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β-Arrestin-Dependent μ-Opioid Receptor-Activated Extracellular Signal-Regulated Kinases (ERKs) Translocate to Nucleus in Contrast to G Protein-Dependent ERK Activation

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ABSTRACT

The cellular location of extracellular signal-regulated kinases (ERKs) activated by a G protein-coupled receptor was shown to be dependent on the pathway that mediated their activation. In general, fast activation of ERKs (2 min) mediated by G proteins resulted in the nuclear translocation of phosphorylated ERKs, whereas a slower activation of ERKs (10 min) mediated by β-arrestins resulted in the cytosolic retention of the phosphorylated ERKs. However, we observed distinct differences from this established ERKs cellular itinerary with the μ-opioid receptor-activated ERKs. Agonists such as morphine and methadone activated ERKs via the protein kinase C-dependent pathway but not the β-arrestin-dependent pathway. The activated ERKs did not translocate into the nucleus, but phosphorylated 90-kDa ribosomal S6 kinase and induced the activity of transcription factor cAMP response element-binding protein. In contrast, agonists such as etorphine and fentanyl activated ERKs in a β-arrestin-dependent manner. The phosphorylated ERKs translocated into the nucleus, resulting in increases in Elk-1 activity and GRK2 and β-arrestin2 transcriptions. Thus, the cellular location of phosphorylated ERKs and subsequent activities on gene transcriptions are dictated by the agonist used to activate the receptor and the subsequent signaling pathway involved.

G protein-dependent and β-arrestin-dependent pathways are two well-established pathways for ERK activation in G_q- and G_s-coupled GPCRs (DeWire et al., 2007). The involvement of the G protein-dependent pathway in ERK activation has been demonstrated by the use of G protein-dependent pathways inhibitors, such as PKC or protein kinase A inhibitors (Ahn et al., 2004; Gesty-Palmer et al., 2006). β-arrestin-dependent ERK activation was illustrated by using siRNA to knockdown β-arrestin levels (Ahn et al., 2004). Although these two pathways could be observed at the same time, they are independent of each other, for ERK activation still could be observed if one of the pathways was blocked (DeWire et al., 2007).

Although the two pathways have been observed with many GPCRs, the kinetics of ERK activation are different. In cells expressing angiotensin II receptors, G protein-dependent ERK activation usually peaked 2 min after stimulation and returned to basal level at the 10-min time point, whereas the β-arrestin-dependent ERK activation peaked 10 min after agonist addition (Ahn et al., 2004). In cells expressing parathyroid receptors, G protein-dependent ERK activation peaked at 10 min, whereas β-arrestin-dependent ERK activation peaked 30 to 60 min after stimulation (Gesty-Palmer et al., 2006). In cells expressing vasopressin receptors, ERK activation mediated by both pathways peaked 2 min after stimulation (Ren et al., 2005).

In addition, not only are there agonists that activate ERKs via both the G protein- and β-arrestin-dependent pathways (DeWire et al., 2007); there are also agonists that use only one of the two pathways. For example, isoproterenol activates ERKs using both pathways, whereas the β2-adrenergic agonist ICI118551 activates ERKs completely via β-arrestin-

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; 90RSK, 90-kDa ribosomal s6 kinase; CREB, cAMP response element-binding; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; MOR, μ-opioid receptor; PKC, protein kinase C; HA, hemagglutinin; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; MEK, mitogen-activated protein kinase kinase; DOR, δ-opioid receptor; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; ICI118551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; Ro-31-8425, 2-[8-(aminomethyl)-6,7,8,9-tetrahydropyrido[1,2-*a*]indol-3-yl]-3-(1-methylindol-3-yl)maleimide, HCl; PD98059, 2′-amino-3′-methoxyflavone; HA-MOR, hemagglutinin-tagged μ-opioid receptor; TIPPψ, H-Tyr-Ticψ[CH_2NH]-Phe-Phe-OH.

dependent pathway, and CCL19 uses only the G protein-dependent pathway in cells expressing the chemokine receptor CCR7 (Azzi et al., 2003; Kohout et al., 2004; Shenoy et al., 2006).

Because the eventual cellular locations of activated ERKs are linked to the pathway mediating the activation and downstream cascades of ERKs involve transcription factors, the pathway for ERK activation becomes critical to overall cellular responses. In cells expressing angiotensin receptors, ERKs activated via G protein-dependent pathway were shown to translocate to the nucleus, whereas β-arrestin-activated ERKs remained in the cytosol (Ahn et al., 2004). However, this pathway-dependent cellular location is not without controversy. Opposite results were reported on β-arrestin's influence on nuclear translocation of ERKs. By overexpressing β-arrestin2 in COS-7 cells with angiotensin II type 1A receptors, Tohgo et al. (2002) reported that nuclear translocation of ERKs was inhibited. In contrast, when β-arrestin2 was overexpressed in COS-7 cells with β2-adrenergic receptors, nuclear translocation of ERKs was enhanced (Kobayashi et al., 2005). These two apparently contrasting studies suggest that the eventual cellular location of the activated ERKs should relate to the type of GPCR.

μ-Opioid receptor (MOR), which couples to $G_{i/o}$, has been shown to activate ERKs (Li and Chang, 1996). On the one hand, by using the PKC inhibitor bisindolylmaleimide I, Belcheva et al. (2005) were able to block both DAMGO- and morphine-induced ERK activation in the cortical astrocytes cultures, suggesting the involvement of the G protein-dependent pathway in MOR-mediated ERK activation. On the other hand, Ignatova et al. (1999) reported that MOR-mediated ERK activation was attenuated by the blockade of receptor internalization (Ignatova et al., 1999). Although subsequent reports have challenged the linkage between MOR-mediated ERK activation and receptor internalization (Kramer and Simon, 2000), these studies suggest the possible involvement of β-arrestin-dependent pathways in MOR-mediated ERK activation. A recent report with Chinese hamster ovary cells proved the involvement of both β-arrestin- and G protein-dependent activation of ERKs by MOR (Rozenfeld and Devi, 2007).

However, the details of β-arrestin- and G protein-dependent ERK activation by MOR have not been resolved. For example, the kinetics of ERK activation and the location of phosphorylated ERKs related to the two pathways are unclear. In addition, whether all MOR agonists activate ERKs similarly has not been demonstrated, especially in the situation in which morphine-MOR complex has been shown to have a low affinity for β-arrestin (Keith et al., 1996). To address these questions, we monitored the activation of ERKs and subsequent cellular location of the activated kinases in HEK293 cells expressing high levels of MOR, in human neuroblastoma SHSY5Y cells expressing relatively low levels of endogenous MOR, and in the primary neuronal culture of rat hippocampus, which is one of the regions that contain highest levels of MOR (Arvidsson et al., 1995). In addition, we examined pathway selectivity for ERK activation with four MOR agonists (morphine, etorphine, methadone, and fentanyl).

Materials and Methods

Cell Culture and Chemicals. HEK293 cells stably expressing hemagglutinin (HA)-tagged μ-opioid receptor were cultured in Eagle's minimal essential medium with Earle's salt supplement, 10% fetal bovine serum (FBS), and 200 ng/ml G418 sulfate. Wild-type, β-arrestin2$^{-/-}$ and β-arrestin1/2$^{-/-}$ mouse embryonic fibroblast (MEF) cells (generous gifts from Dr. R. Lefkowitz, Duke University, Durham, NC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Human neuroblastoma SHSY5Y cells were cultured in 50% Eagle's minimal essential medium and 50% Ham's F-12 supplemented with 10% FBS. When ERK activities are being monitored, cells are normally cultured in a serum-free medium overnight before agonist treatment. Effectene (QIAGEN, Valencia, CA) was used to transfect β-arrestin2 and Dynamin-K44E into HEK293 cells, and the adenovirus system was used for MOR expression in MEF cells. The agonists or inhibitors were added for the desired concentration and time as described in each figure. PKC inhibitor Ro-31-8425 and mitogen-activated protein kinase inhibitor PD98059 were purchased from LClab (Woburn, MA) and Calbiochem (EMD Biosciences, La Jolla, CA).

Fluorescence Flow Cytometry. The HA-tagged μ-opioid receptor (HA-MOR) expressed on the plasma membrane was quantified by FACS analysis of the cell surface immunofluorescence. In brief, HEK293 cells stably expressing HA-MOR were transfected with different plasmids using an Effectene transfection reagent from QIAGEN. After 36 h, the cells were treated with agonists or inhibitors for the desired time as described in each figure. After rapidly rinsing twice with PBS at 4°C, the cells were incubated at 4°C for 2 h in PBS with the anti-HA antibody (1:1000 dilution). Afterward, the cells were washed twice with PBS at 4°C and then incubated with Alexa 488-labeled goat anti-mouse IgG secondary antibody (1:1000) at 4°C for 1 additional hour. After washing the cells to remove the excess secondary antibodies, the cells were fixed with 3.7% formaldehyde before FACS analysis. Receptor immunofluorescence was measured by FACScan (BD Biosciences, Palo Alto, CA). Fluorescence intensity of 10,000 cells was collected for each sample. Cell Quest software (BD Biosciences) was used to calculate the mean fluorescence intensity of the cell population.

Immunoblotting. Cells from 35-mm dishes were washed with PBS at 4°C twice and 0.1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and 1× protease inhibitor cocktail; Roche, Indianapolis, IN) was added. After centrifugation, the supernatant was transferred to a new tube, and SDS-polyacrylamide gel electrophoresis sample buffer was added to the supernatant. Approximately 100 μg of protein from each lysate was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane for immunoblotting. Primary antibody was added after 1 h of 10% milk blocking, and the cells were incubated for 1 h. After washing three times with 0.1% Tween 20, 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl, secondary antibodies conjugated with alkaline phosphate were added, the cells were incubated for 2 h, and then the membrane was washed three times with 0.1% Tween 20, 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. After developing, the fluorescence intensity of each band was measured with Storm 860. The intensity of individual bands was determined with the analysis software ImageQuant (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). For ERK activity, the amount of phosphorylated ERK was monitored by a monoclonal antibody for phosphorylated ERKs (Cell Signaling Technology, Danvers, MA) and was normalized to total ERKs surveyed with total ERK antibodies (Cell Signaling Technology). For the measurement of PKC activity, the phosphor-(Ser) PKC substrate antibody (Cell Signaling Technology) was used and was normalized to the immunoreactivity of Rab4. For the measurement of 90RSK, the antibody for phosphorylated 90RSK (Cell Sig-

naling Technology) was used, and the result was normalized to the immunoreactivity of Rab4 used as a cytosol marker.

