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Melatonin Receptor Type 1 Signals to Extracellular Signal-Regulated Kinase 1 and 2 via G_i and G_s Dually Coupled Pathways in HEK-293 Cells

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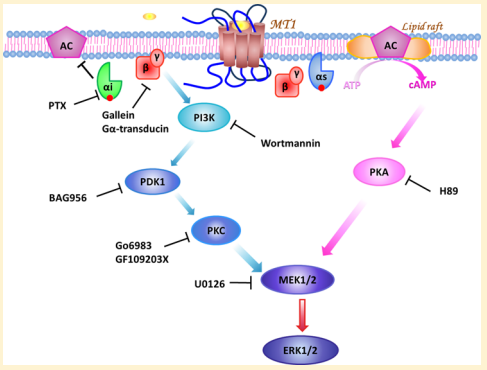
Supporting Information

ABSTRACT: The pineal gland hormone melatonin exerts its regulatory roles in a variety of physiological and pathological responses through two G protein-coupled receptors, melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2), which have been recognized as promising targets in the treatment of a number of human diseases and disorders. The MT1 receptor was identified nearly 20 years ago; however, the molecular mechanisms by which MT1-mediated signaling affects physiology remain to be further elucidated. In this study, using HEK293 cells stably expressing the human MT1 receptor, melatonin induced a concentration-dependent activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2). The melatonin-mediated phosphorylation of ERK1/2 at later time points (≥5 min) was strongly suppressed by pretreatment with pertussis toxin, but only a slight, if any, inhibition of ERK1/2 activation at early time points (≤2 min) was detected. Further experiments demonstrated that the Gβγ subunit, phosphoinositide 3-kinase, and calcium-insensitive protein kinase C were involved in the MT1-mediated activation of ERK1/2 at later time points (≥5 min). Moreover, results derived from cAMP assays combined with a MT1 mutant indicated that the human MT1 receptor could also couple to G_s protein, stimulating intracellular cAMP formation, and that the MT1-induced activation of ERK1/2 at early time points (≤2 min) was mediated by the G_s/cAMP/PKA cascade. Our findings may provide new insights into the pharmacological effects and physiological functions modulated by the MT1-mediated activation of ERK1/2.



Melatonin (5-methoxy-*N*-acetyltryptamine) was initially characterized as a chemical mediator of photoperiodic information. It plays a primary role in the regulation of the circadian day−night rhythm[1] and seasonal biorhythm.[2,3] In addition to being produced in the pineal gland, melatonin is also synthesized in the gastrointestinal tract, retina and lens, skin, immune and hematopoietic cells, some reproductive organs, and endocrine glands.[4−6] Melatonin plays essential roles in a variety of physiological and pathological responses, including metabolism, cardiovascular regulation, antioxidative activity, and oncogenesis.[7−10]

Indeed, melatonin exerts some of its physiological effects in the periphery through two membrane-bound G protein-coupled receptors (GPCRs): melatonin receptor type 1 (MT1) and melatonin receptor type 2 (MT2).[11,12] However, the responsibility of melatonin for a wide range of the characteristic behavioral and physiological functions implied the existence of other not yet identified melatonin receptor types, and studies to identify the melatonin receptor subtypes will continue.[13] The MT1 receptor was first cloned from frog melanophores using a bioassay to monitor the movement of melanin-containing pigment granules within the amphibian skin,[14] and then subsequently from sheep and humans.[11] The MT1 receptor has been shown to be widely distributed in the brain, retina, cardiovascular system, immune system, reproductive system, kidney, pancreas, and liver.[4] Using heterologous expression systems and native tissues based on human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells, the MT1 melatonin receptor has been demonstrated to couple, primarily, to a pertussis toxin (PTX)-sensitive G_i protein, leading to the inhibition of adenylate cyclase.[15−18] Previous studies provided compelling evidence that the MT1 receptor elicits a rapid increase in the intracellular Ca^{2+} concentration via either a PTX-sensitive or a PTX-insensitive G protein-dependent pathway.[19−21] Other studies also showed that the PTX-insensitive G protein is capable of linking activated melatonin receptors to the regulation of other G protein signal transduction pathways.[16,22−24] Recent studies have documented that melatonin exerts its antiproliferative effect on prostate epithelial cells via

