculated as the long wavelength emission divided by the short wavelength emission, and the net BRET was calculated as an increase of BRET ratio, as compared with cells that do not express exogenous GRKs. The expression of Venus-arrestins was evaluated using fluorescence at 535 nm upon excitation at 485 nm. The Venus-arrestin fluorescence, which is directly proportional to the expression levels, was normalized by the basal luminescence from β2AR-RLuc8 construct (fluorescence/luminescence ratio) to account for variations in cell number and expression levels.

Arrestin Recruitment Assay—Chimeric β2AR with V2 C terminus (β2/V2) (34) was co-expressed arrestin-3-GFP (32) without exogenous GRKs or with GRK2 or GRK5. The cells were serum-starved overnight and then imaged live without agonists or following exposure to 10 μM β-agonist isoproterenol. Arrestin-3-GFP was visualized by its fluorescence, and the images were acquired using Nikon LC2000 automated microscope with ×60 oil immersion objective.

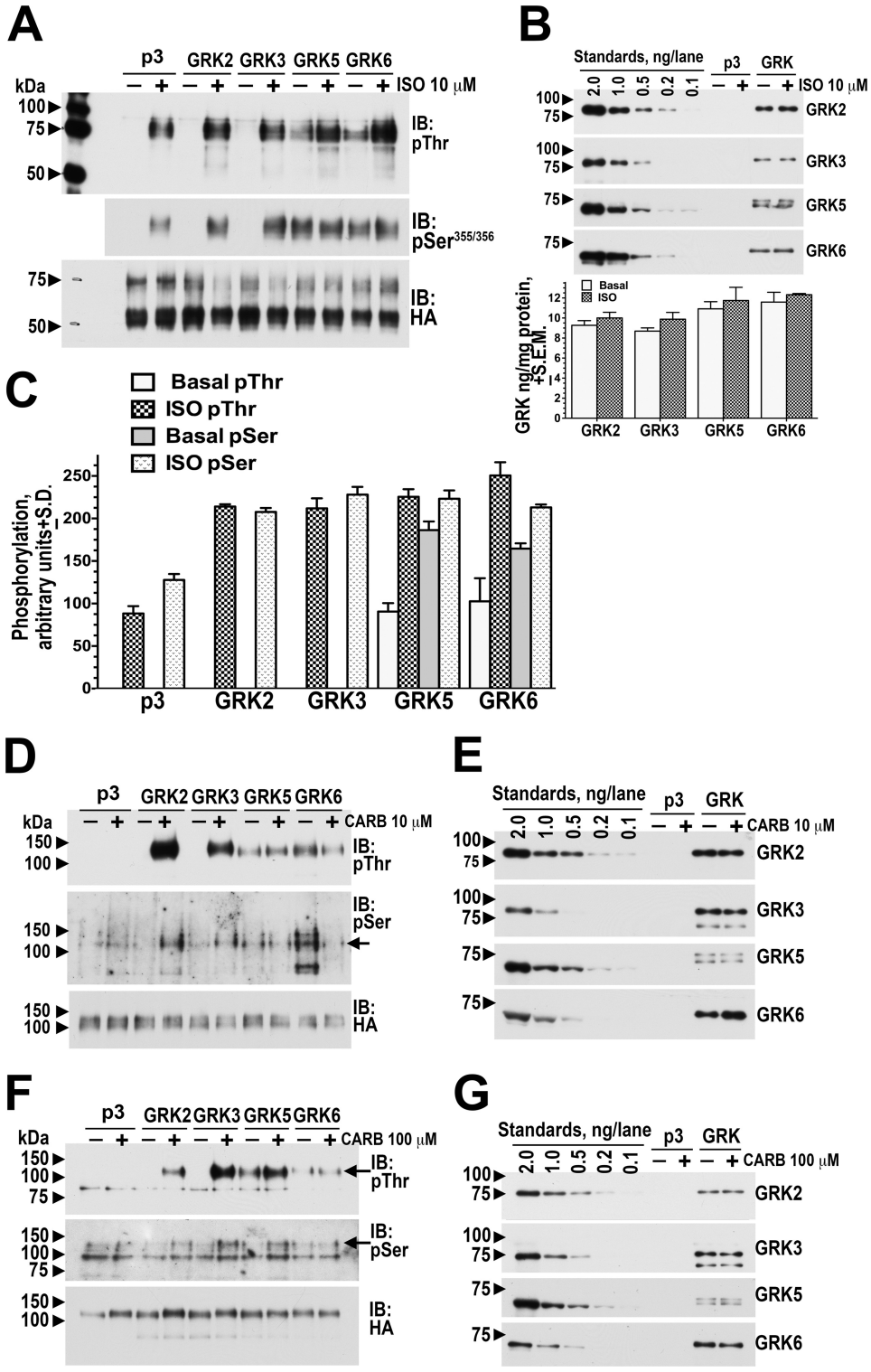
Statistical Analysis—StatView (SAS Institute) software was used for statistical analysis of quantitative data. The data were analyzed by analysis of variance or Student's *t* test where appropriate. In all cases, $p < 0.05$ was considered significant.

RESULTS

GRK5 and -6 but Not GRK2 and -3 Phosphorylate GPCRs Independently of Agonist Stimulation—One distinguishing characteristic of GRKs is their selectivity toward activated GPCRs (6, 7, 10–12). Based on sequence homology, GRKs are divided into three major subfamilies All members of two subfamilies, GRK2/3 and GRK4/5/6, except GRK4, are ubiquitously expressed and capable of phosphorylating multiple GPCRs (15). GRK isoforms from the GRK4/5/6 subfamily were reported to phosphorylate at least some GPCRs in an agonist-independent manner with reasonable efficacy (16–18). To determine whether this ability is an inherent characteristic of the GRK4 subfamily, we tested GRK-mediated phosphorylation of six different GPCRs (β2AR, D1R, M2R, D2R, M3R, and 5-HT2C) in cultured cells by two GRK isoforms from each subfamily, GRK2 and GRK3 from the first and GRK5 and GRK6 from the second. To measure receptor phosphorylation in HEK292FT cells transfected with expression vectors encoding one GRK and one GPCR, we immunoprecipitated receptors, all of which were tagged with triple HA at the N terminus, and we detected phosphorylation with anti-phospho-Thr and anti-phospho-Ser antibodies. Using β2AR, we found that although its phosphorylation in the absence of agonist by GRK2 and GRK3 was negligible, both GRK5 and GRK6 exhibited ~40 and ~80% of maximum phosphorylation of threonines and serines in the receptor in the absence of agonist (Fig. 1, *A* and *C*). All four GRKs were expressed at virtually the same levels, as assessed by quantitative Western blot with calibration curves constructed with known amounts of each purified GRK (Fig. 1*B*). Note that in the presence of isoproterenol, β2AR is phosphorylated by endogenous GRKs (Fig. 1*A*). Because GRK2 is the predominant isoform expressed in HEK293 cells (Figs. 1–5), this result is consistent with strict agonist dependence of phosphorylation in the absence of exogenous GRKs (Fig. 1*A*).

