

Ligand Directed Signaling Differences between Rodent and Human κ -Opioid Receptors*

Received for publication, May 13, 2012, and in revised form, October 6, 2012. Published, JBC Papers in Press, October 19, 2012, DOI 10.1074/jbc.M112.381368

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Background: Dysphoric effects of κ -opioid receptor (KOR) agonists require p38 MAPK activation in mice, but sequence differences in human KOR may affect this mechanism.

Results: Differences in p38 activation were observed between human and rodent KOR for pentazocine and butorphanol.

Conclusion: Species differences affect signaling.

Significance: Rodent models may not predict adverse effects of KOR agonists in humans.

KOR activation of $G\beta\gamma$ dependent signaling results in analgesia, whereas the dysphoric effects of KOR agonists are mediated by a different pathway involving G protein receptor kinase and non-visual arrestin. Based on this distinction, a partial KOR agonist that does not efficiently activate arrestin-dependent biased signaling may produce analgesia without dysphoria. No KOR-selective partial agonists are currently available, and preclinical assessment is complicated by sequence differences between rodent (r) and human (h) KOR. In this study, we compared the signaling initiated by the available partial agonists. Pentazocine was significantly more potent at activating p38 MAPK in hKOR than rKOR expressed in HEK293 cells but equally potent at arrestin-independent activation of ERK1/2 in hKOR and rKOR. Similarly, butorphanol increased phospho-p38-ir in hKOR-expressing cells but did not activate p38 in rKOR-HEK293. Like pentazocine, butorphanol was equally efficacious at activating ERK1/2 in rKOR and hKOR. In contrast, levorphanol, nalorphine, and U50,488 did not distinguish between hKOR and rKOR in p38 MAPK activation. Consistent with its low potency at p38 activation, pentazocine did not produce conditioned place aversion in mice. hKOR lacks the Ser-369 phosphorylation site in rKOR required for G protein receptor kinase/arrestin-dependent p38 activation, but mutation of the Ser-358 to asparagine in hKOR blocked p38 activation without affecting the acute arrestin-independent activation of ERK1/2. This study shows that hKOR activates p38 MAPK through a phosphorylation and arrestin-dependent mechanism; however, activation differs between hKOR and rKOR for some ligands. These functional selectivity differences have important implications for preclinical screening of partial KOR agonists.

κ -Opioid receptor (KOR)² agonists are effective analgesics and are thought to have a lower addictive potential than μ -opioid receptor (MOR) agonists (1–3). However, KOR agonists also have dysphoric effects in humans (4) and produce aversion in experimental animals (5–9), unlike MOR agonists, which are euphorogenic. These negative affective responses to available KOR agonists have limited their therapeutic use (10, 11). The cellular and molecular mechanisms of κ -opioid-induced dysphoria (measured operationally as conditioned place aversion in rodents) are not completely clear, but recent results suggest that KOR activation of p38 α MAPK in serotonergic neurons innervating the ventral striatum is required (12–14). KOR activation of p38 MAPK follows from G protein receptor kinase 3 (GRK3) phosphorylation of the serine 369 residue within the C-terminal tail of the rodent sequence (rKOR) and subsequent recruitment of nonvisual arrestins that act as a scaffold linking a kinase signaling cascade to p38 MAPK phosphorylation (12, 15). Disruption of this signaling pathway in mice through receptor mutation (KORS369A), GRK3 deletion, or conditional deletion of p38 α MAPK blocks the aversive effects of KOR agonists without reducing their analgesic effects (13, 14, 16). These findings have potentially important therapeutic implications, because a selective, partial KOR agonist that does not efficiently activate arrestin-dependent signaling might produce analgesia without significant dysphoria (17). This type of “ligand-directed signaling” or “biased agonism,” where certain ligands preferentially activate a subset of a receptor signaling pathways, has been demonstrated for other receptor systems (18) including MOR; some MOR agonists, such as fentanyl, effectively recruit arrestin, whereas others such as the partial agonist morphine do not; however, both are highly effective analgesics (19).

Development of biased KOR agonists for human treatment is complicated by sequence differences between rKOR and hKOR. Whereas hKOR and rKOR share 94% amino acid sequence homology overall, the amino acid residues involved in

* This work was supported, in whole or in part, by National Institutes of Health Grants T32DA07278 (to H. S., J. R. L., and S. S. S.), KO5 DA020570 (to C. C.), and RO1DA030074 (to C. C.).

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² The abbreviations used are: KOR, κ -opioid receptor; MOR, μ -opioid receptor; ir, immunoreactivity; C.I., confidence interval; SNP, single nucleotide polymorphism; GRK, G protein receptor kinase 3; h-, human; r-, rodent; Bis-Tris, Bis-Tris; ANOVA, analysis of variance; norBNI, norbinaltorphimine.

entire arena. During this time the amount of time spent in each of the three chambers was video-recorded (ZR90, Cannon) and analyzed using Ethovision (v.3.0, Noldus, Wageningen, The Netherlands). Mice were assigned to either vertical- or horizontal-striped chambers based on pretest times. The morning of each of the two training days was paired with vehicle (10 ml/kg intraperitoneal of 15% DMSO, 5% Cremophor and 80% sterile saline), and the afternoon session was paired with drug (pentazocine 10 mg/kg intraperitoneal, pentazocine 10 mg/kg + nor-BNI 10 mg/kg intraperitoneal, or pentazocine 10 mg/kg + naloxone 5 mg/kg intraperitoneal) with a minimum of 4 h elapsing between training sessions. On the fourth day, mice were allowed to roam freely between all three compartments, and the amount of time spent in each compartment was recorded. Test sessions took place midday. Conditioned place preference or aversion scores were calculated by subtracting the time spent in the drug-paired side during the post-test from time spent in the saline-paired side during the post-test.

Locomotor Activity—Locomotor activity during conditioned place preference training was video-recorded (ZR90, Cannon) and analyzed using Ethovision (v.3.0, Noldus, Wageningen, The Netherlands). Scores represent total distance moved (cm) during the 30-min session.

Antinociceptive Testing—Antinociceptive responses were measured using the warm-water tail-withdrawal assay (8). The response latency for an animal to withdraw its tail after being immersed in 52.5 °C water was measured before treatment with (–)pentazocine (10 mg/kg, intraperitoneal). After drug administration, responses were measured every 10 min for the first 30 min and every 30 min after that for 3 h post drug administration. A 15-s maximal immersion duration was used as a cut-off to prevent tissue damage.

Cell Culture—HEK293 cells expressing rKOR-GFP were generated as described previously (8). HEK293 cells stably expressing FLAG-tagged hKOR or mutant hKOR (S358N) were generated by transfecting HEK293 cells using FuGENE 6 according to the manufacturer's instructions. Plasmids expressing FLAG-tagged human KOR- or FLAG-tagged human KOR (S358N) were previously described (20). HEK293 cells were maintained in Dulbecco's modified medium/F-12 with 10% fetal bovine serum. HEK293 cells expressing KOR were grown in medium supplemented with G418 (200 µg/ml). HEK293 cells expressed comparable levels of receptor: 1.6 and 4.1 pmol of [³H]U69,593 specifically bound per mg of protein, rKOR and hKOR, respectively.

siRNA—HEK293 cells were transfected with siRNA directed against arrestin-2 or arrestin-3 or scrambled siRNA control (Dharmacon Research) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Transfected cells were passaged onto 6-well plates after 48 h and 24 h later (72 h post transfection) treated as described.

Dominant Positive Arrestin—HEK293 expressing hKOR-(S358N) were transiently transfected with phosphorylation independent arrestin-2(R169E) or arrestin-3(R170E) (31, 32) tagged with mCherry at the C terminus as described (33) using FuGENE HD (Promega) according to the manufacturer's instructions. Transfected cells were passaged onto 6-well plates

after 48 h, and 24 h later (72 h post-transfection) treated as described.