Received: January 21, 2014
Revised: April 10, 2014
Published: April 11, 2014

   dx.doi.org/10.1021/bi500092e | *Biochemistry* 2014, 53, 2827−2839

the dual activation of G_s and G_q proteins through the MT1 receptor.[25,26]

It is well-established that most GPCRs signal through mitogen-activated protein kinase (MAPK) cascades via distinct G_i-, G_s-, and G_q-dependent signaling pathways. The MAPK cascade is traditionally associated with growth factor receptor signaling and involved in the control of cell proliferation and growth, mobility, differentiation, and apoptosis.[27,28] The MT1 receptor was also reported to activate the MEK/ERK pathway for regulating hypothalamus, ovarian, pancreatic islet function, and osteoblastic differentiation *in vitro* and mouse cortical bone formation *in vivo*.[29−32] However, the precise signaling pathways for the MT1 receptor-mediated activation of MEK/ERK remain largely unknown. In addition, the MT1 receptor is of great importance as a target in the treatment of a number of melatonin-related diseases, including sleep disorders, depression, diabetes, and cancer.[33,34] Therefore, in this study, we aimed to investigate MEK/ERK signaling pathways mediated by the MT1 receptor using the HEK293 model cell system, which heterologously expresses the human MT1 melatonin receptor. Our results clearly demonstrate the involvement of both G_i- and G_s-dependent signaling pathways in the human MT1 receptor-mediated MEK/ERK cascade in HEK293 cells.

MATERIALS AND METHODS

Materials. Lipofectamine 2000, G418, and Opti-MEM I reduced serum medium were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Beijing, China). The pFLAG-CMV-3 expression vector and monoclonal anti-FLAG M2-FITC antibody were purchased from Sigma (St. Louis, MO). RIPA lysis buffer was obtained from Beyotime (Haimen, China). Pertussis toxin (PTX), Go6983, bisindolylmaleimide (GF109203X), forskolin, and BAPTA-AM were purchased from Tocris (Bristol, U.K.). U0126, tyrphostin AG1478, and wortmannin were purchased from Calbiochem (Darmstadt, Germany). Anti-phospho-ERK1/2 (Thr-202/Tyr-204) and anti-ERK1/2 antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA). The human embryonic kidney cell line (HEK293) was kindly provided by the National Institutes of Health (Bethesda, MD).

Molecular Cloning and Plasmid Construction. Melatonin receptors MT1 (NCBI Reference Sequence NM_005958.3) and MT2 (NCBI Reference Sequence NM_005959.3) were cloned by polymerase chain reaction (PCR) using human genomic DNA as a template. The primers for MT1 were 5′-CCC AAG CTT ATG CAG GGC AAC GGC AGC-3′ (forward) and 5′-CCC AAG CTT ATG CAG GGC AAC GGC AGC-3′ (reverse). The primers for MT2 were 5′-CCC AAG CTT ATG TCA GAG AAC GGC TCC-3′ (forward) and 5′-CCC AAG CTT ATG TCA GAG AAC GGC TCC-3′ (reverse). The MT1 point mutant MT1-C289F was constructed using overlap extension PCR strategies. The PCR product was inserted into *Hin*dIII and *Kpn*I sites in the pFLAG-CMV-3 expression vector. All constructs were sequenced to verify the correct sequences and orientations.

Cell Culture and Transfection. HEK293 cells were grown in DMEM supplemented with 10% FBS. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. For establishing stable cell line HEK-MT1, HEK293 cells were seeded in a six-well plate and transfected with 2 μg of MT1 plasmids using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours later, transfected cells were reseeded in 100 mm dishes and selected with complete medium and 800 mg/L G418. Most of the experiment was conducted on the stable cell line except when needed to overexpress a functional or function-deficient protein to detect receptor signaling. HEK293 cells were transiently transfected with 1.5 μg of MT1, MT2, or MT1-C289F plasmids and 1 μg of the corresponding plasmid (pCRE-luc, Gα-transducin, β-arrestin1, β-arrestin2, or β-arrestin2 V54D). pCDNA3.1 was the control plasmid.