Because this finding was unexpected, we tested the phosphorylation of two receptors available in purified form, rhodopsin and β2AR, measuring phosphorylation by a totally different method, the incorporation of radioactive phosphate (Fig. 2). Three forms of rhodopsin were used as follows: dark, light-activated (Rho*), and opsin lacking retinal, all in native disc membranes. Purified GRK1 (rhodopsin kinase), GRK2, and GRK6 phosphorylated rhodopsin strictly in an activation-dependent manner, demonstrating virtually no activity toward dark rhodopsin and less than 5% of activity toward opsin, as compared with Rho* (Fig. 2, *A* and *B*). In contrast, the activity of GRK5 toward dark rhodopsin (which has covalently bound inverse agonist 11-*cis*-retinal) was ~8% of that toward Rho*,

whereas the activity toward opsin reached ~40% of that toward Rho* (Fig. 2, *A* and *B*). Overall, GRK6 demonstrated the lowest ability to phosphorylate any form of rhodopsin, including Rho* (Fig. 2*A*). In the same paradigm, we compared the activity of GRK2 and GRK5 toward purified β2AR reconstituted into a monomeric form in HDL particles (29) in the presence of high affinity agonist BI-167107 (30) or the potent inverse agonist carazolol (Fig. 2*B*) (31). In agreement with previous reports, we found that the phosphorylation of agonist-activated β2AR by GRK2 is ~15-fold higher than of inactive receptor. However, this difference was less than 3-fold in the case of GRK5 (Fig. 2*B*), suggesting that either GRK5 has a higher constitutive activity than GRK2 or that GRK5 can interact with and become activated by even the inactive form of β2AR in HDL particles. These data, obtained in a totally different experimental paradigm, where the level of receptor phosphorylation is measured



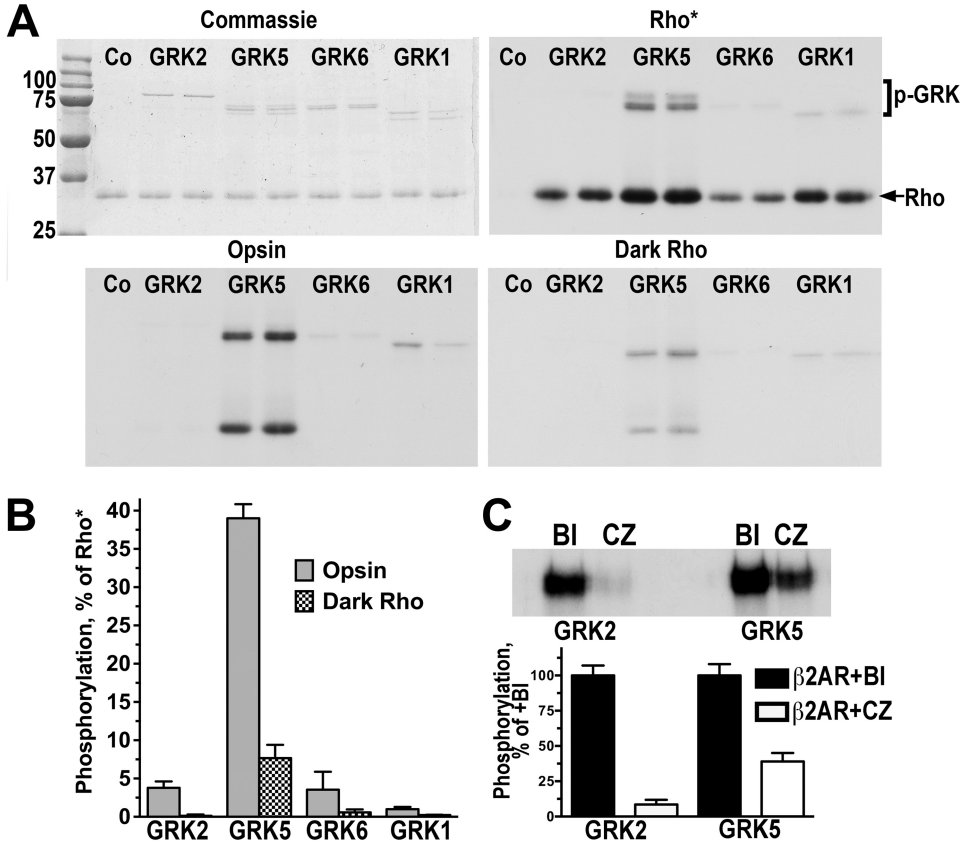


FIGURE 2. **GRK5 phosphorylates inactive rhodopsin and β2AR.** *A,* representative autoradiography of *in vitro* phosphorylation with [γ-^{32}P]ATP of purified light-activated (*Rho**), dark (*Dark Rho*) rhodopsin, and opsin. *B,* quantification of the phosphorylation of different functional forms of rhodopsin by purified GRK1, -2, -5, and -6. The levels of phosphorylation of dark Rho and opsin are expressed as % of Rho* phosphorylation by the same GRK. *C,* representative autoradiography of *in vitro* phosphorylation with [γ-^{32}P]ATP of purified β2AR by purified GRK2 or GRK5 in the presence of selective agonist BI-167107 (*BI*) or inverse agonist carazolol (*CZ*). The phosphorylation experiments were performed as described under "Experimental Procedures." The *bar graph* shows quantification of the phosphorylation data from three independent experiments.

directly by phosphate incorporation, are in remarkably good agreement with in-cell data (Fig. 1, *A* and *C*), suggesting that the ability to phosphorylate inactive rhodopsin and β2AR is an inherent characteristic of GRK5.

Next, we performed similar experiments with the M2R (Fig. 1*D*) and the M3R (Fig. 1*F*), ascertaining equal expression of all GRKs in each case (Fig. 1, *E* and *G*). In contrast to the results with the β2AR, phosphorylation of these receptors by endogenous GRKs was minimal (Fig. 1, *D* and *F*), suggesting that GRK2, at least expressed at the endogenous level, is insufficient for phosphorylation. Expressed GRK2 and GRK3 phosphorylated the M2R very effectively and only in the presence of an agonist, whereas GRK5 and GRK6 demonstrated virtually equal phosphorylation of active and inactive M2R (Fig. 1*D*). In the case of the M3R, GRK3 showed higher phosphorylation levels than other isoforms, with GRK5 being the second most active.

FIGURE 1. **Agonist-independent GPCR phosphorylation by GRK5 and GRK6.** *A,* representative Western blots of in-cell GRK-dependent phosphorylation of β2AR. HEK293FT cells transfected with triple HA-tagged β2AR and co-transfected with indicated GRKs (or empty vector as control; *p3*) were stimulated with isoproterenol (*ISO*; 10 μM) for 10 min as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*) or with antibody directed against phospho-Ser$^{355/356}$ of β2AR (*middle panel*). *Lower panel* shows the level of immunoprecipitated β2AR in samples detected with anti-HA antibody. *IB*, immunoblot. *B,* quantification of the level of GRK expression in phosphorylation experiments. Representative blots show the expression of each GRK isoform. *Left five lanes* show standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform in absolute units. *Bar graph below* shows the data from four independent experiments. *C,* quantification of the level of βAR phosphorylation at Thr(P) and Ser(P)-$^{355/366}$ mediated by each GRK. Note considerable phosphorylation of β2AR by GRK5 and -6 but not GRK2 or -3 in the absence of the agonist. *D,* representative Western blots of in-cell GRK-dependent phosphorylation of M2R. HEK293FT cells transfected with triple HA-tagged M2R and co-transfected with indicated GRKs (or empty vector; *p3*) were stimulated with carbachol (*CARB*; 10 μM) for 10 min as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*) or with antibody directed against phospho-Ser (*pSer, middle panel*). *Lower panel* shows the level of immunoprecipitated M2R detected with anti-HA antibody. *E,* quantification of the level of GRK expression in phosphorylation experiments shown in *D*. Representative blots show the expression of each GRK. *Left five lanes*, standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform in absolute units. *F,* representative Western blots of in-cell GRK-dependent phosphorylation of M3R. HEK293FT cells transfected with triple HA-tagged M3R and co-transfected with GRKs (or empty vector control; *p3*) were stimulated with carbachol (*CARB*; 100 μM) for 10 min as described under "Experimental Procedures." Cells were collected, and the receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*) or with antibody directed against phospho-Ser (*pSer*, *middle panel*). *Lower panel* shows the level of immunoprecipitated M3R detected with anti-HA antibody. *G,* quantification of the level of GRK expression in experiments shown in *F*. Representative blots show the expression of each GRK. *Left five lanes*, standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform in absolute units.