Nuclear Extraction. After washing with PBS at 4°C twice, 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 10% 0.1 mM EDTA, 0.1 mM EGTA, 0.6% Nonidet P-40, 10 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and 1×protease inhibitor cocktail; Roche) was added to the cells in 35-mm dishes, and the cells were incubated on ice for 15 min. After centrifugation, the supernatant was transferred to a fresh tube and designated as the cytosolic fraction. To fractionate the nuclear proteins further, the nuclear pellet was resuspended in an extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and 1× protease inhibitor cocktail; Roche) at 4°C. After incubation at 4°C for 15 min with vigorous shaking, the nuclear extract was centrifuged at 14,000 rpm for 5 min, and the supernatant was removed and designated as the nucleus fraction. The amount of phosphorylated ERK in the nucleus was determined by immunoblotting and normalized to the immunoreactivity of Histone 3, which was used as a nuclear marker.

Luciferase Reporter Assays. Elk-1 and CREB activities were measured by using the Elk-1 or CREB-driven luciferase reporter system (Stratagene, La Jolla, CA). In brief, HEK293 cells stably expressing HA-MOR were transfected with GAL4-Elk-1 or GAL4-CREB-1, pFR-luc, and pRL-tk-luc. The GAL4-Elk-1 or GAL-CREB-1 encodes a fusion protein containing the GAL4 DNA binding domain, the transactivation domain of Elk-1 or CREB. pFR-luc encodes the firefly luciferase gene under the control of the GAL4 DNA binding element, and pRL-tk-luc encodes *Renilla reniformis* luciferase under the control of the thymidine kinase promoter. One day after transfection, the cells were incubated in serum-free media overnight. Stimulations with agonists were carried out for 12 h. Luciferase activities were determined using a dual luciferase assay kit (Promega, Madison, WI). Cells were extracted and assayed sequentially for firefly and *R. reniformis* luciferase activities. Firefly activities were normalized to *R. reniformis* luciferase activity.

RT-PCR. After culturing in a serum-free medium overnight, the cells were stimulated with agonists for 12 h. Total RNA was extracted using the RNeasy kit from Qiagen, and the level of specific mRNA was measured using the one-step RT-PCR kit from Qiagen. The results were normalized to the mRNA level of actin.

Neuronal Culture. Dissociated neuronal cultures from rats (the hippocampus) at postnatal days 1 and 2 were prepared as described previously (Ghosh and Greenberg, 1995; Liao et al., 2005). Neurons were plated onto 35-mm Petri dishes at a density of 1×10^6 cells/dish. The age of cultured neurons was counted from the day of plating (day 1 in vitro), and the cultures at day 21 were used in our current studies.

Results

Morphine and Etorphine Induced Similar Maximum Phosphorylation of ERKs with Similar Kinetics. To determine the characteristics of ERK activation, we examined the time courses of morphine- and etorphine-induced ERK phosphorylation in HEK293 cells stably expressing MOR. The phosphorylation of ERKs peaked to 2.4 ± 0.2-fold ($p = 0.0065$) of the control 10 min after morphine treatment. Then, the stimulatory effect gradually declined, and phosphorylation returned to the basal level after 1 h of agonist incubation (Fig. 1A). We obtained similar results when etorphine was used as the agonist to activate MOR. The maximum phosphorylation of ERKs (2.3 ± 0.2-fold, $p = 0.0008$) appeared 10 min after etorphine incubation and returned to the basal level within 1 h (Fig. 1A). Because the maximal ERK phosphorylation after either morphine or etorphine treatment was observed at 10 min, the concentration-dependent studies were carried out 10 min after the initiation of agonist treatment. The immunoblotting data showed that the EC_{50} values of the two agonists were significantly different from each other: $3.0 \pm 2.2 \times 10^{-8}$ M for morphine and $3.2 \pm 1.7 \times 10^{-10}$ M for etorphine, but the maximum levels of ERK phosphorylation induced by the two agonists were not significantly different (2.5 ± 0.1-fold for morphine and 2.6 ± 0.2-fold for etorphine; Fig. 1B).

Etorphine, but Not Morphine, Preferred the β-Arrestin-Dependent Pathway in ERK Phosphorylation. Because the β-arrestin-dependent pathway is one of the two major pathways in the GPCR activation of ERKs, it was investigated in morphine- and etorphine-mediated ERK

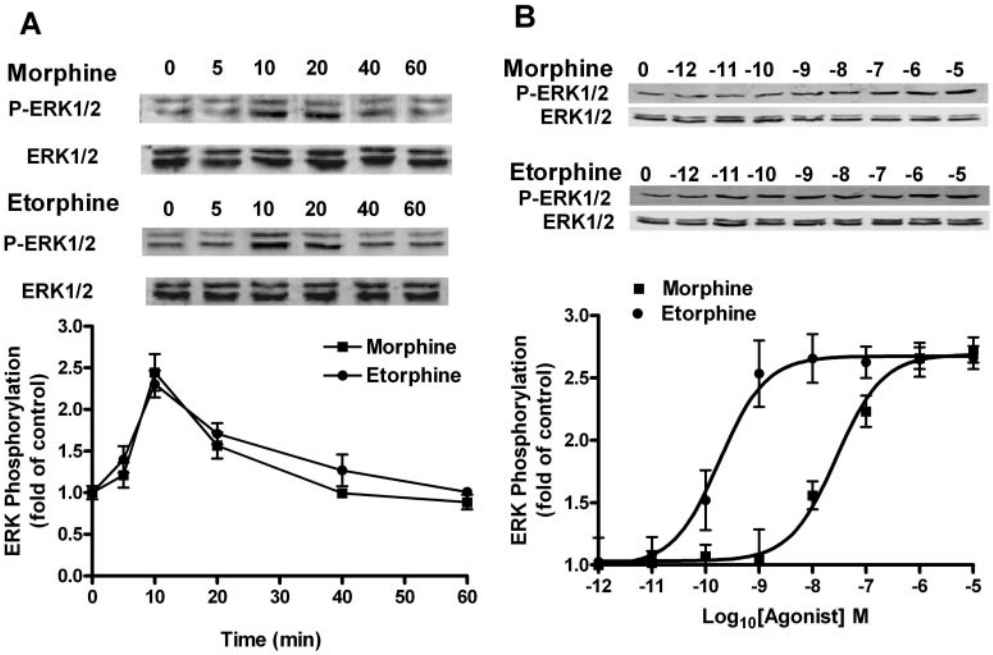

Fig. 1. Time- and concentration-dependent activation of ERK. A, HEK293 cells were prepared as described under *Materials and Methods*. The ERK phosphorylation was monitored by immunoblotting after 1 μM morphine or etorphine treatment at various time points. B, HEK293 cells were incubated with different concentrations of morphine and etorphine for 10 min. The phosphorylation of ERKs was assayed as described under *Materials and Methods*. *, $p < 0.05$, and experiments were repeated a minimum of three times.

phosphorylation. Because MOR was shown to have a higher affinity for β-arrestin2 than β-arrestin1 (Oakley et al., 2000), we focused on the roles of β-arrestin2 in our current studies. We were surprised to find that morphine- and etorphine-induced ERK phosphorylations were affected differently by the overexpression of β-arrestin2. With β-arrestin2 overexpressed, morphine-induced ERK phosphorylation was attenuated to 1.5 ± 0.3-fold compared with 2.3 ± 0.1-fold in the cells transfected with the vector control ($p = 0.0006$). In contrast, etorphine-induced ERK phosphorylation increased to 2.8 ± 0.2-fold compared with 2.3 ± 0.1-fold in the control cells ($p = 0.0026$) (Fig. 2A).

The differential effects of β-arrestins on morphine- and etorphine-induced ERK phosphorylation were confirmed in three types of MEF cells: MEF cells from wild-type mice, MEF cells from β-arrestin2 null mice (β-arrestin2$^{-/-}$), and MEF cells from β-arrestin1 and -2 null mice (β-arrestin1/2$^{-/-}$). These MEF cells were infected with adenovirus containing the MOR gene so as to transiently express MOR. The amount of adenovirus used to infect the MEF cells was controlled to express a similar level of MOR in the three cell types. [^{3}H]Diprenorphine binding assays revealed that the amount of MOR expressed was 0.5 ± 0.1 pmol/mg of protein without any significant difference in the amount of receptor expressed among the three cell lines. In the wild-type MEF cells, a 10-min incubation with both morphine and etorphine led to significant ERK phosphorylation, 1.6 ± 0.1-fold ($p = 0.0421$) and 1.7 ± 0.1-fold ($p = 0.0357$), respectively (Fig. 2B). In β-arrestin2$^{-/-}$ MEF cells, morphine induced significant ERK phosphorylation (1.8 ± 0.2-fold, $p = 0.0367$), but etorphine did not (1.1 ± 0.1-fold, $p = 0.2342$). However, because β-arrestin1 was present in the β-arrestin2$^{-/-}$MEF cells, we could not eliminate the possibility that the morphine-induced ERK phosphorylation was β-arrestin1-dependent. Therefore, we used β-arrestin1/2$^{-/-}$ MEF cells (Fig. 2B). Similar to the observations with β-arrestin2$^{-/-}$ MEF cells, morphine induced ERK phosphorylation in the β-arrestin1/2$^{-/-}$ MEF cells (1.7 ± 0.1-fold, $p = 0.0215$), whereas etorphine did not (1.1 ± 0.1-fold, $p = 0.347$; Fig. 2B). Therefore, etorphine-induced ERK phosphorylation was β-arrestin-dependent, whereas morphine-mediated ERK phosphorylation was β-arrestin-independent.