Detection of the Receptor on the Membrane Surface. HEK293 cells expressing MT1 were seeded in cover glass-bottom six-well plates. After 24 h, cells were prelabeled with the anti-FLAG-FITC (M2) antibody (1:100 dilution) for 1 h at 4 °C in DMEM supplemented with 1% BSA. Cells were washed twice with phosphate-buffered saline (PBS) and then incubated in medium alone (control) or with ligands for the appropriate amount of time at 37 °C. Cells were then fixed with a 4% paraformaldehyde/PBS mixture for 10 min at room temperature, Receptor−antibody complexes were visualized at an excitation wavelength of 488 nm with a Zeiss LSM 510 microscope. Images were collected using QED camera software and processed with Adobe Photoshop.

ERK1/2 Activation Assay. HEK293 cells transiently or stably expressing MT1 were seeded in 24-well plates and starved for 4 h in serum-free medium to reduce background ERK1/2 activation and to eliminate the effects of the change of medium. After being treated with drugs and an agonist, the cells were lysed with RIPA buffer. Equal amounts of the total cell lysate were size-fractionated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (10 to 12%) and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in blocking buffer (TBS containing 0.05−0.1% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature and then incubated with a rabbit monoclonal anti-phospho-ERK1/2 antibody and an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody according to the manufacturer's protocols. Total ERK1/2 was assessed as a loading control after p-ERK1/2 chemiluminescence detection using an HRP substrate.

Measurement of the Accumulation of cAMP. Cells were cultured in 24-well plates overnight and treated with PDE inhibitor IBMX (300 μM) for 1 h and forskolin (10 μM) for 15 min. Cells were stimulated with melatonin for 15 min. The reaction was terminated by the removal of medium and the addition of ice-cold PBS, and the mixture was washed once. Cells were lysed, and cAMP formation was assessed by a competitive binding technique-based enzyme-linked immunosorbent assay (ELISA) (Parameter cAMP assay, R&D, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as the concentration of cAMP in the supernatants.

Detection of Cyclic AMP Response Element Binding Protein (CREB) Activity. The activation of CREB was assessed by the cAMP response element (CRE)-drived firefly luciferase.[35] Cells stably or transiently expressing cotransfected pCRE-luc and MT1, MT2, or the MT1 mutant were seeded in a 96-well plate and incubated overnight. Cells were stimulated with melatonin for 4 h, and 10 μM forskolin was added as needed. Firefly luciferase activity was detected with a luciferase kit (KenReal, Shanghai, China).

Arrestin Translocation Assay. β-Arrestin1 and β-arrestin2 were tagged with enhanced green fluorescent protein (EGFP)