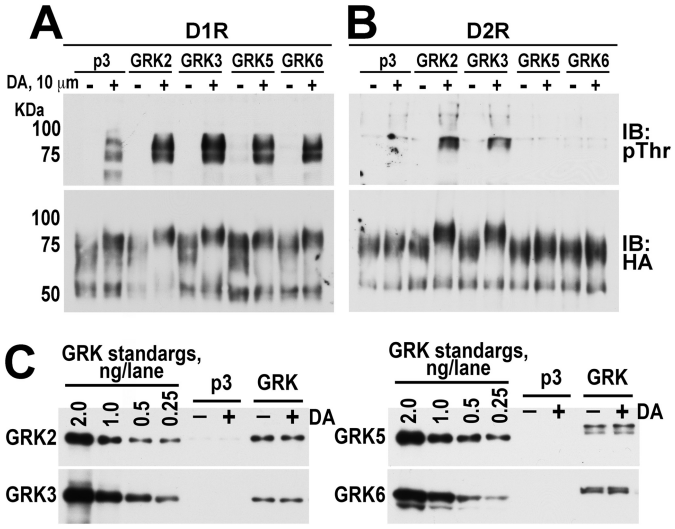


FIGURE 3. **Differential phosphorylation of active and inactive dopamine D1 and D2 receptor by GRK isoforms.** *A,* representative Western blots of in-cell GRK-dependent phosphorylation of D1R. HEK293FT cells transfected with triple HA-tagged D1R and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with dopamine (*DA*; 10 μM) for 10 min as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr, upper panel*). *Lower panel* shows the level of immunoprecipitated D1R detected with anti-HA antibody. *B,* representative Western blots of in-cell GRK-dependent phosphorylation of D2R. HEK293FT cells transfected with triple HA-tagged D2R and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with dopamine (*DA*; 10 μM) for 10 min as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*). *Lower panel* shows the level of immunoprecipitated D2R detected with anti-HA antibody. *IB*, immunoblot. *C,* quantification of the level of GRK expression in phosphorylation experiments shown in *A* and *B*. Representative blots show the expression of each GRK. *Left five lanes*, standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform.

Despite a clear difference in the GRK preference of the M2 and M3 receptors, the pattern remained the same; phosphorylation by GRK2 and GRK3 was strictly dependent on the presence of agonist, whereas both GRK5 and GRK6 showed significant activation-independent phosphorylation (Fig. 1, *D* and *F*).

Similar experiments with D1R yielded different results. All GRKs demonstrated comparable levels of agonist-induced phosphorylation and very low activation-independent phosphorylation (Fig. 3*A*). Interestingly, the phosphorylation of D1R by endogenous GRKs, without GRK overexpression, was strictly activation-dependent, in agreement with the predominance of GRK2 among endogenous GRKs in these cells. Experiments with D2R revealed yet another pattern; only overexpressed GRK2 and GRK3 yielded measurable phosphorylation, which was strictly agonist-dependent (Fig. 3*B*), as was always the case with these two GRKs (Figs. 1–3). Thus, in agreement with the previous report (41), D2R appears to have selectivity for GRK2/3 and is largely resistant to phosphorylation by GRK5/6 (Fig. 3*B*).

Agonist-independent Receptor Phosphorylation Is Not Inhibited by Inverse Agonists—Many GPCRs demonstrate significant constitutive activity, *i.e.* the ability to assume active conformation even in the absence of an agonist. This is explicitly taken into account in the extended ternary complex model of GPCR signaling (42), now widely accepted in the field. Therefore, agonist-independent phosphorylation by GRK5 and GRK6 could reflect constitutive activity of the GPCRs tested, as suggested previously for β2AR (17). However, constitutive receptor activity would likely be "sensed" by all GRKs, which was not the case (Fig. 1). In fact, the same receptors that were phosphorylated by GRK5 and GRK6 in the absence of agonists were phosphorylated in a strictly agonist-dependent manner by GRK2 and GRK3 expressed at the same levels (Fig. 1). However, it is conceivable that receptors could be "biased" toward particular GRKs, similar to known bias toward particular G proteins or arrestins (43). Inverse agonists "freeze" receptors in inactive conformation and thus should inhibit GRK5/6-dependent phosphorylation if it is due to constitutive receptor activity.

Therefore, to determine whether significant agonist-independent phosphorylation of β2AR by GRK5 and GRK6 relies on constitutive activity of the receptor, we compared β2AR phosphorylation in the absence of ligands, as well as in the presence of classical full agonist isoproterenol and two different inverse agonists, ICI118551 and carazolol (Fig. 4). We found that endogenous GRKs, as well as exogenously expressed GRK2 and GRK3, phosphorylated β2AR only in the presence of isoproterenol, whereas levels of phosphorylation by GRK5 and GRK6 in the absence of ligands and in the presence of either inverse agonist were similar, reaching about half or more of the maximum level achieved in the presence of isoproterenol (Fig. 4). These data strongly argue against constitutive activity of the receptor playing a role in its agonist-independent phosphorylation by GRK5 and GRK6. Thus, the ability to phosphorylate an inactive receptor, as well as a receptor inactivated by inverse agonists, is an inherent property of these GRKs.

Activity of GRK5 and -6 toward Inactive GPCRs Is Not Solely Because of Their Association with the Membrane—GRK5 and -6 are equipped with structural elements ensuring their semi-permanent attachment to the plasma membrane. GRK5 possesses an amphipathic helix in its C terminus (44), whereas the GRK6A splice variant in addition to this helix also has palmitoylation sites (45–47). In contrast, the recruitment of GRK2 and GRK3 to the membrane is believed to depend on the interaction of their pleckstrin homology domain with Gβγ released upon G protein activation (48, 49). To test whether the constant presence at the membrane facilitates agonist-independent receptor phosphorylation, we constructed mutants of GRK2 that incorporated the amphipathic helix of GRK5 or GRK6 along with the geranylgeranylation motif of GRK7 (50) to ensure constitutive membrane localization. Cell fractionation revealed a significant increase in the fraction of these mutants associated with plasma membrane, as compared with largely cytosolic WT GRK2 (Fig. 5*B*). Membrane-tethered GRK2 mutants demonstrated somewhat higher agonist-independent activity than WT GRK2 (Fig. 5, *A* and *C*). However, agonist-independent phosphorylation of β2AR by these mutants remained well below that produced by WT GRK5, which has a similar degree of preferential membrane localization (Fig. 5*B*) and was expressed at the same overall level as GRK2 and its mutants (Fig. 5*D*).

Experiments with membrane-tethered GRK2 mutants and the M3R (Fig. 5*E*) demonstrated an even smaller effect of mem-

