THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 41, pp. 35051–35061, October 14, 2005

Chemical Genetic Engineering of G Protein-coupled Receptor Kinase 2*

Received for publication, July 13, 2005, and in revised form, August 3, 2005 Published, JBC Papers in Press, August 4, 2005, DOI 10.1074/jbc.M507594200

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G protein-coupled receptor kinases (GRKs) play a pivotal role in receptor regulation. Efforts to study the acute effects of GRKs in intact cells have been limited by a lack of specific inhibitors. In the present study we have developed an engineered version of GRK2 that is specifically and reversibly inhibited by the substituted nucleotide analog 1-naphthyl-PP1 (1Na-PP1), and we explored GRK2 function in regulated internalization of the μ-opioid receptor (μOR). A previously described method that conferred analog sensitivity on various kinases, by introducing a space-creating mutation in the conserved active site, failed when applied to GRK2 because the corresponding mutation (L271G) rendered the mutant kinase (GRK2-*as*1) catalytically inactive. A sequence homology-based approach was used to design second-site suppressor mutations. A C221V second-site mutation produced a mutant kinase (GRK2-*as*5) with full functional activity and analog sensitivity as compared with wild-type GRK2 *in vitro* and in intact cells. The role of GRK2-as5 activity in the membrane trafficking of the μOR was also characterized. Morphine-induced internalization was completely blocked when GRK2-as5 activity was inhibited before morphine application. However, inhibition of GRK2-as5 during recycling and reinternalization of the μOR did not attenuate these processes. These results suggest there is a difference in the GRK requirement for initial ligand-induced internalization of a G protein-coupled receptor compared with subsequent rounds of reinternalization.

G protein-coupled receptors (GPCRs)[2] detect a large variety of extracellular stimuli and initiate intracellular signaling pathways that regulate a diverse array of physiological processes (1–4). Upon agonist binding, conformational changes occur to the receptor that activate heterotrimeric G proteins. An important mechanism that regulates this process is termed receptor desensitization, which results in a reduction in the rate of G protein activation by the agonist-bound receptor complex (5, 6). Desensitization is thought to be mediated through phosphorylation by G protein-coupled receptor kinases (GRKs) and second messenger-dependent protein kinases such as cAMP-dependent kinase (PKA), protein kinase C (PKC), and calmodulin-dependent kinase (7–11). Only GRKs selectively phosphorylate agonist-activated receptors, whereas second messenger-dependent kinases can phosphorylate receptors in the presence or absence of agonist. Receptor phosphorylation by GRKs leads to recruitment of the cytoplasmic accessory proteins, arrestins, to the plasma membrane to target receptors for internalization via clathrin-coated pits (12, 13). The internalized receptors can be subsequently dephosphorylated and recycled back to the plasma membrane or targeted to the lysosome for degradation (14, 15).

Opioid receptors are GPCRs that are well known to be phosphorylated and desensitized by a number of kinases, including GRKs and second messenger-dependent kinases such as PKC (7, 16–23). Internalization of opioid receptors has been linked to GRK-mediated phosphorylation. For example, in response to morphine, internalization of the μ-opioid receptor (μOR) is strongly dependent on cellular GRK activity. Morphine fails to promote rapid internalization of the μOR in HEK293 cells expressing GRKs at endogenous levels. Overexpression of GRK2 renders morphine capable of promoting rapid phosphorylation and endocytosis of this GPCR (24).

A major limitation to more definitive studies of agonist-dependent phosphorylation is our inability to specifically manipulate GRK or second messenger kinase activity on a rapid time scale. Phosphorylation and internalization of GPCRs typically occur within minutes after agonist activation. However, there are no specific inhibitors of GRKs or second messenger kinases to assess the role of each kinase in these processes. The inhibitor, staurosporine, which is used to inhibit PKC, can bind with high affinity to over 50 other protein kinases (25). The only known inhibitor of GRK2, heparin, requires incubation overnight in the cell media with lipids in order to effect GRK activity (26). However, the effects of heparin can only block GRK activity before agonist is applied; the role of GRK2 during other membrane trafficking events such as internalization, recycling, and resensitization has not been evaluated. Available genetic techniques to manipulate kinase function *in vivo*, such as overexpression, gene knock-out, and knock-down are specific but also suffer from lack of temporal control. Thus, an ideal tool for studying the functional consequences of agonist-induced kinase activity *in vivo* would be a cell-permeable small molecule drug that could specifically inhibit a single kinase isozyme in intact cells, without producing off-target or biochemical effects.

Our laboratory has developed a chemical genetic approach to specifically inhibit any protein kinase. Substitution of a single conserved amino acid, termed the gatekeeper residue, in the active site creates an analog-sensitive (*as*) kinase that is uniquely sensitive to inhibition by cell-permeable modified PP1 analog inhibitors while maintaining its ability to phosphorylate the same physiological substrates as the wild type (27–30). This approach has been applied successfully to over 24 kinases (27–33). We have chosen to inhibit specifically GRK2 in order to study its agonist-dependent phosphorylation. Because our small molecule inhibitors are designed only for analog-sensitive kinases, they have no effect on wild-type second messenger kinases such as PKA, calmodulin-dependent kinase, and tyrosine kinases, as well as other kinases implicated in GPCR regulation (29, 31, 34).

* This work was supported by National Institutes of Health Grant AI44009. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HEK, human embryonic kidney; μOR, μ-opioid receptor; DAMGO, [D-Ala[2],*N*-Me-Phe[4],Gly[5]-ol]enkephalin; 1Na-PP1, 4-amino-1-*tert*-butyl-3-(1′naphthyl)pyrazolo[3,4-*d*]pyrimidine; PKA, cAMP-dependent kinase; PKC, protein kinase C; HA, hemagglutinin; as, analog-sensitive; PBS, phosphate-buffered saline.

In this study we specifically inhibit the kinase activity of GRK2 and investigate its role in morphine regulatory endocytosis of the μOR. In our efforts to create an analog-sensitive version of GRK2, based on a single point mutation in the ATP binding pocket, a significant loss of catalytic activity of the mutant kinase occurred. A sequence-based approach was developed for rescuing full enzymatic activity while still allowing for potent inhibition by an analog of PP1. To our knowledge, the present results are the first to develop a highly selective small molecule inhibitor for any member of the GRK family. By using this approach, we have identified an unexpected temporal function of μOR regulation by morphine.

MATERIALS AND METHODS

Inhibitor Synthesis—1Na-PP1 was synthesized as described previously (35).

Plasmid Construction—Bovine GRK2 in pcDNA3.0 (a gift from J. Benovic) was used for transient cell expression. A carboxyl-terminal hemagglutinin (HA)-tagged version was constructed by cloning into pcDNA 3.1 and inserting a carboxyl-terminal HA tag (YPYDVPDYA) by standard site-directed mutagenesis. An amino-terminally His_6-tagged GRK2, used for expression in baculovirus, was constructed using the Gateway and Bac-to-Bac systems (Invitrogen). Point mutations were introduced using oligonucleotide-directed mutagenesis (QuickChange system, Stratagene). HA-tagged GRK2-as1 and GRK2-as5 were also cloned into PIRES hyg3 vector (Clontech) in order to generate hygromycin-resistant stably transfected cells. All cDNAs were verified by dideoxynucleotide sequencing. The amino-terminally FLAG-tagged murine μOR-1 (36), cloned into in pcDNA3.0, has been described previously (14).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (University of California, San Francisco Cell Culture Facility). For studies of receptor trafficking in transiently transfected cells, cells were transfected using Lipofectamine 2000 (Invitrogen) and used for flow cytometry assay 48 h thereafter. Stably transfected cells expressing epitope-tagged μOR and GRK2 were generated by selection for neomycin resistance using 50 μg/ml G418 (Geneticin, Invitrogen) and hygromycin resistance using 200 μg/ml hygromycin (Roche Applied Science). Colonies representing individual neoR and *hygroR* clones were isolated and selected for further study based on receptor and GRK2 expression levels (assessed by fluorescence flow cytometry).

Fluorescence Flow Cytometry—Internalization of epitope-tagged μOR was estimated by using fluorescence flow cytometry of μOR and GRK2 transient and stably transfected cells to measure changes in the relative amount of FLAG-tagged receptors present in the plasma membrane after surface labeling with Alexa488-conjugated M1 antibody as described previously (37). Fluorescence flow cytometry was performed using a FACScan instrument (BD Biosciences). 10,000 cells were collected for each sample. Triplicate samples were analyzed for each condition in each experiment. The mean fluorescence values for each experiment (n = 3–5 experiments) were averaged, and the S.D. was calculated across all experiments.