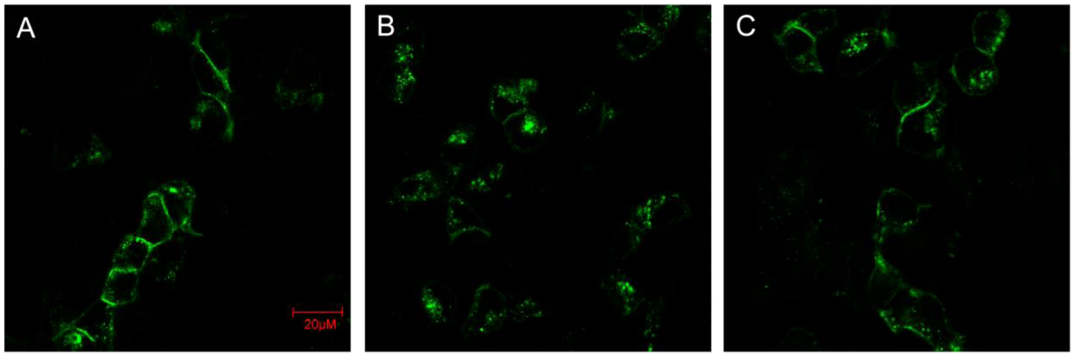


Figure 1. Expression and cellular localization of recombinant MT1 in HEK293 cells. HEK293 cells stably expressing Flag-tagged MT1 were prelabeled with the anti-FLAG-FITC (M2) antibody. Cells were washed and incubated in medium alone (A) or with 100 nM melatonin (B) for 30 min at 37 °C. When needed, cells were pretreated with 10 μM luzindole for 30 min first and then stimulated with 100 nM melatonin for an additional 30 min (C). Finally, cells were fixed, and the receptor−antibody complexes were visualized by confocal microscopy. The data shown are representative of at least three independent experiments.

as described previously.[36] HEK293 cells were cotransfected with FLAG-MT1 and β-arrestin1-EGFP or β-arrestin2-EGFP using Lipofectamine 2000 according to the manufacturer's instructions. The following day, the cells were seeded in covered glass-bottom six-well plates, and after 24 h, the cells were treated with melatonin for 5 min. After the agonist had been removed, the cells were fixed with 3% paraformaldehyde for 15 min. Confocal images were taken on a Zeiss LSM 510 microscope with an attached Axiovert 200 microscope and an LSM5 computer system. Excitation was performed at 488 nm, and a 525 ± 25 nm bandpass filter was used for fluorescence detection. Images were collected using QED camera software and processed with Adobe Photoshop. All pictures shown are representative of at least three independent experiments.

Data Analysis. All experiments were repeated a minimum of two times on different days in separate experiments. Data points in assays are the mean and range of duplicates or the mean and standard error of the mean (SEM) of three to six determinations. EC_{50} values are means ± SEM from three to five experiments. EC_{50} values were determined with Prism. The statistical significance of differences was analyzed by an unpaired Student's t test or analysis of variance with Dunnett's post-test analysis. p values of <0.05 were considered to indicate a significant difference.

RESULTS

Functional Expression of Recombinant Melatonin Receptor MT1 in HEK293 Cells. MT1 (GenBank entry NM_005958.3) was cloned by PCR from human genomic DNA as a template and inserted into the pFLAG-CMV-3-expressing vector. After transfection of HEK293 cells with the recombinant expression vector, a stable cell line expressing MT1 (HEK293-MT1) was established by the addition of 800 mg/L G418. As shown in Figure 1, observation with confocal microscopy revealed that MT1 receptors were mainly expressed and localized to the plasma membrane in HEK293 cells, but with certain intracellular accumulation in the absence of ligand. Upon stimulation with melatonin (100 nM), MT1 receptors underwent a rapid internalization from the cell surface to the cytoplasm, a key process for regulating the strength and duration of receptor-induced signaling, and this agonist-induced internalization was blocked by treatment with melatonin antagonist luzindole (10 μM, 30 min), suggesting that the MT1 receptors are expressed and function normally in HEK293 cells.

MT1 Activates the MAPK/ERK Pathway. The MT1 receptor has been identified as a receptor with a high affinity for melatonin (pK_d = 9.4−9.9).[16] We first determined whether the activated MT1 receptor in HEK293 cells signals to the ERK1/2 pathway using a phospho-specific antibody against phosphorylated ERK1/2. As indicated in Figure 2A, data for the analysis of the concentration−response curve showed that melatonin (10^{-15} to 10^{-5} M) treatment triggered a concentration-dependent activation of ERK1/2 with an EC_{50} value of 5 nM in HEK293-MT1 cells. To further investigate the time course of MT1 receptor-mediated ERK1/2 phosphorylation, HEK293-MT1 cells seeded in 24-well plates overnight were treated with 100 nM melatonin for the indicated periods of time. As shown in Figure 2B, melatonin stimulation elicited an increase in the level of ERK1/2 phosphorylation in a time-dependent manner with maximal activity at 5 min and a return to almost basal levels by 30 min. The melatonin-elicited ERK1/2 phosphorylation was significantly inhibited by pretreatment with 10 μM luzindole (Figure 2C). Moreover, a known MEK1/2 inhibitor, U0126 (1 μM), was found to effectively inhibit melatonin-induced ERK1/2 activation (Figure 2D). Taken together, these results suggest that the phosphorylation of ERK1/2 in HEK293-MT1 cells is truly mediated through the human melatonin MT1 receptor, and upstream MEK1/2 is required for MT1-mediated ERK1/2 activation.