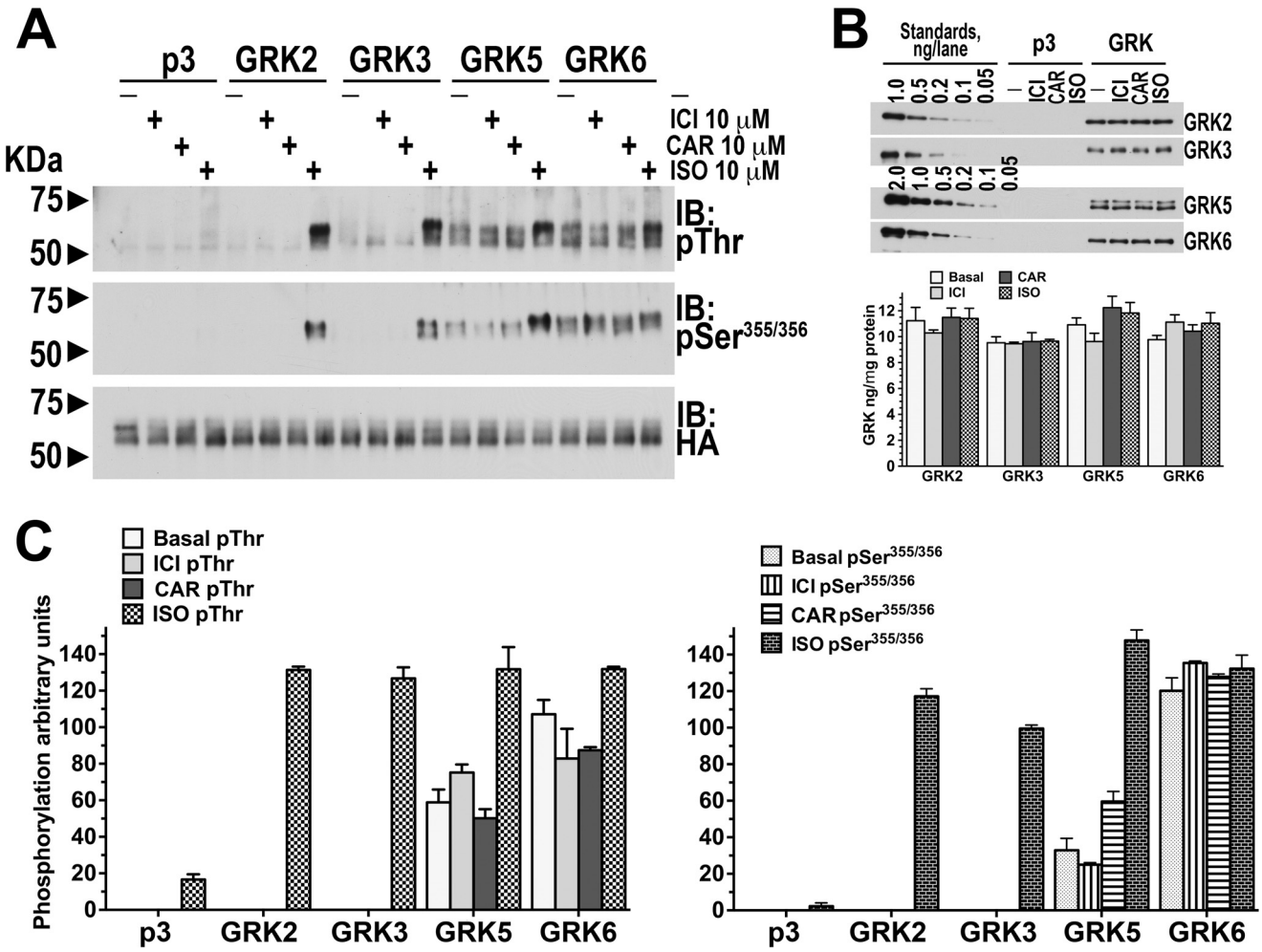


FIGURE 4. **Inverse agonists do not inhibit agonist-independent phosphorylation of β2AR by GRK5 and -6.** *A,* representative Western blots of in-cell GRK-dependent phosphorylation of β2AR in the presence of inverse agonists ICI118551 (*ICI*) and carazolol (*CAR*), as compared with full agonist isoproterenol (*ISO*). HEK293FT cells transfected with triple HA-tagged β2AR and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with the indicated drugs for 10 min, as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*) or with antibody directed against phospho-Ser$^{355/356}$ of β2AR (*middle panel*). *Lower panel* shows the level of immunoprecipitated β2AR detected with anti-HA antibody. *IB*, immunoblot. *B,* quantification of GRK expression in experiments shown in *A*. Representative blots show the expression of each GRK. *Left five lanes*, standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform in absolute units. *Graph* presents the data from four independent experiments. *C,* quantification of the level of β2AR phosphorylation at Thr(P) (*left graph*) and Ser(P)-$^{355/366}$ (*right graph*) mediated by each GRK. Note considerable phosphorylation of β2AR by GRK5 and -6, but not GRK2 or -3, which is not inhibited by inverse agonists.

brane anchors; the phosphorylation by all forms of GRK2 was strictly dependent on receptor activation, whereas GRK5 showed significant agonist-independent phosphorylation (Fig. 5*E*). In these experiments GRK5, GRK2, and its mutant forms were expressed at essentially the same level (Fig. 5, *F* and *G*). Thus, constitutive membrane association of GRK5 and GRK6 *per se* does not explain agonist-independent receptor phosphorylation by these kinases. Apparently, phosphorylation of inactive receptors by these kinases reflects their inherent functional characteristics, which cannot be reproduced in GRK2 even by constitutive membrane localization.

Phosphorylation of Serotonin 5-HT2C Receptor by GRK5/6 Is Independent of Its Editing- and Ligand-dependent Activity—The mRNA of serotonin 5-HT2C receptor was shown to be edited by adenosine deaminases, which leads to changes in the protein sequence of the third cytoplasmic loop (51). Interestingly, the receptor encoded by unedited mRNA (containing sequence Ile-Asn-Ile, or INI) has fairly high constitutive activity, whereas constitutive activity of the fully edited receptor carrying the sequence Val-Gly-Val (VGV) is very low (52). Thus, these two variants of the 5-HT2C receptor, which contain exactly the same potential phosphorylation sites, differ primarily by the level of agonist-independent activity. We used these receptors to determine the role of constitutive activity in phosphorylation by GRKs in intact cells and found that GRK2 and GRK3 do not appreciably phosphorylate either receptor, whereas GRK5 and GRK6 expressed at the same level phosphorylate both comparably (Fig. 6, *A–C*). Similar to our observations with M2 and M3 muscarinic receptors (Fig. 1, *D* and *F*), natural full agonist 5-HT does not significantly increase phosphorylation level of either INI or VGV form by GRK5 or GRK6 (Fig. 6*A*). Next, we tested whether SB206553, a known inverse agonist of the 5-HT2C receptor (53), can suppress its phosphorylation by GRK5/6 and detected the same level of phosphothreonine in both INI and VGV, which have high and low constitutive activity, respectively, in the absence of ligands, the