Radioligand Binding—HEK293 cells were homogenized in ice-cold 50 mM Tris buffer, pH 7.4, followed by centrifugation at 26,000 × *g* for 30 min at 4 °C. Three 10-cm plates were pooled for each independent replicate. Homogenization of the pelleted membrane fraction and centrifugation were repeated twice. After the final centrifugation step, excess buffer was removed, and the membranes were stored at –20 °C until use. Membranes were resuspended in ice-cold Tris buffer. Protein concentrations were determined by BCA assay. Samples were incubated for 90 min at room temperature with 1 nM KOR ligand [³H]U69,543. GF/B glass fiber filters (Brandel) were preincubated for 90 min at room temperature with Tris buffer, 0.3% polyethyleneimine. After 90 min of incubation at room temperature, samples were placed on ice and collected with the filters with a Brandel 24 well harvester. Filters were washed with 3× with 3 ml of cold Tris buffer and counted in 5 ml of Ecoscint scintillation fluid. Radioligand concentrations were confirmed by scintillation count of free ligand. *K_i* values were calculated for each experiment; these values were used to calculate *K_i* and confidence intervals. Statistical significance was calculated by Student's *t* test using GraphPad Prism.

Immunoblotting—HEK293 cells were serum-starved for 6 h and then treated as described and lysed in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, 10% glycerol, phosphatase inhibitors, and protease inhibitors). Lysates were sonicated and centrifuged (15,000 × *g*, 20 min, 4 °C), and the supernatant was stored at –20 °C. Total protein concentration was determined by BCA assay (Pierce) with bovine serum albumin standards before loading 30 µg (phospho-ERK1/2) or 45 µg (phospho-p38 MAPK) onto 10% Bis-Tris precast gels (Invitrogen) and running at 130 V for 1.5–2 h. Blots were transferred to nitrocellulose (Whatman, Middlesex, UK) for 1.5–2 h at 30 V. The nitrocellulose was blocked with 5% BSA-TBS 1 h at room temperature and stained overnight at 4 °C for phospho-ERK1/2-immunoreactivity (ir) or phospho-p38-ir (Cell Signaling, 1:1000) and actin-ir (Abcam, 1:5000) in 5% BSA-TBS. Blots were incubated in IRdye secondary (Li-Cor Biosciences, 1:10,000 for phospho-p38-ir and phospho-ERK1/2-ir or 1:20,000 for actin) in 1:1 Odyssey buffer (Li-Cor) and 5% milk-TBS 1 h at room temperature then scanned on the Odyssey Infrared Imaging System (Li-Cor Biosciences). Band intensity was measured using the Odyssey software and expressed as phospho-ERK1/2-ir or phospho-p38 MAPK-ir band intensity over actin-ir band intensity. Data were normalized to percentage of control sample (basal, 100%) and plotted using GraphPad Prism. Statistical significance (*p* < 0.05) was determined by Student's *t* test or analysis of variance followed by Bonferroni's post-hoc test.

Dose-response Curves—Dose-response curves for p38 and ERK1/2 phosphorylation were calculated in GraphPad Prism using a three-parameter least squares nonlinear regression with a 1/Y weighting and a bottom constrained at 100%. Significant differences between EC₅₀ values and *E*_{max} were determined by extra sum-of-squares F test. To calculate relative efficacy and confidence interval (C.I.), maximal drug-induced ERK1/2 or p38 phosphorylation was normalized to the increase in phos-

Signaling Differences between Rodent and Human KOR

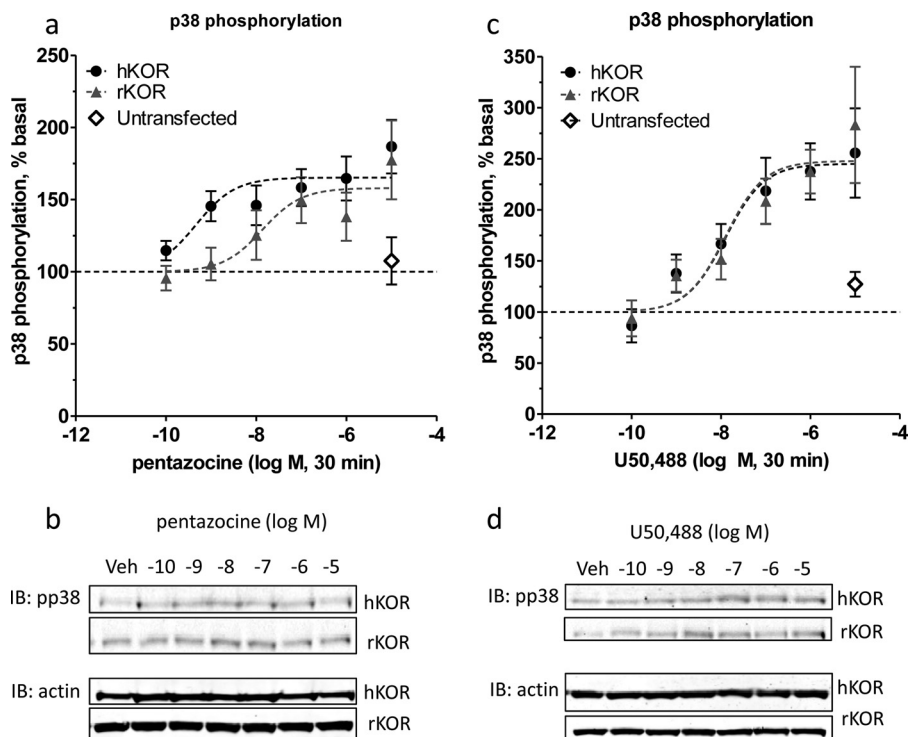


FIGURE 2. Pentazocine is less efficacious than U50,488 in activation of p38. Pentazocine, but not U50,488, is more potent for activation of the p38 pathway in hKOR as compared with rKOR. *a* and *b*, HEK293 cells expressing hKOR or rKOR were treated for 30 min with the indicated concentrations of pentazocine (*A*) or (–)U50,488 (*C*) before lysis and immunoblotted for phospho-p38-ir ($n = 9–14$). Dose-response curves are shown. *b* and *d*, representative immunoblots (*IB*) are shown for *a* and *c*.

phorylation stimulated by U50,488 (10 μM) for each experiment. For relative efficacy, statistical significance ($p < 0.05$) was calculated by Student's *t* test using GraphPad Prism.

SNP Analysis—To identify common nucleotide sequence polymorphisms, we performed re-sequencing on DNA samples from a discovery panel of 95 individuals from several diverse populations (African American, Caucasian, African, Hispanic, Asian). Polymerase chain reaction (PCR) and sequencing methods for SNP discovery have been published previously (34). For the present study, all of the coding regions of *OPRK1* were re-sequenced using M13-tailed PCR amplification of overlapping amplicons. PCR primer sequences are available upon request. All amplicons were purified, diluted, and used in standard Big-Dye terminator sequencing reactions under standard conditions (v3.1 Cycle Sequencing Kit Protocol Manual; Applied Biosystems, Foster City, CA) and run on an ABI 3730XL. Each chromatogram was trimmed of low quality sequence (Phred quality score < 25), assembled, and edited in Consed to ensure accuracy. SNPs were identified using the PolyPhred program (Version 5.0) (35). Putative polymorphic sites were flagged by PolyPhred and reviewed for genotype accuracy to remove false positives.

RESULTS

Do hKOR and rKOR Differ in Their Activation of p38 MAPK?—HEK293 cells stably expressing either rKOR or hKOR were treated for 30 min with either U50,488 or pentazocine at concentrations from 100 μM to 10 μM before lysis, and phospho-p38-ir was measured by Western blot (Fig. 2, Table 1). The EC_{50} values for pentazocine-induced p38 phosphorylation for

rKOR and hKOR were 14 nM (C.I. 1.3–140 nM, $n = 10$) and 0.47 nM (88 μM to 2.5 nM, $n = 14$), respectively. The 30-fold greater potency of pentazocine for activating p38 MAPK in cells expressing hKOR was statistically significant ($p < 0.05$). The EC_{50} values for U50,488-induced p38 phosphorylation for rKOR and hKOR were 21 nM (confidence interval 4.8 nM to 88 nM, $n = 9$) and 8.6 nM (1.9 nM to 39 nM, $n = 12$) respectively. In contrast to pentazocine, the potencies of U50,488 stimulation of phospho-p38 for hKOR and rKOR were not significantly different. Neither pentazocine nor U50,488 at the highest concentration tested increased p38 MAPK phosphorylation in untransfected HEK293 (Fig. 2). Consistent with previous *in vivo* assays (22, 36), pentazocine also appears to be a partial agonist in activating the p38 pathway (having 35–47% of the activity of U50,488 in both hKOR and rKOR). Pentazocine increased phospho-p38-ir to 165 and 158% of basal levels in hKOR and rKOR expressing cells, whereas stimulation with U50,488 resulted in p38 phosphorylation of 240 and 255% over basal. This difference in E_{max} was significant for both hKOR ($p < 0.001$) and rKOR ($p < 0.001$).