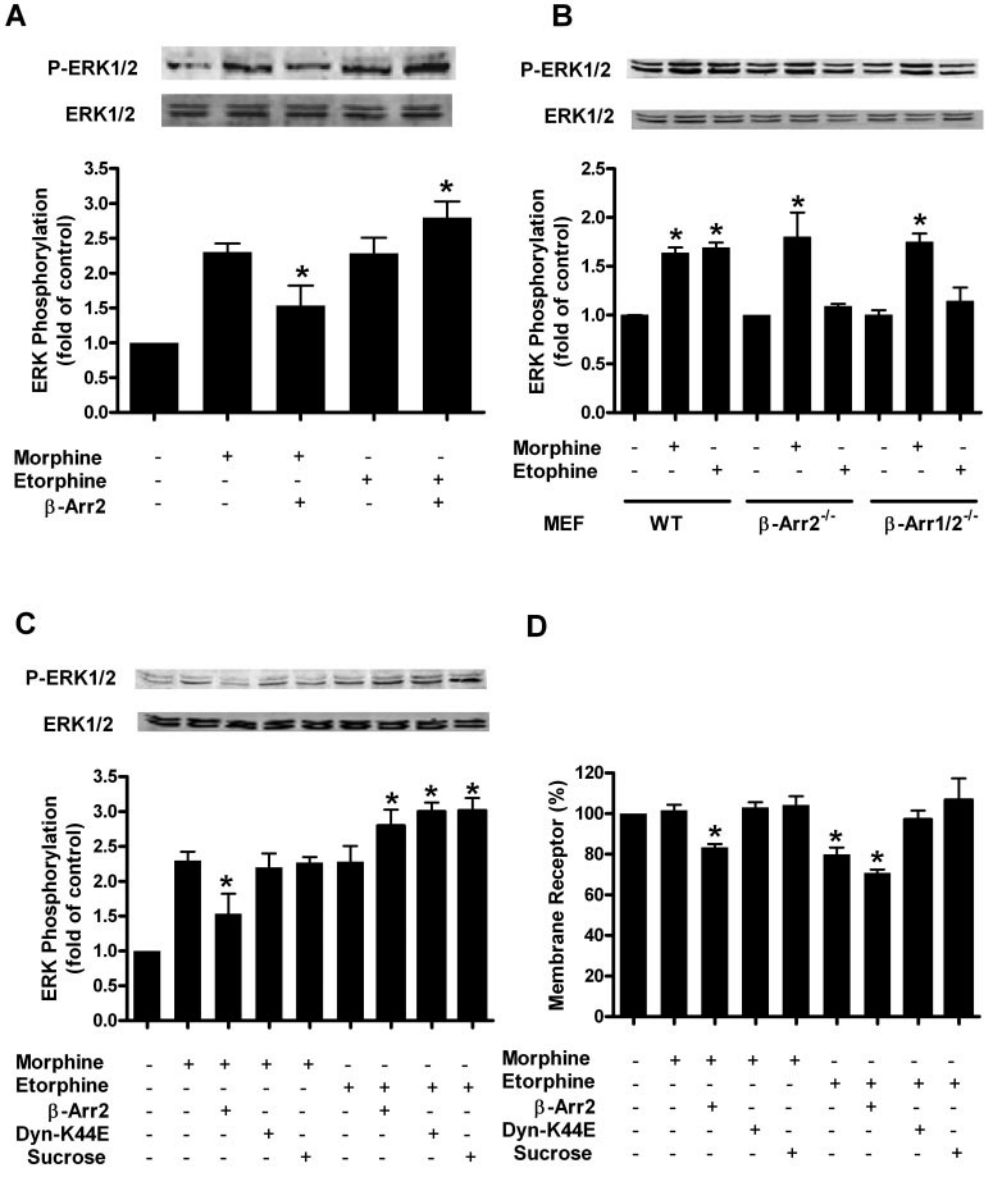

Fig. 2. Etorphine activated ERKs via β-arrestin-dependent pathway and independent of MOR internalization A, 1 μg of β-arrestin2 or control vector was transfected into HEK293 cells cultured in 35-mm dishes. After culturing in serum-free medium overnight, 1 μM agonists were added for 10 min. ERK phosphorylation then was measured. B, MEF cells were infected with adenovirus-containing MOR with HA epitope. Twenty-four hours after virus infection, the cells were cultured in a serum-free medium overnight. Cells then were incubated with 1 μM agonists for 10 min, and ERK phosphorylation was analyzed by immunoblotting. C, HEK293 cells were transfected with β-arrestin2, Dynamin-K44E, or control vector for 24 h. After culturing the cells in a serum-free medium overnight, 1 μM agonists were added for 10 min, and ERK phosphorylation was examined. In the sucrose group, 0.4 M sucrose was used to pretreat the cells 10 min before agonist incubation. D, to monitor receptor internalization, the HEK293 cells were treated as mentioned in C but without the serum-free treatment. The level of receptor internalization 10 min after agonist treatment was analyzed by FACS as described under *Materials and Methods*. *, $p < 0.05$, and experiments were repeated a minimum of three times.

Because β-arrestins are keys to agonist-induced GPCR internalization, we assessed the relation between ERK phosphorylation and β-arrestin-mediated receptor internalization by blocking the receptor internalization with the dominant-negative mutant of dynamin I, dynamin K44E, or with a hypertonic sucrose medium, 0.4 M sucrose. The possibility that a hypertonic medium could lead to ERK phosphorylation was eliminated before these experiments (data not shown). Consistent with previous reports, both dynamin K44E and 0.4 M sucrose blocked the agonist-induced receptor internalization (Fig. 2D). Such attenuation in receptor internalization did not affect the morphine-induced ERK phosphorylation but enhanced etorphine-induced ERK phosphorylation significantly (Fig. 2C). Dynamin-K44E and sucrose increased the etorphine-induced ERK phosphorylation from 2.3 ± 0.1- to 3.0 ± 0.1-fold ($p = 0.0004$) and 3.0 ± 0.2-fold ($p = 0.0016$), respectively. These data and those with MEF cells suggest that the etorphine-induced ERK phosphorylation required β-arrestin2 independent of the β-arrestin2's activity in mediating agonist-induced receptor internalization.

Morphine, but Not Etorphine, Preferred to Use the G Protein-Dependent Pathway to Activate ERKs. The decrease of morphine-induced ERK phosphorylation in HEK293 cells overexpressing β-arrestin2 and the ability of morphine to activate ERKs in β-arrestin1/2$^{-/-}$ MEF cells suggest that morphine-induced ERK phosphorylation is not mediated by the β-arrestin-dependent pathway. As one of the two major pathways mediating ERK phosphorylation, G-protein-dependent pathway is the likely candidate pathway in mediating morphine-induced ERK phosphorylation. Because PKC is one of the normal intermediates within G-protein-dependent pathway (Ahn et al., 2004), and MOR activation of ERKs has been reported to be inhibited by a PKC inhibitor (Belcheva et al., 2005), we used a selective PKC inhibitor, Ro-31-8425. In HEK293 cells pretreated with 1 μM Ro-31-8425, morphine-induced ERK phosphorylation decreased to 1.3 ± 0.1-fold from the 2.3 ± 0.1-fold observed in control cells ($p = 0.0003$) (Fig. 3A). In contrast, this PKC inhibitor did not significantly affect either the kinetics or the magnitude of etorphine-induced ERK phosphorylation, which were 2.5 ± 0.1- and 2.4 ± 0.1-fold in the absence and presence of the PKC inhibitor, respectively ($p = 0.7443$) (Fig. 3B).

The role of PKC on morphine-induced ERK phosphorylation was further demonstrated by examining the enzymatic activity of PKC. PKC activity was assessed by determining the amount of phosphorylated PKC substrates. We observed

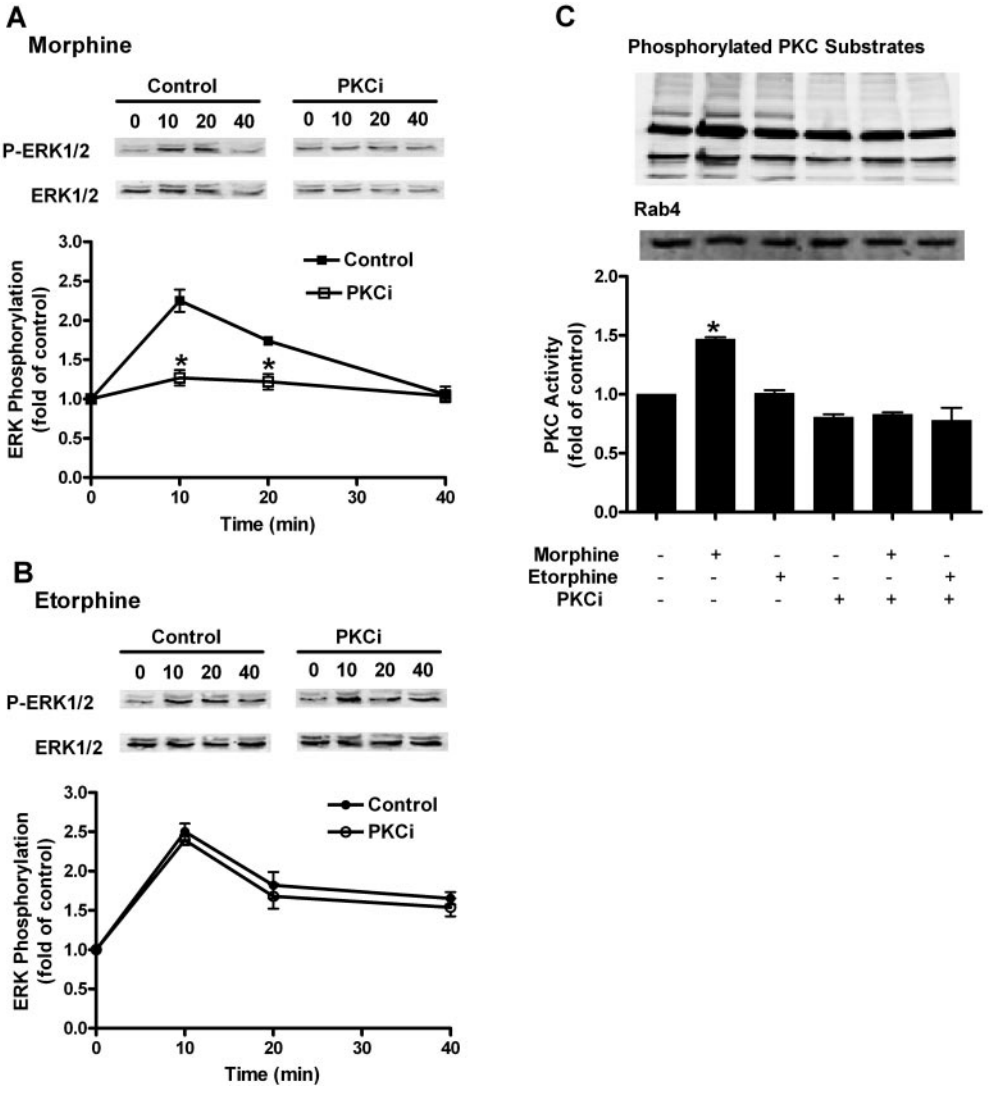

Fig. 3. Morphine activated ERKs via G protein-dependent pathway (A and B), 1 μM PKC inhibitor Ro-31-8425, or DMSO (control) was used to pretreat the cells for 1.5 h, and then 1 μM morphine (A) or etorphine (B) was added for various times as indicated. The samples were subjected to immunoblotting for ERK phosphorylation. C, HEK293 cells were treated with 1 μM PKC inhibitor Ro-31-8425 or DMSO (control) for 1.5 h followed by a 10-min treatment of 1 μM agonists. PKC activities were determined with immunoblotting using antibody specific for PKC phosphorylated substrates. *, $p < 0.05$, and experiments were repeated a minimum of three times.

that etorphine treatment did not increase the amount of phosphorylated PKC substrates, whereas the amount of phosphorylated PKC substrates increased significantly after morphine treatment (1.5 ± 0.5-fold, $p = 0.0167$) (Fig. 3C). These results suggest that morphine-induced ERK phosphorylation requires the activation of PKC and therefore is G-protein-dependent.