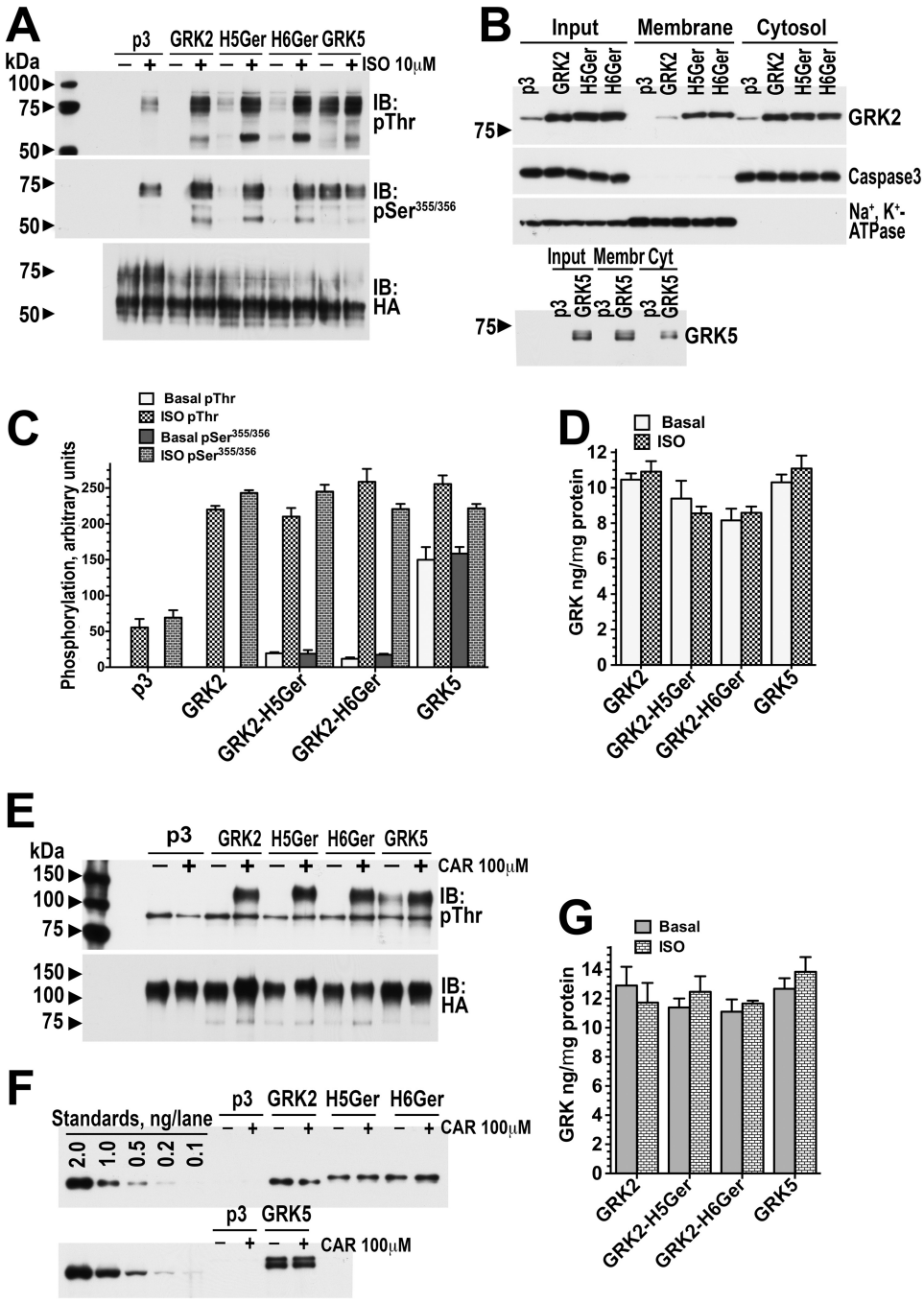


FIGURE 5. **Constitutive membrane association does not facilitate agonist-independent receptor phosphorylation by GRK2.** *A,* representative Western blots of in-cell phosphorylation of β2AR by wild type GRK2, GRK5, and GRK2 mutants with lipid modifications. HEK293FT cells transfected with triple HA-tagged β2AR and co-transfected with the indicated GRKs (or empty vector control; *p3*) were stimulated with isoproterenol (*ISO*; 10 μM) for 10 min, as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*) or with antibody directed against phospho-Ser$^{355/356}$ of β2AR (*middle panel*). *Lower panel* shows the level of immunoprecipitated β2AR detected with anti-HA antibody. *IB*, immunoblot. *B,* subcellular distribution of WT GRK2, GRK2 mutants, and GRK5. Caspase3 was used as a marker for cytosol and Na^+,K^+-ATPase as a marker for the membrane fraction. Note higher membrane association of GRK5 and lipid-modified GRK2 mutants, as compared with wild type GRK2. *C,* quantification of the level of β2AR phosphorylation at Thr(P) and Ser(P)-$^{355/366}$ mediated by each GRK. Note considerable phosphorylation of β2AR by GRK5 in the absence of the agonist. Means ± S.D. of three experiments are shown. The phosphorylation of β2AR in the absence of the agonist by membrane-associated GRK2 mutants, although slightly increased in comparison with wild type GRK2, remains considerably lower than that mediated by GRK5. *D,* quantification of the expression of WT and mutant GRKs in phosphorylation experiments shown in *A* and *C*. The data from three independent experiments are shown as mean ± S.E. *E,* representative Western blots of in-cell phosphorylation of M3R by wild type GRK2, GRK5, and GRK2 mutants with lipid modifications. HEK293FT cells transfected with triple HA-tagged M3R and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with carbachol, as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptor was blotted with anti-phospho-Thr (*pThr*, *upper panel*). *Lower panel* shows the level of immunoprecipitated M2R detected with anti-HA antibody. *F,* expression of wild type and mutant GRK2 and GRK5 in experiments shown in *D*. *G,* quantification of the expression of WT and mutant GRKs in phosphorylation experiments shown in *E*. The data from three independent experiments are shown as mean ± S.E.

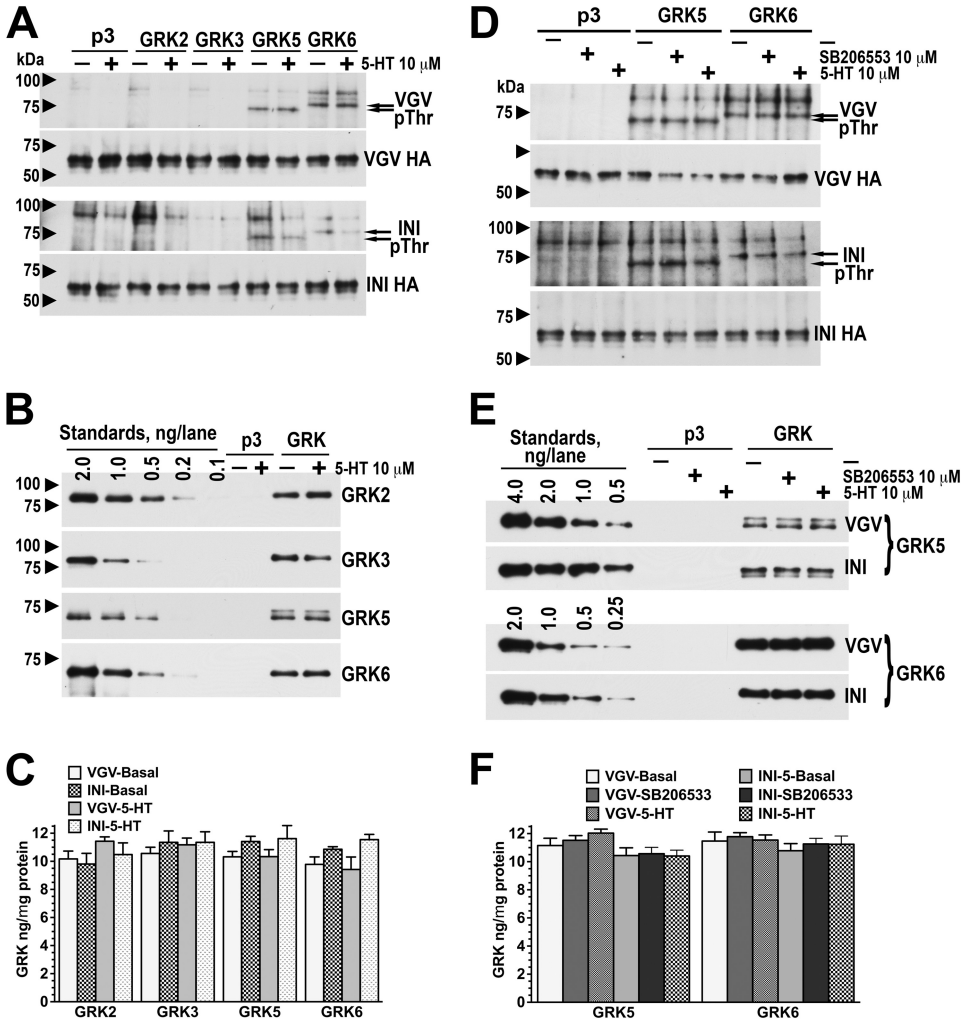


FIGURE 6. **Differential phosphorylation of unedited (INI) or fully edited (VGV) 5-HT2C receptor by GRKs.** *A,* representative Western blots of in-cell GRK-dependent phosphorylation of 5-HT2C by GRKs. HEK293FT cells transfected with triple HA-tagged VGV-5-HT2C or INI-5-HT2C and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with 5-HT, as described under "Experimental Procedures." Cells were collected, and receptor was immunoprecipitated with anti-HA antibody. The receptors were blotted with anti-phospho-Thr (*pThr*). *Lower panels* in each pair show levels of immunoprecipitated receptors detected with anti-HA antibody. *B,* representative blots show the expression of GRK isoforms in phosphorylation experiments shown in *A*. *Left five lanes*, standard dilutions of purified recombinant GRKs used to construct calibration curves for quantification of each GRK isoform in absolute units. *C,* quantification of the expression of the GRK isoforms in phosphorylation experiments shown in *A*. *D,* representative Western blots of in-cell GRK-dependent phosphorylation of 5-HT2C in the presence of inverse agonist SB206533 and full agonist 5-HT. HEK293FT cells transfected with triple HA-tagged VGV-5-HT2C or INI-5-HT2C and co-transfected with indicated GRKs (or empty vector control; *p3*) were stimulated with the indicated drugs for 10 min, as described under "Experimental Procedures." Cells were collected; receptors were immunoprecipitated with anti-HA antibody and blotted with anti-phospho-Thr (*pThr*, *upper panel*) or anti-HA antibody. *E,* representative blots show the expression of GRK5 and GRK6 in phosphorylation experiments shown in *D*. *Left four lanes*, standard dilutions of purified recombinant GRKs. *F,* quantification of the expression of the GRK5 and -6 in phosphorylation experiments shown in *D*.

presence of the agonist 5-HT, or the inverse agonist SB206533 (Fig. 6, *D–F*). Thus, neither mRNA editing nor the presence of activating or inactivating ligands affects 5-HT2C receptor phosphorylation by GRK5 and GRK6 (Fig. 6). These data demonstrate activation-independent phosphorylation of two forms of yet another GPCR, 5-HT2C, by GRK5 and GRK6. To summarize, we detected phosphorylation by GRK5/6 of many inactive GPCRs as follows: β2AR, M2, M3, and the INI and VGV variants of the 5-HT2C receptor (Figs. 1, 3–6). Interestingly, the D1R was the only receptor that was phosphorylated by GRK5/6 in a largely activation-dependent manner.