MT1 Triggers ERK1/2 Phosphorylation via the PTX-Sensitive G_i Protein-Dependent Pathway. Upon activation by melatonin, MT1 receptors mediate the inhibition of intracellular cAMP accumulation via a pertussis toxin (PTX)-sensitive G_i protein.[37,38] Therefore, we sought to assess the role of G_i protein in the MT1-mediated activation of ERK1/2. HEK293-MT1 cells were cultured in the presence or absence of 50 ng/mL PTX in serum-free DMEM for 16 h, followed by melatonin stimulation. As indicated in panels A and B of Figure 3, pretreatment of cells with PTX led to a significant inhibition of ERK1/2 phosphorylation compared to that upon addition of agonist alone, demonstrating that the MT1 receptor signals through the ERK1/2 pathway via a PTX-sensitive G_i protein-dependent mechanism.

For most G_i-coupled GPCRs, the $G_{\beta\gamma}$ subunits that are released from G_i proteins have been demonstrated to play a central role in signaling from the activated receptor to ERK1/2

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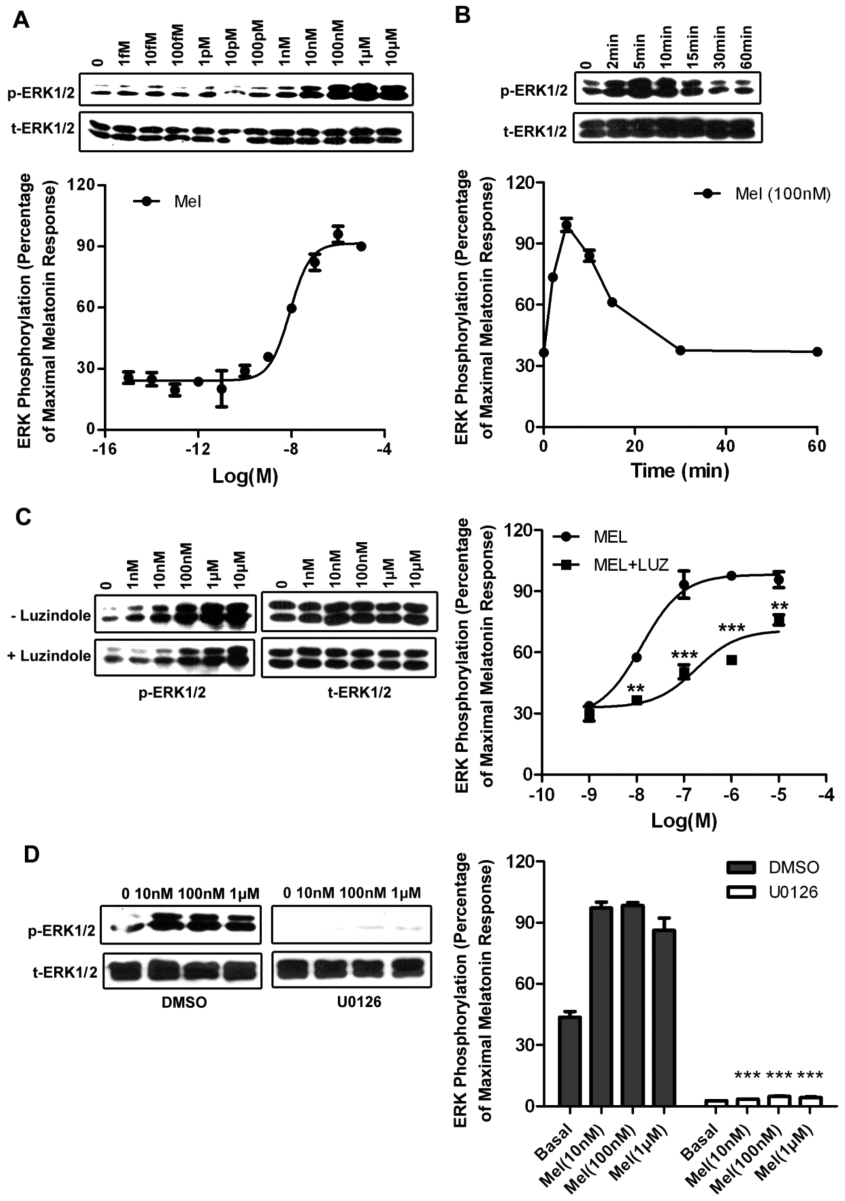