Are the Differences between hKOR and rKOR Pathway Dependent?—Activation of KOR has been reported to increase phospho-ERK1/2-ir by two separate mechanisms; a G-protein-mediated early phase of ERK1/2 phosphorylation and a slower arrestin-mediated late phase (37). To measure the early phase of ERK1/2 activation through the G-protein pathway, HEK293 expressing rKOR or hKOR were treated for 5 min with pentazocine or U50,488 at concentrations from 100 μM to 10 μM before lysis, and phospho-ERK1/2-ir was measured by Western

TABLE 1
Pharmacological data for opioid agonists in hKOR and rKOR expressing HEK293

Shown is a summary of the pharmacological properties of opioid agonists in this study. Shown are EC₅₀ and relative efficacy values and confidence intervals for ERK1/2 phosphorylation after pentazocine and U50,488 treatment and for p38 phosphorylation after all drugs were calculated based on three parameter dose-response curves. Relative efficacy and confidence intervals for p38 MAPK and ERK1/2 phosphorylation after treatment with nalorphine, butorphanol, or levorphanol (30 min) were calculated based on maximal response to each drug normalized to the 10 μM U50,488 response within an experiment. K_i values are an average of K_i values from 4–5 competition binding experiments. Significance differences (*p* < 0.05) between hKOR and rKOR is indicated (Student's *t* test for relative efficacy and K_i; extra sum-of-squares F test for EC₅₀). Data rows shown in bold highlight the statistically significant differences noted. n.s., not significant.

Opioid agonist	hKOR	rKOR	rKOR/hKOR ratio	rKOR vs. hKOR
	<i>Confidence interval; n</i>	<i>Confidence interval; n</i>		
Butorphanol				
pp38 relative efficacy	1.1 (0.65-1.6; 6)	0.12 (−0.41-0.65; 10) ^a	0.1 ^a	<i>p</i> < 0.05
pERK relative efficacy	0.23 (0.10-0.37; 5)	0.30 (0.01-0.59; 4)	1.3	n.s.
pp38/pERK ratio	4.8	0.4 ^a		
pp38 EC ₅₀	0.11 nM (0.004-3.1 nM; 6)	No significant activation ^a		
K _i ([³ H]U69 binding)	2.8 nM (0.55-5.0 nM; 5)	1.9 nM (1.2–2.7 nM; 5)	0.7	n.s.
Levorphanol				
pp38 relative efficacy	1.4 (−0.01-2.8; 6)	1.3 (−0.33-2.9; 5)	0.9	n.s.
pERK relative efficacy	0.11 (0.08-0.15; 4)	0.33 (0.09-0.58; 4)	3.0	<i>p</i> < 0.05
pp38/pERK ratio	12.6	3.9		
pp38 EC ₅₀	15 nM (1.5-150 nM; 6)	16 nM (1.3-160 nM; 5)	1.1	n.s.
K _i ([³ H]U69 binding)	11 nM (5.4-17 nM; 5)	26 nM (16-37; 5)	2.4	<i>p</i> < 0.01
Nalorphine				
pp38 relative efficacy	0.97 (0.77-1.2; 7)	1.1 (0.43-1.7; 7)	1.1	n.s.
pERK relative efficacy	0.23 (0.10-0.35; 5)	0.56 (0.19-0.93; 4)	2.4	<i>p</i> < 0.05
pp38/pERK ratio	4.2	1.9		
pp38 EC ₅₀	0.18 nM (0.004-7.6 nM; 7)	0.16 nM (0.02-1.4 nM; 7)	0.9	n.s.
K _i ([³ H]U69 binding)	4.1 nM (1.6-6.5 nM; 5)	9.5 nM (1.3-18 nM; 5)	2.1	n.s.
Pentazocine				
pp38 relative efficacy	0.47 (0.36-0.56; 14)	0.35 (0.21-0.51; 10)	0.7	n.s.
pERK relative efficacy	0.27 (0.23-0.39; 12)	0.40 (0.29-0.52; 13)	1.5	n.s.
pp38/pERK ratio	1.7	0.9		
pp38 EC₅₀	0.47 nM (0.088-2.5 nM; 14)	14 nM (1.3-140 nM; 10)	29.8	<i>p</i> < 0.05
pERK EC ₅₀	2.3 nM (0.52-10 nM; 12)	7.2 nM (1.6-32 nM; 13)	3.1	n.s.
pp38/pERK ratio	0.2	1.9		
K _i ([³ H]U69 binding)	9.4 (4.1-15 nM; 5)	15 nM (10-19 nM; 5)	1.6	n.s.
U50,488				
pp38 relative efficacy	1.0 (0.73-1.3; 12)	1.0 (0.72-1.3; 9)	1.0	n.s.
pERK relative efficacy	1.0 (0.72-1.3; 12)	1.0 (0.82-1.2; 10)	1.0	n.s.
pp38/pERK ratio	1.0	1.0		
pp38 EC ₅₀	8.6 nM (1.9-39 nM; 12)	21 nM (4.8-88 nM; 9)	2.4	n.s.
pERK EC ₅₀	16 nM (4.4-56 nM; 12)	16 nM (7.2-35 nM; 10)	1.0	n.s.
pp38/pERK ratio	0.5	1.3		
K _i ([³ H]U69 binding)	2.1 nM (1.1-3.0 nM; 4)	2.4 nM (0.08-4.9 nM; 4)	1.1	n.s.

^a *p* < 0.05.

blot (Fig. 3, Table 1). The EC₅₀ values for pentazocine-induced phospho-ERK1/2-ir for rKOR and hKOR were 7.2 nM (C.I. 1.6–32 nM, *n* = 13) and 2.3 nM (C.I. 520 pM to 10 nM, *n* = 12), respectively, with no significant difference. The EC₅₀ values for U50,488-induced ERK1/2 phosphorylation for rKOR and hKOR were 16 nM (C.I. 7.2–35 nM, *n* = 10) and 16 nM (C.I. 4.4–56 nM, *n* = 12), respectively, with no significant difference. As was observed for p38 pathway activation, pentazocine appears to be a partial agonist (with 27–40% of U50,488 activity) for stimulation of the early phase of the ERK1/2 pathway. Stimulation of hKOR- and rKOR-expressing cells increased phospho-ERK1/2-ir by 297 and 330% of basal levels, respectively, whereas stimulation with U50,488 increased phospho-ERK1/2-ir to 742 and 670% of basal. This difference in *E*_{max} was significant for both hKOR (*p* < 0.001) and rKOR (*p* < 0.001).

The difference between pentazocine effects on p38 and ERK activation in hKOR and rKOR is striking and suggests that the sequence differences between hKOR and rKOR more strongly affect p38 activation than the early phase of the ERK1/2 signaling cascade. Furthermore, because U50,488 affected hKOR and rKOR similarly and there were no differences between hKOR and rKOR in pentazocine affinity (Table 1), the results suggest that the differences in pathway activation were a consequence

of pentazocine-KOR interaction differences rather than differences in expression or drug affinity.

What Is the Relevance of in Vitro Pharmacological Data for Pentazocine to Behavioral Effects?—The relatively weak p38 activation in rKOR by pentazocine suggests that it may not produce aversion in mice through KOR activation. To assess this, mice were trained in a balanced, unbiased three chamber conditioning apparatus, receiving saline, 10 mg/kg pentazocine or pentazocine in combination with 10 mg/kg norBNI and/or 5 mg/kg naloxone. Mice showed a 200-s place preference for the pentazocine-paired chamber that was not affected by pretreatment with the KOR-selective antagonist norBNI. Pretreatment with naloxone reversed the preference for the pentazocine-paired chamber and unveiled an aversion that was not blocked by pretreatment with norBNI (Fig. 4a). These results suggest that pentazocine did not produce a KOR-mediated aversion in mice, in contrast to reports of KOR-mediated aversive effects in humans.