Agonist-independent Receptor Phosphorylation by GRK5 Promotes Arrestin Recruitment—The best known functional result of GPCR phosphorylation by GRKs is increased affinity for arrestin proteins (54, 55). Most GPCRs are subject to the two-step homologous desensitization as follows: phosphorylation of active receptor by GRK, followed by high affinity arrestin binding to active phosphoreceptor (14, 56). However, only visual arrestin-1 is exquisitely selective for the active and phosphorylated form of its cognate receptor, rhodopsin (57–59). Direct binding studies with arrestin-2 and arrestin-3 revealed that both nonvisual subtypes bind with fairly high affinity to inactive phosphorylated GPCRs, with this binding reaching 70–80% of maximum achieved with corresponding active phosphoreceptors (60–62). Therefore, to determine whether agonist-independent phosphorylation of GPCRs by GRK5 has any functional consequences, we examined arrestin recruitment in intact cells, using BRET between β2AR tagged with *Renilla*

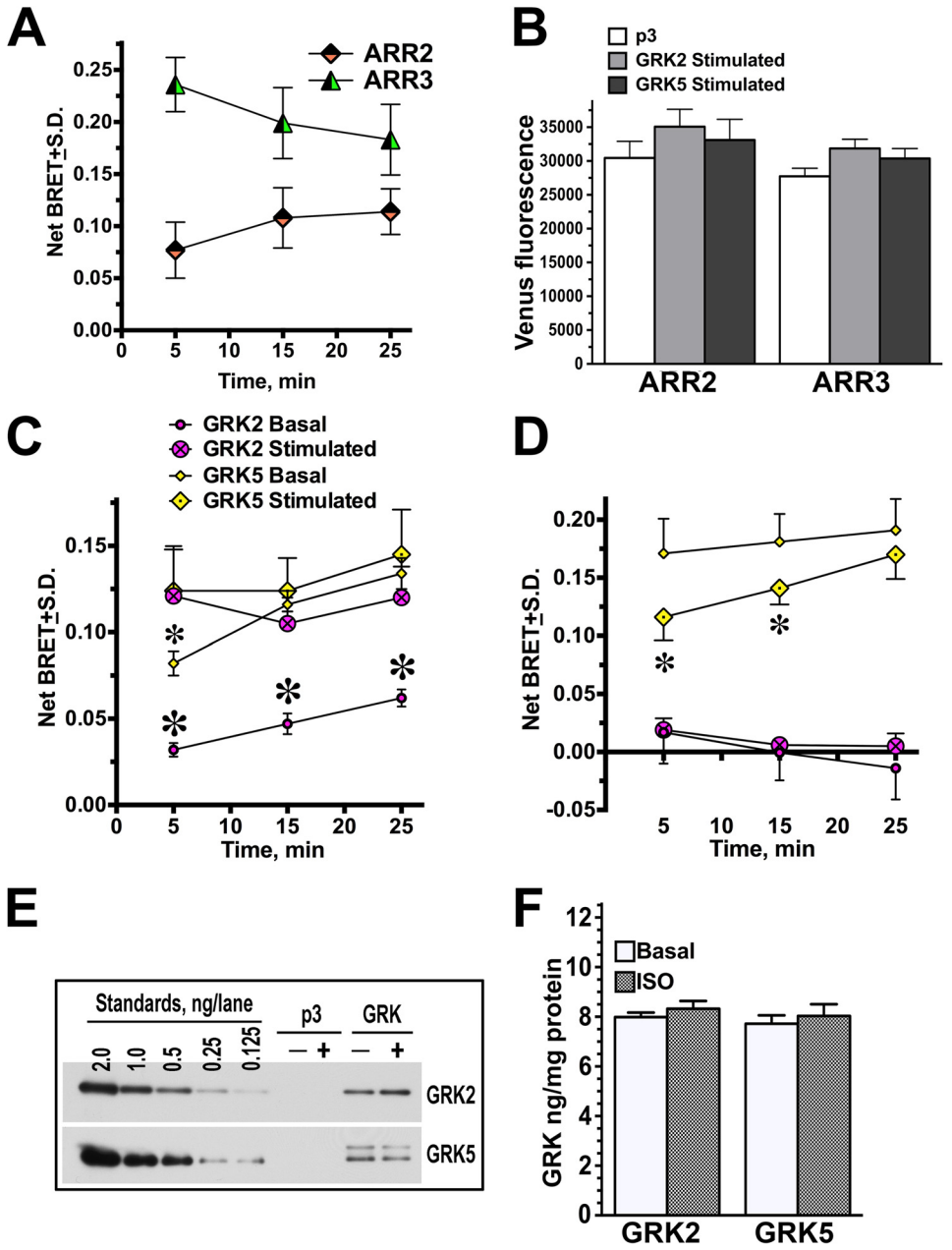


FIGURE 7. **Agonist-independent phosphorylation of β2AR by GRK5 promotes arrestin binding.** HEK293FT cells were transfected with RLuc8-tagged β2AR, Venus-arrestin, and co-transfected with empty vector (*p3*), GRK2, or GRK5. BRET was measured at 5, 15, and 25 min after the addition of agonist or vehicle, as described under "Experimental Procedures." Net BRET (increase in BRET induced by additional GRK as compared with p3) is shown. *A,* net BRET of Venus-arrestin-2 as a function of time following agonist stimulation without exogenous GRKs. *B,* expression of Venus-tagged arrestins as measured by Venus fluorescence across experiments (mean ± S.E.). *C,* net BRET of Venus-arrestin-2 as a function of time with or without (basal) agonist stimulation in the presence of GRK2 or GRK5. *D,* net BRET of Venus-arrestin-3 as a function of time with or without (basal) agonist stimulation in the presence of GRK2 or GRK5. Means ± S.D. of four independent experiments are shown. *, $p < 0.05$ to respective basal condition using an unpaired Student's *t* test. *E,* representative blots; *F,* quantification showing equal expression of GRK2 and -5 in BRET experiments shown in *C* and *D*.

luciferase at the C terminus and arrestins tagged with Venus at the N terminus (35, 36, 40, 63, 64). The BRET ratio in these experiments was determined as described previously (35, 36, 63), but because we were primarily interested in GRK-dependent increase of arrestin recruitment, we calculated net BRET as an increase in BRET ratio in the presence of exogenous GRK, as compared with cells that only express endogenous GRKs along with tagged β2AR and arrestins. In agreement with agonist-dependent increase in β2AR phosphorylation in the presence of exogenous GRK2 (Fig. 1*A*), we observed only a minimal increase in arrestin-2 recruitment to the β2AR in the absence of receptor activation. In contrast, a significant increase was observed upon β2AR activation by isoproterenol (Fig. 7*A*). Unlike GRK2, overexpressed GRK5 increased receptor phosphorylation levels both in the presence and absence of agonists (Fig. 1*A*). This translates into a comparable increase in arrestin-2 recruitment in both cases (Fig. 7*A*). Arrestin-3, because of structural peculiarities of the receptor-binding surface unique for this subtype (65), is the most promiscuous of the two nonvisual arrestins, demonstrating the least pronounced

selectivity for active receptor over inactive and for phosphorylated over nonphosphorylated forms (60–62, 65). Overexpression of GRK2 does not enhance the recruitment of arrestin-3 to β2AR regardless of activation (Fig. 7*B*), suggesting that the phosphorylation by endogenous GRKs (Fig. 1*A*) is sufficient for arrestin-3 binding. Interestingly, upon agonist activation the overall increase in the level of phosphoserine and phosphothreonine in cells overexpressing GRK5 is similar to that observed upon GRK2 overexpression (Fig. 1*A*). However, GRK5 induced a profound increase in arrestin-3 recruitment to β2AR that GRK2 did not have (Fig. 7*B*). We ascertained in these experiments that both nonvisual arrestins (Fig. 7*C*) and both GRK2 and GRK5 (Fig. 7*D*) were expressed at the same level, which makes direct comparisons of BRET signal across all experiments valid. These data strongly suggest that GRK5 likely phosphorylates Ser and/or Thr residues distinct from those targeted by GRK2, and this differential phosphorylation by the two GRKs is differentially "read" by arrestin-3, which apparently prefers phosphates at positions targeted by GRK5. Importantly, arrestin-3 recruitment to unstimulated β2AR phosphorylated by GRK5 is essentially the same as to the agonist-activated β2AR (Fig. 7*B*). Thus, the presence of GRK5 induces the formation of the arrestin-2 and arrestin-3 complexes with the receptor regardless of agonist stimulation (Fig. 7).