Fluorescence Microscopy—Endocytic trafficking of μOR initially labeled in the plasma membrane was visualized by fluorescence microscopy using a previously described "antibody feeding" method (38). For initial internalization experiments, stably transfected 293 cells expressing μOR and GRK2 were grown on glass coverslips (Corning Glass), and μOR was specifically labeled by incubating intact cells with M1 anti-FLAG antibody (2.0 μg/ml, Sigma) with or without 1Na-PP1 (2.5 μM). Surface-labeled μOR cells were subsequently incubated (at 37 °C for 30 min) in the presence of the appropriate opioid (10 μM morphine). Following this incubation, cells were fixed immediately in 4% formaldehyde freshly prepared in phosphate-buffered saline (PBS) (pH 7.4) for 15 min and then quenched with three washes of Tris-buffered saline (pH 7.5), supplemented with 1 mM $CaCl_2$. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in a blocking solution (3% dry milk in Tris-buffered saline plus 1 mM $CaCl_2$) and incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibody (1:500 dilution, Jackson ImmunoResearch) for 30 min to detect antibody-labeled receptors. For recycling and reinternalization experiments, μORs were specifically labeled by incubating intact cells with Alexa488 M1 anti-FLAG antibody (2.0 μg/ml, Sigma). Surface-labeled μOR cells were subsequently incubated (at 37 °C for 30 min) in the presence of morphine. Following this incubation, the M1 antibody was stripped using PBS/EDTA, Dulbecco's modified Eagle's medium applied along with Cy3 anti-mouse, opioid agonist or antagonist, and inhibitor. Cells were fixed immediately in 4% formaldehyde freshly prepared in PBS (pH 7.4) for 15 min and then quenched with three washes of Tris-buffered saline (pH 7.5) supplemented with 1 mM $CaCl_2$. Epifluorescence microscopy was performed using an inverted microscope (Nikon Diaphot) equipped with a Nikon 60×NA1.4 objective and standard interference filter sets (Omega Optical). Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer running IPLab Spectrum software (Scanalytics).

Purification of Recombinant GRK2—His_6-tagged versions of wild-type and mutant GRK2 were overexpressed and purified from Sf9 cells using the baculovirus expression system as detailed previously (39). Briefly, cells were harvested 72 h after infection by low speed centrifugation. Following homogenization in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, and a protease inhibitor tablet (Roche Applied Science), a high speed supernatant was prepared. The soluble fraction was diluted and incubated with nickel-nitrilotriacetic acid-agarose (Qiagen) for 1 h. The agarose was washed with 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole. GRK2 was eluted with 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole and stored at −80 °C in 30% glycerol under which the protein was stable for 2 months. Protein concentration was determined by the method of Bradford (40) using purified bovine serum albumin as a standard. Purity of the GRK2 preparation was determined by Coomassie staining to be greater than 95%.

Assay of GRK2 Activity—Phosphorylation reactions to determine the kinetics of ATP used tubulin (gift from R. Vale) as a substrate. Reactions contained 5 μM tubulin, 0.2 μM GRK2, 20 mM HEPES (pH 7.8), 2 mM EDTA, 7 mM $MgCl_2$, and varying concentrations of [γ-^{32}P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences) in a total volume of 20 μl. Incubations were at 23 °C for 30 min. Reactions to determine the kinetics of tubulin or rhodopsin were the same as above except using 0.025 μM GRK2, 700 μM [γ-^{32}P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences), and varying concentrations of tubulin or rhodopsin. Incubations were at 23 °C for 10 min. Phosphorylation reactions for inhibition of rhodopsin contained 400 nM rhodopsin (rod outer segments from Calbiochem), 75 nM GRK2, 20 mM Tris (pH 7.5), 2 mM EDTA, 7 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences) in a total volume of 20 μl. Incubations were at 23 °C for 3 min under exposure to laboratory lighting. Reactions for tubulin and rhodopsin were quenched with SDS sample buffer and subjected to 10% SDS-PAGE and autoradiography. Stoichiometry of phosphorylation was determined by PhosphorImager analysis (Amersham Biosciences).

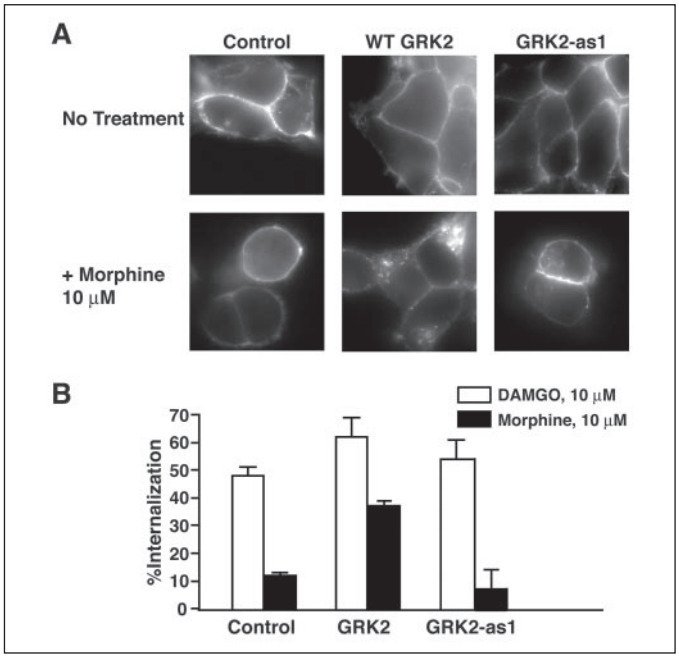

FIGURE 1. **GRK2-as1 is not tolerant to the gatekeeper mutation, L271G.** *A,* HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2 or GRK2-as1 were treated with 10 μM morphine for 30 min at 37 °C and then fixed and stained with M1 anti-FLAG primary and Cy3 anti-mouse secondary antibody. In the presence of morphine only, overexpression of GRK2 redistributes receptors from the plasma membrane to numerous endocytic vesicles in the cytoplasm. *WT,* wild type. *B,* quantitation of morphine-induced μOR internalization by GRK2 or GRK2-as1 by flow cytometry. Cells were treated with 10 μM DAMGO or morphine and then surface-stained with M1 Alexa488 anti-FLAG. 10,000 cells per sample were counted. Overexpression of GRK2 enhances morphine-induced endocytosis over 4-fold.

RESULTS

Design and Analysis of GRK2-as1—Through sequence alignment with other kinases, L271 in GRK2 was determined to be the gatekeeper residue, and this residue was mutated to glycine (L271G) (41).

To begin to assess the activity of GRK2 L271G (GRK2-as1) in intact HEK293 cells, we assessed the ability of the overexpressed kinase to promote morphine-induced internalization (24, 42). Morphine did not produce detectable μOR internalization in the absence of GRK2 overexpression, consistent with earlier reports (Fig. 1*A*). Receptor internalization was assessed by fluorescence microscopy, using anti-FLAG antibodies to detect FLAG-tagged μOR also expressed in HEK293 cells. The FLAG-μOR remained at the plasma membrane after incubation either in the absence or presence of a saturating concentration (10 μM) of morphine for 30 min in the absence of GRK2. When wild-type GRK2 was overexpressed, a pronounced redistribution of μOR from the plasma membrane to punctate intracellular vesicles occurred in response to morphine (Fig. 1*A*). This punctate pattern has been shown previously to reflect endocytosis of the μOR through clathrin-coated pits (24, 42, 43). Comparison of GRK2 *versus* GRK2-as1-mediated μOR internalization revealed that overexpression of GRK2-as1 failed to induce morphine-dependent endocytosis, even in cells expressing this mutant kinase at high levels (as estimated by fluorescence staining intensity) relative to those at which wild-type GRK2 promoted morphine-induced endocytosis.

In order to quantitate receptor internalization on a larger population of cells, we utilized flow cytometry. Flow cytometry was used to measure the level of FLAG-μOR cell surface fluorescence from 10,000 cells using Alexa488-conjugated anti-FLAG antibody both in the presence and absence of overexpressed GRK2 or GRK2-as1. The mean fluorescence intensity measured in agonist-treated cells was normalized to that measured in untreated cells in order to compute fractional internalization of receptors induced by agonist. In cells not overexpressing GRK2, morphine induced little (~12%) μOR internalization (Fig. 1*B*). Overexpression of GRK2 produced substantially increased, morphine-induced μOR internalization (~31%). Yet, overexpression of GRK2-as1 under similar conditions failed to promote morphine-induced internalization of receptors above the background produced by endogenous levels of GRK2, further suggesting GRK2-as1 is a severely compromised GRK2 allele.