To assess the receptor selectivity of pentazocine in mice, wild type, KOR^{−/−}, and MOR^{−/−} mice were injected with pentazocine 10 mg/kg intraperitoneal, and tail flick withdrawal latency was measured over 180 min (Fig. 4b). There was a significant time-dependent (*p* < 0.0001) increase in tail withdrawal

Signaling Differences between Rodent and Human KOR

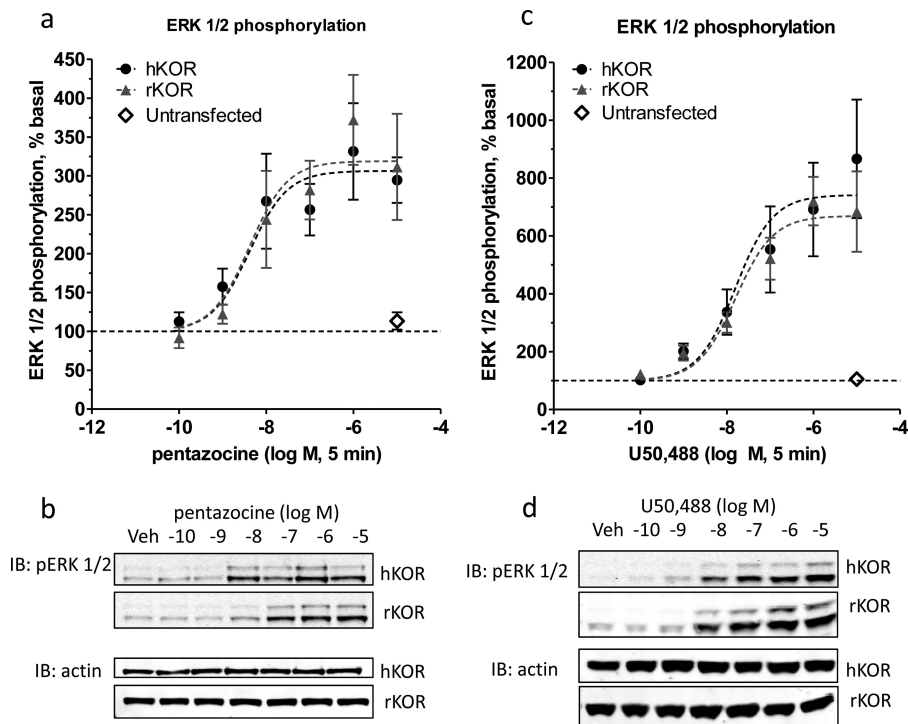


FIGURE 3. **Pentazocine is less efficacious than U50,488 for activation of ERK1/2.** hKOR and rKOR do not differ in potency for the ERK1/2 pathway. *a* and *c*, HEK293 cells expressing hKOR or rKOR were treated 5 min with pentazocine (*a*) or (–)U50,488 (*c*) before lysis and immunoblotted for phospho-ERK1/2-ir ($n = 10–13$). Dose-response curves are shown. *b* and *d*, representative immunoblots (IB) are shown for *a* and *c*.

latency and a specific effect of subject pairing ($p < 0.0001$), genotype ($p < 0.05$), and interaction ($p < 0.05$). This antinociceptive response was not significantly different between wild type and $KOR^{-/-}$ mice, whereas $MOR^{-/-}$ had significantly reduced analgesia as compared with wild type and $KOR^{-/-}$ mice. Furthermore, pretreatment with norBNI produced no reduction in pentazocine-induced analgesia in $MOR^{-/-}$ mice (data not shown).

To determine the locomotor effects of pentazocine, total distance traveled was measured during drug training sessions for conditioned place preference. Pentazocine produced neither hypolocomotor effects (typical of κ agonists) nor hyperlocomotor effects (typical of μ agonists) (Fig. 4c).

Do Other Partial KOR Agonists Differ in p38 Activation by hKOR and rKOR?—To determine whether other partial KOR agonists differ in activation of p38 by hKOR and rKOR, HEK293 cells stably expressing rKOR or hKOR were treated 30 min with nalorphine, levorphanol, or butorphanol at concentrations from 100 μ M to 10 μ M before lysis, and phospho-p38-ir was measured by Western blot. To determine relative efficacy, maximal drug-induced p38 phosphorylation in each experiment was normalized to the increase in phospho-p38-ir stimulated by U50,488 (10 μ M) (Table 1). Nalorphine treatment stimulated p38 activation in rKOR and hKOR with relative efficacies of 1.1 (C.I. 0.43–1.7, $n = 7$) and 0.97 (C.I. 0.77–1.2, $n = 7$) and EC_{50} values of 0.16 nM (C.I. 0.02–1.4 nM) and 0.18 nM (C.I. 0.004–7.6 nM), respectively. The relative efficacies of levorphanol for p38 activation by rKOR and hKOR relative to U50,488 were 1.3 (C.I. –0.33–2.9, $n = 5$) and 0.38 (C.I. –0.01–2.8, $n = 6$), with EC_{50} values of 16 nM (C.I. 1.3–160 nM) and 15 nM (C.I. 1.5–150 nM), respectively. No significant difference was

observed between hKOR and rKOR in efficacy or potency of nalorphine- or levorphanol-stimulated p38 phosphorylation. Butorphanol increased phospho-p38-ir in hKOR expressing HEK293 cells with an EC_{50} of 0.11 nM (C.I. 0.004–3.1 nM, $n = 6$) and an efficacy of 1.1 relative to U50,488 (C.I. 0.65–1.6). In contrast, butorphanol was significantly less efficacious (~10-fold) for stimulation of p38 phosphorylation in rKOR-expressing cells as compared with hKOR express cells (Student's *t* test, $p < 0.05$), with a relative efficacy of 0.12 (C.I. –0.41–0.65, $n = 10$) and did not stimulate a significant increase in p38 phosphorylation as compared with untransfected HEK293 cells. No increase in p38 phosphorylation was observed in untransfected HEK293 treated with 10 μ M nalorphine, levorphanol, or butorphanol (data not shown).

To determine if nalorphine, levorphanol, or butorphanol differ in the acute, G-protein-dependent phase of ERK1/2 activation by hKOR and rKOR, HEK293 cells expressing rKOR or hKOR were treated for 5 min with 10 μ M nalorphine, butorphanol, levorphanol, or U50,488 before lysis, and phospho-ERK1/2-ir was measured by Western blot (Table 1). No significant differences were observed between hKOR and rKOR in the relative efficacy of ERK1/2 phosphorylation stimulated by butorphanol. The relative efficacies of levorphanol and nalorphine for ERK1/2 activation were significantly higher in rKOR as compared with hKOR (3.0- and 2.4-fold, respectively). Competition radioligand binding with [3 H]U69,593 showed no significant differences between hKOR and rKOR in binding affinity for nalorphine or butorphanol. Levorphanol had a significant 2.4-fold lower affinity for rKOR as compared with hKOR.

