THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 14, pp. 12724–12733, April 8, 2011

Modulating μ-Opioid Receptor Phosphorylation Switches Agonist-dependent Signaling as Reflected in PKCε Activation and Dendritic Spine Stability*

Received for publication, August 19, 2010, and in revised form, February 1, 2011 Published, JBC Papers in Press, February 3, 2011, DOI 10.1074/jbc.M110.177089

Hui Zheng[‡§1], **Ji Chu**[‡], **Yuhan Zhang**[‡], **Horace H. Loh**[‡2], **and Ping-Yee Law**[‡3]

From the [‡]*Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455-0217 and the* [§]*Stem Cell and Cancer Biology Group, Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kaiyuan Road, Guangzhou 510530, China*

A new role of G protein-coupled receptor (GPCR) phosphorylation was demonstrated in the current studies by using the μ-opioid receptor (OPRM1) as a model. Morphine induces a low level of receptor phosphorylation and uses the PKCε pathway to induce ERK phosphorylation and receptor desensitization, whereas etorphine, fentanyl, and [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) induce extensive receptor phosphorylation and use the β-arrestin2 pathway. Blocking OPRM1 phosphorylation (by mutating Ser^{363}, Thr^{370} and Ser^{375} to Ala) enabled etorphine, fentanyl, and DAMGO to use the PKCε pathway. This was not due to the decreased recruitment of β-arrestin2 to the receptor signaling complex, because these agonists were unable to use the PKCε pathway when β-arrestin2 was absent. In addition, overexpressing G protein-coupled receptor kinase 2 (GRK2) decreased the ability of morphine to activate PKCε, whereas overexpressing dominant-negative GRK2 enabled etorphine, fentanyl, and DAMGO to activate PKCε. Furthermore, by overexpressing wild-type OPRM1 and a phosphorylation-deficient mutant in primary cultures of hippocampal neurons, we demonstrated that receptor phosphorylation contributes to the differential effects of agonists on dendritic spine stability. Phosphorylation blockage made etorphine, fentanyl, and DAMGO function as morphine in the primary cultures. Therefore, agonist-dependent phosphorylation of GPCR regulates the activation of the PKC pathway and the subsequent responses.

Agonist-dependent or agonist-biased signaling is a new concept in understanding the signaling of the G protein-coupled receptor (GPCR)[4] (1). It suggests that GPCR agonists activate the downstream signaling pathways in a selective manner. In agonist-biased signaling, some agonists activate one set of pathways and other agonists activate a different set of pathways. A well studied example is GPCR-mediated ERK phosphorylation (2, 3). Generally, two distinct pathways, the PKC and β-arrestin pathways, mediate ERK phosphorylation in the GPCR system. Although both pathways can be observed with nearly all GPCRs, agonists do have preference. Some agonists can use only the PKC pathway to induce ERK phosphorylation, whereas some other agonists can use only the β-arrestin pathway (3). ERK phosphorylated via the PKC pathway stays in the cytosol and activates p90 ribosomal S6 kinase, whereas ERK phosphorylated via the β-arrestin pathway translocates into the nucleus and activates Elk1 (4, 5).

The binding of an agonist to a GPCR leads to receptor phosphorylation, which subsequently increases the affinity of the agonist-receptor complex for the cytosolic protein β-arrestin. Translocation of β-arrestin to the receptor complex disrupts receptor-G protein coupling, ceases G protein-mediated signaling, and initiates β-arrestin-mediated signaling (6). Because the activation of the PKC pathway requires G protein activation, it has been suggested that the affinity of the agonist-receptor complex for β-arrestin determines the selection between the PKC and β-arrestin pathways (7). However, this interpretation cannot explain why agonists that normally use the β-arrestin pathway do not use the PKC pathway when β-arrestin is removed (4, 7, 8). Thus we propose that receptor phosphorylation, rather than increased affinity for β-arrestin after phosphorylation, determines the selection between the PKC and β-arrestin pathways.

To prove this hypothesis, the μ-opioid receptor (OPRM1), of which agonist-dependent signaling has been reported, was used as a model. Unlike agonists such as etorphine, fentanyl, and [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), morphine has lower abilities to induce receptor phosphorylation, β-arrestin2 recruitment, and receptor internalization (9–11). In addition, morphine uses the PKC pathway to induce ERK phosphorylation, whereas etorphine, fentanyl, and DAMGO utilize the β-arrestin pathway (4). Agonist-dependent signaling was also observed with the desensitization of intracellular calcium ($[Ca^{2+}]_i$) release: morphine via the PKC pathway and the other three agonists via the β-arrestin pathway (8, 12).

* This work was supported, in whole or in part, by National Institutes of Health Grants DA007339, DA016674, DA000564, and DA011806.

[1] To whom correspondence should be addressed: 6-120 Jackson Hall, 321 Church St. S. E., Minneapolis, MN 55455-0217. Tel.: 6126266539; Fax: 6126258408; E-mail: zhenhui2055@hotmail.com.

[2] Recipient of NIH Grant K05-DA70544.

[3] Recipient of NIH Grant K05-DA00513.

[4] The abbreviations used are: GPCR, G protein-coupled receptors; $[Ca^{2+}]_i$, intracellular calcium; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; HEK3A, HEK cells stably expressing a phosphorylation-deficient mutant of OPRM1; HEKOPRM1, HEK cells stably expressing the HA-tagged wild-type OPRM1; IL, intracellular loop; MEF, mouse embryonic fibroblast; miR-190, microRNA-190; OPRM1, μ-opioid receptor.

In addition, chronic morphine treatment decreases dendritic spine density in primary hippocampal cultures (13). Agonists that initiate high receptor phosphorylation, such as etorphine, fentanyl, and DAMGO, do not decrease spine density (14, 15). Because β-arrestin and ERK mediate this difference (16–18), whether receptor phosphorylation also contributes to these differential responses was tested.

Hence, HEK cells stably expressing HA-tagged wild type OPRM1 (HEKOPRM1) and the phosphorylation-deficient OPRM1 mutant (3A, which has the Ser^{363}, Thr^{370}, and Ser^{375} residues mutated to Ala) (HEK3A) were used to determine the contribution of receptor phosphorylation to the selection between the PKC and β-arrestin pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—HEKOPRM1, HEK3A, and mouse embryonic fibroblast cells (MEF, wild type, and β-arrestin2$^{-/-}$ from Dr. Lefkowitz, Duke University) were cultured in MEM supplied with 10% FBS and 200 ng/ml G418. Primary cultures of hippocampus neurons were generated from wild-type C57BL/J6 mice (Charles River Laboratories, Portage, MI) and β-arrestin2$^{-/-}$ mice (C57B/J6 background, from Dr. Lefkowitz's Laboratory) as described previously (13). Experiments started 21 days after plating.

PKC subtype-specific inhibitors were ordered from Biomatik Corporation (Cambridge, Ontario, Canada): PKCαi (Myr-FARKGALRQ-OH), PKCγi (Myr-EAVSLKPT-OH), and PKCεi (Myr-CRLVLASC-OH). The peptides were myristoylated on the N-terminal for penetration across the cell membrane. Adenoviruses were titrated precisely to reach a receptor level of ~0.5 pmol/mg protein in MEF cells and primary cultures. The antibodies against phosphorylated ERK, total ERK, phosphorylated Ser^{375} of OPRM1, and β-actin were purchased from Cell Signaling (Danvers, MA). PKC subtype antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against phosphorylated amino acids was from Sigma. The antibody against HA was from Covance Research Products (Emeryville, CA). The AP-conjugated secondary antibodies were from Bio-Rad. Gαi2 antibodies were generated as described previously (19).

Intracellular Calcium Measurement—Intracellular calcium was measured using the FLIPR® calcium assay kit (Molecular Devices Corp.) as described previously (8). Briefly, FLEXstation (Molecular Devices Corp.) was used to monitor intracellular calcium levels. The $[Ca^{2+}]_i$ release in 10 to 12 wells were averaged. For desensitization, the potentiation of ADP-induced $[Ca^{2+}]_i$ released after agonist pretreatment was normalized to that without pretreatment. The percentage decrease in potentiation intensity reflected the percentage of desensitization of OPRM1.