TABLE ONE
ATP kinetic parameters of GRK2-mediated tubulin phosphorylation

Kinase	ATP		
	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1}\ \mu M^{-1}$
GRK2	17 ± 5	0.174 ± 0.01	1.02×10^{-2}
GRK2-as1	62 ± 24	0.006 ± 0.002	9.67×10^{-5}
GRK2-as5	149 ± 42	0.246 ± 0.04	1.65×10^{-3}

We analyzed internalization of the μOR in response to a second agonist, the peptide (enkephalin analog) DAMGO, by microscopy and flow cytometry when GRK2-as1 is expressed. DAMGO is a high affinity peptide agonist that, unlike morphine, is not dependent on the overexpression of GRK2 to induce internalization (43, 44). In cells overexpressing GRK2-as1, the μOR did internalize in response to DAMGO (Fig. 1*B*). This result suggests that the failure of GRK2-as1 to induce morphine-based internalization by the *as* allele is likely due to a lack of catalytic activity and not disruption of the endogenous endocytic machinery.

To test this hypothesis directly, the catalytic activities of GRK2 and GRK2-as1 were assessed biochemically following purification, from a baculovirus expression system, and kinetic constants were determined for ATP using tubulin as a substrate (TABLE ONE) (39, 45). The $K_{m\ (ATP)app}$ for purified GRK2-as1 was increased ~4-fold relative to that of the wild-type GRK2. An increase in K_m of this magnitude is typical for *as* kinases, since the gatekeeper mutation creates a larger ATP-binding site resulting in weaker ATP binding (30). This change is unlikely to be physiologically significant because of the relatively high ATP concentration (~1–5 mM) present in the cytoplasm (30, 46, 47). More significantly, the k_{cat} was sharply decreased, almost 30-fold, for GRK2-as1 relative to wild-type GRK2.

Second Site Mutations Restore Activity to GRK2-as1—This drastic decrease in catalytic activity is unusual as the corresponding gatekeeper mutation is tolerated by over 24 different kinases. However, in the cAMP-dependent kinase (PKA) family, although PKAα was able to tolerate the gatekeeper mutation, PKAβ had a profound decrease in activity (29). Because PKAα and PKAβ have a high degree of sequence conservation, sequence alignments were able to identify a small number of amino acids that differ between them. Rescue of catalytic activity of PKAβ was achieved by replacing the only nonconserved residue between PKAα and PKAβ. This residue is known as a second-site suppressor mutation. Because the L271G gatekeeper mutation in GRK2 also resulted in loss of catalytic activity, we investigated whether a similar sequence-based approach could also be applied to restore functional activity to GRK2-as1.

However, because no other GRK family kinases have been engineered successfully, we decided to take a broader, genome-wide approach using sequence data from all known protein kinase families. We hypothesized that, because many protein kinases from various families can tolerate the as mutation, we could identify the conserved amino acids in them by sequence alignment (41). We could then align GRK2-as1 with the *as-*

tolerant kinases, identify which residues were nonconserved, and make those changes to GRK2-as1. These changes would potentially make GRK2-as1 more like the tolerant kinases and restore catalytic activity. We recently have reported another approach for the identification of second-site suppressor mutations of weak as kinase alleles using random mutagenesis (48).

Here we used a purely sequence-based analysis approach to compare the sequences of several kinases that have been successfully engineered in order to identify sequence similarities between them (Fig. 2*A*). The catalytic domain of kinases can be grouped into 12 smaller subdomains, 7 which contain residues that directly contact the ATP binding pocket (49). Because the gatekeeper mutation is in the ATP binding pocket and all protein kinases have highly conserved ATP binding regions, we hypothesized that we could identify conserved regions in the tolerant *as* kinases by aligning the areas in the subdomains that contain residues in the ATP binding region. For example, in kinase subdomain II, which is located in the β-sheet structure of the amino-terminal domain, there are several residues that are in the ATP binding region. Sequence alignment of the *as*-tolerant kinases revealed that the most common residue at position 221 is a β-branched hydrophobic residue such as threonine, valine, or isoleuceine, which occurs in 50% of kinases. Subdomain II also contains the highly conserved alanine at position 218 that contributes to the hydrophobic adenine ring pocket and the invariant catalytic lysine at position 220 that is essential for maximal kinase activity.

After identifying the most conserved residues in all the subdomains among the *as*-tolerant kinases, we then compared these trends to the corresponding residues in GRK2-as1 in order to identify specific amino acids for candidate second-site suppressor mutations. Alignment of GRK2-as1 with the *as*-tolerant kinases revealed several nonconserved residues. In subdomain II the residue immediately following the catalytic lysine was found to be cysteine (Cys-221) in GRK2-as1. This residue is located in the center of the β-sheet in the kinase domain (Fig. 2*B*) (50). Because the most conserved residue at position 221 in the *as*-tolerant kinases is a β-branched residue, we mutated Cys-221 to valine. In subdomain V, which links the large and small lobes of the kinase catalytic subunit, GRK2-as1 contains a leucine at position 273, whereas more than 50% of *as*-tolerant kinases contain an tyrosine or phenylalanine residue at this position. Subdomain VII folds into a β-sheet structure and contains the DFG triplet, which is invariant in *as*-tolerant kinases. However, GRK2-as1contains a leucine residue at position 336 instead of a phenylalanine. Therefore, the C221V, L273Y, and L336F mutations were introduced individually into GRK2-as1.

These candidate second-site suppressor mutations for GRK2-as1 were tested in transiently transfected cells expressing the μOR using the flow cytometry assay for morphine-induced μOR internalization in the presence of overexpressed GRK2 (Fig. 2*C*). All of the second-site suppressor mutations exhibited greater ability than GRK2-as1 to promote morphine-induced μOR internalization. GRK2-as1 exhibited 25% of the activity of wild-type GRK2, whereas C221V, L273Y, and L336F demonstrated 110, 53, and 82% activity, respectively. Because the activity of GRK2-as1 C221V (herein referred to as GRK2-as5) was essentially identical to wild-type GRK2, this mutant was characterized further.

GRK2-as5 Is Functionally Equivalent to GRK2 in Vitro and in Vivo—We purified GRK2-as5 using a baculovirus expression system and measured kinetic parameters for in vitro phosphorylation of tubulin. The kinetics of GRK2-as5 with ATP were similar to wild-type GRK2. The k_{cat} values were similar, 0.174 and 0.246 min^{-1} for GRK2 and GRK2-as5, respectively. In comparison to the single mutant GRK2-as1, this represents 40-fold improvement in k_{cat} for GRK2-as5 (TABLE ONE). The K_m value for ATP was increased for