Figure 2. Melatonin receptor MT1 mediates MAPK/ERK pathway activation. HEK293 cells stably expressing MT1 were starved with DMEM for 1 h. Cells were then treated with drugs and melatonin and then harvested for the detection of ERK1/2 phosphorylation by Western blotting. (A) HEK293-MT1 cells were stimulated with the indicated concentrations of melatonin for 5 min. (B) HEK293-MT1 cells were stimulated with 100 nM melatonin for the indicated periods of time. (C) HEK293-MT1 cells were pretreated with vehicle (DMSO) or 10 μM luzindole for 1 h, and the cells were stimulated with indicated concentration of melatonin for 5 min. (D) HEK293-MT1 cells were pretreated with vehicle (DMSO) or 1 μM U0126 for 1 h, and the cells were stimulated with 100 nM melatonin for 5 min. Data were analyzed using the Student's *t* test (**$p < 0.01$; ***$p < 0.001$). All data shown are representative of at least three independent experiments.

through the activation of PLC and phosphoinositide 3-kinase (PI3K).[39−41] To further define the role of the $G_{\beta\gamma}$ subunit in MT1 receptor-induced ERK1/2 phosphorylation, HEK293-MT1 cells were transfected with a putative $G_{\beta\gamma}$ scavenger, the Gα subunit of transducin (Gα-transducin). Overexpression of the putative $G_{\beta\gamma}$ scavenger resulted in significant inhibition of MT1-induced ERK1/2 phosphorylation (Figure 3C). This result was confirmed by the observation that treatment of HEK293-MT1 cells with gallein (10 μM, 1 h), a specific $G_{\beta\gamma}$ small molecule inhibitor, led to a significant decrease in the level of melatonin-triggered ERK1/2 phosphorylation (Figure 3D). Hence, the $G_{\beta\gamma}$ subunit of $G_{\alpha i\beta\gamma}$ is likely to play a central role in MT1 receptor-induced ERK1/2 activation.

The PI3K/PDK1/PKC Pathway Is Involved in MT1-Mediated ERK1/2 Phosphorylation. Accumulating evidence suggests that G_i-coupled GPCRs activate the ERK1/2 pathway via a $G_{\beta\gamma}$/PLC/Ca^{2+}/PKC signaling pathway.[42] Thus, we investigated whether the MT1 receptor triggers ERK1/2 phosphorylation through a $G_{\beta\gamma}$/PLC/Ca^{2+}/PKC signaling pathway. HEK293-MT1 cells were treated with melatonin in the presence or absence of a PLC inhibitor, an intracellular calcium chelator, and protein kinase C (PKC) inhibitors. As shown in Figure 4, the PLC inhibitor U73122 (10 μM, 1 h), the intracellular calcium chelator BAPTA-AM (20 μM, 1 h), and the calcium-sensitive PKC inhibitor Go6976 exhibited no effect on melatonin-induced ERK1/2 phosphorylation, whereas treatment with the broad spectrum PKC inhibitor Go6983