PKC Subtype Activity Assay—Activity of the PKC subtypes was determined by using the PKC activity assay kit from Cell Signaling as described previously (12). PKCε, PKRα, or PKCγ was immunoprecipitated from the supernatant from total cell lysate by subtype-specific antibodies and protein G-agarose beads. Reaction solution (Cell Signaling) containing biotin-linked PKC substrate was added to the beads. Reaction lasted 15 min at 37 °C with rotation, and was stopped by adding an equal volume of EDTA (50 mM, pH 8.0). Then the substrates in supernatant were precipitated with 30 μl of streptavidin-linked beads. The phosphorylated substrates were identified by specific antibodies and Alexa 488-conjugated goat-anti-rabbit antibody (Invitrogen). The PKC subtype activity was determined by measuring the fluorescence intensity using a α-Fusion plate reader (PerkinElmer Life Sciences, Boston, MA).

Lipid Raft Separation and Continuous Sucrose Gradient—Continuous sucrose gradient was used to separate the microdomains on the cell membrane according to their densities as described previously (20). Briefly, the samples were placed at the bottom of a 5–30% continuous sucrose gradient, which was centrifuged at 32,000 rpm for 16 h in a SW41 rotor at 4 °C. Twelve fractions (1 ml volume each) were further concentrated to 100 μl with trichloroacetic acid precipitation and analyzed with immunoblotting.

Immunoblotting and Immunoprecipitation—ERK phosphorylation was determined by immunoblotting (4). Immunoblotting and immunoprecipitation were performed as reported previously (18). Immunofluorescence was performed with a BD CARV II™ Confocal Imager (including Leica DMIRE2 fluorescence microscope), Hamamatsu EM-CCD C9100 camera, and IPlab 4.0 (BD Biosciences, San Jose, CA) (16).

Statistical Analysis—Experiments were repeated at least four times, and at least eight individual cells were used for images analyses. The results were analyzed by using the two-tailed Student's *t* test and ANOVA test, except for data depicted in Figs. 1, 2, *D* and *E*, and 7, which were analyzed by the one-way ANOVA test with the Dunnett-test as a post-hoc test for comparisons. The error bars present the standard derivations and * indicates a significant difference between the marked result and the basal or control result (indicated in the legends of the *x*-axis).

RESULTS

Receptor Phosphorylation Attenuates PKCε Activation—To determine the roles played by receptor phosphorylation in activating the PKC pathway, two cell lines (HEKOPRM1 and HEK3A) and four agonists (morphine, etorphine, fentanyl, and DAMGO) were used. HEK3A cells express a phosphorylation-deficient mutant of OPRM1 with S363A, T370A, and S375A mutations. For consistency, "OPRM1" will be used for wild-type OPRM1, "3A" will be used for the phosphorylation-deficient mutant, and "receptor" will indicate both the wild-type and mutants in this discussion. HEKOPRM1 and HEK3A cells have similar receptor expression levels: 6.3 ± 0.4 and 5.8 ± 0.2 pmol/mg protein. In addition, there is no significant difference between the binding affinities of the agonists for OPRM1 and 3A (21).

Morphine induces maximum ERK phosphorylation and adenylyl cyclase inhibition at 1 μM (20). Considering the relative affinity of the four agonists for OPRM1 and their abilities to induce maximum ERK phosphorylation and adenylyl cyclase inhibition (4, 22), DAMGO was used at 1 μM, whereas etorphine and fentanyl were used at 10 nM, to achieved equivalent concentrations. At these concentrations, the agonists induce ERK phosphorylation and adenylyl cyclase inhibition to similar levels.

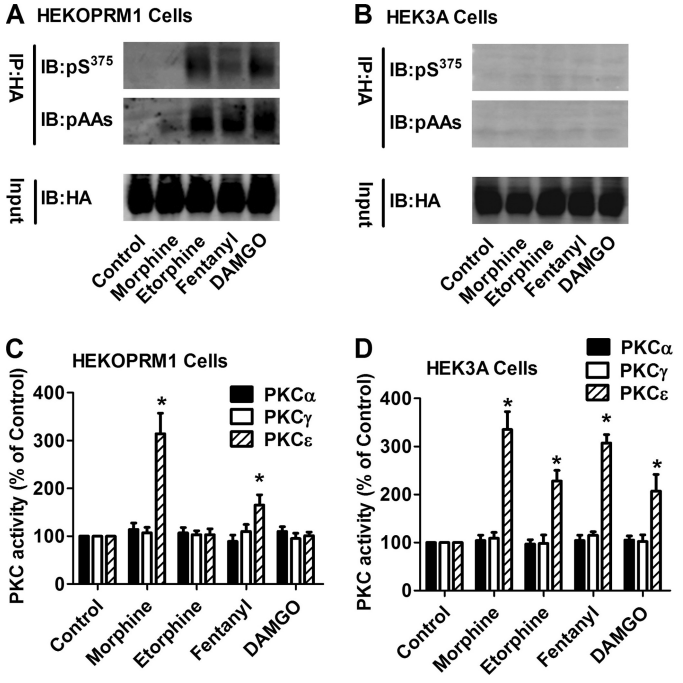

FIGURE 1. **Receptor phosphorylation attenuates PKCε activation.** HEKOPRM1 (*A* and *C*) and HEK3A cells (*B* and *D*) were treated with PBS (Control), 1 μM morphine, 10 nM etorphine, 10 nM fentanyl and 1 μM DAMGO for 5 min. *A* and *B*, receptors were immunoprecipitated with HA antibody. The phosphorylated Ser^{375} on OPRM1 (pS^{375}) and phosphorylated amino acid (*pAAs*) were determined in the immunoprecipitated receptor. *C* and *D*, activities of three PKC subtypes were determined as described under "Experimental Procedures." The results were normalized against that in the control in each group.

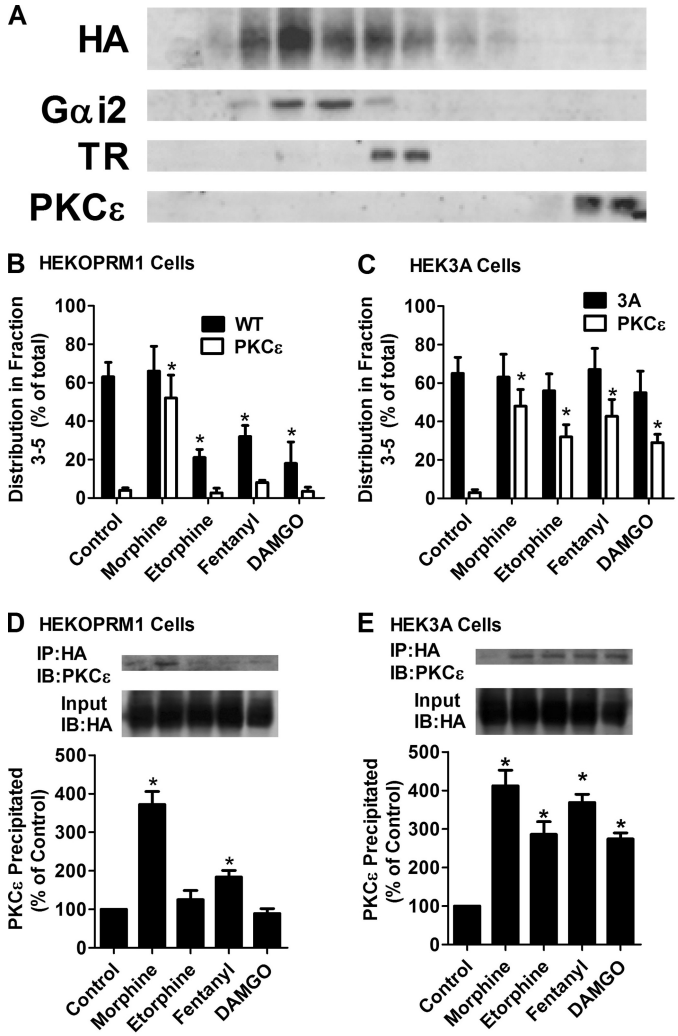

FIGURE 2. **Receptor phosphorylation attenuates the recruitment of PKCε.** *A*, distributions of OPRM1, Gαi2, transferrin receptor (TR), and PKCε in continuous sucrose gradient in untreated HEKOPRM1 cells. *B–E*, HEKOPRM1 (*A* and *C*) and HEK3A cells (*B* and *D*) were treated as in Fig. 1. *B* and *C*, cells were subjected to continuous sucrose gradient and immunoblotting as described. The amounts of receptor and PKCε from fraction 3 to fraction 5 were normalized to the total amounts (from fraction 1 to fraction 12). *C* and *D*, receptors were immunoprecipitated with HA antibody. The amounts of PKCε co-immunoprecipitated with receptor were determined. The results were normalized against that in the control.