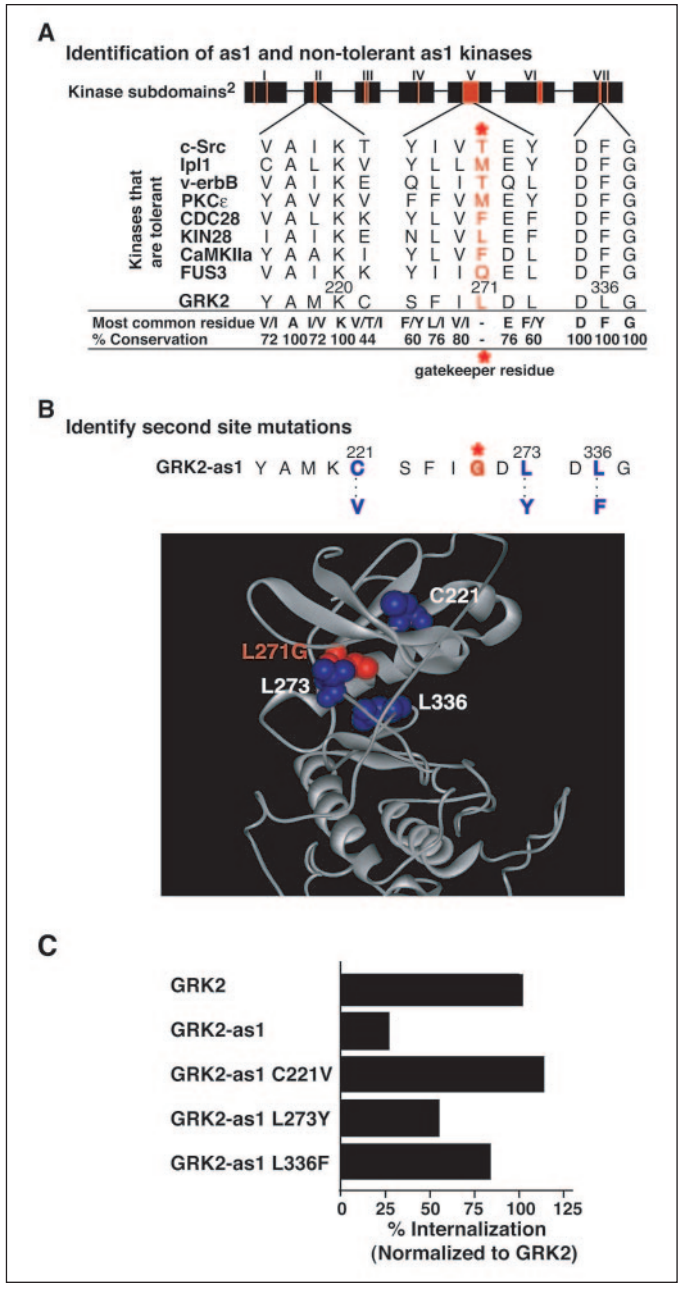

FIGURE 2. **Second-site suppressor mutations for rescue of GRK2-as1 function.** Kinase subdomains are shown in *black boxes* with the residues lining the ATP binding pocket residues in *red*. *A*, analog-sensitive mutants are created by mutating the large, conserved gatekeeper residue, shown in *red*, in the ATP binding pocket to an alanine or glycine. This mutation, L271G, reduced activity in GRK2. Sequence alignments of ATP binding pocket residues from representative *as*-tolerant kinases are given in the *upper part* of the *table*. The *bottom part* of the table identifies the most common residues and the percent conservation for all kinases in the ATP binding pocket based upon sequence alignment of all *as*-tolerant kinases. This identifies possible residues to change in GRK2-as1 to restore catalytic activity while maintaining inhibitor specificity. *B*, the residues identified by sequence comparison and what they were changed to are shown in the GRK2-as1 sequence and in the GRK2 crystal structure. *C*, activity of the mutants was determined through flow cytometry. GRK2-as1 second-site suppressor mutants were transiently transfected in μOR-expressing HEK293 cells. Cells were surface-stained with M1 Alexa488 anti-FLAG antibody. 10,000 cells per sample were counted. All second-site mutations were able to recover varying levels of activity. GRK2-as1 C221V was chosen for further characterization.

GRK2-as5 relative to wild-type GRK2 (as well as GRK2-as1). However, the K_m value measured (~150 μM) was still much less than the estimated cytoplasmic ATP concentration, so it is not likely to negatively impact the cellular activity of the mutant (51). This suggests that the second-site suppressor mutation was

TABLE TWO

Substrate specificity of GRK2 and GRK2-as5 for tubulin and rhodopsin

Kinase	Tubulin			Rhodopsin,
	K_m	k_{cat}	k_{cat}/K_m	k_{cat}/K_m
	μM	min^{-1}	$min^{-1}\ \mu M^{-1}$	$min^{-1}\ \mu M^{-1}$
GRK2	0.147 ± 0.04	0.738 ± 0.04	5.03	0.275 ± 0.07
GRK2-as5	0.051 ± 0.02	0.305 ± 0.03	6.01	0.305 ± 0.03

sufficient to restore physiologically relevant catalytic ability back to the GRK2-as1 allele.

Whereas the C221V second site suppressor mutation restored ATP binding and turnover, the protein substrate binding pocket of GRK2-as5 could have been structurally disrupted by the mutation. Therefore, substrate specificity of tubulin and light-activated rhodopsin for GRK2 and GRK2-as5 was also assessed (TABLE TWO). For tubulin, there was less than a 3-fold difference for either K_m or k_{cat} between GRK2 and GRK2-as5. Because V_{max} was not reached for light-activated rhodopsin due to its low stock concentration, only a k_{cat}/K_m value was obtained from the slope of a linear plot. The k_{cat}/K_m values for rhodopsin were similar, 0.275 and 0.305 μM^{-1} min^{-1} for GRK2 and GRK2-as5, respectively. These results suggest that the *as* and second-site mutations in GRK2-as5 did not disrupt its ability to phosphorylate either a prototypic GPCR substrate, in a conformation-dependent manner, or a cytosolic protein substrate *in vitro*.

To investigate in more detail the functional activity of GRK2-as5 in intact cells, we again used the morphine-induced internalization assay (Fig. 3) (24). Stably transfected HEK293 cells were generated expressing either HA-tagged GRK2 or GRK2-as5, together with FLAG-tagged μOR. GRK2 and GRK2-as5 were expressed at similar levels as assessed by Western blotting (Fig. 3*A*). Internalization in response to morphine was indistinguishable for GRK2 and GRK2-as5 with an EC_{50} of 148 and 191 nM, respectively (Fig. 3*B*). To compare further the functional activity of the GRK2-as5 mutant kinase to wild-type GRK2 in intact cells, we compared the time course of μOR internalization induced by a saturating concentration (10 μM) of agonist. The kinetics of μOR internalization mediated by GRK2-as5 were similar to wild-type GRK2 and reached a steady state value within 20 min after morphine addition to the culture medium. These results demonstrate that wild-type GRK2 and GRK2-as5 are functionally identical *in vitro* and in cells.

GRK2-as5 Is Specifically and Reversibly Inhibited by 1Na-PP1 in Vitro and in Vivo—A panel of PP1-based inhibitors were screened in order to identify a potent inhibitor of GRK2-as5 that does not inhibit the wild-type kinase (data not shown). The PP1 analog, 1Na-PP1, exhibited >1000-fold selectivity for GRK2-as5 over wild-type GRK2-catalyzed tubulin phosphorylation (IC_{50} ~24 nM and >25 μM, respectively).

To assess the inhibitory activity of 1Na-PP1 in the context of direct receptor phosphorylation, we utilized isolated bovine rod outer segments as a source of rhodopsin. When isolated bovine rod outer segments were incubated with GRK2 or GRK2-as5 in the dark, minimal phosphorylation of rhodopsin was observed (Fig. 4*A*). Following brief exposure to room light, GRK2 and GRK2-as5 both mediated efficient phosphorylation of rhodopsin, consistent with ligand-specific phosphorylation by GRK2. GRK2-mediated phosphorylation of rhodopsin was not detectably inhibited when 1 μM 1Na-PP1 was included in the assay. However, 1Na-PP1 completely abrogated rhodopsin phosphorylation mediated by GRK2-as5. Therefore, by using either tubulin or light-activated rhodopsin as a substrate, GRK2-as5 phosphorylation was completely inhibited by 1 μM 1Na-PP1, without any effect on wild-type GRK2 activity.

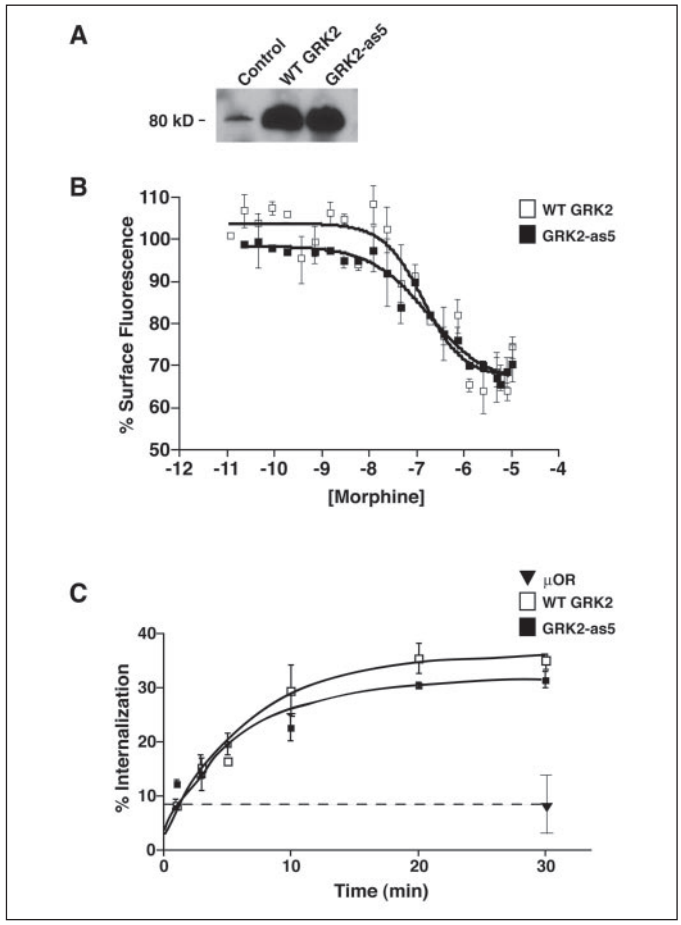

FIGURE 3. **GRK2-as5 is functionally similar to GRK2 *in vivo*.** *A*, Western blot analysis of HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2 or GRK2-as5. *B*, dose-response curves of morphine-induced internalization for GRK2 and GRK2-as5 as evaluated by flow cytometry. Cells were treated with varying concentrations of morphine at 37 °C for the time indicated and then surface-stained with M1 Alexa488 anti-FLAG antibody. 10,000 cells per sample were counted. EC_{50} values for GRK2 and GRK2-as5 are 141 and 191 nM, respectively. *C*, time course of morphine-induced internalization. Cells were treated with 10 μM morphine at 37 °C for the time indicated and then analyzed as in *B*. *WT*, wild type.