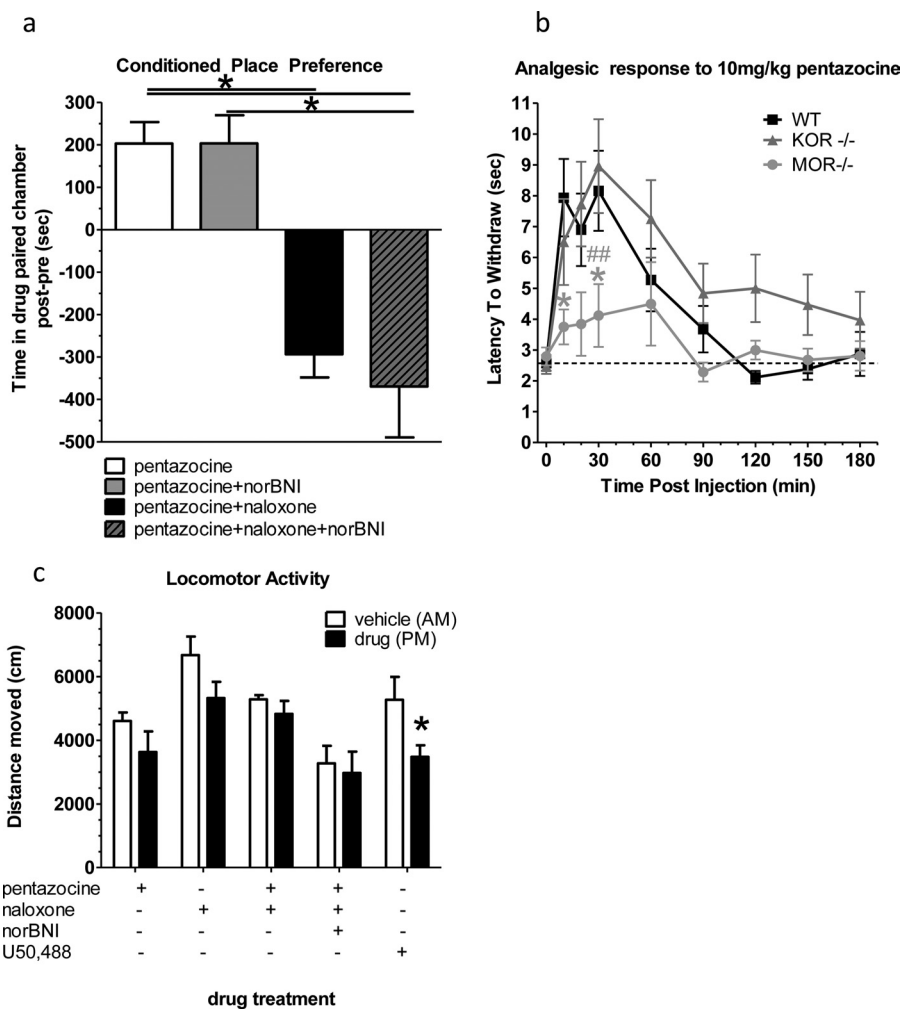


FIGURE 4. *a*, C57BL/6 mice were trained in a balanced, unbiased three-chamber conditioning apparatus. The morning of each of the two training days was paired with vehicle (10 ml/kg intraperitoneal of 15% DMSO, 5% Cremophor, and 80% sterile saline), and the afternoon session was paired with drugs (pentazocine, 10 mg/kg intraperitoneal, pentazocine + norBNI 10 mg/kg intraperitoneal, pentazocine 10 mg/kg + naloxone 5 mg/kg intraperitoneal, or pentazocine 10 mg/kg + norBNI 10 mg/kg + naloxone 5 mg/kg). On the test day mice were allowed to roam freely between all three compartments, and the amount of time spent in each compartment was recorded. Scores were calculated by subtracting the time spent in the drug-paired side of the posttest from time spent in the saline-paired side during the post-test. Significant effect of drug treatment was based on one-way ANOVA ($p < 0.0001$, $n = 4-10$), with the Bonferroni-Dunn post-hoc test with correction for multiple comparisons. *b*, wild type, KOR^{-/-}, and MOR^{-/-} C57BL/6 mice were injected with 10 mg/kg pentazocine intraperitoneally, and tail flick withdrawal latency was recorded over 180 min post-injection. Two-way repeated measures ANOVA revealed a significant effect of interaction ($p < 0.05$), time ($p < 0.0001$), genotype ($p < 0.05$), and subject matching ($p < 0.0001$) ($n = 13-19$). Significance according to the Bonferroni-Dunn post-hoc test of the differences as compared with wild type or KOR^{-/-} is indicated by * or #, respectively ($p < 0.05$ for one mark, $p < 0.01$ for two marks). *c*, total distance traveled during training sessions for conditioned place preference testing in *a* was recorded. Significant effect of the specific drug treatment ($p < 0.01$) and drug versus vehicle ($p < 0.01$) based on two-way repeated measures ANOVA is shown. Significance according to Bonferroni-Dunn post-hoc test of the differences for drug versus vehicle ($p < 0.05$) is indicated by the *.

Is the Ser-358 Site of hKOR Required for hKOR Activation of p38 upon U50,488 Stimulation?—Because hKOR and rKOR differ in p38 activation and in GRK phosphorylation sites, we next asked if the mechanism for hKOR activation of p38 requires phosphorylation of hKOR by GRK, similar to rKOR. To determine if activation of hKOR induced p38 phosphorylation requires the Ser-358 residue, HEK293 cells expressing hKOR, rKOR, or hKOR(S358N) were treated with 10 μ M U50,488 for 5–120 min at 37 °C. This resulted in a significant increase in phospho-p38-ir by 30 min in both hKOR- and rKOR-expressing HEK293, whereas no increases in p38 phosphorylation were detected in HEK293 expressing hKOR-(S358N) or in untransfected HEK293 cells (Fig. 5). These data suggest that hKOR activation promotes p38 phosphorylation in

a receptor phosphorylation-dependent manner as has previously been reported for rKOR (12).

Is Arrestin Required for hKOR to Activate p38?—Phosphorylation of p38 in rKOR requires recruitment of arrestin-3 after receptor phosphorylation by GRK3 (12, 16). To determine if hKOR-stimulated p38 phosphorylation also requires arrestin, we transiently transfected hKOR expressing HEK293 with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA control. 72 h after transfection, cells were treated with vehicle or 10 μ M U50,488 for 30 min, before lysis and immunoblotting for phospho-p38-ir. To confirm the selectivity of arrestin knockdown, lysates were immunoblotted against arrestin-2 or arrestin-3, and arrestin expression was compared with expression in cells transfected with scrambled siRNA control. Arres-

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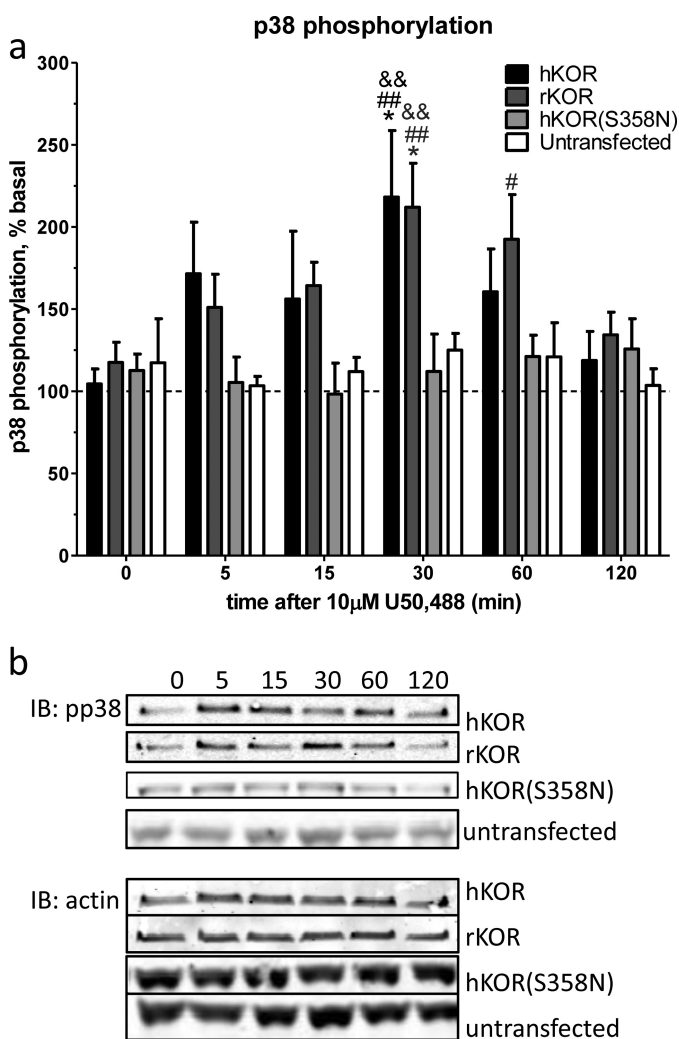


FIGURE 5. The Ser-358 residue is required for hKOR activation of p38 MAPK. *a*, HEK293 cells expressing hKOR, hKOR(S358N), rKOR, or untransfected wild type HEK293 were treated 5–120 min with U50,488 (10 μ M) before cell lysis and immunoblotted for phospho-p38-ir. Values represent the mean \pm S.E. Significant effect of cell type ($p < 0.05$) and U50,488 treatment ($p < 0.01$) based on two-way ANOVA ($n = 8-16$) is shown. Significance according to the Bonferroni-Dunn post-hoc test, as compared with hKOR(S358N), untransfected, or vehicle is indicated by *, &, or #, respectively ($p < 0.05$ for single mark or $p < 0.01$ for double mark). *b*, representative immunoblots (IB) for *a* are shown.

tin-2-ir levels were reduced 43% by arrestin-2 siRNA but were unchanged by arrestin-3 siRNA. Arrestin-3-ir levels were reduced 53% by arrestin-3 siRNA but were unchanged by arrestin-2 siRNA (Fig. 6, *a-c*). U50,488 treatment resulted in a significant increase in phospho-p38-ir in cells transfected with scrambled siRNA (one sample *t* test, $p < 0.05$). However, a reduction in the U50,488-stimulated increase in phospho-p38-ir was observed compared with cells not exposed to siRNA and transfection reagent. Knockdown of arrestin-3 but not arrestin-2 prevented the U50,488-stimulated increase in p38 phosphorylation (Fig. 6*d*).