Because the phosphorylation sites of OPRM1 have been demonstrated to be the Ser^{363}, Thr^{370}, and Ser^{375} residues of the C terminus (21), antibodies against phosphorylated Ser^{375} (pS^{375}) and phosphorylated amino acids (pAAs) were used to monitor receptor phosphorylation. In HEKOPRM1 cells, all the agonists except for morphine induced significant receptor phosphorylation as detected with the pS^{375} and pAAs antibodies (Fig. 1*A*). Fentanyl-induced Ser^{375} phosphorylation was lower than that induced by etorphine or DAMGO. In HEK3A cells, none of the four agonists induced receptor phosphorylation (Fig. 1*B*), confirming the ability of 3A to serve as a phosphorylation-deficient mutant.

PKC is a large kinase family and is divided into three subfamilies, conventional, atypical, and novel, depending on their activation mechanisms (23). PKCα, PKCγ, and PKCε contribute to morphine-induced tolerance (24). Thus, the activities of PKCα, PKCγ, and PKCε were determined after agonist treatment. Morphine induced PKCε activation (314 ± 43% of control, n = 4) in HEKOPRM1 cells. Fentanyl induced a much lower activation of PKCε (158 ± 21%, n = 4). Neither etorphine (103 ± 12%, n = 4) nor DAMGO (101 ± 8%, n = 4) induced activation of PKCε (Fig. 1*C*). In the HEK3A cells, all four agonists activated PKCε (Fig. 1*D*). Etorphine and DAMGO induced lower activation of PKCε (228 ± 22% and 207 ± 35% of control, n = 4) than did morphine and fentanyl (335 ± 37% and 307 ± 18%, respectively, n = 4).

In HEKOPRM1 cells, the intensity of agonist-induced PKCε activation was such that morphine was greater than fentanyl, which was greater than etorphine or DAMGO. Intensity of receptor phosphorylation, on the other hand, was such that etorphine and DAMGO were greater than fentanyl, which was greater than morphine. In HEK3A cells, all four agonists activated PKCε but did not induce receptor phosphorylation. Thus, the ability of the agonists to activate PKCε inversely correlates with their ability to induce receptor phosphorylation. Therefore, it is reasonable to suggest that receptor phosphorylation attenuates PKCε activation.

Receptor Phosphorylation Attenuates the Recruitment of PKCε—After being activated, PKCs translocate from cytosol to the cell membrane (23). Therefore, we measured the translocation of PKCε to the cell membrane to confirm the influence of receptor phosphorylation on PKCε activation. Continuous sucrose gradient was used to separate the microdomains on the cell membrane as described previously (20). The distribution of lipid raft microdomains was marked by Gαi2 (25) and that of

non-raft microdomains was marked by transferrin receptor (26). The amounts of Gαi2, transferrin receptor, and PKCε peaked at fraction 4, 6, and 12, respectively (Fig. 2*A*). The percentage amounts of target proteins in fractions 3–5 were used to indicate their distribution in the lipid raft microdomains.

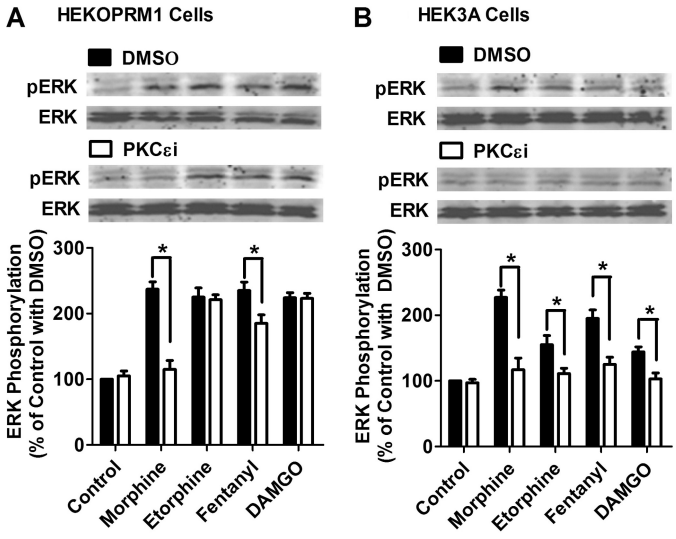

FIGURE 3. **Phosphorylation blockage enables agonists to use PKCε for ERK phosphorylation.** Cells were treated with DMSO or 50 μM PKCε-specific inhibitor (PKCεi) for 3 h. HEKOPRM1 (*A*) and HEK3A cells (*B*) were then treated with agonists as in Fig. 1. ERK phosphorylation was determined by normalizing the immunoreactivities of phosphorylated ERK against the immunoreactivities of total ERK. The results were further normalized against that in the control with DMSO.

In the absence of an agonist, the majority of the receptor was in the lipid raft microdomains in both HEKOPRM1 and HEK3A cells (Fig. 2, *B* and *C*). PKCε was enriched in fractions 11 and 12, suggesting the cytosolic location of PKCε under the control condition. In HEKOPRM1 cells, morphine induced translocation of PKCε from the cytosol to the lipid raft microdomains (Fig. 2*B*). Fentanyl also induced translocation, but the percentage of PKCε translocated to fractions 3–5 totalled only 8.1 ± 1% ($n = 4$), which is much less than that (52 ± 12%, $n = 4$) induced by morphine. Etorphine and DAMGO did not affect the location of PKCε (2.7 ± 2% ($n = 4$) and 3.4 ± 2% ($n = 4$), respectively). In HEK3A cells, all four agonists induced translocation of PKCε: morphine (48 ± 9%, $n = 4$), etorphine (32 ± 6%, $n = 4$), fentanyl (43 ± 9%, $n = 4$), and DAMGO (29 ± 4%, $n = 4$) (Fig. 2*C*).

Because PKCε translocated to the lipid raft microdomains after receptor activation, the interaction between the receptor signaling complex and PKCε was monitored by immunoprecipitation. In HEKOPRM1 cells, the interaction between the OPRM1 complex and PKCε was identified only after morphine or fentanyl treatment. The morphine-OPRM1 complex precipitated more PKCε (372 ± 34% of control, $n = 4$) than did the fentanyl-OPRM1 receptor complex (184 ± 17% of control, $n = 4$) (Fig. 2*D*). However, all four agonists induced significant