We next asked whether 1Na-PP1 could specifically block GRK2-as5-dependent morphine-induced internalization of the μOR. In the absence of 1Na-PP1, a 30-min exposure to 10 μM morphine produced significant internalization in cells coexpressing either wild-type GRK2 or GRK2-as5, as evidenced by fluorescence microscopic localization of FLAG-μOR (Fig. 4*B*). Morphine-induced μOR internalization was quantified by flow cytometry, which indicated that the extent of FLAG-μOR internalization induced by morphine in GRK2-as5 expressing cells was similar to that observed in cells expressing recombinant wild-type GRK2 (Fig. 4*C*). In the absence of morphine treatment, 1Na-PP1 alone had no effect on receptor localization or internalization (Fig. 4, *B* and *C*). More importantly, 1Na-PP1 did not affect the ability of morphine to induce rapid internalization of μOR in cells expressing wild-type GRK2. In contrast, in cells expressing GRK2-as5, morphine-induced internalization was completely blocked by 1Na-PP1 and occurred only at the base-line levels observed in cells not expressing the recombinant GRK2 construct. These results confirm that GRK2 activity is able to promote morphine-induced endocytosis of μOR at levels equivalent to that achieved by GRK2 and demonstrate that 1Na-PP1 specifically inhibits GRK2-as5-dependent regulation of the μOR, without affecting regulation mediated by wild-type GRK2.

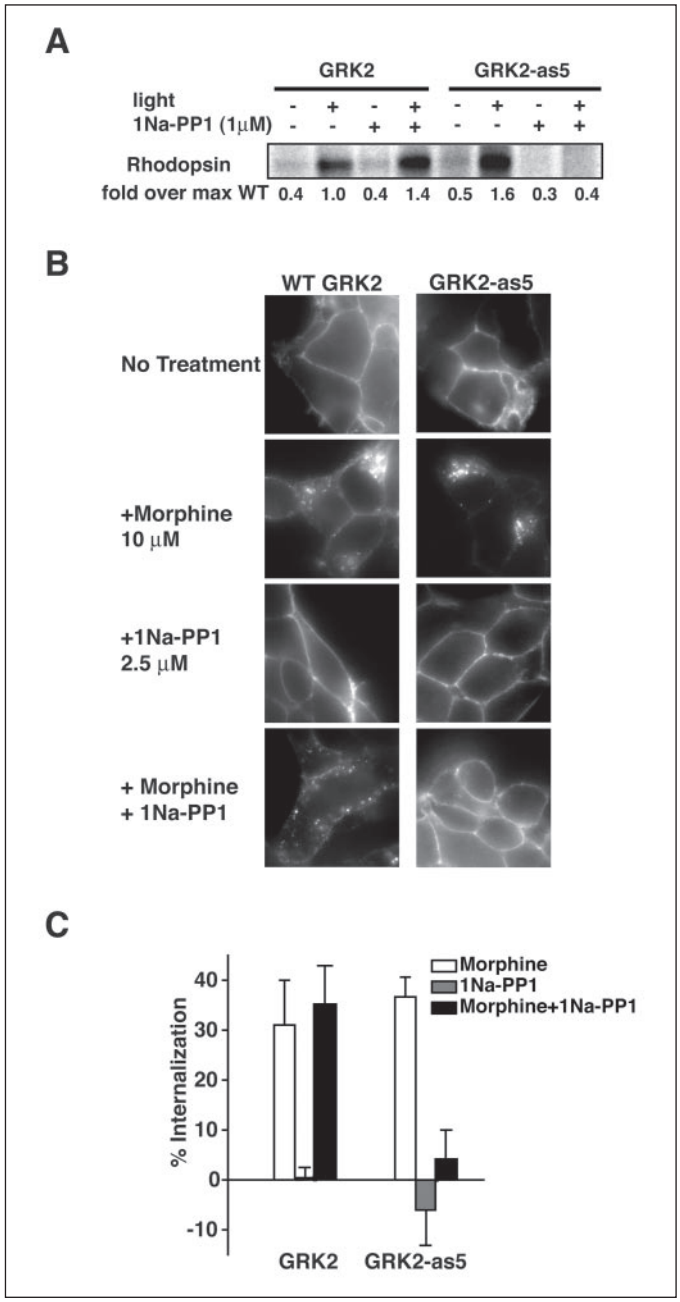

FIGURE 4. **Differential response to 1Na-PP1 by overexpression of GRK2 or GRK2-as5.** *A,* phosphorylation of light-activated rhodopsin by GRK2 and GRK2-as5. GRK2 or GRK2-as5 (75 nM) was incubated with rhodopsin (400 nM) in the dark (−) or light (+) and with (+) or without (−) 1 μM 1Na-PP1 for 3 min. Receptor phosphorylation was analyzed by SDS-PAGE autoradiography. *B,* HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2 or GRK2-as1 were treated with 10 μM morphine at 37 °C and then fixed and stained with M1 anti-FLAG primary and Cy3 anti-mouse secondary antibodies. In the presence of morphine, overexpression of both GRK2 or GRK2-as5 redistributes receptors from the plasma membrane to numerous endocytic vesicles in the cytoplasm. 2.5 μM 1Na-PP1 alone has no effect on any of the cells. However, morphine and 1Na-PP1 treatment block internalization only in GRK2-as5. *C,* quantitation of morphine-induced μOR internalization by GRK2 or GRK2-as5 by flow cytometry. Cells were treated with μOR 10 μM morphine and then surface-stained with M1 Alexa488 anti-FLAG. 10,000 cells per sample were counted. Overexpression of GRK2 and GRK2-as5 enhance morphine-induced endocytosis over 4-fold. 1Na-PP1 treatment with morphine completely inhibits GRK2-as5-induced internalization but has no effect on wild-type (*WT*) GRK2.

We next asked whether the effects of 1Na-PP1 could be fully reversed upon removal of the inhibitor from the cell medium prior to morphine addition. As demonstrated in Fig. 4, a 10-min preincubation with 1Na-PP1 was sufficient to block the GRK2-as5-dependent enhancement of morphine-induced internalization of the μOR. This effect was fully reversible, as indicated by the full recovery of morphine-induced internalization within 30 min after washout of 1Na-PP1 from the culture medium (Fig. 5*A*).

Rapid GRK2-dependent Receptor Regulation Following Morphine Stimulation—The μOR undergoes a series of regulatory events, including GRK2 activation, phosphorylation, arrestin binding, and clathrin-mediated internalization within the first few minutes of agonist exposure. These desensitization mechanisms operate on the second to minute time scale. In order to use the GRK2-as5 as a tool to investigate these rapid regulatory mechanisms, one would like an inhibitor to act on a comparable time scale. To assess the time scale required for 1Na-PP1-mediated inhibition of GRK2-as5-dependent internalization, we carried out a series of execution point experiments. Cells expressing the μOR and GRK2-as5 were preincubated for 1, 3, or 5 min with 1Na-PP1 prior to treatment with morphine. GRK2-as5 strongly promoted morphine-induced internalization of μOR when inhibitor was not present, and this effect was completely blocked in as short as 1 min following addition of 1Na-PP1 to the cultures prior to morphine addition (Fig. 5*B*, *bars 1–3* and *7*, respectively). These results indicate that 1Na-PP1 strongly inhibits GRK2-as5 catalytic activity within 1 min following application to intact cells.

By taking advantage of this rapid inhibition, we conducted execution point experiments by addition of 1Na-PP1 at 1, 3, or 5 min following agonist exposure to cells expressing μOR and GRK2-as5. Maximal internalization of μOR is achieved between 10 and 20 min following morphine treatment with minimal internalization occurring after 1 min of agonist treatment (Fig. 3*C*). Thus, treatment with 1Na-PP1 in the first 5 min following morphine treatment assesses the role of GRK2 at time points *prior* to maximal receptor internalization yet *subsequent* to receptor activation. Addition of 1Na-PP1 just 1 min following morphine addition blocked less than 10% of the maximal amount of receptor internalization achieved in the absence of inhibitor treatment (Fig. 5*B*, *bar 4*). Similarly, 1Na-PP1 treatment 3 or 5 min post-morphine addition did not significantly block receptor internalization (Fig. 5*B*, *bars 5* and *6*). These data demonstrate that GRK2 activity is required for only 1 min following morphine treatment to support maximal internalization 30 min after agonist stimulation. Taken together, these data suggest the existence of two distinct kinetic steps involved in receptor internalization: 1) rapid (<1 min) priming of the μOR by GRK2 (Fig. 5*B*) and 2) subsequent, slower (20 min) actual internalization of the receptor (Fig. 3*C*). The existence of rapid GRK2 effects and slower receptor trafficking events prompted us to investigate the role of GRK2 following initial receptor internalization. These events have been termed receptor recycling and reinternalization (52).