Etorphine- and Morphine-Activated ERKs Had Different Cellular Locations. With other GPCRs, G protein-dependent and β-arrestin-dependent activation of ERKs resulted in different cellular location of the activated enzymes. In general, β-arrestin-activated ERKs were retained in the cytosol, whereas the G protein-activated ERKs translocated to the nucleus (Ahn et al., 2004). Whether MOR-mediated ERK phosphorylation will follow this cellular translocation scenario remains to be demonstrated.

To investigate the cellular location of phosphorylated ERKs, nucleus fractions were separated after agonist treatment as described in *Materials and Methods*. Phosphorylated ERKs in nucleus fractions isolated from etorphine-treated cells was 2.2 ± 0.3-fold of that in nucleus fractions isolated from control cells ($p = 0.0037$; Fig. 4A). In contrast, 1 μM morphine did not lead to a significant nuclear translocation of phosphorylated ERKs at either 10 (1.1 ± 0.2-fold, $p = 0.4131$) or 20 min (1.1 ± 0.2-fold, $p = 0.6173$) after agonist treatment (Fig. 4A). Successful nucleus isolation from cytosol fractions could be demonstrated by the absence of β-actin and Rab4 immunoreactivities in the isolated nucleus fraction and the absence of histone 3 immunoreactivities in the isolated cytosol fractions (Fig. 4A). Concentration-dependent studies with the two agonists illustrated the same phenomena. Morphine did not induce ERK translocation even at 10 μM ($p = 0.8679$), whereas etorphine-induced ERK translocation could be observed at 10 nM (1.7 ± 0.1, $p = 0.0424$) (Fig. 4B). Although receptor internalization was not required for etorphine-induced ERK phosphorylation, the involvement of internalized MOR in the nuclear translocation of phosphorylated ERKs was still possible. Hence, β-arrestin2 and dynamin-K44E were transiently transfected into HEK293 cells. Both proteins enhanced the amount of phosphorylated ERKs observed in nucleus fractions from a 1.5 ± 0.1-fold increase from the basal level in control cells to 1.9 ± 0.1-fold increase ($p = 0.0296$) in cells transfected with β-arrestin and a 1.9 ± 0.2-fold increase ($p = 0.0393$) in dynamin K44E-transfected cells (Fig. 4C). The levels of increase in phosphorylated ERKs within the nucleus fractions paralleled the increase in ERK phosphorylation when β-arrestin2 and dynamin K44E were overexpressed (Fig. 2C). These results suggest that both the

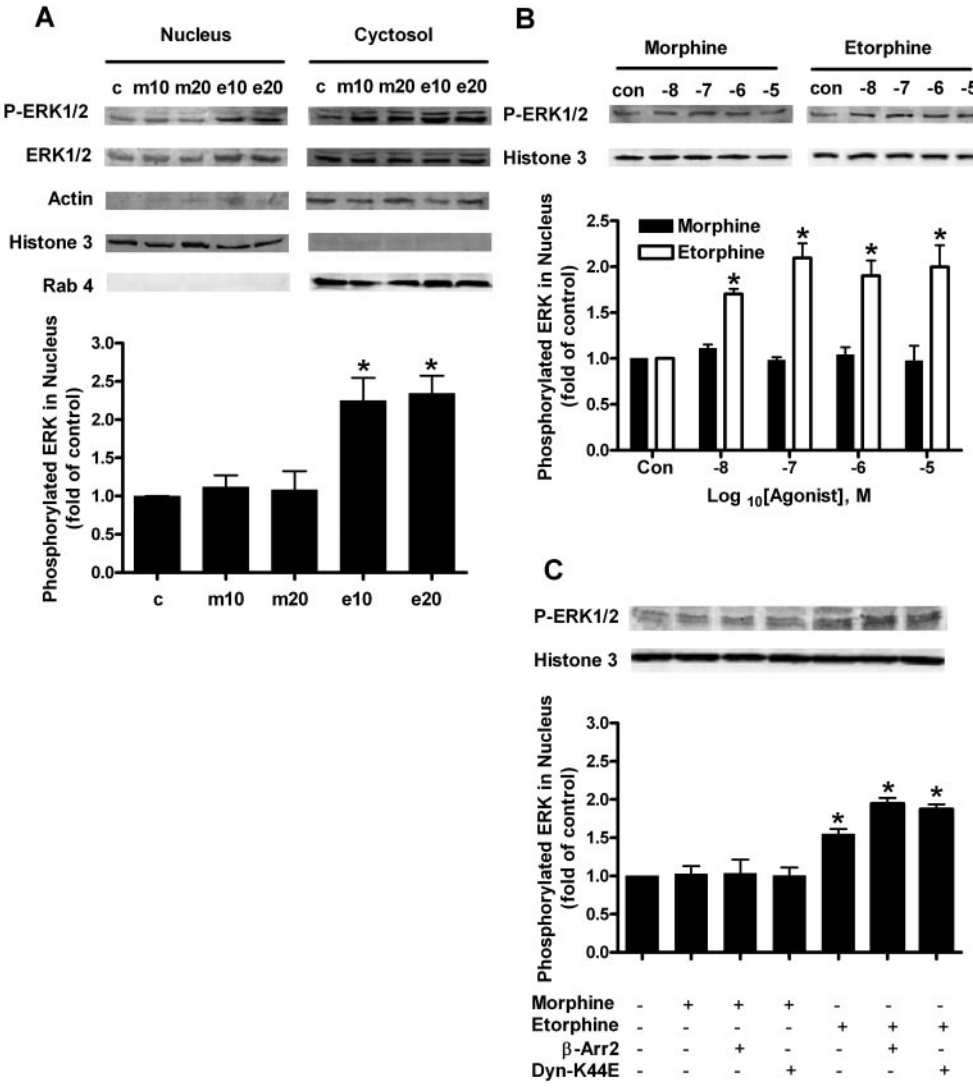

Fig. 4. Differential cellular locations of activated ERKs. A, 1 μM agonists were added to the cells for 10 or 20 min, and the cytoplasm and nucleus fractions were separated as described under *Materials and Methods*. Immunoblotting was used to monitor the phosphorylated ERK level, and antibodies to β-actin, Rab4, and Histone3 were used to examine the success of separating nucleus from cytoplasm. m10 and m20 represents phosphorylated ERKs level 10 and 20 min after morphine treatment, respectively; e10 and e20 represents phosphorylated ERK levels 10 and 20 min after etorphine treatment, respectively. B, different concentrations of agonists were added for 10 min. The level of phosphorylated ERKs in the nucleus was measured as in A. C, HEK293 cells were transfected with 1 μg of β-arrestin2 or Dynamin K44E or vector (control). Then 1 μM agonists were added for 10 min. After nucleus extraction, the level of phosphorylated ERKs in the nucleus was determined by immunoblotting. *, $p < 0.05$, and experiments were repeated a minimum of three times.

etorphine-induced ERK phosphorylation and subsequent ERK translocation are separate events from agonist-induced MOR internalization. In addition, because morphine-activated ERKs did not translocate into the nucleus, the amount of phosphorylated ERKs in the nucleus remained similar to the basal level (Fig. 4C), even when the ERK phosphorylation induced by morphine was decreased in cells overexpressing the β-arrestin2 (Fig. 2A).

Etorphine-Activated ERKs Translocated into the Nucleus and Resulted in Elk-1 Activation and Transcription Increase of GRK2 and β-Arrestin2. Transcription factor Elk-1 localizes predominantly in the nucleus (Janknecht et al., 1994) and is one of the important substrates for the translocated ERKs (Gille et al., 1995; Aplin et al., 2001). To demonstrate the actual nuclear translocation of ERKs after MOR activation, the Elk-1-driven luciferase reporter system was used (Shoda et al., 2001). A 10% FBS treatment was used as the positive control. Treatment of HEK293 cells with FBS increased the activity of Elk-1 by 2.1 ± 0.1 fold ($p = 0.0039$) from the basal level, which were serum-free. A 12-h etorphine treatment increased Elk-1 activity (1.5 ± 0.1-fold; $p = 0.0004$). Under similar conditions, morphine treatment did not result in a significant increase in Elk-1 activity (Fig. 5A). Because 12 h were needed to allow the expression of luciferase proteins, the observed increase in Elk-1 activity could have resulted from the prolonged agonist treatment. This scenario was eliminated by adding the MOR antagonist naloxone to the medium 10 min after initiation of the agonist treatment. Under this paradigm, etorphine remained able to induce the increase of Elk-1 activity (1.5 ± 0.1-fold; $p = 0.0045$), whereas morphine could not (1.1 ± 0.1-fold; $p = 0.3345$) (Fig. 5A). In addition, the increase in Elk-1 activity was shown to be a direct result of the ERK activation. When the HEK293 cells were pretreated with the selective mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD98059 (Dudley et al., 1995), the etorphine-induced increase in Elk-1 activity was blocked completely (Fig. 5B). In addition, transfection of β-arrestin2 (1.7 ± 0.1-fold, $p = 0.0175$) or Dynamin K44E (1.7 ± 0.1-fold, $p = 0.0201$) further increased etorphine-induced Elk-1 activity significantly (Fig. 5C), which correlates with the higher increase in total ERK phosphorylation and the higher amount of phosphorylated ERKs in the nucleus (Figs. 2C and 4C).