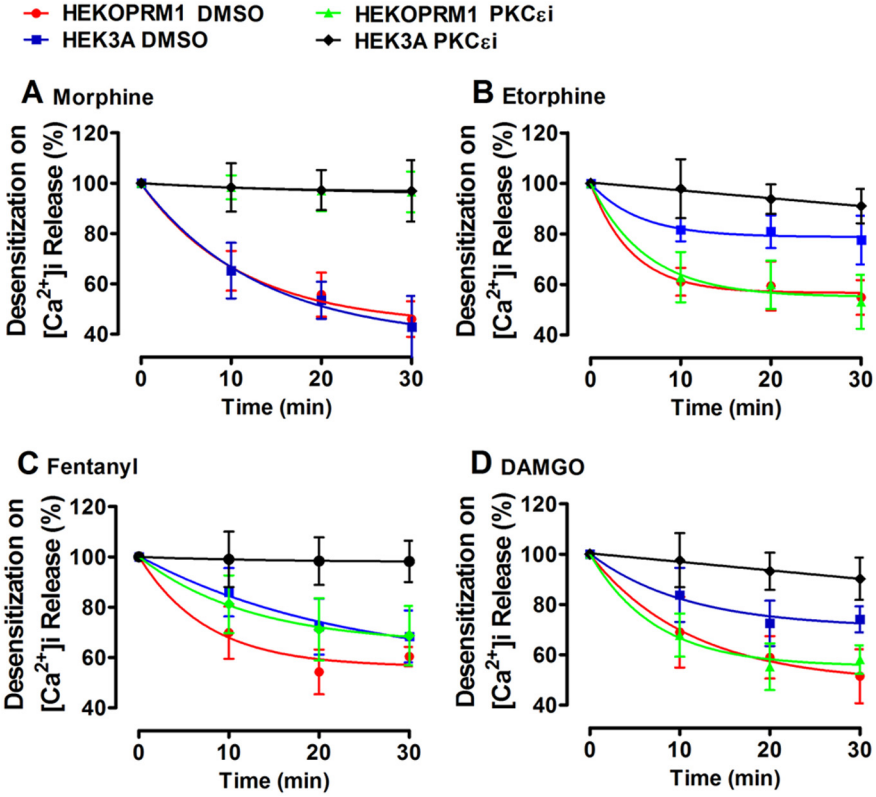

FIGURE 4. **Phosphorylation blockage enables agonists to use PKCε for receptor desensitization.** HEKOPRM1 and HEK3A were treated with DMSO or 50 μM PKCεi for 3 h and were then pretreated with 100 nM morphine (*A*), 1 nM etorphine (*B*), 1 nM fentanyl (*C*), and 100 nM DAMGO (*D*) for the times indicated on the *x* axis. ADP was added after agonist pretreatment. The percentage decrease in the agonist-induced potentiation on the ADP-induced $[Ca^{2+}]_i$ release was used to indicate the receptor desensitization. The decreases were calculated by using the formula: 100% − potentiation on ADP-induced $[Ca^{2+}]_i$ release with pretreatment/potentiation on ADP-induced $[Ca^{2+}]_i$ release without pretreatment.

recruitment of PKCε to the receptor signaling complex in HEK3A cells (Fig. 2*E*).

Blocking Receptor Phosphorylation Switches Agonists from the β-Arrestin2 Pathway to the PKCε Pathway—Morphine-induced ERK phosphorylation and receptor desensitization of $[Ca^{2+}]_i$ release require PKCε activation, whereas etorphine and DAMGO use the β-arrestin2 pathway to initiate these two signaling events (4, 12). Because etorphine and DAMGO activated and recruited PKCε in HEK3A cells, whether phosphorylation blockage could switch these agonists from the β-arrestin2 pathway to the PKCε pathway was investigated. To distinguish the signaling mediated by PKCε from that mediated by β-arrestin2, a PKCε-specific inhibitor (PKCεi) was used.

As predicted, PKCεi attenuated ERK phosphorylation induced by morphine in HEKOPRM1 cells, from 237 ± 11% to 115 ± 14% ($n = 4$) of basal level (Fig. 3*A*). In addition, PKCεi slightly attenuated fentanyl-induced ERK phosphorylation, but did not affect that induced by either etorphine or DAMGO. In HEK3A cells, all four agonists induced ERK phosphorylation (Fig. 3*B*). Because of the low affinity of 3A for β-arrestin2, another pathway is likely involved that mediates ERK phosphorylation, presumably the PKCε pathway. PKCεi reduced the ability of all four agonists to induce ERK phosphorylation in HEK3A cells: morphine from 227 ± 11% to 117 ± 18%; etorphine from 155 ± 14% to 111 ± 8%; fentanyl from 195 ± 13% to 125 ± 11%; DAMGO from 144 ± 8% to 103 ± 9% ($n = 4$) (Fig. 3*B*).

Receptor desensitization of $[Ca^{2+}]_i$ release also was monitored after agonist treatment to further establish the relationship between receptor phosphorylation and PKCε-related signaling. Because the concentrations of agonists used above induce desensitization quickly, morphine and DAMGO were used at 100 nM, and the other two agonists at 1 nM. PKCεi was used to distinguish PKCε-mediated desensitization from β-arrestin2-mediated desensitization. Morphine-induced receptor desensitization was PKCε-mediated in both cell lines (Fig. 4*A*). Etorphine and DAMGO induced less desensitization in HEK3A cells than in HEKOPRM1 cells. Desensitization induced by etorphine and DAMGO in HEKOPRM1 cells was not mediated by PKCε, because PKCεi did not affect the desensitization. However, receptor desensitization induced by etorphine and DAMGO in HEK3A cells was PKCε-mediated (Fig. 4, *B* and *D*). Fentanyl functioned as a combination of morphine and etorphine, although more similarly to etorphine (Fig. 4*C*). Thus, blocking receptor phosphorylation enables etorphine and DAMGO to use the PKCε pathway, although not as efficiently as morphine.

Attenuation of PKCε Activation Is Independent of the Increased Affinity of the Receptor Complex for β-Arrestin2—Receptor phosphorylation increases the affinity of an agonist-receptor complex for β-arrestin2 (6). However, this increased affinity of an agonist-receptor complex for β-arrestin2 is not the reason why receptor phosphorylation prevents PKCε activation. MEF cells from wild-type (WTMEF) and β-arrestin2$^{-/-}$ (BKOMEF) mice were used. The adenoviruses encoding the wild type OPRM1 (AdOPRM1) and 3A mutant (Ad3A) were used to express the receptor in the MEF cells. In order to compare the results from these two types of MEF cells, the amounts of adenoviruses were titrated precisely to express the receptor at 0.5 pmol/mg. The results obtained in the WTMEF cells infected with AdOPRM1 and Ad3A were similar to those observed in HEKOPRM1 and HEK3A cells. The blockage of receptor phosphorylation increased the ability of etorphine, fentanyl, and DAMGO to use the PKCε-pathway, although less efficiently than morphine (Table 1).

In the BKOMEF infected with AdOPRM1, etorphine and DAMGO did not induce ERK phosphorylation or receptor desensitization, indicating they did not activate PKCε pathway (Table 1). Receptor phosphorylation therefore prevents agonists from using the PKCε pathway directly rather than by increasing the affinity of agonist-receptor for β-arrestin2. In

TABLE 1

Receptor phosphorylation but not β-arrestin2 contributes to PKCε activation

WTMEF and BKOMEF cells were used. The AdOPRM1 and Ad3A were used to express receptor to about 0.5 pmol/mg protein. For ERK activation, cells were treated with 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, and 1 μM DAMGO for 5 min. The amounts of phosphorylated ERK were normalized against those of total ERK. The results were further normalized against those under control condition. For receptor desensitization, cells were treated with 100 nM morphine, 1 nM etorphine, 1 nM fentanyl, and 100 nM DAMGO for 30 min. Normally ADP, but not the agonists, can induce $[Ca^{2+}]_i$ release. However, the agonists can potentiate the $[Ca^{2+}]_i$ release induced by ADP. Thus the abilities of agonists to induce this potentiation were used to indicate their abilities to activate receptor, and the percentage decrease in the potentiation was used to indicate the desensitization. The "% inh." was calculated by using the formula: 100% - result in DMSO-treated cells/result in PKCεi-treated cells. N/S suggests the effect of PKCεi was not significant. N/A suggests the agonist did not induce signaling in DMSO group, therefore no decrease can be calculated.

ERK phosphorylation (% of basal level)				
	WTMEF cells		BKOMEF cells	
	AdOPRM1	Ad3A	AdOPRM1	Ad3A
Morphine				
DMSO	178 ± 13	183 ± 7	176 ± 12	173 ± 8
PKCεi	111 ± 7	97 ± 15	105 ± 9	103 ± 11
% Inh.	86	104	93	96
Etorphine				
DMSO	183 ± 12	154 ± 13	113 ± 14	181 ± 16
PKCεi	175 ± 14	116 ± 8	105 ± 12	118 ± 12
% Inh.	N/S	70	N/A	78
Fentanyl				
DMSO	174 ± 8	184 ± 11	138 ± 10	174 ± 9
PKCεi	185 ± 11	108 ± 13	123 ± 17	104 ± 7
% Inh.	N/S	90	N/A	95
DAMGO				
DMSO	181 ± 10	153 ± 8	111 ± 7	183 ± 7
PKCεi	174 ± 8	119 ± 6	109 ± 11	106 ± 11
% Inh.	N/S	64	N/A	93
Receptor Desensitization on $[Ca^{2+}]_i$ (% decrease in basal activity)				
	WTMEF cells		BKOMEF cells	
	AdOPRM1	Ad3A	AdOPRM1	Ad3A
Morphine				
DMSO	36 ± 12	43 ± 7	43 ± 7	38 ± 11
PKCεi	6 ± 5	3 ± 4	2 ± 6	2 ± 7
% Inh.	83	93	95	95
Etorphine				
DMSO	49 ± 7	26 ± 6	1 ± 7	45 ± 6
PKCεi	42 ± 12	9 ± 7	9 ± 10	7 ± 3
% Inh.	N/S	65	N/A	84
Fentanyl				
DMSO	45 ± 6	33 ± 12	17 ± 11	44 ± 5
PKCεi	34 ± 10	11 ± 6	2 ± 5	5 ± 8
% Inh.	N/S	67	N/A	89
DAMGO				
DMSO	37 ± 11	29 ± 12	6 ± 3	41 ± 14
PKCεi	41 ± 9	9 ± 8	5 ± 9	8 ± 10
% Inh.	N/S	69	N/A	80