Assessing μOR Recycling and Reinternalization—The μOR has been shown to recycle rapidly back to the plasma membrane after initial internalization and is capable of undergoing cycles of reinternalization (52). In order to track the μOR in response to morphine, we used a fluorescence-based microscopy assay in which the initially internalized pool of receptors is specifically labeled with a monoclonal antibody that does not interfere with GPCR recycling (Fig. 6*A*) (38). Recycling or reinternalization of the internal receptor pool is detected by surface accessibility to a secondary antibody.

First, we asked whether the μOR was capable of recycling in the presence of overexpressed GRK2 or GRK2-as5. HEK293 cells expressing wild-type GRK2 or GRK2-as5, together with the FLAG-μOR, were treated with morphine in the presence of a mouse monoclonal, calcium-dependent, Alexa488-conjugated anti-FLAG antibody (Fig. 6*B*, *panels 1* and *4*, *green*). This allowed for a pool of internalized Alexa488 FLAG-

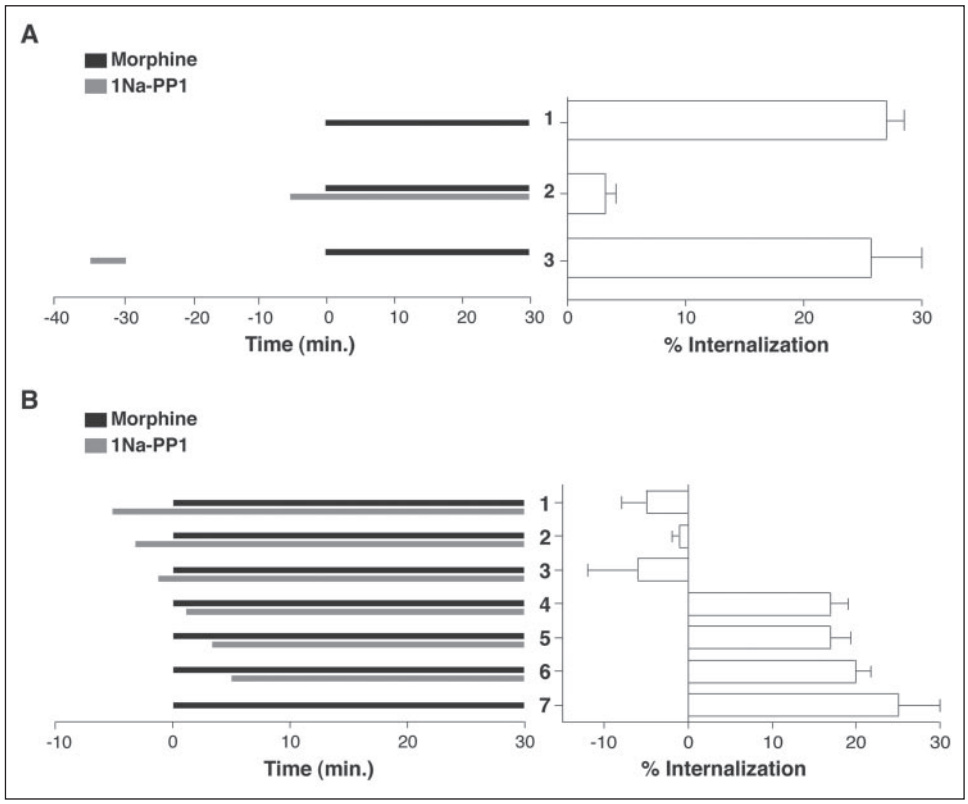

FIGURE 5. **Regulation of initial internalization by GRK2-as5. A model diagram of the effects of timing 1Na-PP1 and morphine application on internalization is to the** *left*. *A,* reversibility of GRK2-as5 inhibition by 1Na-PP1. HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2-as5 were treated with morphine for 30 min or 5 μM 1Na-PP1 for 10 min. Cells treated with 1Na-PP1 were then either exposed to morphine for 30 min or washed with Dulbecco's modified Eagle's medium and then exposed to morphine for 30 min. Quantitation of morphine-induced μOR internalization was analyzed by flow cytometry. Morphine-induced internalization is fully restored to cells after washing away the 1Na-PP1. *B,* HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2-as5 were treated with 1Na-PP1 for 1, 3, or 5 min and then exposed to morphine for an additional 30 min (*bars 1–3*). GRK2-as5 can be inhibited in 1 min. To assess inhibition of GRK2-as5 by 1Na-PP1 after treatment with morphine, HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2-as5 were treated with morphine for 1, 3, or 5 min and then exposed to 1Na-PP1 (while still in the presence of morphine) for an additional 30 min (*bars 4–6*). As a control, cells were treated only with morphine for 30 min (*bar 7*).

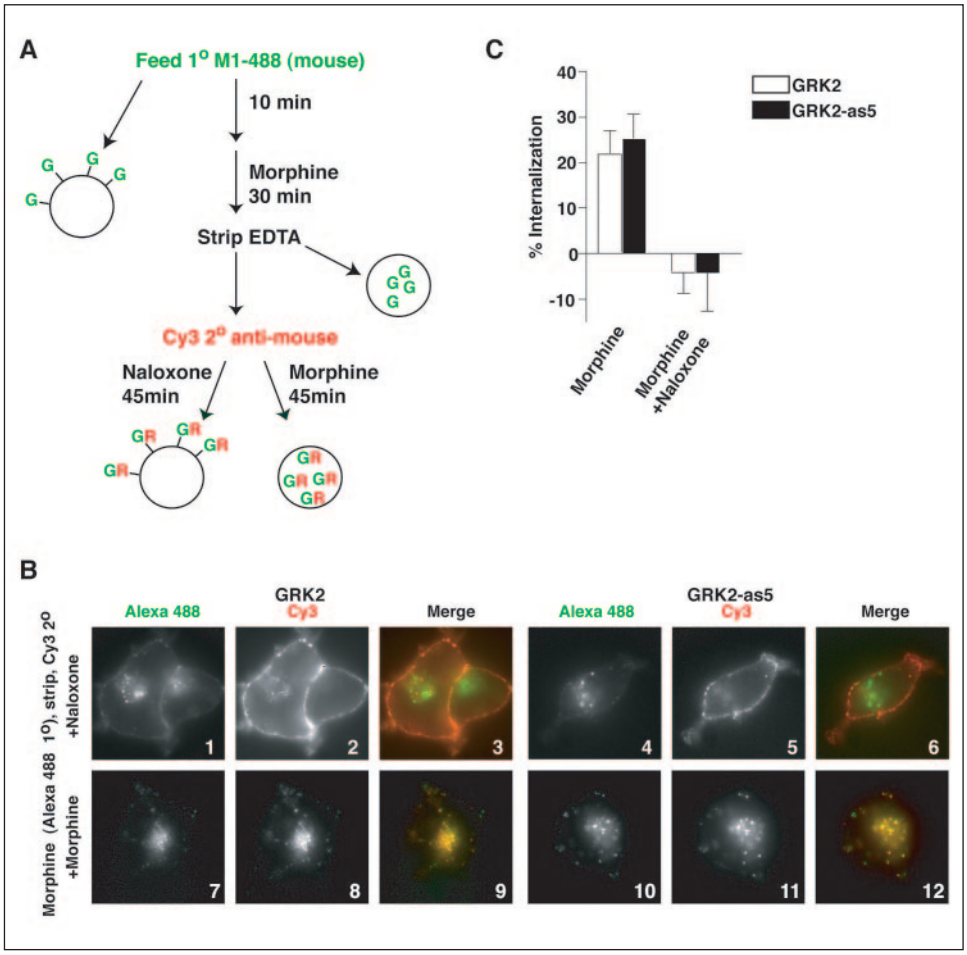

FIGURE 6. **Characterization of μOR recycling and reinternalization by GRK2.** *A,* schematic of an immunofluorescent assay to detect specifically the recycling and reinternalization of internalized receptors to the plasma membrane. *B,* HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2 or GRK2-as5 were treated with anti-FLAG Alexa488 antibody and 10 μM morphine at 37 °C for 30 min, stripped, and treated with anti-mouse Cy3 and naloxone or morphine. With naloxone, the μOR recycles back to the plasma membrane as seen by the accumulation of Cy3 when GRK2 (*panels 1–3*) or GRK2-as5 (*panels 4–6*) is overexpressed. In the presence of morphine, the μOR reinternalizes as seen by the accumulation of Cy3 in the cytoplasm when GRK2 (*panels 7–9*) or GRK2-as5 (*panels 10–12*) is overexpressed. *C,* quantification of this effect by flow cytometry demonstrates that morphine-induced reinternalization and recycling proceeds with overexpression of GRK2 and GRK2-as5.