Because Elk-1 initiates transcription of many genes (Cavigelli et al., 1995; El-Dahr et al., 1998; Stein et al., 1998), and etorphine was reported to induce the GRK2 and β-arrestin2 expression in animals (Narita et al., 2006), the observed translocation of ERKs probably resulted in the increased GRK2 and β-arrestin2 transcript levels. Hence, after the HEK293 cells were treated with agonists for 12 h, RT-PCR was used to determine whether the transcriptions of GRK2 and β-arrestin2 were altered. As expected, GRK2 mRNA level was observed to increase by 1.9 ± 0.1-fold ($p = 0.0146$), whereas the β-arrestin2 mRNA level was increased to 1.3 ± 0.1-fold ($p = 0.0229$) after etorphine treatment (Fig. 5D).

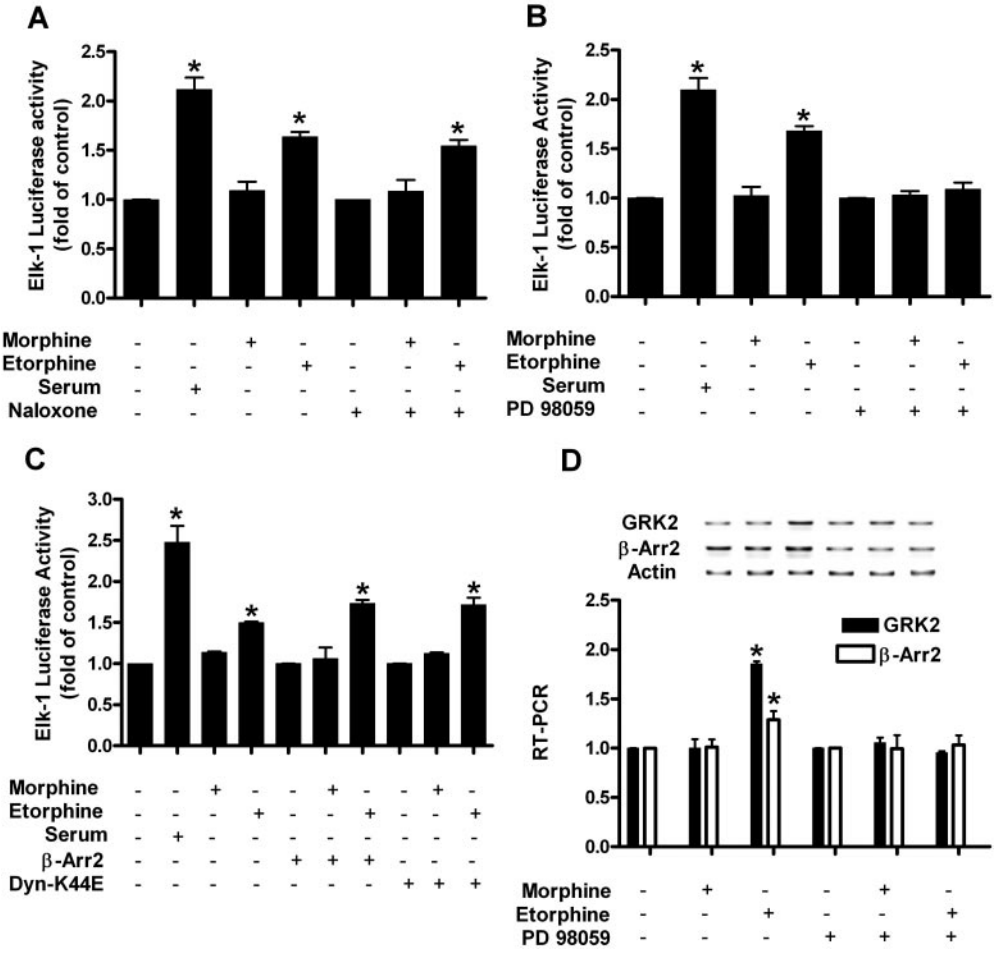

Fig. 5. Etorphine induced an increase in Elk-1 activity and gene transcription. A, HEK293 cells were transfected with the Elk-1 luciferase reporter system. Then the cells were incubated with 10% serum or 1 μM agonists for 12 h or 10 min of agonists followed by 12 h of 10 μM naloxone treatment. Elk-1 luciferase activity was monitored as described under *Materials and Methods*. B, cells were transfected with the Elk-1 luciferase reporter system and pretreated with a 2-h treatment of 40 μM PD98059 or DMSO (control cells). Agonists (1 μM) were then added for 12 h, and Elk-1 luciferase activity was monitored as in A. C, HEK293 cells were transfected with the Elk-1 luciferase reporter system plus vector or β-arrestin2 or Dynamin K44E. Cells were cultured with 1 μM agonists for 12 h, and Elk-1 luciferase activity was measured. D, 1 μM agonists were added to the medium for 12 h after the 2-h 40 μM PD98059 or DMSO pretreatment. The total RNA was extracted, and the mRNA levels of GRK2 and β-arrestin2 were examined as described under *Materials and Methods*. *, $p < 0.05$, and experiments were repeated a minimum of three times.

Consistent with the data on nuclear translocation and Elk-1 activity, morphine did not result in the transcriptional increase of either GRK2 (1.0 ± 0.1-fold, $p = 0.4257$) or β-arrestin2 (1.0 ± 0.1-fold, $p = 0.3342$). Again, the increased GRK2 and β-arrestin2 transcriptions were demonstrated to be consequences of ERK activation for pretreatment of HEK293 cells with PD98059 attenuated these increases (Fig. 5D). These studies clearly indicate that ERKs activated by MOR via the β-arrestin-dependent pathway translocate to the nucleus.

Morphine-Activated ERKs Stayed in Cytosol and Activated 90RSK and CREB. Because morphine-induced phosphorylation of ERKs did not result in the nuclear translocation of the phosphorylated enzymes, we investigated whether such phosphorylated ERKs could phosphorylate cytosolic substrates. 90RSK, a cytosolic protein kinase, is normally one of the common substrates of activated ERKs (Frodin and Gammeltoft, 1999; Sheffler et al., 2006). After a 10-min morphine treatment, the phosphorylation level of 90RSK increased to 1.9 ± 0.2-fold of the basal level ($p = 0.0045$). However, when MOR was activated by etorphine, a similar increase in the 90RSK phosphorylation level was not observed (1.0 ± 0.1-fold, $p = 0.8745$) (Fig. 6A). To demonstrate that 90RSK is the substrate of morphine-activated ERK signal cascades, the PKC inhibitor Ro-31-8425 and the MEK inhibitor PD98059 were used to pretreat the cells. As expected, the two inhibitors attenuated the phosphorylation of 90RSK induced by morphine (Fig. 6A).

Several groups have reported that the transcriptional factor CREB serves as the substrate of activated and nucleus-translocated 90RSK (Frodin and Gammeltoft, 1999; Sheffler et al., 2006). If ERKs activated by MOR via the G protein-dependent pathway controlled transcriptions through 90RSK, an increase in CREB activities should be observed after morphine treatment. Thus, we used a luciferase reporter system to determine the CREB activity. As expected, morphine, but not etorphine, induced an activation of CREB reporter luciferase activities (1.3 ± 0.1-fold; $p = 0.0067$) (Fig. 6B). To confirm the results, several control experiments were carried out. In one group, 10 μM naloxone was added 10 min after the initiation of agonist incubations to eliminate any effects stemming from prolonged agonist treatment. In two other groups, a PKC inhibitor or an MEK inhibitor was used to pretreat the cells to determine whether CREB activation was through a MOR-PKC-ERK pathway. The addition of naloxone 10 min after the initiation of morphine treatment could not block the agonist-induced CREB activation (1.3 ± 0.1-fold, $p = 0.0162$), whereas the PKC inhibitor and the MEK inhibitor attenuated the agonist-induced CREB activation significantly (1.0 ± 0.1-fold, $p = 0.1768$, and 1.0 ± 0.2-fold, $p = 0.9590$, respectively).

Morphine- and Etorphine-Induced ERK Phosphorylation in SHSY5Y Cells also Used Different Pathways. The distinct pathways selected by the two agonists could be due to the relatively high level of MOR heterologously expressed in the HEK293 cells. Thus, we used a human neuroblastoma SHSY5Y cell line, which expresses both MOR and δ-opioid receptor (DOR) endogenously, to examine whether the observed pathway selectivity is restricted to cells with high receptor levels. Morphine and etorphine again were used to treat the cells and ERK phosphorylation was monitored via immunoblotting analysis. As in the case of the HEK293 cells, the two agonists could activate ERKs to similar levels in the SHSY5Y cells (morphine, 2.0 ± 0.1-fold, $p = 0.0006$, and etorphine, 2.0 ± 0.1-fold, $p = 0.0008$) and both agonist-mediated ERK phosphorylations could be attenuated by a MEK inhibitor (Fig. 7A). Because both MOR and DOR are endogenously expressed in the SHSY5Y cells, we used the MOR-specific antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) and the DOR-specific antagonist H-Tyr-Ticψ[CH2NH]-Phe-Phe-OH (TIPPψ) to distinguish the

