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-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) induce extensive receptor phosphorylation and use the β-arrestin2 pathway. Blocking OPRM1 phosphorylation (by mutating Ser³⁶³, Thr³⁷⁰ and Ser³⁷⁵ 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, since these agonists were unable to use the PKCε pathway when β-arrestin2 was absent. In addition, over-expressing G protein-coupled receptor kinase 2 (GRK2) decreased the ability of morphine to activate PKCε, whereas over-expressing dominant negative GRK2 enabled etorphine, fentanyl, and DAMGO to activate PKCε. Furthermore, by over-expressing 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.

Introduction

Agonist-dependent or agonist-biased signaling is a new concept in understanding the signaling of the G protein-coupled receptor (GPCR) (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-Ala²,N-Me-Phe⁴,Gly⁵-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 ([Ca²⁺]i) release: morphine via the PKC pathway and the other three agonists via the β-arrestin pathway (8,12).

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). Since 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 Ser³⁶³, Thr³⁷⁰ and Ser³⁷⁵ 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 200ng/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 lab) 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 approximately 0.5 pmol/mg protein in MEF cells and primary cultures. The antibodies against phosphorylated ERK, total ERK, phosphorylated Ser³⁷⁵ 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 (St. Louis, MO). The antibody against HA was from Covance Research...
Products (Emeryville, CA). The AP conjugated secondary antibodies were from BioRad (Hercules, CA). Goα2 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). PKCe, 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 15min at 37ºC with rotation, and was stopped by adding an equal volume of EDTA (50mM, pH 8.0). Then the substrates in supernatant were precipitated with 30µl 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 and Analytical 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% to 30% continuous sucrose gradient, which was centrifuged at 32,000 rpm for 16 hrs in a SW41 rotor at 4ºC. Twelve fractions (1ml 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).

**Statistic 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 t-test and ANOVA test, except for data depicted in Fig. 1, Fig. 2D-E, and Fig. 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 PKCe 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 1μM (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 1μM, whereas etorphine and fentanyl were used at 10nM, to achieved equivalent concentrations. At these concentrations, the agonists induce ERK phosphorylation and adenylyl cyclase inhibition to similar levels.

Because the phosphorylation sites of OPRM1 have been demonstrated to be the Ser363, Thr370, and Ser375 residues of the C-terminus (21), antibodies against phosphorylated Ser375 (pS375) 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 pS375 and pAAs antibodies (Fig. 1A). Fentanyl-induced Ser375 phosphorylation was lower than that induced by etorphine or DAMGO. In HEK3A cells, none of the four agonists induced receptor phosphorylation (Fig. 1B), 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). PKCa, PKGγ, and PKCe contribute to morphine-induced tolerance (24). Thus, the activities of PKCa, PKGγ, and PKCe were determined after agonist treatment. Morphine induced PKCe activation (314±43% of control, n=4) in HEKOPRM1 cells. Fentanyl induced a much lower activation of PKCe (158±21%, n=4). Neither etorphine (103±12%, n=4) nor DAMGO (101±8%, n=4) induced activation of PKCe (Fig. 1C). In the HEK3A cells, all four agonists activated PKCe (Fig. 1D). Etorphine and DAMGO induced lower activation of PKCe (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 PKCe 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 PKCe but did not induce receptor phosphorylation. Thus, the ability of the agonists to activate PKCe inversely correlates with their ability to induce receptor phosphorylation. Therefore, it is reasonable to suggest that receptor phosphorylation attenuates PKCe activation.

Receptor phosphorylation attenuates the recruitment of PKCe

After being activated, PKCs translocate from cytosol to the cell membrane (23). Therefore, we measured the translocation of PKCe to the cell membrane to confirm the influence of receptor phosphorylation on PKCe activation. Continuous sucrose gradient was used to separate the lipid raft 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 PKCe peaked at fraction 4, 6, and 12, respectively (Fig. 2A). The percentage amounts of target proteins in
fractions 3-5 were used to indicate their distribution in the lipid raft microdomains.

In the absence of an agonist, the majority of the receptor was in the lipid raft microdomains in both HEKOPRM1 and HEK3A cells (Fig. 2B 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. 2B). 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. 2C).

Since 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. 2D). However, all four agonists induced significant recruitment of PKCε to the receptor signaling complex in HEK3A cells (Fig. 2E).