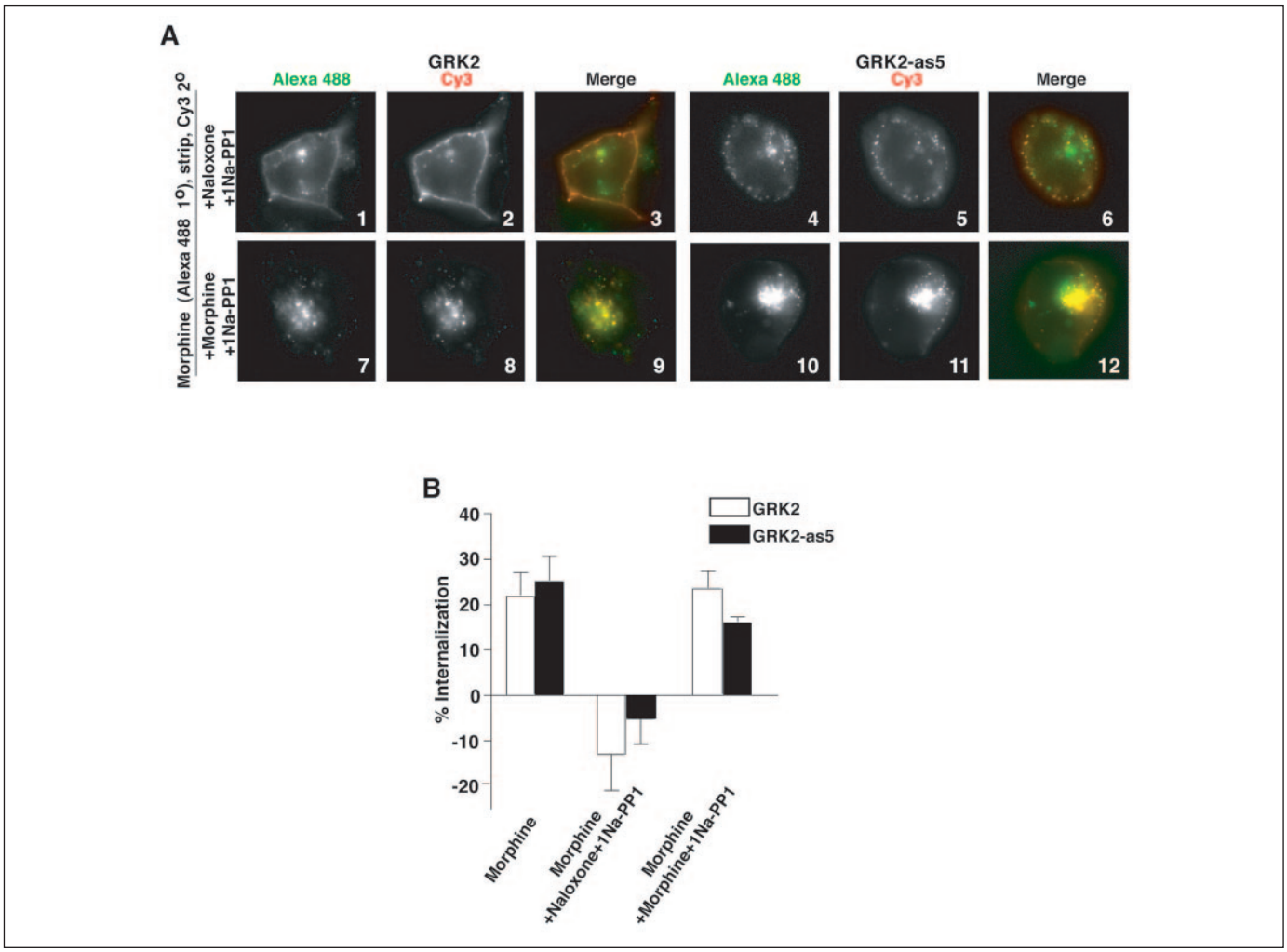

FIGURE 7. **Recycling and reinternalization are not dependent on enhanced expression of GRK2.** *A,* HEK293 cells stably expressing FLAG epitope-tagged μOR and GRK2 or GRK2-as5 were treated with anti-FLAG Alexa488 antibody and 10 μM morphine at 37 °C for 30 min, stripped, and treated with anti-mouse Cy3, naloxone, and 1Na-PP1 (*panels 1–6*) or morphine and 1Na-PP1 (*panels 7–12*). For GRK2, when cells are treated with naloxone and 1Na-PP1, the μOR recycles back to the plasma membrane as seen by the accumulation of Cy3 (*panels 1–3*). Inhibition of GRK2-as5 with 1Na-PP1 also has no effect on recycling (*panels 4–6*). When cells expressing GRK2 are treated with morphine and 1Na-PP1, reinternalization continues (*panels 7–9*). Inhibition of GRK2-as5 with 1Na-PP1 also has no effect on reinternalization (*panels 10–12*). *B,* quantification of this effect by flow cytometry. Treatment of cells expressing GRK2 or GRK2-as5 with 1Na-PP1 had no effect on recycling. 1Na-PP1 treatment with morphine also has no effect on GRK2 or GRK2-as5-induced reinternalization.

μOR to be visualized in endosomes. After 30 min of morphine exposure, EDTA was used to chelate calcium leading to disruption of the binding of the Alexa488 anti-FLAG antibody to the μOR at the plasma membrane. Both GRK2 and GRK2-as5 showed robust initial internalization as seen by the green punctate pattern in the cytoplasm of the cell (Fig. 6*B*, *panels 1* and *4*). A Cy3 anti-mouse secondary antibody was then applied with antagonist naloxone (Fig. 6*B*, *panels 2* and *5, red*). Naloxone does not interfere with receptors recycling to the plasma membrane and does not stimulate receptor internalization (52). Recycling of the FLAG-μOR from endosomes to the cell surface was apparent by the abundance of Cy3-labeled antibody (red) at the plasma membrane. This staining is indicative of the Alexa488 primary antibody (green)-labeled receptors recycling to the cell surface where they were bound by the Cy3 antibody (red) and could no longer internalize due to lack of agonist.

We next demonstrated that the FLAG-μOR is capable of undergoing multiple rounds of internalization, herein known as reinternalization, in response to morphine when GRK2 or GRK2-as5 are overexpressed. Cells were treated with morphine and Alexa488-conjugated anti-FLAG primary antibody and stripped in order to have only the pool of internalized receptors fluorescently labeled (Fig. 6*B*, *panels 7* and *10*). The Cy3 secondary antibody was then applied with morphine (Fig. 6*B*, *panels 8* and *11*). Reinternalization of the FLAG-μOR was visualized by the overlapping primary green and secondary red antibody punctate pattern in the cytoplasm of the cell, indicating the primary labeled receptors in the cytoplasm had returned to the cell surface and reinternalized (Fig. 6*B*, *panels 9* and *12*).

The results obtained using microscopy were validated by flow cytometry (Fig. 6*C*). In this assay only the surface receptor levels were quantitated following the same morphine and naloxone treatment regiment used in Fig. 6*B*. At the end of the assay an Alexa488-conjugated anti-FLAG antibody was used in order to quantitate the receptors at the cell surface. In this way we avoided possible confusing effects of antibody binding on the observed reinternalization. When cells overexpressing GRK2 or GRK2-as5 were treated only with morphine, significant internalization (~25%) of the μOR occurred, as observed by flow cytometry (Figs. 3*C* and 4 and 5). When treated first with morphine, washed, and then treated with the antagonist naloxone, there was very little internalization, indicating that upon removal of morphine the μOR had recycled back to plasma membrane and did not further internalize. In response to morphine, washing, and then morphine again, the FLAG-μOR internalized 22 and 25% with GRK2 and GRK2-as5, respectively (Fig. 6*C*). Because the FLAG-μOR can recycle and reinternalize effi-

ciently in the presence of morphine, we next sought to determine whether these processes were dependent on GRK2 overexpression.