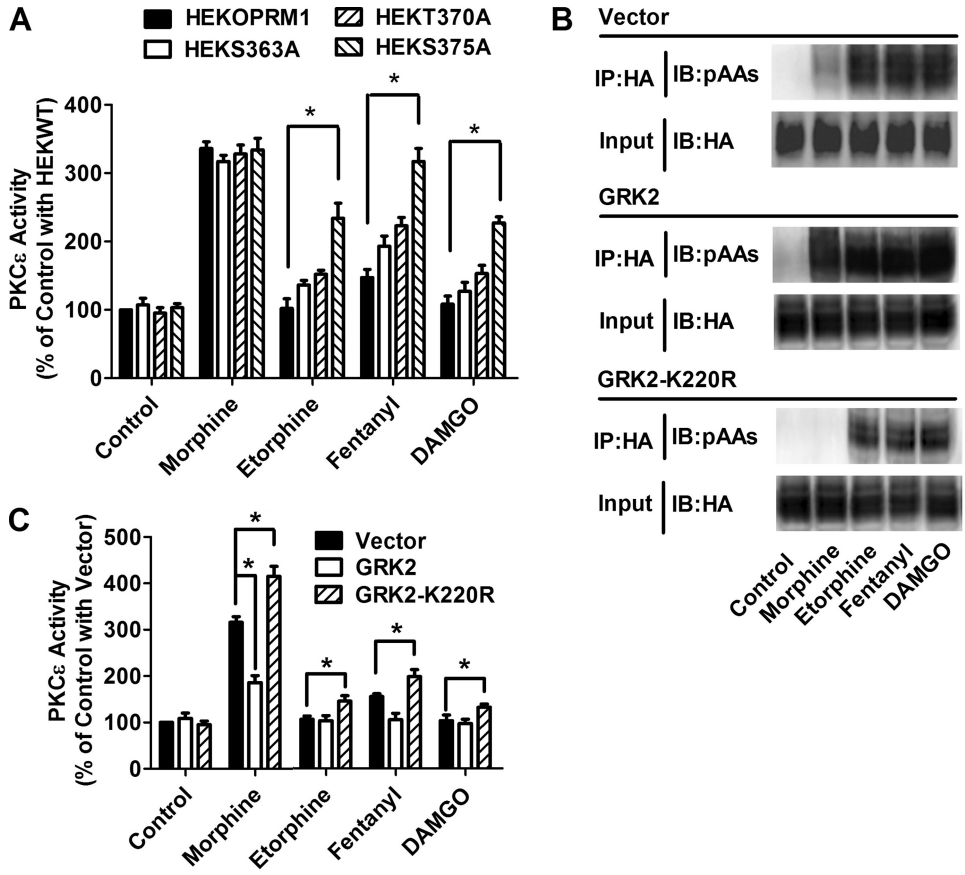

FIGURE 5. **GRK2 phosphorylation on Ser^{375} attenuates PKCε activation.** *A*, HEKOPRM1, HEKS363A, HEKT370A, and HEKS375A cells were treated with PBS (Control), 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, and 1 μM DAMGO for 5min. The activity of PKCε was determined. *B* and *C*, HEKOPRM1 was transfected with a vector, GRK2, and GRK2-K220R. One day after transfection, cells were treated with agonists as in *A*. Receptor phosphorylation on Ser^{375} was determined in *B*. PKCε activities were normalized against that in "Control with Vector" in *C*.

addition, etorphine and DAMGO activated the PKCε pathway in BKOMEF infected with Ad3A as efficiently as morphine. Considering that etorphine and DAMGO activate the PKCε pathway less efficiently than morphine in WTMEF infected with Ad3A, the existence of β-arrestin2 limited the activation of PKCε pathway, presumably through the basal affinity of β-arrestin2 for the non-phosphorylated receptor.

Phosphorylation on Ser^{375} Is the Major Inhibitor of PKCε Activation—Three residues were mutated in the 3A to prevent receptor phosphorylation. Because Ser^{375} has been suggested to be the major site for agonist-induced receptor phosphorylation (8, 21), the contribution of phosphorylation on these three residues to PKCε activation was investigated. Ser^{363}, Thr^{370}, and Ser^{375} were mutated to Ala individually and stably expressed in HEK293 cells. The activity of PKCε was determined in these cells lines after agonist treatment.

Etorphine and DAMGO induced PKCε activation in HEKS363A and HEKT370A cells, but the activation was much less than in HEKS375A cells. Etorphine activated PKCε to 102 ± 14% in HEKOPRM1 cells, while to 136 ± 7%, 152 ± 7%, and 234 ± 22% of basal level in HEKS363A, HEKT370A, and HEKS375A cells, respectively (Fig. 5*A*). DAMGO activated PKCε to 108 ± 12%, 127 ± 13%, 153 ± 12%, and 227 ± 9% of basal level in HEKOPRM1, HEKS363A, HEKT370A, and HEKS375A cells, respectively. In addition, fentanyl-induced PKCε activation was potentiated much more in HEKS375A than in HEKS363A or HEKT370A cells.

Ser^{375} in OPRM1 has been suggested to be the phosphorylation site of G protein-coupled receptor kinase 2 (GRK2) (8, 21), thus wild-type GRK2 and a dominant-negative mutant of GRK2 (GRK2-K220R) were overexpressed in HEKOPRM1 cells to modulate the ability of the agonists to induce receptor phosphorylation. As indicated in Fig. 5*B*, wild-type GRK2 overexpression increased the ability of all four agonists to induce receptor phosphorylation, whereas GRK2-K220R overexpression resulted in the opposite effect. The activities of PKCε were measured after overexpression. Morphine-induced activation of PKCε was potentiated by GRK2-K220R, but impaired by GRK2 (Fig. 5*C*). Although GRK2 overexpression could not reduced PKCε activity to below basal level, GRK2-K220R overexpression did increase the ability of etorphine, fentanyl, and DAMGO to activate PKCε. Because Ser^{375} is the major functional site, an adenovirus expressing a S375A mutant of OPRM1 (AdS375A) was used in further studies.

S375A Mutation Switches Agonists to the PKCε Pathway in Primary Cultures—HEK cells have different characteristics from neuronal cells in which OPRM1 is expressed endogenously. Hence, primary cultures of mouse hippocampal neurons were used to confirm the ability of receptor phosphorylation to impair the activation of the PKCε pathway. As predicted,