**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). Since 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. 3A). 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. 3B). 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. 3B).

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. Since the concentrations of agonists used above induce desensitization quickly, morphine and DAMGO were used at 100nM, 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. 4A). Etorphone 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, since PKCεi did not affect the desensitization. However, receptor
desensitization induced by etorphine and DAMGO in HEK3A cells was PKCε-mediated (Fig. 4B and D). Fentanyl functioned as a combination of morphine and etorphine, although more similarly to etorphine (Fig. 4C). 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 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. Since 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. 5A). 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 over-expressed in HEKOPRM1 cells to modulate the ability of the agonists to induce receptor phosphorylation. As indicated in Fig. 5B, wild type GRK2 over-expression increased the ability of all four
agonists to induce receptor phosphorylation, whereas GRK2-K220R over-expression resulted in the opposite effect. The activities of PKCε were measured after over-expression. Morphine-induced activation of PKCε was potentiated by GRK2-K220R, but impaired by GRK2 (Fig. 5C). Although GRK2 over-expression could not reduce PKCε activity to below basal level, GRK2-K220R over-expression did increase the ability of etorphine, fentanyl, and DAMGO to activate PKCε. Since Ser375 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, morphine, but not etorphine or DAMGO, activated PKCε in the primary cultures, whereas fentanyl activated PKCε to a lower level than morphine (Fig. 6A). When the primary cultures were pretreated with the general opioid receptor antagonist naloxone and the OPRM1 specific antagonist Cys²-Tyr³-Orn⁵-Pen⁷-amide, PKCε activation was attenuated, indicating that agonist-induced PKCε activation is through OPRM1. In addition, pretreatment with PKC subtype-specific inhibitors demonstrated that PKCα, but not PKCα or PKCγ, is activated by OPRM1 in the primary cultures (Fig. 6B).

Hippocampal primary cultures from wild type mice and β-arrestin2−/− mice were prepared as described in 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. 6C 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. 7A). Infection efficiency is indicated in Fig. 7B 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 three days, and the dendritic spine stability was monitored during the next three 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±16%, 61±12%, and 51±9%, respectively, n=4) (Fig. 8A). 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. 8C 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. 8C 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 over-expression 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 over-expression increased the ability of morphine to induce receptor phosphorylation, but decreased morphine’s ability to use the PKCε pathway (Fig. 5B and C). These observations are consistent with the fact that β-arrestin2 over-expression makes morphine switch from the PKCε 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).

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 Gβγ 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 dendritic 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 as adult neurogenesis, long-term potentiation and learning, and overall adaptational responses to chronic drug treatment.

Reference
GPCR Phosphorylation in Biased Agonism


Footnotes
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The abbreviations used are: [Ca2+]i, intracellular calcium; DAMGO, [d-Ala2,N-Me-Phe4,Gly5-ol]-

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enkephalin; GPCR, G protein-coupled receptors; 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.

Figure Legend

Figure 1
Receptor phosphorylation attenuates PKCε activation
HEKOPRM1 (A and C) and HEK3A cells (B and D) were treated with PBS (Control), 1µM morphine, 10nM etorphine, 10nM fentanyl and 1µM DAMGO for 5 min.

(A-B) Receptors were immunoprecipitated with HA antibody. The phosphorylated Ser375 on OPRM1 (pS375) and phosphorylated amino acid (pAAs) were determined in the immunoprecipitated receptor.

(C-D) The activities of three PKC subtypes were determined as described in the Experimental Procedures. The results were normalized against that in the control in each group.

Figure 2
Receptor phosphorylation attenuates the recruitment of PKCε
(A) The 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-C) The 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-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.

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 three hours.

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.

Figure 4
Phosphorylation blockage enables agonists to use PKCε for receptor desensitization
HEKOPRM1 and HEK3A were treated with DMSO or 50 µM PKCεi for three hours and were then pretreated with 100nM morphine (A), 1nM etorphine (B), 1nM fentanyl (C), and 100nM 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.