As these results were rather unexpected, we used an alternative method to observe receptor-arrestin interactions. To this end, we used β2AR with the V2 C terminus that was previously shown to form stable complexes with nonvisual arrestins (34), along with arrestin-3-GFP, the recruitment of which to the plasma membrane in response to receptor activation can be easily visualized (32). In HEK293 cells expressing only endogenous GRKs (where the predominant form is GRK2), arrestin-3-GFP is evenly distributed throughout the cytoplasm (Fig. 8*A*), is recruited to the membrane upon receptor stimulation (Fig. 8*B*), and after 20–30 min of isoproterenol treatment is found in the endosomes, visible as intracellular puncta (Fig. 8*C*), as reported previously (34). Co-expression of GRK2 does not change this pattern (Fig. 8, *D–F*). In contrast, in a significant proportion of cells tranfected with GRK5, the recruitment of arrestin-3-GFP to the membrane and even its accumulation in the intracellular puncta can be observed in the absence of agonist (Fig. 8, *G1–G3*), similar to its localization upon brief (Fig. 8*H*) or extended (Fig. 8*I*) agonist treatment. Thus, it appears that agonist-independent phosphorylation of the receptor by GRK5, which can be easily observed using receptor immunoprecipitation and Western blotting for Thr(P) (Fig. 8*J*), leads to the recruitment of arrestin to the receptor even in the absence of an agonist (Fig. 8, *G1–G3*). Thus, two different methods show that agonist-independent GRCR phosphorylation by GRK5 translates into the recruitment of arrestin to inactive receptor (Figs. 7 and 8).

Because the formation of the arrestin-receptor complex appears to initiate several branches of cell signaling (56, 66, 67), these data suggest that the cellular complement of GRKs likely determines the level of arrestin-mediated signaling, which can be regulated by the level of GPCR and/or GRK4/5/6 expression, not only via receptor activation by agonists. Because bound nonvisual arrestins also serve as the bridge between GPCR and clathrin (68) and AP2 (69), arrestin binding to inactive GPCRs phosphorylated by GRK5/6 might also drive basal cycling of these receptors between plasma membrane and endocytic compartments.

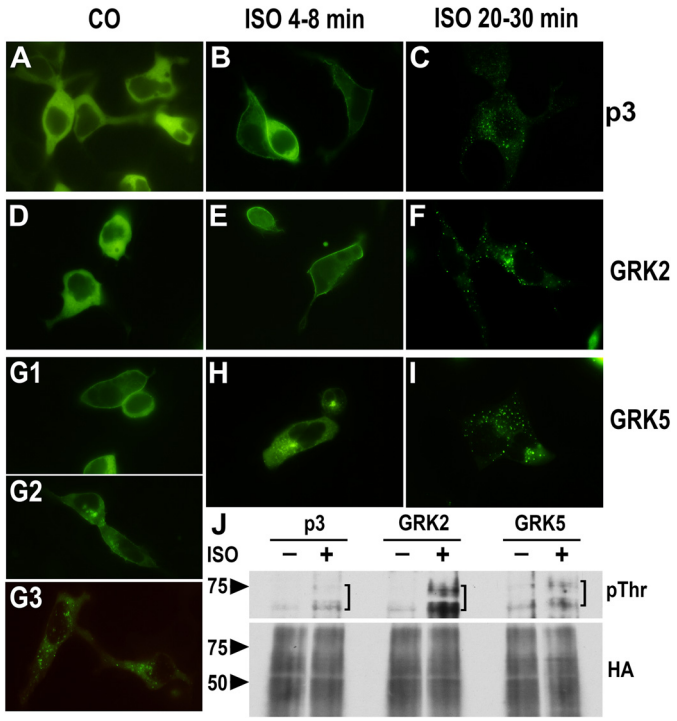


FIGURE 8. **Phosphorylation of inactive receptor by GRK5 induces arrestin recruitment.** HEK293FT cells were co-transfected with β2AR with V2 C terminus, GFP-tagged arretin-3, and empty pcDNA3 vector, GRK2, or GRK5, as described under "Experimental Procedures." Cells were serum-starved overnight and imaged live 48 h post-transfection either in the absence of agonist (*CO*) or at indicated time points following the addition of 10 μM of isoproterenol (*ISO*). Imaging was performed using Nikon LC2000 automated microscope equipped with NLS software with ×60 oil immersion objective. *A–C,* cells did not express exogenous GRKs. *D–F,* cells expressed exogenous GRK2. *G1–G3, H,* and *I,* cells expressed exogenous GRK5. *G1–G3,* representative images of different types of control cells with distinct distribution of expressed GFP-tagged arretin-3. *J,* cells co-expressing HA-tagged β2AR with V2 C terminus with indicated GRKs or control empty vector (*p3*) were incubated with (+) or without (−) 10 μM isoproterenol (*ISO*) for 10 min, as described under "Experimental Procedures." Cells were collected; receptors were immunoprecipitated with anti-HA antibody and blotted with anti-phospho-Thr (*pThr*, *upper panel*) or anti-HA (*lower panel*) antibody.

DISCUSSION

Despite relatively limited experimental evidence, all GRKs were widely believed to specifically phosphorylate only active GPCRs (15). Here, using seven structurally distinct GPCRs that couple to three different types of G proteins, we demonstrate that although this notion is correct for the GRK2/3 subfamily, it is not strictly true for GRK5/6. GRK5/6 belong to the GRK4 subfamily that separated from the GRK2/3 branch very early in the evolution of these kinases, apparently before the emergence of Metazoa (23). The fact that all GRKs share the very unusual positioning of the kinase domain inserted in a loop within its regulator of G protein homology domain, as well as high overall sequence similarity, suggests that ancestors of both subfamilies emerged by gene duplication (15, 23). As a rule, two such genes are retained only when they acquire sufficient functional specialization (70–73). Because only members of the GRK4 sub-

family have been reported to phosphorylate inactive GPCRs (Figs. 1–5, 7, and 8) (16–18), it is possible that differential dependence of their activity on receptor activation was one of the functional distinctions that resulted in retention of both GRK subfamilies.

Our data indicate that both GRK5 and GRK6 effectively phosphorylate inactive β2AR, M2R, and M3R (Fig. 1), in sharp contrast to GRK2 and GRK3, which phosphorylate the same receptors in a strictly activation-dependent manner. Naturally, Ser(P) and Thr(P) antibodies used in many cases would not discriminate between receptors phosphorylated by GRKs or second messenger-activated PKA or PKC. However, the most striking differences were detected in the absence of agonists (Figs. 1–5, 7, and 8), when activation of PKA or PKC is unlikely. If any of the receptors tested signal in the absence of agonists due to constitutive activity, then this signaling would similarly activate cellular kinases in all cases, and as with all GRKs, we used receptors expressed at the same level (Figs. 1 and 2–6). There is also no reason to expect significant changes in the activity of these or other kinases upon the expression of individual GRK subtypes. However, observed levels of receptor phosphorylation clearly were dependent on the type of GRK expressed (Figs. 1 and 2–6). Using a completely different method of detection, incorporation of radioactive phosphate *in vitro*, we observed significant activity of GRK5, but not GRK1 and 2, toward retinal-free opsin (Fig. 2, *A* and *B*), the form of rhodopsin that most closely mimics ligand-free nonvisual GPCRs. In case of β2AR, we confirmed our in-cell findings using purified receptor in the presence of full agonist or the potent inverse agonists and GRK2 or GRK5 *in vitro*. In these experiments, the phosphorylation was measured by direct phosphate incorporation into the receptor, which excludes any artifacts associated with immunodetection (Fig. 2*C*). We also found that GRK5 and GRK6 phosphorylate edited and unedited forms of the 5-HT2C receptor, regardless of the presence of ligand or its nature (Fig. 6). As far as the GRK4 subfamily is concerned, selective phosphorylation of active receptors appears to be an exception, rather than the rule; in this study, we observed it only in case of D1R (Fig. 3). Considering that each mammalian species has at least 400 nonodorant GPCRs, many other receptors may also require activation before phosphorylation by this GRK subfamily. Our data suggest that phosphorylation by GRK5 and GRK6 does not necessarily reflect constitutive activity of the receptor because, in the case of the β2AR, this phosphorylation is not suppressed by two structurally distinct inverse agonists (Fig. 4), and in case of the 5-HT2C receptor, it is not affected by two distinct mechanisms suppressing constitutive activity: editing and inverse agonist (Fig. 6). We also found that the activity of GRKs toward inactive GPCRs is not driven by their constitutive membrane association; membrane-tethered forms of GRK2, which localize to the membrane at least as effectively as GRK5 (Fig. 5), remain activation-dependent in case of both β2AR and M3R (Fig. 5).