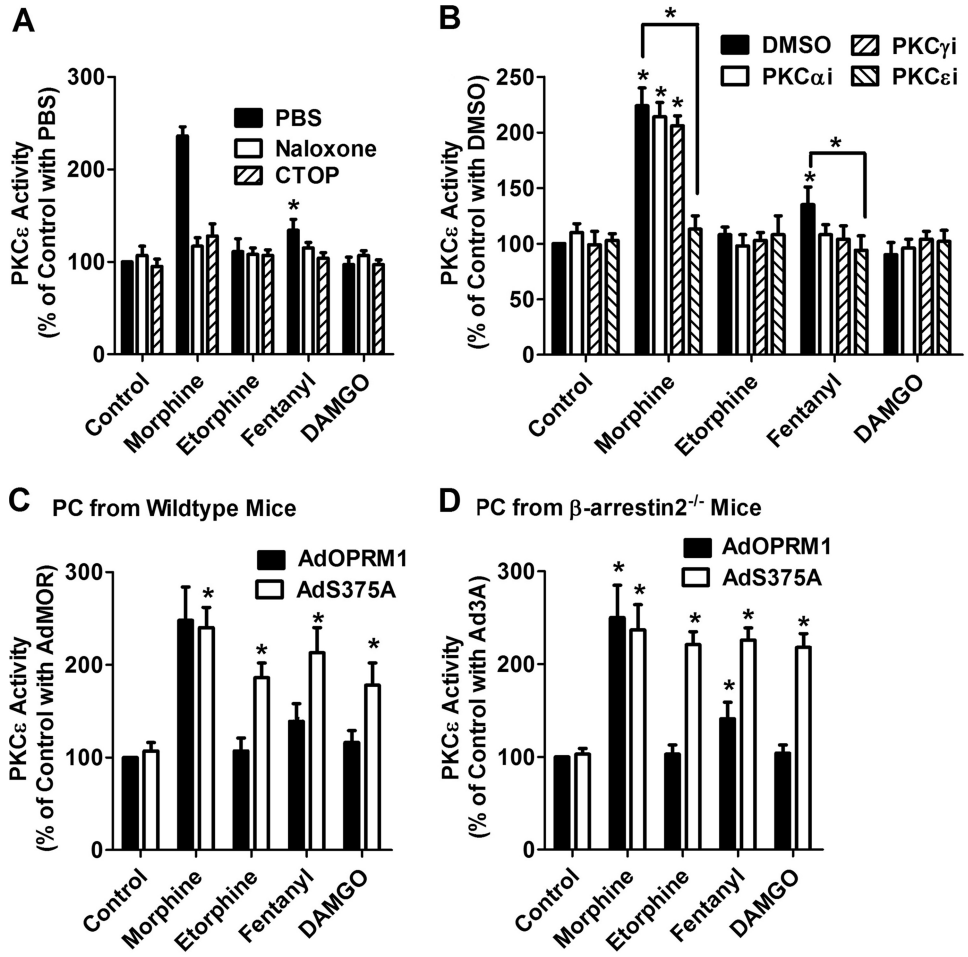

FIGURE 6. **Receptor phosphorylation attenuates PKCε activation in primary cultures.** *A* and *B*, primary cultures of hippocampal neurons from wild-type mice were pretreated with PBS, 10 μM naloxone, or 10 μM CTOP for 10 min (*A*). The cultures were pretreated with DMSO or 50 μM PKC subtype-specific inhibitors (PKCαi, PKCγI, and PKCεi) for 3 h (*B*). Then the primary cultures were incubated with 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, or 1 μM DAMGO for 5 min, and the activities of PKC subtypes were determined as described under "Experimental Procedures." The results were normalized against that of the control with PBS (*A*) and the control with DMSO (*B*). *C*, primary hippocampal neurons from wild-type mice were infected with AdOPRM1 or AdS375A for 3 days. Then the primary cultures were incubated with 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, or 1 μM DAMGO for 5 min, and the activity of PKCε was determined. The results were normalized against that of the control with AdOPRM1. *D*, primary hippocampal neurons from βarrestin2$^{-/-}$ mice were prepared as in *C*, and PKCε activity was determined.

morphine, but not etorphine or DAMGO, activated PKCε in the primary cultures, whereas fentanyl activated PKCε to a lower level than morphine (Fig. 6*A*). When the primary cultures were pretreated with the general opioid receptor antagonist naloxone and the OPRM1 specific antagonist Cys^2-Tyr^3-Orn^5-Pen^7-amide, PKCε activation was attenuated, indicating that agonist-induced PKCε activation is through OPRM1. In addition, pretreatment with PKC subtype-specific inhibitors demonstrated that PKCε, but not PKCα or PKCγ, is activated by OPRM1 in the primary cultures (Fig. 6*B*).

Hippocampal primary cultures from wild type mice and β-arrestin2$^{-/-}$ mice were prepared as described under "Experimental Procedures." AdOPRM1 and AdS375A were used to express exogenous receptors and to determine the effect of phosphorylation blockage on PKCε activation. In both primary cultures, phosphorylation blockage increased the ability of etorphine, DAMGO, and fentanyl to activate PKCε (Fig. 6, *C* and *D*). Therefore, receptor phosphorylation also directly prevented the agonists from using the PKCε pathway in primary cultures.

The adenoviruses generated a receptor expression level at about 0.45 pmol/mg protein, which is much higher than the endogenous OPRM1 level (about 0.048 pmol/mg protein) (Fig. 7*A*). Infection efficiency is indicated in Fig. 7, *B* and *C*, and the percentage of the infected primary cultures (HA-positive) in DAPI-positive cells was close to 90%. Therefore, after adenovirus infection, the primary cultures express much more exogenous receptor than endogenous OPRM1, resulting in for the ability to determine agonist-dependent signaling in primary cultures.

S375A Mutation Reversed the Effects of Agonists on Dendritic Spine Stability—It has been reported that agonist-dependent activation of ERK resulted in differential regulation of miR-190, NeuroD, and dendritic spines stability (16–18). Primary cultures from wild-type mice were infected with AdOPRM1 or AdS375A for 3 days, and the dendritic spine stability was monitored during the next 3 days with agonist incubation. In addition, the level of miR-190 and NeuroD mRNA was quantified in the infected primary cultures.

In AdOPRM1-infected primary cultures, etorphine, fentanyl, and DAMGO induced decreases in miR-190 expression (54 ±

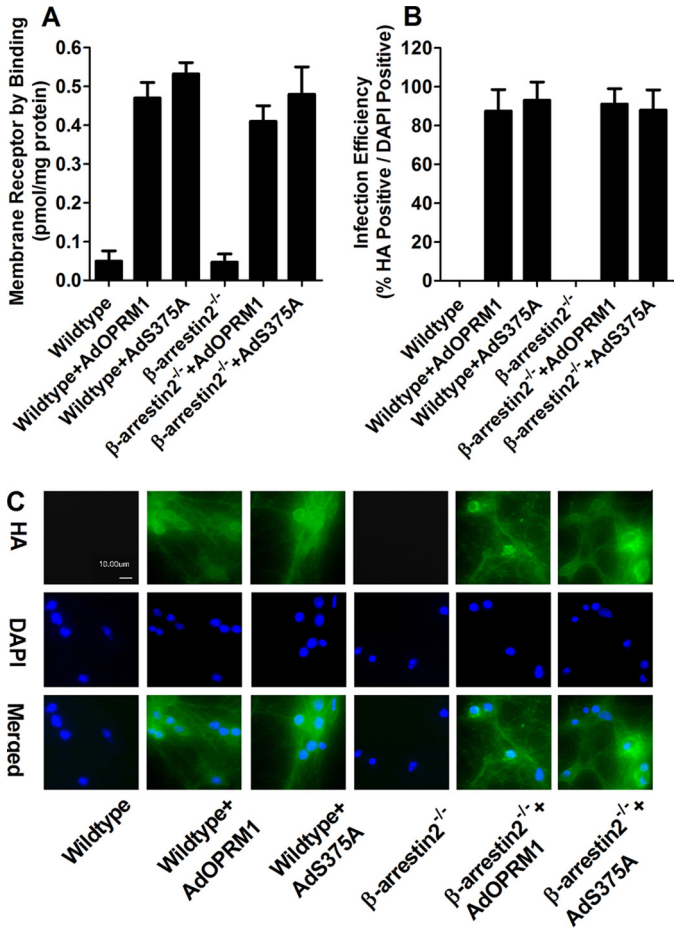

FIGURE 7. **High efficiency of virus infection leads to high receptor expression.** Primary cultures of hippocampal neurons from wild-type or β-arrestin2$^{-/-}$ mice were infected with AdOPRM1 or AdS375A for 3 days. The receptor expression was determined with binding assay after infection (*A*). The efficiency of the infection was indicated by the percentage of HA-positive (*green* in *C*) cells in DAPI-positive (*blue* in *C*) cells (*B* and *C*).

16%, 61 ± 12%, and 51 ± 9%, respectively, $n = 4$) (Fig. 8*A*). However, in AdS375A-infected primary cultures, the effects of these three agonists on miR-190 were attenuated. In addition, etorphine, fentanyl, and DAMGO increased NeuroD mRNA level in AdOPRM1-infected, but not in AdS375A-infected, primary cultures.