Figure 5
**GRK2 phosphorylation on S\textsuperscript{375} attenuates PKC\varepsilon activation**
(A) HEKOPRM1, HEKS363A, HEKT370A and HEKS375A cells were treated with PBS (Control), 1\(\mu\)M morphine, 10nM etorphine, 10nM fentanyl and 1\(\mu\)M DAMGO for 5min. The activity of PKC\varepsilon was determined.
(B-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 S\textsuperscript{375} was determined in (B). PKC\varepsilon activities were normalized against that in “Control with Vector” in (C).

Figure 6
**Receptor phosphorylation attenuates PKC\varepsilon activation in primary cultures**
(A-B) Primary cultures of hippocampal neurons from wild type mice were pretreated with PBS, 10\(\mu\)M naloxone, or 10\(\mu\)M CTOP for 10 min (A). The cultures were pretreated with DMSO or 50 \(\mu\)M PKC subtypes-specific inhibitors (PKC\(\alpha\), PKC\(\gamma\), and PKC\(\varepsilon\)) for three hours (B). Then the primary cultures were incubated with 1\(\mu\)M morphine, 10nM etorphine, 10nM fentanyl, or 1\(\mu\)M DAMGO for five minutes, and the activities of PKC subtypes were determined as described in the *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 three days. Then the primary cultures were incubated with 1\(\mu\)M morphine, 10nM etorphine, 10nM fentanyl, or 1\(\mu\)M DAMGO for five minutes, and the activity of PKC\varepsilon was determined. The results were normalized against that of the control with AdOPRM1.
(D) Primary hippocampal neurons from \(\beta\)-arrestin2\(^{-/-}\) mice were prepared as in (C) and PKC\varepsilon activity was determined.

Figure 7
**High efficiency of virus infection leads to high receptor expression**
Primary cultures of hippocampal neurons from wild type or \(\beta\)-arrestin2\(^{-/-}\) mice were infected with AdOPRM1 or AdS375A for three 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 (Fig. B and C).

Figure 8
**Phosphorylation blockage switches the effects of agonist on spine stability**
(A-B) Primary hippocampal neurons from wild type mice were infected with AdOPRM1 or AdS373A for three days. Then the primary cultures were incubated with 1\(\mu\)M morphine, 10nM etorphine, 10nM fentanyl, or 1\(\mu\)M DAMGO for additional three 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-D) Primary hippocampal neurons from wild type mice were transfected with DsRed in pRK5 seven days after plating. Two weeks later, the cultures were infected with AdOPRM1 or AdS375A for three days. Then the primary cultures were incubated with 1\(\mu\)M morphine, 10nM etorphine, 10nM fentanyl, or 1\(\mu\)M DAMGO for additional three days. At Day 0 and Day 3 of agonist treatment,
GPCR Phosphorylation in Biased Agonism

dendritic spine stability was examined in confocal images as described in the *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).
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 1μM morphine, 10nM etorphine, 10nM fentanyl, and 1μM DAMGO for 5 min. The amounts of phosphorylated ERK were normalized against total ERK. The results were further normalized against those under control condition. For receptor desensitization, cells were treated with 100nM morphine, 1nM etorphine, 1nM fentanyl, and 100nM DAMGO for 30 min. Normally ADP, but not the agonists, can induce $[\text{Ca}^{2+}]_i$ release. However, the agonists can potentiate the $[\text{Ca}^{2+}]_i$ 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 PKCε-treated cells. N/S suggests the effect of PKCε was not significant. N/A suggests the agonist did not induce signaling in DMSO group, therefore no decrease can be calculated.

<table>
<thead>
<tr>
<th></th>
<th>ERK Phosphorylation (% of basal level)</th>
<th>Receptor Desensitization on$[\text{Ca}^{2+}]_i$ (% decrease in basal activity)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WTMEF Cells</td>
<td>BKOMEF Cells</td>
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<tr>
<td></td>
<td>AdOPRM1</td>
<td>Ad3A</td>
</tr>
<tr>
<td>Morphine</td>
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<tr>
<td>DMSO</td>
<td>178±13</td>
<td>183±7</td>
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<tr>
<td>PKCεi</td>
<td>111±7</td>
<td>97±15</td>
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<td>% Inh.</td>
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<td>Etorphine</td>
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<td>DMSO</td>
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<tr>
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<td>116±8</td>
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<tr>
<td>% Inh.</td>
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<tr>
<td>Fentanyl</td>
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<tr>
<td>DMSO</td>
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<td>184±11</td>
</tr>
<tr>
<td>PKCεi</td>
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<td>108±13</td>
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<td>% Inh.</td>
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<td>DAMGO</td>
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<tr>
<td>DMSO</td>
<td>181±10</td>
<td>153±8</td>
</tr>
<tr>
<td>PKCεi</td>
<td>174±8</td>
<td>119±6</td>
</tr>
<tr>
<td>% Inh.</td>
<td>N/S</td>
<td>64</td>
</tr>
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</table>
Figure 1