To confirm that the absence of p38 phosphorylation in hKOR(S358N) was due to an inability to recruit arrestin, hKOR(S358N)-expressing HEK293 cells were transiently transfected with constructs expressing arrestin-2(R169E) or arrestin-3(R170E). These mutations allow the arrestin mole-

cule to bind to active-state G protein coupled receptors in the absence of receptor phosphorylation, resulting in a dominant-positive phosphorylation-independent arrestin (31, 32). Cells were treated for 30 min with 10 μ M U50,488 or vehicle 72 h after transfection before lysis for immunoblot analysis (Fig. 6*e*). Phosphorylated p38 was measured by immunoblot, and the amount of phospho-p38-ir was normalized to the level in transfection reagent control (no DNA) cells treated with vehicle. Treatment with U50,488 had no significant effect on phospho-p38-ir in transfection reagent control hKOR(S358N) cells. A basal increase in p38 phosphorylation was observed after transfection with arrestin-2(R169E) with no significant further increase in p38 phosphorylation after U50,488. No basal increase was observed in vehicle-treated hKOR(S358N) cells transfected with arrestin-3(R170E), but a significant increase in phospho-p38-ir was observed after U50,488. These data show that expression of arrestin-3(R170E), which does not require receptor phosphorylation to be recruited to the receptor, was sufficient to allow agonist-induced p38 phosphorylation by hKOR (S358N).

Is the Ser-358 Site of hKOR Required for ERK1/2 Activation?—Rapid agonist-induced, GRK/arrestin-independent ERK1/2 activation has been previously reported for both hKOR and rKOR, and a slower GRK/arrestin-dependent phase of phosphorylation after rKOR stimulation has been reported in some cell lines (37). We, therefore, sought to assess the time course of ERK1/2 phosphorylation after hKOR stimulation and to determine if the Ser-358 site plays a role in ERK1/2 phosphorylation after hKOR activation. ERK1/2 phosphorylation levels were analyzed by Western blot after treatment with 10 μ M U50,488 for 5–120 min at 37 $^{\circ}$ C. This treatment resulted in a rapid and significant increase in phospho-ERK1/2-ir at 5 min in HEK293 cells expressing rKOR, hKOR, or hKOR(S358N) but not in untransfected HEK293 cells (Fig. 7). Although there was no significant difference in phospho-ERK1/2-ir between hKOR, rKOR, or hKOR(S358N) at 5 min, ERK1/2 phosphorylation was significantly higher for hKOR compared with hKOR(S358N) at 15 and 30 min after U50,488. Surprisingly, ERK1/2 phosphorylation also remained significantly higher for hKOR as compared with rKOR. ERK1/2 phosphorylation remained significantly elevated in hKOR but not rKOR- or hKOR(S358N)-expressing HEK293 cells up to 60 min after agonist stimulation. This suggests that in addition to the established rapid, receptor phosphorylation-independent phase of ERK1/2 phosphorylation, hKOR has a slower phase of ERK1/2 phosphorylation in HEK293 that requires the Ser-358 site. Furthermore, this late phase of ERK1/2 phosphorylation was more evident in HEK293 cells expressing hKOR than rKOR.

Is Arrestin Required for hKOR Activation of Late Phase ERK1/2?—Arrestin recruitment has been implicated in the late phase of ERK1/2 activation by rKOR (37). We hypothesized that arrestin is also required for the late phase of hKOR-stimulated ERK1/2 phosphorylation, which is significantly reduced by mutation of the Ser-358 site. To address this question, we transiently transfected hKOR-expressing HEK293 cells with siRNA against arrestin-2, arrestin-3, or a scrambled siRNA control. 72 h after transfection, cells were treated with vehicle or 10 μ M U50,488 for 30 min before lysis and immunoblotting

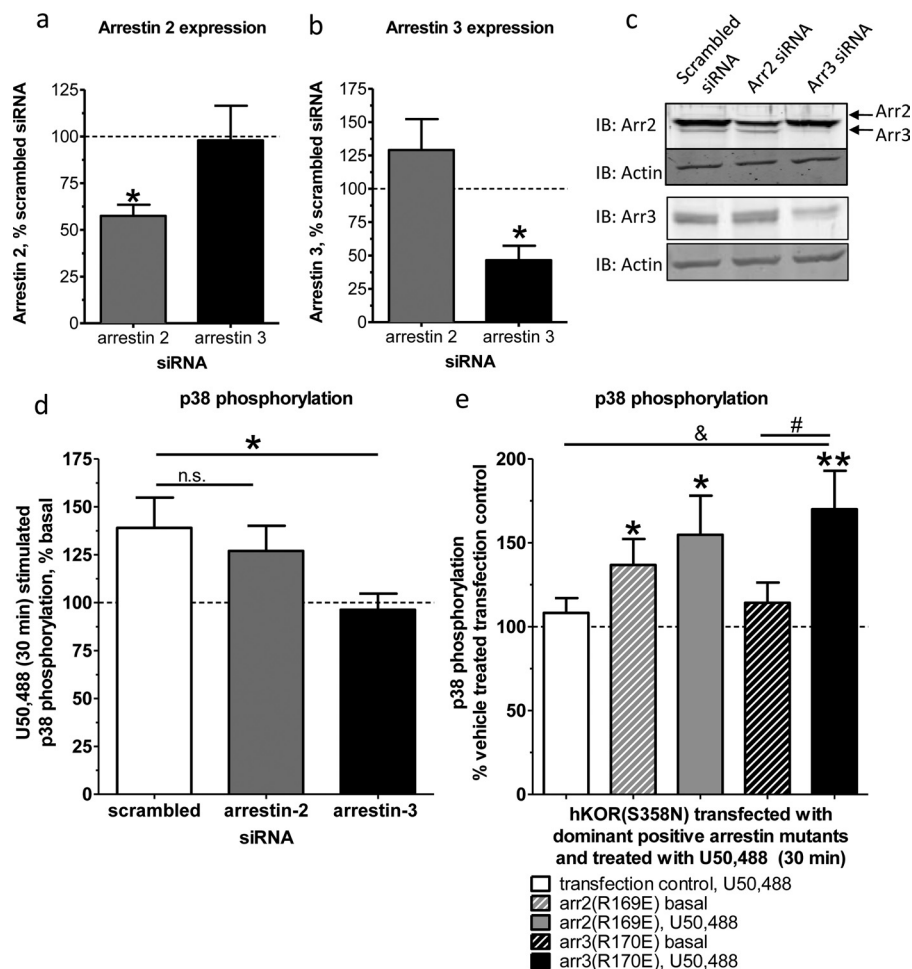


FIGURE 6. Arrestin-3 is required for hKOR induced p38 MAPK phosphorylation. *a*, HEK293 cells expressing hKOR were transiently transfected with siRNA against arrestin-2, arrestin-3, or scrambled siRNA control. After 72 h, cells were treated with U50,488 ($10 \mu\text{M}$) 30 min before cell lysis and immunoblotted for arrestin-2-ir. Significant decrease in arrestin-2 expression as compared with scrambled siRNA controls is indicated by *; one sample *t* test ($p < 0.001$, $n = 9$). *b*, cells were treated as in *a* but immunoblotted for arrestin-3-ir. Significant decrease in arrestin-3 expression as compared with scrambled siRNA controls is indicated by *; one sample *t* test ($p < 0.01$, $n = 5$). *c*, representative immunoblots are shown for *a* and *b*. *d*, cells were treated as in *a* but immunoblotted against phospho-p38-ir. Significant decrease of U50,488-induced p38 phosphorylation as compared with scrambled siRNA controls is indicated by *; Student's *t* test ($p < 0.05$, $n = 14$ – 16). *e*, HEK293 cells expressing hKOR(S358N) were transiently transfected with arrestin-2(R169E), arrestin-3(R170E), or no DNA (transfection reagent control). After 72 h, cells were treated with U50,488 ($10 \mu\text{M}$) 30 min before cell lysis and immunoblotted for phospho-p38-ir. Significant increase in p38 phosphorylation relative to vehicle-treated transfection reagent control hKOR(S358N) cells is indicated by *; one sample *t* test ($p < 0.05$ for single mark or $p < 0.01$ for double mark, $n = 21$ – 23). Significant increase in p38 phosphorylation relative to U50,488 treated transfection reagent control or vehicle treatment for same transfection is indicated by & or #, respectively, Student's *t* test ($p < 0.05$, $n = 21$ – 23).

for phospho-ERK1/2-ir. The 30-min time point after agonist stimulation was selected because the greatest difference in ERK phosphorylation between hKOR- and hKOR(S358N)-expressing cells was detected then (Fig. 5*a*). U50,488 treatment resulted in a significant increase in phospho-ERK1/2-ir in cells transfected with scrambled siRNA (one sample *t* test, $p < 0.0001$). However, a reduction in the U50,488-stimulated increase in phospho-ERK1/2-ir was observed compared with cells not exposed to siRNA and transfection reagent. Increased basal phospho-ERK1/2-ir and reduced U50,488 stimulated increase in phospho-ERK1/2-ir were observed as compared cells not exposed to scrambled siRNA and transfection reagent. Knockdown of either arrestin-2 or arrestin-3 significantly reduced the level of ERK1/2 phosphorylation (Fig. 8*a*). This suggests that both arrestin-2 and arrestin-3 were required for the increased ERK1/2 phosphorylation relative to hKOR(S358N).