In AdOPRM1-infected primary cultures, similar observations were obtained as reported previously. A three-day morphine treatment decreased the volume of dendritic spines by 40 ± 12%, where as treatments with etorphine, fentanyl, and DAMGO did not decrease spine volume (Fig. 8, *C* and *D*).

When used to treat the AdS375A-infected primary cultures, all four agonists significantly decreased spine volume during the three-day treatments. Morphine, etorphine, fentanyl, and DAMGO decreased spine volume by 47 ± 12%, 24 ± 7%, 23 ± 11%, and 27 ± 9% from that on Day 0, respectively (Fig. 8, *C* and *D*). Thus, phosphorylation blockage can regulate the effects of agonists, such as etorphine, fentanyl, and DAMGO, on dendritic spine stability.

DISCUSSION

Four agonists (morphine, fentanyl, etorphine, and DAMGO) and two signaling events (ERK phosphorylation and desensitization) were examined in the current studies. In summary, three types of agonists were represented. Morphine activated the PKCϵ pathway and used only the PKCϵ pathway for ERK phosphorylation. Etorphine and DMAGO did not activate the PKCϵ pathway and used only the β-arrestin2 pathway for ERK phosphorylation. Fentanyl acted more like etorphine and DAMGO, but it could activate the PKCϵ pathway. Thus it represented agonists that can use both pathways for ERK phosphorylation.

Etorphine and DAMGO used the β-arrestin2 pathway for signaling in the presence of both receptor phosphorylation and β-arrestin2. If β-arrestin2 was removed from the system, no activation of ERK was observed. In contrast, if receptor phosphorylation was blocked, the two agonists utilize the PKCϵ pathway for signaling. Thus, receptor phosphorylation, rather than the increased affinity of the receptor complex for β-arrestin2, attenuates activation of the PKCϵ pathway. These observations were consistent with the report that phosphorylation of the δ-opioid receptor regulates agonist-dependent signaling (27). However, the basal affinity between a non-phosphorylated receptor and β-arrestin2 should still limit activation of the PKCϵ pathway. Morphine induces little receptor phosphorylation and no receptor internalization under normal conditions (9), but overexpression of β-arrestin2 enables morphine to induce significant receptor internalization (28). In addition, morphine induces significant receptor internalization in striatal neurons, but not in hippocampal neurons, probably due to the differential amounts and basal activities of β-arrestin in these systems (29). This hypothesis about the basal affinity was also confirmed in the current study. Blocking receptor phosphorylation made etorphine and DAMGO utilize the PKCϵ pathway, but not as efficiently as morphine. Removing β-arrestin2 additionally enabled etorphine and DAMGO to activate the PKCϵ pathway to levels similar to that induced by morphine. Therefore, although not the major blocker of PKCϵ activation, the existence of β-arrestin2 and its affinity for non-phosphorylated receptor also prevents agonists from using the PKCϵ pathway.

GRK2 overexpression increased the ability of morphine to induce receptor phosphorylation, but decreased morphine ability to use the PKCϵ pathway (Fig. 5, *B* and *C*). These observations are consistent with the fact that β-arrestin2 overexpression makes morphine switch from the PKC pathway to the β-arrestin2 pathway (4, 8). These results also provide a possible mechanism for morphine to use the β-arrestin pathway. In addition, the agonists switching from one pathway to another provides a useful tool in controlling the downstream responses to the agonists. On the one hand, the most critical regions on GPCRs for G protein coupling are the second intracellular loop (IL), the N terminus, and the C terminus of IL3 (30). On the other hand, in addition to the C terminus facilitating the binding of β-arrestin by mediating phosphorylation (31), the IL2, the N terminus, and the C terminus of the IL3 are also essential for the binding of β-arrestin (32–36). Due to this overlap of binding sites, receptor phosphorylation can be suggested as regulating the competition between G proteins and β-arrestin for the binding site on GPCRs (7).

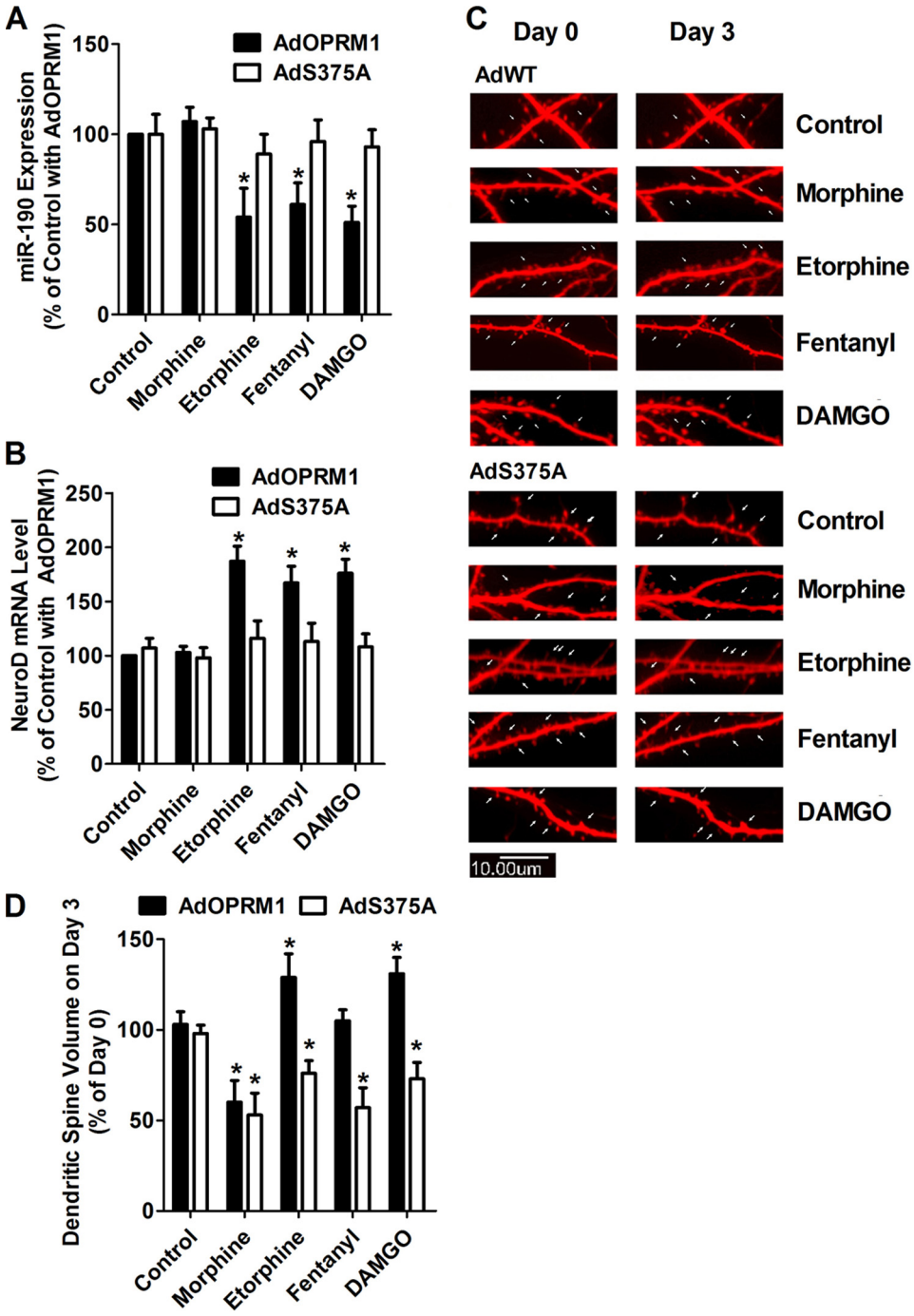

FIGURE 8. **Phosphorylation blockage switches the effects of agonist on spine stability.** *A* and *B*, primary hippocampal neurons from wild-type mice were infected with AdOPRM1 or AdS373A for 3 days. Then the primary cultures were incubated with 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, or 1 μM DAMGO for additional 3 days. The levels of miR-190 (*A*) and NeuroD mRNA (*B*) were determined. The results were normalized against that of the control with AdOPRM1. *C* and *D*, primary hippocampal neurons from wild-type mice were transfected with DsRed in pRK5 7 days after plating. Two weeks later, the cultures were infected with AdOPRM1 or AdS375A for 3 days. Then the primary cultures were incubated with 1 μM morphine, 10 nM etorphine, 10 nM fentanyl, or 1 μM DAMGO for additional 3 days. At Day 0 and Day 3 of agonist treatment, dendritic spine stability was examined in confocal images as described under "Experimental Procedures." The spine densities (spine volume) on Day 3 were normalized against that on Day 0 and summarized (*C*). The images indicate the changes on spine morphology (*D*).