Enhanced GRK2 Activity Is Not Required for Recycling of the μOR—To determine whether enhanced GRK2 activity is required for μOR recycling, 1Na-PP1 was added with naloxone after morphine-induced internalization, and the ability of antibody-labeled receptors to return to the plasma membrane was assessed by fluorescence microscopy (Fig. 7*A*, *panels 1–6*). For wild-type GRK2 treated with 1Na-PP1, FLAG-μOR recycling was observed by the abundance of red-labeled receptors that gathered on the cell membrane as the FLAG-μOR recycled back to the cell membrane (Fig. 7*A*, *panels 2* and *3*). These results are similar to those with wild-type GRK2 expressing cells in the absence of 1Na-PP1, as wild-type GRK2 cannot be inhibited by 1Na-PP1 (Fig. 6*B*, *panels 2* and *3*). Upon addition of 1Na-PP1 to GRK2-as5-expressing cells, recycling was still observed (Fig. 7*A*, *panels 4–6*). Analysis by flow cytometry demonstrated that GRK2- and GRK2-as5-expressing cells exhibited no significant internalization when treated with morphine followed by naloxone and 1Na-PP1 (Fig. 7*B*). These results demonstrate that FLAG-μOR recycling, after the initial morphine-induced internalization, does not require enhanced GRK2 activity.

Reinternalization of the μOR Does Not Require GRK2—For reinternalization studies with GRK2, cells were first treated with morphine followed by morphine and 1Na-PP1. Upon treatment with morphine and 1Na-PP1, the FLAG-μOR could either recycle to the plasma membrane and no longer internalize, which would be visualized by accumulation of red Cy3 antibody on the plasma membrane, or alternatively, the receptor could continue internalizing in a GRK2-independent manner, which would appear as Cy3 accumulation in the cytoplasm. When cells expressing GRK2 were treated with morphine, 1Na-PP1 and Cy3 secondary after stripping the green primary antibody from the cell surface, wild-type GRK2 cells showed a punctate pattern of Cy3 antibody staining in the cytoplasm of the cell, indicating reinternalization had occurred (Fig. 7*A*, *panels 7–9*) This was similar to what was observed without 1Na-PP1 (Fig. 6*B*, *panels 7–9*). Most surprisingly, when GRK2-as5 was inhibited with 1Na-PP1, reinternalization of the FLAG-μOR still occurred (Fig. 7*A*, *panels 10–12*). Therefore, there is a difference in the GRK2 dependence of μOR internalization in drug-naive cells compared with cells previously exposed to morphine. Although enhanced GRK2 activity is required for initial morphine-induced internalization of the μOR, it is not required for subsequent rounds of receptor internalization (Fig. 4*C versus* Fig. 7*B*).

DISCUSSION

We have used a chemical genetic strategy to develop, to our knowledge, the first specific inhibitor of a G protein-coupled receptor kinase. A standard protein kinase engineering strategy, successfully applied to over 24 kinases, resulted in a severe loss of catalytic activity when applied to GRK2. We modified our strategy to include a genome-wide comparison across all known kinase families. This strategy led to identification of a number of second-site mutations in the ATP binding region of GRK2-as1 that restored functional GRK2 activity, as indicated by a rapid cell-based assay. The inhibitable form of GRK2 was then used as a tool to study the morphine-induced membrane trafficking of the μOR. This approach, in addition to its specific utility in guiding the engineering of a regulated GRK isoform, could in principle be applied to any kinase for which the standard gatekeeper mutation results in a loss of activity.

An additional mutation in GRK2-as1, identified using a genetic screen, GRK2 (L271G/S268V), was shown to rescue function of GRK2-as1 (48). Thus, multiple approaches can be used to identify second-site suppressors of kinases with a small gatekeeper residue, greatly expanding the utility of the chemical genetic approach.

Although our strategy was successful in producing a specifically inhibited GRK isoform, which has comparable activity to the wild-type GRK2 both *in vitro* and in intact cells, our efforts to understand the biophysical basis of these mutational effects have so far been unsuccessful. Biophysical studies to examine the structure-function relationship between GRK2, GRK2-as1, and GRK2-as5 included thermal and guanidinium hydrochloride denaturation to measure structural differences and further thermal denaturation studies to measure activity by *in vitro* phosphorylation of tubulin. Unfortunately, because GRK2 is a large, multidomain protein, none of the data obtained from these methods provided a single well defined transition between folded and unfolded states, making the data difficult to interpret. Further studies such as crystal structure or hydrogen-deuterium exchange could help elucidate the presumed differences in stability and folding between GRK2, GRK2-as1, and GRK2-as5.

Previous studies have demonstrated that phosphorylation of the μOR occurs in less than a minute, whereas internalization takes minutes to hours. The major requirement for temporal resolution of GPCR internalization is an assay that measures receptor activity over discrete time points over a period of minutes rather than the integrated activity at the end of this period. The use of small molecule inhibition of GRK2 has provided temporal resolution of the regulatory events governing morphine-induced internalization of the μOR. Using a small molecule inhibitor of GRK2 that works on a rapid time scale, we have been able to demonstrate that enhanced GRK2 activity appears to be only required within the first minute of agonist stimulation. The present results confirm the ability of GRK2 to enhance morphine-induced internalization of μOR when overexpressed and to establish unequivocally that this endocytic effect is a specific and acutely reversible consequence of increased GRK2 catalytic activity.

We have gone on to use the GRK2-as5 mutant kinase as a tool to investigate temporal aspects of GRK activity in opioid receptor trafficking. Our results indicate that, although enhanced GRK2 activity is required for the ability of morphine to promote rapid endocytosis of receptors when initially applied, continuous rounds of recycling and re-endocytosis of receptors can occur in the absence of enhanced GRK2 activity. These results suggest an additional feature of GPCR endocytic trafficking, which extends beyond the current "resensitization" view of GPCR recycling as a process that fully reverses the effects of previous phosphorylation. Execution point experiments suggest that the altered GRK dependence for reinternalization persists for up to 3 h after initial receptor activation by morphine (data not shown), a time far in excess of that required for complete recycling of internalized receptors (<45 min). Thus our results argue strongly for a type of functional hysteresis in the GRK2 dependence of μOR endocytosis.

One important question for future study is to elucidate the precise mechanistic basis of the observed GRK2-induced hysteresis of μOR regulation. The most likely hypothesis is that the cellular memory for previous opioid exposure represents a GRK2-mediated phosphorylation event, which persists long after receptor recycling has occurred. Indeed, there is some evidence that opioid receptors can remain persistently phosphorylated, and there may also be similar effects on other GPCRs such as the β_2-adrenergic receptor (53, 54). It will be interesting in future studies to determine whether the present results can be explained by persistent phosphorylation of opioid receptors themselves or perhaps distinct (non-GPCR) substrates.

Another important question is to investigate the possible physiological importance of the observed GRK2 effect in physiologically relevant

systems. For example, there is considerable debate about the degree to which morphine can induce GRK-mediated phosphorylation and internalization in physiologically relevant systems, and if such regulatory processes play an important role in the induction or maintenance of physiological drug tolerance and dependence. Although the present observations are fully consistent with previous results from heterologous cell models, regarding the relatively weak ability of morphine to induce regulated internalization of opioid receptors, morphine appears to produce rapid internalization of μOR-specific neuronal cell types and even in specific neuronal membrane domains (55). The availability of a GRK2-selective inhibitor now provides an important tool for exploring the potential utility of GRKs as therapeutic targets in disorders involving altered GPCR-mediated signaling.

The GRK2-as5 allele could be very useful in addressing this question in genetically engineered mice. Recently, analog-sensitive knock-in mice were created for all three members of the Trk receptor tyrosine kinase family (56). The Trk-as mice were viable and had the same neuronal responses as wild-type Trk. Because 1Na-PP1 can cross the blood-brain barrier, the Trk-as mice provided for selective, rapid, and reversible inhibition of neurotrophin signaling in the brain, whereas no such Trk inhibition was observed in wild-type neurons. These results indicate that a knock-in mouse of GRK2-as5 could be pharmacologically regulated by 1Na-PP1.

In future studies it will be important to examine more broadly the role of GRK2, as well as other GRK family kinases, in the regulation of other receptors and agonists. It will also be of interest to apply this strategy to investigate recent evidence that certain GRKs can mediate positive rather than negative effects on specific signaling processes, perhaps by functioning as direct mediators of signaling via phosphorylation of non-GPCR substrates (57, 58). The ability to selectively inhibit only one GRK in cells that express many other kinases, often including multiple GRK isoforms (59), is anticipated to accelerate the understanding of specific GRK signaling and regulatory mechanisms, as well as to identify specific substrates relevant to these mechanisms.

Acknowledgments—We thank Jeff Benovic for providing the bovine GRK2 construct, rhodopsin, and valuable advice regarding GRK biochemistry. We thank Ron Vale for purified tubulin. We also thank Henry Bourne, Michael Cohen, and all members of the Shokat and Von Zastrow laboratories, especially Mike Gage, Zachary Knight, and Dustin Maly, for helpful comments and discussions.

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