To determine if expression of phosphorylation-independent arrestin-2 or -3 was sufficient to increase the late phase of U50,488-induced ERK1/2 activation in hKOR(S358N)-expressing cells to levels observed in hKOR cells, hKOR(S358N)-expressing HEK293 cells were transiently transfected with constructs expressing mCherry tagged arrestin-2(R169E), arrestin-3(R170E), or a no-DNA transfection reagent control. Cells were treated 72 h after transfection for 30 min with $10 \mu\text{M}$ U50,488 or vehicle before lysis for immunoblot analysis. Phosphorylated ERK1/2 was measured by immunoblot, and phospho-ERK1/2 was normalized to the level in transfection reagent control (no DNA) cells treated with vehicle. The results obtained showed that ERK1/2 phosphorylation was increased after U50,488 treatment regardless of transfection condition, but transfection with arrestin-2(R169E) or arrestin-3(R170E) alone did not further increase ERK1/2 phosphorylation to the higher ERK1/2 phosphorylation levels observed in cell express-

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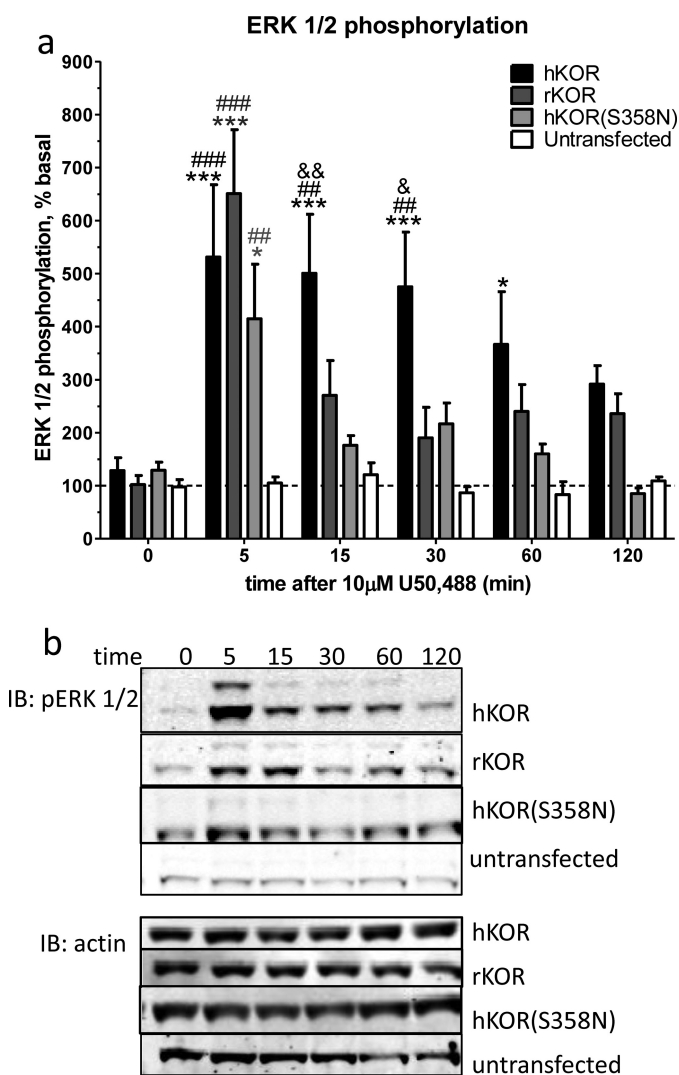


FIGURE 7. Ser-358 is not required for acute hKOR activation of ERK1/2 but is required for late phase ERK1/2 activation. *a*, HEK293 cells expressing hKOR, hKOR(S358N), rKOR, and untransfected wild type HEK293 were treated 5–120 min with U50,488 (10 μ M) before cell lysis and immunoblotted for phospho-ERK1/2-ir. Significant effect of cell type ($p < 0.01$), U50,488 treatment ($p < 0.01$), and interaction ($p < 0.05$) was revealed by two-way ANOVA ($n = 6–11$). Significance of the differences according to Bonferroni-Dunn post-hoc test as compared with hKOR(S358N), untransfected, or vehicle is indicated by *, &, or #, respectively ($p < 0.05$ for single mark, $p < .01$ for double mark, or $p < 0.001$ for triple mark). *b*, representative immunoblots (IB) are shown for *a*.

ing wild type hKOR at this time point (Fig. 8*b*). These data show that expression of phosphorylation independent mutants of arrestin-2 or arrestin-3 is not sufficient to recover the late phase of ERK1/2 phosphorylation by hKOR(S358N).

Are Polymorphisms at the Ser-358 Residue Common in the Human Population?—The NCBI Single Nucleotide Polymorphism Database (dbSNP accession number ss43313677; www.ncbi.nlm.nih.gov) reports 10 missense mutations for the *OPRK1* gene. This includes a guanine-to-thymine mutation at mRNA position 1308 (rs34369022) that results in mutation of S358I, which would prevent receptor phosphorylation at that residue and subsequent activation of the p38 pathway. To assess if this is a common SNP within the human population, we performed re-sequencing of the *OPRK1* gene in 95 individ-

uals from diverse populations (Table 2). Of the 14 SNPs identified, 10 were in coding regions. Three non-synonymous SNPs were found, two of which had been previously reported in dbSNP. The identified missense SNPs were a valine-to-methionine within the second intracellular loop (not previously reported), a lysine-to-glutamate within the second extracellular loop, and an aspartate-to-asparagine in the C tail. No SNPs were identified within codon 358, suggesting that the reported variant is a rare allele ($<1/100$) or a sequencing mistake. To further explore the frequency of this variant, we accessed the NHLBI Exome Sequencing Project (Exome Variant Server, Seattle, WA; May, 2012), which screened nearly 5500 individuals. A search of this database reveals 23 non-synonymous SNPs within the coding region of the *OPRK1* gene but no report of a variant at amino acid position 358, also suggesting that previously reported mutation was extremely rare ($<1/10,000$) or a false report.

DISCUSSION

The primary findings of this study were that the basic mechanism of p38 MAPK activation is conserved between rKOR and hKOR but that the relative abilities of different agonists to activate specific pathways are not consistent between rKOR and hKOR. This has important implications for the development of biased KOR agonists for therapeutic use, including a limitation on the ability to assess ligand-directed signaling in people based on rodent models.

We found that the nonselective KOR agonist pentazocine is 30-fold more potent for p38 activation in hKOR as compared with rKOR. In contrast, pentazocine was equally potent for arrestin-independent activation of ERK1/2 in hKOR and rKOR, and there was no difference in potency for U50,488 activation of ERK1/2 or p38. Consistent with other studies examining the efficacy of KOR agonists in GTP γ S and cAMP inhibition assays (22, 36), we found pentazocine to be a partial agonist for both hKOR and rKOR in both pathways studied.

The effects of pentazocine in mice were complex and not consistent with the model of pentazocine functioning primarily as a KOR agonist. We found that pentazocine produces conditioned place preference and thermal analgesia. Both of these effects were mediated by MOR and unaffected by the disruption of KOR through genetic knock-out or administration of the KOR-selective antagonist norBNI. The opioid receptor mediating pentazocine analgesia in mice is not consistent across studies and seems to be dependent on multiple factors including mouse strain and pain modality (38, 42–44). Similar to other studies (42, 44), we found that in C57Bl/6 mice pentazocine thermal analgesia is mediated through the partial agonism at MOR rather than KOR. Although it has been assumed that pentazocine analgesia is mediated through KOR in humans, few studies have tested this (21), and it is not clear to what extent the mechanisms of pentazocine analgesia are similar between mice and humans.