Although the inhibitory effects of receptor phosphorylation on PKCϵ activation were demonstrated in the current studies, the detailed mechanism has not been elucidated completely. One possible mechanism is that conformational changes of the receptor during phosphorylation prevent activation of PKCϵ. The different affinities between phosphorylated and non-phosphorylated receptors for β-arrestin support the existence of conformational differences. In addition, GRK2 is recruited to the cell membrane and binds with the receptor after activation by free G$\beta\gamma$ subunits (37–39). The binding of GRK2 to the receptor signaling complex also may be the reason that activation of PKCϵ is prevented.

As reported previously, the different abilities of morphine and fentanyl to affect miR-190, NeuroD, and subsequent den-

dritic spine stability are due to the different pathways used by them to induce ERK phosphorylation (18). Thus, phosphorylation blockage enabled etorphine, fentanyl, and DAMGO to activate PKCϵ and to function like morphine. In addition, since NeuroD plays an essential role in hippocampus development, adult neurogenesis, and maintaining dendritic spine stability (40–42), the effects of agonists on NeuroD should have board implications. Controlling agonist-dependent signaling by altering GRK activities or the receptor phosphorylation stage not only implicates controlling ERK phosphorylation, receptor desensitization, and dendritic spine stability, but also may be useful in other aspects, such asadult neurogenesis, long-term potentiation and learning, and overall adaptational responses to chronic drug treatment.

REFERENCES

1. Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., Javitch, J. A., Roth, B. L., Christopoulos, A., Sexton, P. M., Miller, K. J., Spedding, M., and Mailman, R. B. (2007) *J. Pharmacol. Exp. Ther.* **320,** 1–13
2. Violin, J. D., and Lefkowitz, R. J. (2007) *Trends Pharmacol. Sci.* **28,** 416–422
3. DeWire, S. M., Ahn, S., Lefkowitz, R. J., and Shenoy, S. K. (2007) *Annu. Rev. Physiol.* **69,** 483–510
4. Zheng, H., Loh, H. H., and Law, P. Y. (2008) *Mol. Pharmacol.* **73,** 178–190
5. Kobayashi, H., Narita, Y., Nishida, M., and Kurose, H. (2005) *Cell Signal.* **17,** 1248–1253
6. Lefkowitz, R. J. (1998) *J. Biol. Chem.* **273,** 18677–18680
7. Zheng, H., Loh, H. H., and Law, P. Y. (2010) *IUBMB Life* **62,** 112–119
8. Chu, J., Zheng, H., Loh, H. H., and Law, P. Y. (2008) *Cell Signal.* **20,** 1616–1624
9. Keith, D. E., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Kang, L., Evans, C. J., and von Zastrow, M. (1996) *J. Biol. Chem.* **271,** 19021–19024
10. Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., and Caron, M. G. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7157–7162
11. Koch, T., Widera, A., Bartzsch, K., Schulz, S., Brandenburg, L. O., Wundrack, N., Beyer, A., Grecksch, G., and Höllt, V. (2005) *Mol. Pharmacol.* **67,** 280–287
12. Chu, J., Zheng, H., Zhang, Y., Loh, H. H., and Law, P. Y. (2010) *Cell Signal.* **22,** 684–696
13. Liao, D., Lin, H., Law, P. Y., and Loh, H. H. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 1725–1730
14. Liao, D., Grigoriants, O. O., Wang, W., Wiens, K., Loh, H. H., and Law, P. Y. (2007) *Mol. Cell. Neurosci.* **35,** 456–469
15. Lin, H., Higgins, P., Loh, H. H., Law, P. Y., and Liao, D. (2009) *Neuropsychopharmacology* **34,** 2097–2111
16. Zheng, H., Zeng, Y., Chu, J., Kam, A. Y., Loh, H. H., and Law, P. Y. (2010) *J. Neurosci.* **30,** 8102–8110
17. Zheng, H., Zeng, Y., Zhang, X., Chu, J., Loh, H. H., and Law, P. Y. (2010) *Mol. Pharmacol.* **77,** 102–109
18. Zheng, H., Chu, J., Zeng, Y., Loh, H. H., and Law, P. Y. (2010) *J. Biol. Chem.* **285,** 21994–22002
19. Roerig, S. C., Loh, H. H., and Law, P. Y. (1992) *Mol. Pharmacol.* **41,** 822–831
20. Zheng, H., Chu, J., Qiu, Y., Loh, H. H., and Law, P. Y. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 9421–9426
21. El Kouhen, R., Burd, A. L., Erickson-Herbrandson, L. J., Chang, C. Y., Law, P. Y., and Loh, H. H. (2001) *J. Biol. Chem.* **276,** 12774–12780
22. Chakrabarti, S., Law, P. Y., and Loh, H. H. (1995) *Brain Res. Mol. Brain Res.* **30,** 269–278
23. Newton, A. C. (2001) *Chem. Rev.* **101,** 2353–2364
24. Smith, F. L., Gabra, B. H., Smith, P. A., Redwood, M. C., and Dewey, W. L. (2007) *Pain* **127,** 129–139
25. Foster, L. J., De Hoog, C. L., and Mann, M. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 5813–5818
26. Macdonald, J. L., and Pike, L. J. (2005) *J. Lipid Res.* **46,** 1061–1067
27. Xu, C., Hong, M. H., Zhang, L. S., Hou, Y. Y., Wang, Y. H., Wang, F. F., Chen, Y. J., Xu, X. J., Chen, J., Xie, X., Ma, L., Chi, Z. Q., and Liu, J. G. (2010) *J. Cell Sci.* **123,** 4259–4270
28. Law, P. Y., Loh, H. H., and Wei, L. N. (2004) *Neuropharmacology* **47,** Suppl. 1, 300–311
29. Haberstock-Debic, H., Kim, K. A., Yu, Y. J., and von Zastrow, M. (2005) *J. Neurosci.* **25,** 7847–7857
30. Wess, J. (1997) *Faseb J.* **11,** 346–354
31. Gurevich, V. V., and Gurevich, E. V. (2006) *Pharmacol. Ther.* **110,** 465–502
32. Cen, B., Xiong, Y., Ma, L., and Pei, G. (2001) *Mol. Pharmacol.* **59,** 758–764
33. DeGraff, J. L., Gurevich, V. V., and Benovic, J. L. (2002) *J. Biol. Chem.* **277,** 43247–43252
34. Han, M., Gurevich, V. V., Vishnivetskiy, S. A., Sigler, P. B., and Schubert, C. (2001) *Structure* **9,** 869–880
35. Lan, H., Liu, Y., Bell, M. I., Gurevich, V. V., and Neve, K. A. (2009) *Mol. Pharmacol.* **75,** 113–123
36. Macey, T. A., Liu, Y., Gurevich, V. V., and Neve, K. A. (2005) *J. Neurochem.* **93,** 128–134
37. Kozasa, T., and Gilman, A. G. (1996) *J. Biol. Chem.* **271,** 12562–12567
38. Li, J., Xiang, B., Su, W., Zhang, X., Huang, Y., and Ma, L. (2003) *J. Biol. Chem.* **278,** 30219–30226
39. Métayé, T., Gibelin, H., Perdrisot, R., and Kraimps, J. L. (2005) *Cell Signal.* **17,** 917–928
40. Chae, J. H., Stein, G. H., and Lee, J. E. (2004) *Mol. Cells* **18,** 271–288
41. Gaudillière, B., Konishi, Y., de la Iglesia, N., Yao, G., and Bonni, A. (2004) *Neuron* **41,** 229–241
42. von Bohlen Und Halbach, O. (2007) *Cell Tissue Res.* **329,** 409–420