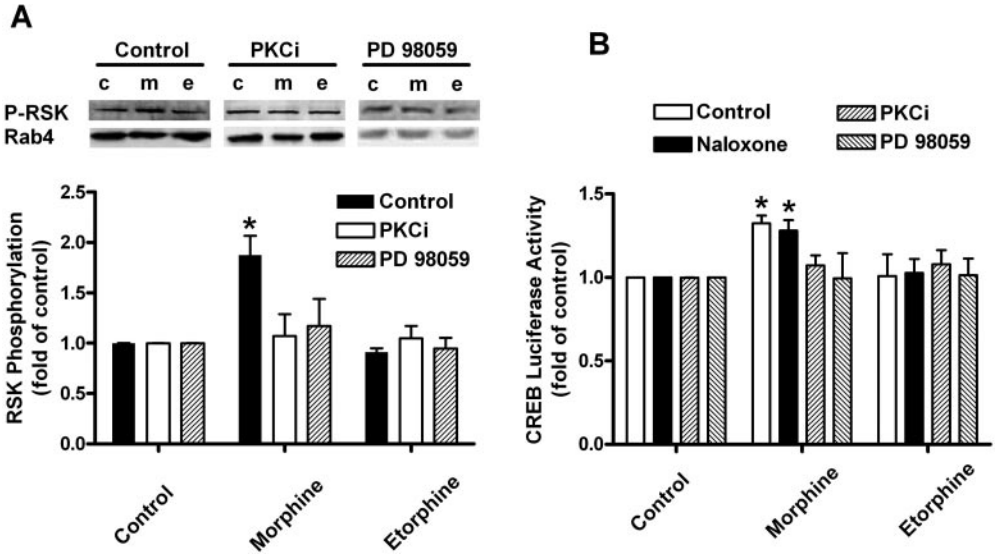

Fig. 6. Morphine induced 90RSK and CREB activation. A, HEK293 cells were cultured as described previously and were pretreated with 1 μM PKC inhibitor Ro-31-8425 for 1.5 h or with 40 μM PD98059 for 2 h before 1 μM morphine or etorphine was added to the cells. Then the immunoreactivity of phosphorylated 90RSK was monitored by immunoblotting and normalized to the amount of Rab4. In each group, "c" stands for no drug treatment, "m" stands for morphine treatment, and "e" stands for etorphine treatment. B, CREB luciferase reporter system was transfected into HEK293 cells. PKC inhibitor Ro-31-8425 and 40 μM PD98059 (1 μM each) were added 1.5 and 2 h, respectively, before 1 μM morphine or etorphine treatment. Naloxone (10 μM) was added 10 min after agonist treatment. After a 12-h incubation of drugs, CREB luciferase activity was monitored as mentioned under *Materials and Methods*. *, $p < 0.05$, and experiments were repeated a minimum of three times.

receptor type involved in the ERK phosphorylation. Pretreatment with 10 μM CTOP blocked both morphine- and etorphine-induced ERK phosphorylation, whereas TIPPψ did not have a significant effect on the actions of agonists (Fig. 7B), suggesting that the ERK phosphorylation under this paradigm was mainly mediated by the interaction between agonists and MOR.

Similar to the observations with HEK293 cells, there was agonist selectivity toward pathways for ERK phosphorylation and cellular locations of phosphorylated ERKs in the SHSY5Y cells. Pretreatment of the SHSY5Y cells with the PKC inhibitor Ro-31-8425 resulted in the attenuation of morphine-induced but not etorphine-induced ERK phosphorylation (Fig. 7A). In addition, morphine-induced but not etorphine-induced ERK phosphorylation resulted in an increase in the phosphorylation of 90RSK (1.7 $\pm$ 0.2-fold, $p = 0.0134$) (Fig. 7D). The nuclear translocation of etorphine-induced but not morphine-induced phosphorylated ERKs was demonstrated by the increases in GRK2 (1.7 $\pm$ 0.1-fold, $p = 0.0234$) and β-arrestin2 (1.4 $\pm$ 0.1-fold, $p = 0.0156$) transcriptions in SHSY5Y cells in the presence of etorphine but not in the presence of morphine (Fig. 7C). Therefore, even at a low receptor level expressed endogenously, MOR agonists select distinct signaling pathways in ERK phosphorylation, and the subsequent cellular locations of the phosphorylated enzymes are also agonist-selective.

Morphine- and Etorphine-Induced ERK Phosphorylation in Primary Neuronal Culture of Rat Hippocampus also Used Different Pathways. To demonstrate the existence of similar phenomena in MOR-expressing neurons, we monitored ERK phosphorylation in the primary neuronal culture of rat hippocampus. We used the MOR-specific antagonist CTOP and the DOR-specific antagonist TIPPψ to eliminate the interaction between the two types of receptors in this primary neuronal culture. CTOP attenuated ERK phosphorylation induced by both morphine and etorphine, whereas TIPPψ had little effect on the phosphorylation of the kinases (Fig. 8B). The existence of agonist-selective signaling in the primary culture was demonstrated by using a PKC inhibitor, determining the phosphorylation of 90RSK, and measuring the mRNA levels of GRK2 and β-arrestin2. Pretreating the primary culture with the PKC inhibitor Ro-31-8425 blocked the ERK phosphorylation induced by morphine (1.2 $\pm$ 0.3-fold, $p = 0.5158$) but did not affect the etorphine-induced ERK phosphorylation (2.0 $\pm$ 0.1-fold, $p = 0.0084$) (Fig. 8A). When the mRNA levels of the GRK2 and β-arrestin2 were measured after 12-h agonist treatment, only the neurons treated by etorphine had significant increases in

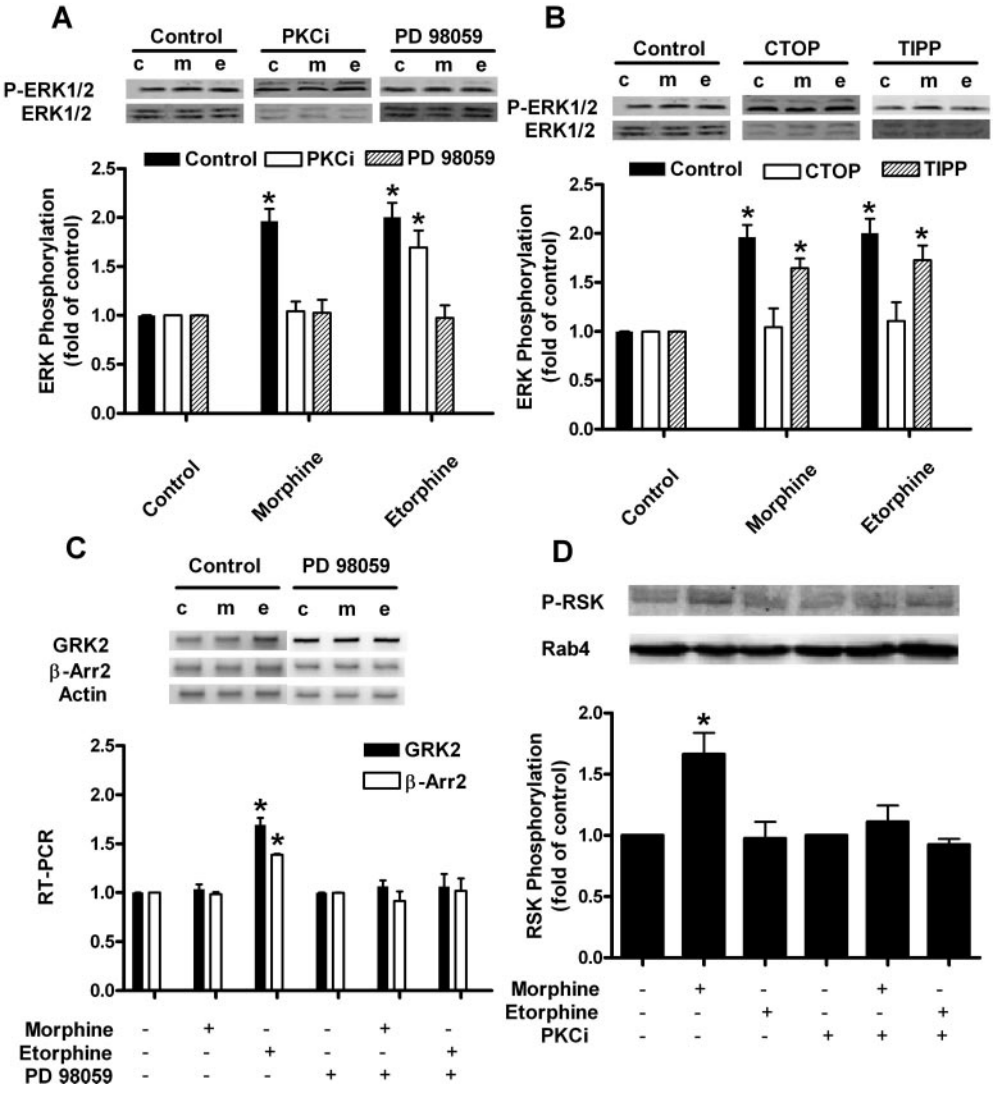

Fig. 7. Agonist-selective ERK phosphorylation in human neuroblastoma SHSY5Y cells. A, SHSY5Y cells were treated with 1 μM PKC inhibitor Ro-31-8425 for 1.5 h or with 40 μM PD98059 or DMSO for 2 h. Then ERK phosphorylations were determined after 10 min of 1 μM morphine or 10 nM etorphine treatment. In each group, "c" stands for no drug treatment, "m" stands for morphine treatment, and "e" stands for etorphine treatment. B, SHSY5Y cells were treated with 10 μM CTOP or 10 μM TIPPψ for 10 min. Then ERK phosphorylations were determined after 10-min 1 μM morphine or 10 nM etorphine treatment. In each group, "c" stands for no drug treatment, "m" stands for morphine treatment, and "e" stands for etorphine treatment. C, SHSY5Y cells were treated with 40 μM PD98059 or DMSO for 2 h. Then the total RNA was extracted after 12 h of 1 μM morphine or 10 nM etorphine treatment. The transcription levels of GRK2 and β-arrestin2 were examined as described under *Materials and Methods*. D, SHSY5Y cells were treated with 1 μM PKC inhibitor Ro-31-8425 or DMSO for 1.5 h. Then 90RSK activities were determined after 10 min of 1 μM morphine or 10 nM etorphine treatment. $*$, $p < 0.05$, and experiments were repeated a minimum of three times.

these two transcripts levels (1.7 ± 0.1-fold, $p = 0.0334$, for GRK2, and 1.5 ± 0.1-fold, $p = 0.0176$, for β-arrestin2) (Fig. 8C). At the same time, the phosphorylation of 90RSK was shown to be consistent with our observations with the HEK293 and SHSY5Y cells. In these primary hippocampal neuronal cultures, morphine-induced but not etorphine-induced ERK phosphorylation resulted in an increase in 90RSK phosphorylation (1.7 ± 0.2-fold, $p = 0.0257$) (Fig. 8D). Therefore, agonist selectivity for pathways in ERK phosphorylation and cellular locations of phosphorylated ERKs exist in primary neuronal culture of rat hippocampus, as in the case of the two cell models that we investigated.