In the presence of naloxone, mice demonstrated a conditioned place aversion to pentazocine, but this aversion was not blocked by norBNI, showing that it was not KOR-mediated. The mechanism of this aversion is not clear but may be a result of pentazocine activation of δ -receptors (40, 41). These findings

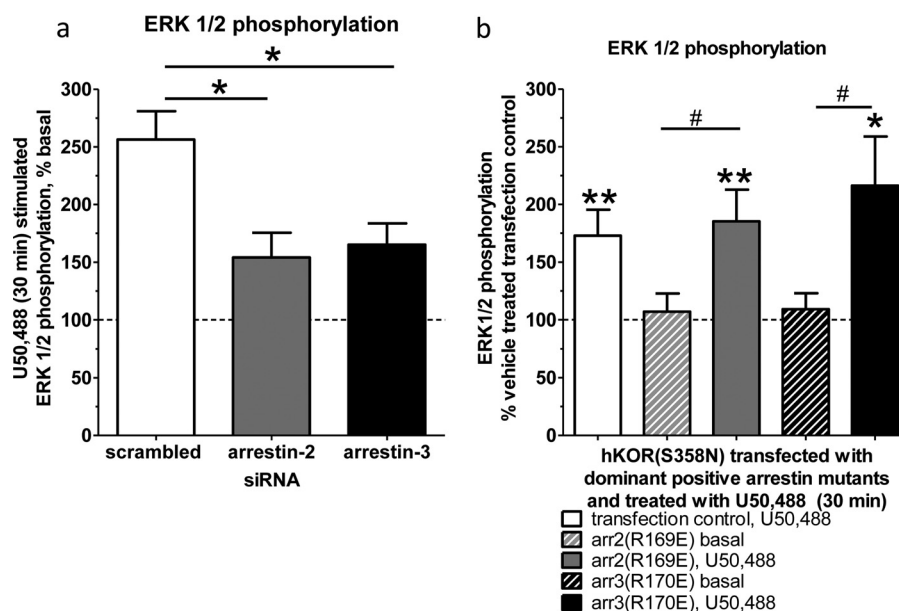


FIGURE 8. Both arrestin-2 and arrestin-3 are required for late phase hKOR activation of ERK1/2. *a*, HEK293 cells expressing hKOR were transiently transfected with siRNA against arrestin-2, arrestin-3, or scrambled siRNA control. After 72 h cells were treated with U50,488 (10 μ M) 30 min before cell lysis and immunoblotted for phospho-ERK1/2-ir. Significant decrease in U50,488 induced ERK1/2 phosphorylation as compared with scrambled siRNA controls is indicated by *, Student's *t* test ($p < 0.01$, $n = 15-16$). *b*, HEK293 cells expressing hKOR(S358N) were transiently transfected with arrestin-2(R169E), arrestin-3(R170E), or no DNA (transfection reagent control). After 72 h, cells were treated with U50,488 (10 μ M) 30 min before cell lysis and immunoblotted for phospho-ERK1/2-ir. Significant increase in p38 phosphorylation relative to vehicle-treated transfection control hKOR(S358N) cells is indicated by *, one sample *t* test ($p < 0.05$ for the single mark or $p < 0.01$ for the double mark, $n = 20-22$). Significant increase in ERK1/2 phosphorylation relative to vehicle treatment for same transfection is indicated by #, Student's *t* test ($p < 0.05$, $n = 21-23$).

TABLE 2

Polymorphisms identified in the human κ -opioid receptor gene

Polymorphisms identified in KOR in human population. Data rows shown in bold highlight variants resulting in missense mutations.

Chromosome 8 position	cDNA position	SNP	Amino acid position	Effect	Minor allele frequency	Variant ID
54163669	intron	Cys > Thr			0.038	rs9282806
54163562	271	Gly > Thr	12	Synonymous	0.108	rs1051660
54147470	694	Cys > Thr	153	Synonymous	0.053	rs7815824
54147451	713	Gly > Ala	160	Val > Met	0.005	
54147368	796	Ala > Gly	187	Synonymous	0.005	
54147331	833	Ala > Gly	200	Lys > Glu	0.005	rs77476023
54147250	Intron	Gly > Ala			0.005	
54147242	Intron	Gly > Ala			0.021	rs16918900
54142157	1078	Ala > Gly	281	Synonymous	0.258	rs702764
54142154	1081	Cys > Thr	282	Synonymous	0.047	rs16918875
54142148	1087	Cys > Thr	284	Synonymous	0.005	rs60353047
54142052	1183	Cys > Thr	316	Synonymous	0.016	rs78517449
54141880	1355	Gly > Ala	374	Asp > Asn	0.016	rs9282808
54141824	1411	Gly > Ala	3'-UTR		0.245	

in mice are distinctly different from human studies on pentazocine, in which pentazocine produced KOR-dependent dysphoric effects that were enhanced by blocking MOR (28). These results may reflect differences in measurements and assays between humans and mice, although other KOR agonists have been shown to cause KOR-dependent conditioned place aversion in mice. It is possible that the differences between the KOR-dependent aversive properties of pentazocine in humans and mice are partially due to the sequence differences between hKOR and rKOR, as hKOR and rKOR differ in pentazocine potency of p38 activation, which has been shown to play a role in the aversive properties of KOR agonists. Interpretation is limited, however, by the lack of KOR-selectivity, and more selective KOR partial agonists lacking activity at the μ -opioid and δ -receptors will be valuable in understanding the *in vivo* consequences of these sequence differences.

Differential p38 phosphorylation by hKOR and rKOR was found not to be unique to pentazocine. Butorphanol was similarly efficacious for acute ERK1/2 phosphorylation in hKOR and rKOR expressing HEK293 but did not induce any significant p38 phosphorylation in rKOR-expressing HEK293. In contrast, butorphanol was a potent agonist for p38 activation in hKOR-expressing HEK293, with an efficacy similar to U50,488. Although nalorphine and levorphanol were found to have similar efficacies and potencies for p38 phosphorylation in hKOR- and rKOR-expressing HEK293, these drugs were 2–3-fold more efficacious in rKOR activation of ERK1/2.

Based on these results, there appear to be ligand-specific differences between rKOR and hKOR in activation of MAPKs and of p38 in particular. This may be a result of the difference in the GRK phosphorylation site between hKOR and rKOR and the resulting difference in the molecular environment of the phos-

phorylated residue. Arrestin binding has previously been shown to be affected by the distribution of charged amino acids and other phosphorylatable residues adjacent to a GRK phosphorylation site (39) that are altered by the differences in location of the GRK site within the KOR C tail. We conclude from this that mice, rats, and rKOR expression systems are not appropriate models for screening for biased KOR ligands for human therapeutics.

Although the ligand-directed signaling properties of KOR agonists are not consistent between hKOR and rKOR, the signaling mechanisms are conserved. p38 activation through hKOR requires the GRK phosphorylation site on the C tail and arrestin-3, whereas the early phase of ERK1/2 phosphorylation is independent of either. There is also evidence that hKOR has a second, slower phase of ERK1/2 phosphorylation that is receptor phosphorylation- and arrestin-dependent, as has previously been shown in some cell lines with rKOR (37). Interestingly, our data show that this late phase of ERK1/2 requires both arrestin-2 and arrestin-3, although how arrestin-2 and -3 cooperate in promoting ERK1/2 signaling is not clear.

Although we cannot extrapolate biased signaling properties of individual agonists from mice and rats to humans, these results show that our understanding of KOR signaling mechanisms in these models likely applies to humans as well. It will be important not to draw conclusions about the signaling properties of particular KOR agonists in humans based purely on rKOR models, but the approach of identifying partial KOR agonists with lower potency for arrestin-mediated pathways than G-protein-mediated pathways holds promise for developing analgesics that lack the abuse potential of MOR agonists and the aversive properties of classic KOR agonists. It is worth noting that the guinea pig KOR is closer in homology to hKOR, containing the Ser-358 residue similar to hKOR. Further studies are needed, but guinea pigs may be a better model system than rats or mice for *in vivo* screening of biased KOR ligands with human therapeutic potential.

Acknowledgments—We thank Dr. John Pintar for providing the original MOR^{-/-} and KOR^{-/-} mice. We thank Dan Messinger for mouse genotyping.

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