These data argue against the notion that GRK5/6-dependent phosphorylation of inactive receptors is simply an artifact caused by overexpression of both receptors and kinases. It is worth noting that the concentration of overexpressed GRKs in HEK293 cells used here still falls short of concentrations of endogenous GRK5 or -6 in some cell types, such as neurons, where the GRK5/6 expression reaches 50–150 ng/mg protein (depending of the brain area) (74–79) as compared with 10–12 ng/mg used in this study. Furthermore, considering that GRK5 and, particularly, GRK6 are concentrated in specific membrane compartments (*e.g.* synaptic membranes), their effective concentrations in these compartments could be even higher (74, 75, 78, 97). The behavior of membrane-tethered GRK2 mutants is reminiscent of strict dependence on receptor activation of visual GRK1- and GRK7-dependent phosphorylation of rhodopsin and cone opsins, even though both visual GRKs are constitutively membrane-associated via C-terminal prenylation (50, 80). Although GRK1 was shown to phosphorylate many molecules of inactive rhodopsin upon activation of each rhodopsin molecule by light (19, 20), this phenomenon could be explained within the framework of the general model that GRK1 activation requires its physical interaction with an active GPCR (8). Because of the very high density of rhodopsin in rod discs membranes, the Rho*-GRK1 complex during its lifetime could encounter many inactive rhodopsins, allowing GRK1 to phosphorylate their disordered cytoplasmic tails (22). It is tempting to speculate that the ability to phosphorylate GPCRs in their inactive form appears to be an inherent characteristic of at least GRK5 and -6 in the GRK4 subfamily. However, because of the limited number of GPCRs tested, we cannot exclude that there are many receptors similar to D1R (Fig. 3), where the phosphorylation by GRK5/6 is highly activation-dependent. It is intriguing, however, that although in all reported structures of GRK1 and GRK2 these kinases are in an "inactive" conformation with misaligned large and small lobes of the kinase domain (37, 81–85), in three out of four structures of GRK5 and -6 the two lobes are much closer to being aligned (10, 13),[3,4] as one would expect in an active kinase (88).

Increased affinity for arrestins is the best characterized functional consequence of GPCR phosphorylation by GRKs (54, 56). Therefore, we tested whether phosphorylation of inactive receptors by members of the GRK4 subfamily affects arrestin recruitment. To this end, we chose the β2AR and the most catalytically active member of the GRK4 subfamily, GRK5 (Fig. 7). We found that the phosphorylation of active β2AR by either GRK2 or GRK5, as well as phosphorylation of inactive β2AR by GRK5, increases recruitment of arrestin-2 (89, 90), to a comparable extent in all cases (Fig. 7*A*). Interestingly, this experimental paradigm revealed an important functional difference between closely related arrestin-2 and -3 (57, 91). In contrast to arrestin-2 (Fig. 7*A*), similar increases in receptor phosphorylation by overexpressed GRK2 and GRK5 had differential effects on arrestin-3 recruitment; GRK5 enhanced it, but GRK2 did not (Fig. 7*B*). These results indicate that although β2AR phosphorylation by these two GRKs is similar in magnitude (Fig. 1*A*), it is not equivalent. The most parsimonious interpretation of the data is that GRK2 and GRK5 target different serines and threonines in β2AR, the phosphorylation of which is equally effective in "attracting" arrestin-2, but is read differentially by

[3] K. T. Homan, H. Waldschmidt, A. Glukhova, A. Cannavo, J. Song, J. Y. Cheung, W. J. Koch, S. D. Larsen, and J. J. Tesmer, submitted for publication.
[4] K. E. Komolov, A. Bhardwaj, and J. L. Benovic, submitted for publication.

arrestin-3, which prefers phosphates at the sites targeted by GRK5 (Fig. 7). These data constitute yet another piece of indirect evidence that GRK2/3 and GRK5/6 target distinct residues on the same receptor, potentially allowing for phosphorylation by these GRK subfamilies to lead to different biological outcomes (92, 93). These results are consistent with the "barcode" hypothesis (94), which posits that different GRK subtypes specifically target distinct residues in the same receptor (95, 96). We were also able to detect arrestin recruitment to inactive GPCR (*i.e.* without agonist stimulation) in cells expressing GRK5 by an alternative method, using GFP-tagged arrestin-2 and imaging detection (Fig. 8). Some unstimulated cells had internalized receptors. Neither arrestin recruitment nor internalization was observed in the absence of agonists in cells overexpressing GRK2. These data suggest that in the presence of a high enough level of GRK5, arrestin is constitutively recruited to the receptor, and the receptor is constitutively desensitized. It remains unclear whether this is the case in physiological conditions without overexpression. This could be a local event in some cells where the local concentration of receptors and GRK5/6 is high. Alternatively, receptor phosphorylation by GRK5/6 could promote arrestin recruitment and arrestin-mediated signaling rather than receptor desensitization/internalization, as has been suggested for GRK6 (95).

Direct comparison of the effectiveness of the four ubiquitously expressed GRK subtypes (15) in phosphorylating the active forms of six different GPCRs is also interesting. All four GRKs phosphorylate some receptors comparably (*e.g.* agonist-activated β2AR and D1R) (Figs. 1 and 3), but their ability to phosphorylate other GPCRs differs dramatically. GRK2 is by far the most effective kinase for active M2R; GRK3 is a distant second; whereas both GRK5/6 demonstrate low activity (Fig. 1*D*). In contrast, GRK3 phosphorylates active M3R better than the others, followed by GRK5, whereas GRK2 and GRK6 are relatively ineffective (Fig. 1*F*). Two receptor subtypes were phosphorylated by members of only one GRK subfamily. D2R was only phosphorylated by GRK2/3 but not by GRK5/6 (Fig. 3). In contrast, both unedited (INI) and edited (VGV) forms of the 5-HT2C receptor were phosphorylated by GRK5/6 but not GRK2/3 (Fig. 6). Thus, each GPCR has its own preference for a particular GRK, and some GRKs do not appreciably phosphorylate certain receptors. Although the heterologous expression employed in this study may not be physiological in non-disease states, it still provides useful information. If overexpressed GRK is unable to phosphorylate a particular receptor subtype under these conditions, it seems unlikely to be able to do so *in vivo*.

To summarize, our data reveal several unexpected aspects of GRK function. First, in the in-cell receptor phosphorylation experimental paradigm, they clearly show surprising receptor specificity of the four most ubiquitous GRKs (Figs. 1, 3, and 6) expressed at virtually identical levels. Second, we found that GRK5/6, which belong to the GRK4 subfamily, effectively phosphorylate inactive forms of several GPCRs (Figs. 1, 4–6, and 8), which cannot be explained by constitutive activity of the receptor (Fig. 4 and 6) or constitutive membrane association of these GRKs (Fig. 5). Last but not least, we showed that the phosphorylation of the inactive receptor by GRK5 significantly enhances arrestin recruitment (Figs. 7 and 8). Because the formation of the arrestin-receptor complex initiates a number of signaling events (66, 67, 98), this finding suggests that arrestin-mediated signaling of certain GPCRs, as well as receptor trafficking, can be regulated not only by the presence of an agonist but also by the expression level of the receptor and GRK5/6. The biological significance of agonist-independent phosphorylation of at least some GPCRs by GRK5 and -6, likely followed by arrestin binding and possibly internalization and/or arrestin-mediated signaling, remains to be elucidated.

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