Other Opioid Agonists also Exhibited Pathway Selectivity. To obtain broader understanding of agonist-selective pathway-dependent ERK phosphorylation, we tested additional clinical relevant MOR agonists such as methadone and fentanyl. The four agonists equally activated ERKs at concentrations that resulted in maximal receptor occupation but induced the nuclear translocation of phosphorylated ERKs differentially (Fig. 9A). Similar to etorphine, fentanyl increased the amount of phosphorylated ERKs in the nucleus to 1.5 ± 0.1-fold ($p = 0.0274$) of the basal level, whereas morphine (1.0 ± 0.1-fold, $p = 0.4234$) and methadone (1.2 ± 0.2-fold, $p = 0.2154$) could not induce significant changes in the phosphorylated ERK level within the nucleus (Fig. 9B). Likewise, the Elk-1 activity increased significantly after etorphine (1.8 ± 0.2-fold, $p = 0.0127$) and fentanyl (1.6 ± 0.2-fold, $p = 0.0285$) treatment, but no such increase was observed after morphine or methadone incubation (Fig. 9C). Morphine and methadone stimulated PKC activity to 1.4 ± 0.1-fold ($p = 0.0084$) and 1.3 ± 0.1-fold ($p = 0.0156$), respectively, whereas etorphine and fentanyl did not (Fig. 9D). Thus, the four agonists were divided into two groups. Morphine and methadone induced phosphorylation of ERKs via a PKC-dependent pathway, with the phosphorylated ERKs remaining in the cytosol, whereas etorphine and fentanyl used β-arrestin-dependent pathway and resulted in nuclear translocation of phosphorylated ERKs.

Discussion

The current studies demonstrate that MOR agonists select for either the G protein-dependent or the β-arrestin-dependent pathway in their activation of ERKs. This selectivity was observed with a heterologous expression cell system (HEK293 cells) expressing high levels of the receptor, a neu-

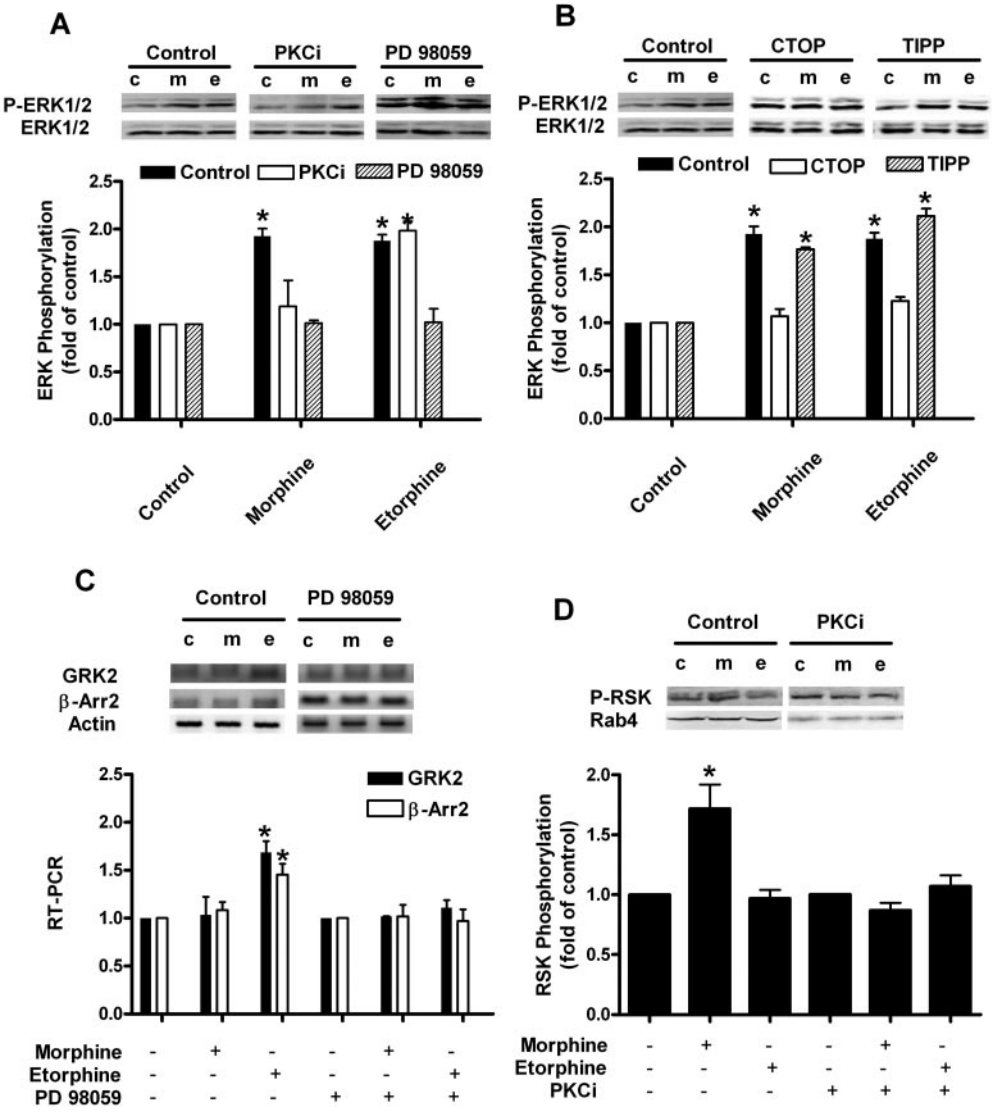

Fig. 8. Agonist-selective ERK phosphorylation in primary neuronal culture of rat hippocampus. The primary culture of rat hippocampal neurons was prepared as described under *Materials and Methods*. A, primary cultures of hippocampal neurons were treated with 1 μM PKC inhibitor Ro-31-8425 for 1.5 h or 40 μM PD98059 or DMSO for 2 h. ERK phosphorylations then were determined after 10 min of 1 μM morphine or 10 nM etorphine treatment. In each group, "c" stands for no drug treatment, "m" stands for morphine treatment, and "e" stands for etorphine treatment. B, primary cultures were treated with 10 μM CTOP or 10 μM TIPPψ for 10 min. Then ERK phosphorylations were determined after 10-min 1 μM morphine or 10 nM etorphine treatment. In each group, "c" stands for no drug treatment, "m" stands for morphine treatment, and "e" stands for etorphine treatment. C, primary cultures were treated with 40 μM PD98059 or DMSO for 2 h. The total RNA then was extracted after 12 h of 1 μM morphine or 10 nM etorphine treatment. The transcription levels of GRK2 and β-arrestin2 were examined as described under *Materials and Methods*. D, primary culture were treated with 1 μM PKC inhibitor Ro-31-8425 or DMSO for 1.5 h. Then 90RSK activities were determined after 10 min of 1 μM morphine or 10 nM etorphine treatment. *, $p < 0.05$, and experiments were repeated a minimum of three times.

roblastoma cell line (SHSY5Y cells) expressing low levels of the receptor endogenously and a primary culture system of rat hippocampal neurons. Thus, the agonist-dependent pathway selectivity was not a consequence of receptor reserves or specific cellular backgrounds and should reflect the in vivo conditions. In our studies, both etorphine- and morphine-induced ERK phosphorylation exhibited similar time courses and maximal increases. Blockade of the G protein-dependent pathway with a PKC inhibitor or elimination of the β-arrestin-dependent pathway in the MEF cells resulted in the attenuation of one agonist's activities without altering either the time course or maximal response of the other agonist. Clearly, the agonists tested in our current studies did not seem to use both the G protein-dependent and β-arrestin-dependent pathways in their activation of ERKs. This is a distinct difference from reported observations that other GPCRs could use both pathways (Azzi et al., 2003; Kohout et al., 2004; Shenoy et al., 2006). The current studies, however, could not preclude the possibility that other MOR agonists could activate both pathways. With the distinct differences in DAMGO's and morphine's ability to induce receptor internalization and β-arrestin recruitment, and because the PKC inhibitor bisindolylmaleimide I could inhibit both the morphine- and DAMGO-induced ERK activation, an agonist such as DAMGO possibly could activate both pathways (Belcheva et al., 2005). Whether DAMGO could activate ERKs under the current paradigm and model systems via multiple pathways remains to be demonstrated. Nevertheless, our observation that morphine uses the G protein-dependent pathway and etorphine uses the β-arrestin-dependent pathway to activate ERKs provides a clear demonstration of the agonist-selective signaling theory, which suggests that the efficacies of a particular agonist on different signaling pathways can be different (Urban et al., 2007).

The nuclear translocation of ERKs is normally regulated by the phosphorylation status of MEK1 (Whitmarsh and