A HEKOPRM1 Cells

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<thead>
<tr>
<th>IB: pS\textsuperscript{375}</th>
<th>IB: pAA\textsubscript{S}</th>
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</thead>
<tbody>
<tr>
<td>\textit{Input}</td>
<td>IB: HA</td>
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<tr>
<td>Control, Morphine, Etorphine, Fentanyl, DAMGO</td>
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</tr>
</tbody>
</table>

B HEK3A Cells

<table>
<thead>
<tr>
<th>IB: pS\textsuperscript{375}</th>
<th>IB: pAA\textsubscript{S}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Input}</td>
<td>IB: HA</td>
</tr>
<tr>
<td>Control, Morphine, Etorphine, Fentanyl, DAMGO</td>
<td></td>
</tr>
</tbody>
</table>

C HEKOPRM1 Cells

PKC activity (% of Control)

D HEK3A Cells

PKC activity (% of Control)
Figure 2

A

HA
Gαi2
TR
PKCε

B HEKOPRM1 Cells

Distribution in Fraction 3-5 (% of total)

WT
PKCε

Control
Morphine
Etorphine
Fentanyl
DAMGO

C HEK3A Cells

Distribution in Fraction 3-5 (% of total)

3A
PKCε

Control
Morphine
Etorphine
Fentanyl
DAMGO

D HEKOPRM1 Cells

IP: HA
IB: PKCε
Input
IB: HA

PKCε Precipitated (% of Control)

Control
Morphine
Etorphine
Fentanyl
DAMGO

E HEK3A Cells

IP: HA
IB: PKCε
Input
IB: HA

PKCε Precipitated (% of Control)

Control
Morphine
Etorphine
Fentanyl
DAMGO
Figure 3

A HEKOPRM1 Cells

B HEK3A Cells

**ERK Phosphorylation (% of Control with DMSO)**

- **A**
  - Control
  - Morphine
  - Etorphine
  - Fentanyl
  - DAMGO

- **B**
  - Control
  - Morphine
  - Etorphine
  - Fentanyl
  - DAMGO

* indicates statistically significant difference from control.
Figure 4

A Morphine

B Etorphine

C Fentanyl

D DAMGO

Desensitization on [Ca^{2+}]_i Release (%) vs. Time (min)
Figure 5

A

PKC\(_\varepsilon\) Activity (% of Control with HEKWT)

- HEKOPRM1
- HEKT370A
- HEKS363A
- HEKS375A

Control, Morphine, Etorphine, Fentanyl, DAMGO

B

Vector

- IP: HA
- IB: pAAs
- Input
- IB: HA

GRK2

- IP: HA
- IB: pAAs
- Input
- IB: HA

GRK2-K220R

- IP: HA
- IB: pAAs
- Input
- IB: HA

C

PKC\(_\varepsilon\) Activity (% of Control with Vector)

- Vector
- GRK2
- GRK2-K220R

Control, Morphine, Etorphine, Fentanyl, DAMGO
Figure 6

A

PKCα Activity (% of Control with PBS)

B

PKCα Activity (% of Control with DMSO)

C

PC from Wildtype Mice

D

PC from β-arrestin2−/− Mice

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Figure 8

A

![Graph showing miR-190 expression (% of control with AdOPRM1) for different treatments.](image)

B

![Graph showing NeuroD mRNA level (% of control with AdOPRM1) for different treatments.](image)

C

Day 0

Day 3

Control

Morphine

Etorphine

Fentanyl

DAMGO

AdWT

AdS375A

D

![Graph showing dendritic spine volume on Day 3 (% of Day 0) for different treatments.](image)
Modulating μ-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKCδ activation and dendritic spine stability

Hui Zheng, Ji Chu, Yuhan Zhang, Horace H. Loh and Ping-Yee Law

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