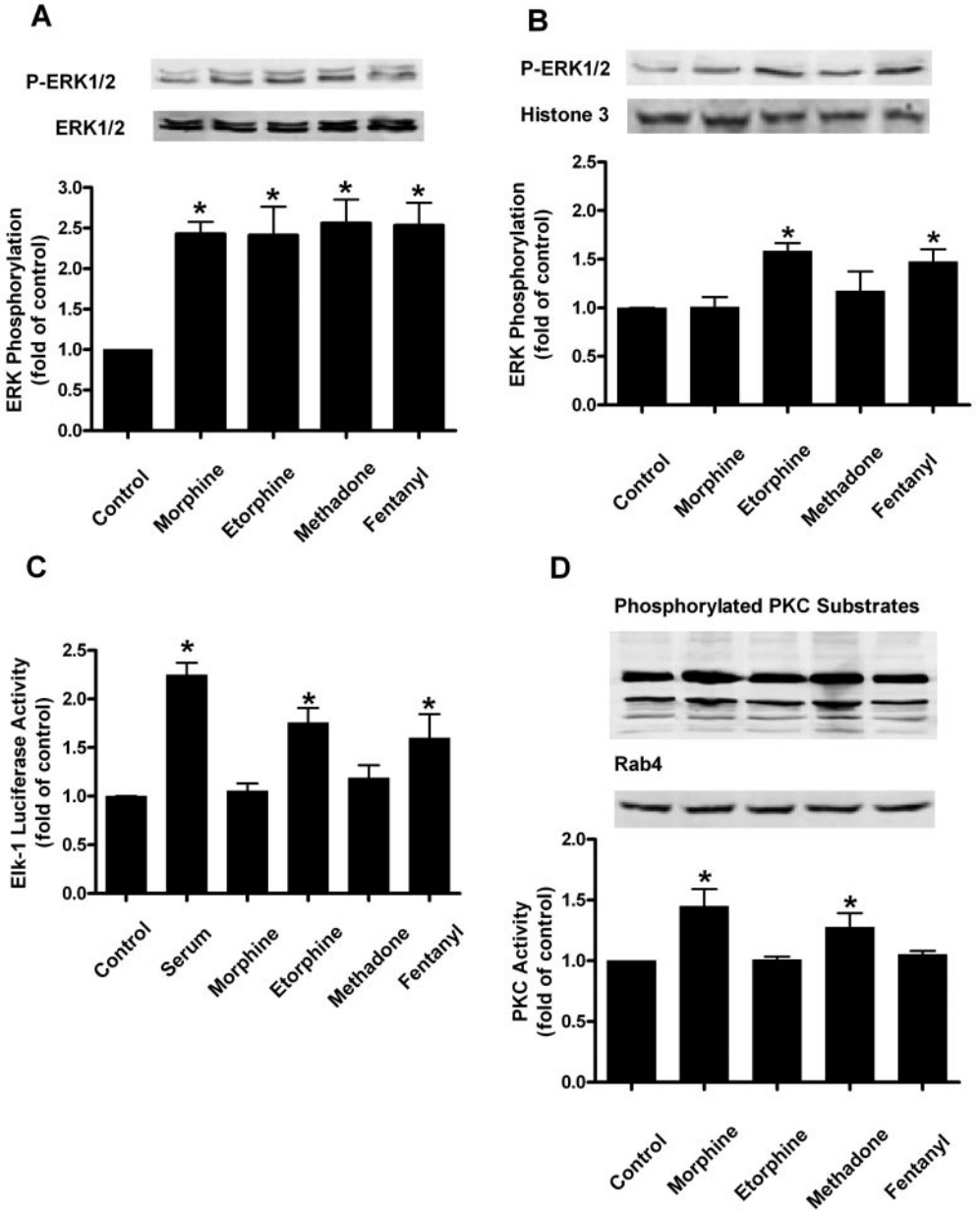

Fig. 9. ERK phosphorylation and cellular location of pERKs induced by opioid agonists. A, HEK293 cells were treated with morphine (1 μM), etorphine (1 μM), methadone (10 μM), or fentanyl (1 μM) for 10 min. The cells were lysed, and the levels of phosphorylated ERKs were determined by immunoblotting as described under *Materials and Methods*. B, HEK293 cells were treated with the four agonists, and the nucleus fractions were isolated to determine the amount of phosphorylated ERKs in the nucleus by immunoblotting. C, HEK293 cells were transfected with the Elk-1 luciferase report system and incubated with 10% serum, morphine (1 μM), etorphine (1 μM), methadone (10 μM), or fentanyl (1 μM) for 12 h. Elk-1 activity was examined as described under *Materials and Methods*. D, HEK293 cells were incubated with the same concentration of agonists as in A for 10 min. The PKC activity was determined by measuring the amount of PKC phosphorylated substrates by immunoblotting. *, $p < 0.05$, and experiments were repeated a minimum of three times.

Davis, 1999). MEK1 binds to ERKs and prevents nuclear translocation of ERKs by its nucleus export signal. Phosphorylation of MEK1 leads to the activation of ERKs and the dissociation of MEK1-ERK complexes, which results in the subsequent nuclear translocation of activated ERKs. In several G_q-coupled GPCR systems, the G protein-dependent pathway activates ERKs through PKC, and the activated ERKs translocate into the nucleus, whereas the β-arrestin functions as a scaffold for both MEK1 and ERKs, thereby preventing the nuclear translocation of β-arrestin-activated ERKs (Tohgo et al., 2002; Shenoy and Lefkowitz, 2005). In addition, the prevention of the nuclear translocation of β-arrestin-activated ERKs is related to the interaction between receptor and β-arrestin. With the reduction in receptor-β-arrestin interaction allowing the recycling of the internalized receptor, a certain amount of activated ERKs can translocate into the nucleus (Tohgo et al., 2003). In this scenario, nuclear translocation of the activated ERKs probably could occur even with the β-arrestin-dependent activation.

In our studies, the β-arrestin-dependent activation of ERKs occurred without the agonist-induced receptor internalization. Blockade of the receptor internalization with either the dominant-negative dynamin or with 0.4 M sucrose resulted in an increase in the amount of phosphorylated ERKs in the nucleus. Whichever process is involved in MOR-induced ERK nuclear translocation, it does not involve the agonist-induced internalization of the receptor-β-arrestin complex. Because β-arrestin has been shown to translocate into the nucleus (Scott et al., 2002; DeWire et al., 2007; Ma and Pei, 2007), it is reasonable to propose that the β-arrestin-ERK complex is the cause for the observed increase in the phosphorylated ERKs within the nucleus fractions after etorphine treatment. This hypothesis could explain why overexpression of β-arrestin could increase the nuclear translocation of ERKs activated by etorphine in cells expressing MOR and by a β2-adrenergic agonist in COS7 cells expressing a β2-adrenergic receptor (Kobayashi et al., 2005). In addition, because a reduction in the β-arrestin receptor interaction increases nuclear translocation of phosphorylated ERKs (Tohgo et al., 2003), the receptor possibly will retain the β-arrestin-ERK complex in cytosol when there is a strong β-arrestin-receptor interaction. The β-arrestin-ERK complex, on the other hand, will translocate to the nucleus when the β-arrestin receptor interaction is diminished, as in the case of deubiquitination of the β-arrestin, allowing the GPCR to recycle (Shenoy and Lefkowitz, 2005). However, this hypothesis does not explain all of our observations. Similar to studies reported previously (Zuo, 2005), overexpression of β-arrestin enabled morphine to induce rapid β-arrestin-mediated receptor internalization. However, even with β-arrestin overexpressed, morphine did not select a β-arrestin-dependent pathway to activate ERKs. The switching of pathways was not observed when ERK phosphorylation induced by morphine was completely blocked by PKC inhibitor Ro-31-8425. Therefore, the recruitment of β-arrestin to the vicinity of MOR is not sufficient for scaffolding, activation, and subsequent nuclear translocation of ERKs. A yet-to-be identified cellular factor that is recruited by the etorphine-MOR but not by the morphine-MOR complex must be involved in pathway selectivity and subsequent cellular location of the phosphorylated ERKs. The deviation of the current observations from the established pathway-dependent nuclear translocation of phosphorylated ERKs also points to the complexity of the problem. Most likely, the final cellular location of phosphorylated ERKs will depend on both the pathway and GPCR involved in the enzymes' activations.

Our current observations also present an alternative mechanism for the differences in opioid agonists' abilities to induce in vivo tolerance (Duttaroy and Yoburn, 1995). An existing hypothesis attributes the higher ability of morphine to induce tolerance than etorphine in relative equivalent doses reflects the different abilities of MOR agonists to induce receptor internalization, receptor phosphorylation, receptor desensitization, and β-arrestin2 recruitment (Yu et al., 1997; Whistler and von Zastrow, 1998; Zhang et al., 1998; He et al., 2002). However, this hypothesis does not reconcile the relative rapid desensitization of receptor signaling (in minutes or in hours) with the slow development of tolerance in vivo (in days). To solve the inconsistency between the time courses of the two processes, we propose that gene expression changes during long-term agonist treatment may link these two processes. Our current observations that illustrate the different cellular locations and hence different targets of the phosphorylated ERKs present an alternative mechanism of differential tolerance development. It is reasonable to hypothesize that both the G protein-dependent pathway, used by agonists such as morphine, and the β-arrestin2-dependent pathway, used by agonists such as etorphine, to activate ERKs contribute to the development of tolerance. Because the cellular locations of the ERKs activated by these two pathways and subsequent gene expression changes are different, the levels of agonist-induced tolerance also should be different. By translocating into the nucleus and activating transcription factors such as Elk-1, etorphine-, and fentanyl-activated ERKs induce the enhanced transcription of GRK2 and β-arrestin2. The overexpression of GRK2 and β-arrestin2 accelerate the rate of receptor uncoupling from G proteins and desensitization of MOR signaling (Aplin et al., 2001). Our current studies cannot address whether these accelerations in receptor desensitization are related to tolerance development in vivo. However, the morphine-activated ERKs retained in the cytosol could activate transcription factors such as CREB and Fos, which have been shown to be regulated by in vivo morphine treatment (Zhou and Zhu, 2006). The relation between the G protein-dependent pathway and morphine tolerance can be best illustrated by the ability of specific PKC inhibitors to block the development of morphine tolerance in vivo (Bohn et al., 2002; Smith et al., 2007). Therefore, if the agonist-dependent pathway selectivity of ERKs activation is one of many facets of development of tolerance in vivo, it would be prudent to not treat all opioid agonists as a single class of compounds. Genomic and proteomic studies of specific drugs, which reveal targets for the treatment of long-term drug effects, must be taken into consideration.

In conclusion, our current studies indicate that with the four agonists tested and with three separate models, MOR agonists select distinctive nonoverlapping pathways in their activation of ERKs. In contrast to previous reports indicating that the nuclear translocation of the phosphorylated ERKs is modulated by the β-arrestin scaffolding of ERKs, the nuclear translocation of the MOR-activated ERKs was via the β-arrestin-dependent pathway. It is interesting that the G protein-dependent pathway was used by agonists such as mor-

phine, and the phosphorylated ERKs remained in cytosol, contrary to the accepted dogma of ERK activation. Therefore, for such a scenario to happen, we hypothesize that cellular factor(s) other than β-arrestin recruited by the morphine-MOR complex must be involved in the retention of activated ERKs in the cytosol, and cellular factor(s) other than β-arrestin recruited by etorphine-MOR complex must be responsible for the subsequent nuclear translocation of the activated ERK. The identities of such factors will enable us to elucidate the mechanism of agonist-dependent pathway selectivity of GPCR signaling.

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Dr. Robert Lefkowitz (Duke University) generously provided the β-arrestin and β-arrestin2-V54D constructs, the wild-type, β-arrestin2$^{-/-}$, and β-arrestin1/2$^{-/-}$ mouse embryonic fibroblasts cells used in current studies. Dr. Mark von Zastrow (University of California at San Francisco) generously provided the Dynamin 1 and Dynamin K44E plasmids.

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