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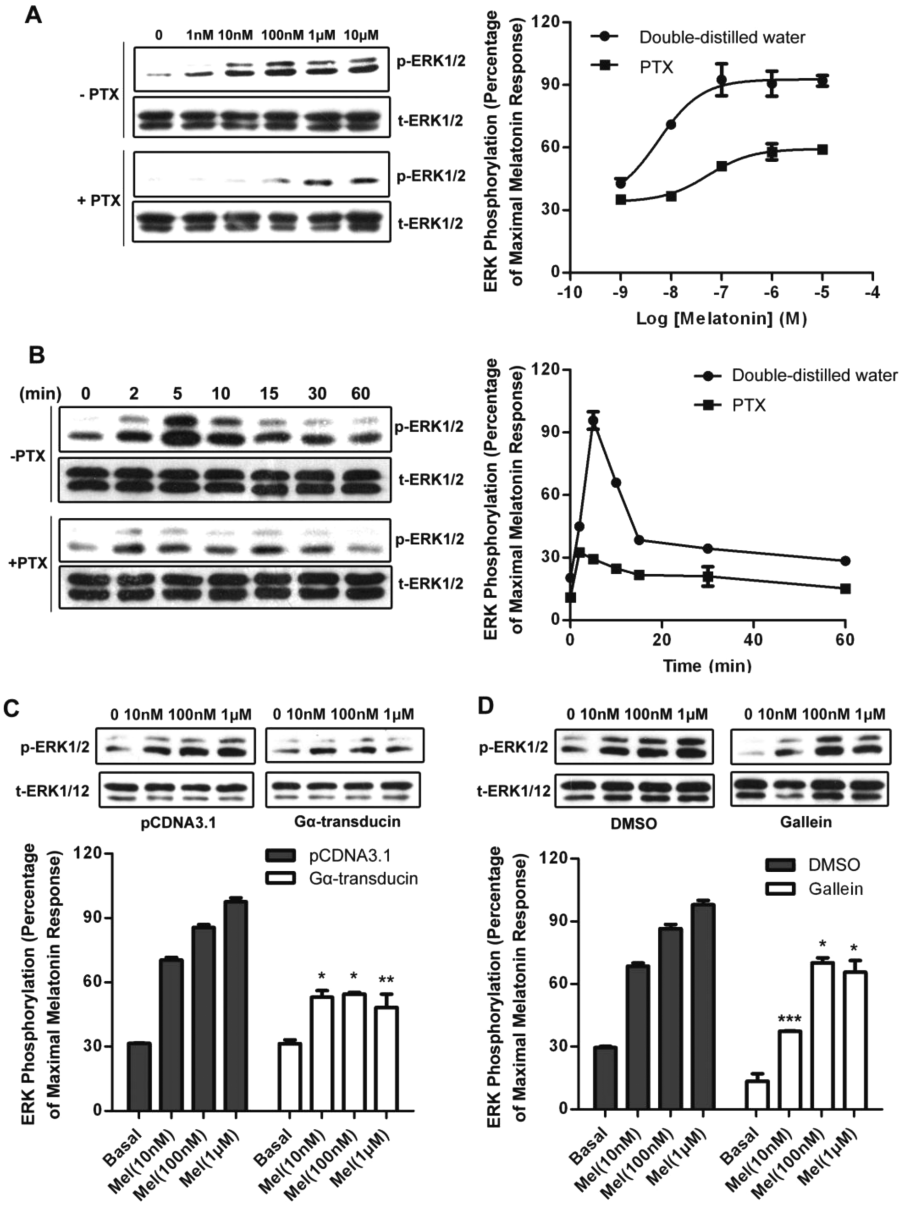

Figure 3. G_i protein is involved in MT1-mediated ERK1/2 phosphorylation. Serum-starved HEK293-MT1 cells were treated with drugs and melatonin and then harvested for the detection of ERK1/2 phosphorylation. (A) HEK293-MT1 cells were pretreated with vehicle (doubly distilled water) or 50 ng/mL PTX for 16 h, and cells were stimulated with the indicated concentrations of melatonin for 5 min. (B) HEK293-MT1 cells were pretreated with vehicle (doubly distilled water) or 50 ng/mL PTX for 16 h, and the cells were stimulated with 100 nM melatonin for the indicated periods of time. (C) HEK293 cells were transiently transfected with MT1 and pCDNA3.1 or G$\beta\gamma$ scavenger Gα-transducin. Forty-eight hours later, cells were stimulated with the indicated concentrations of melatonin for 5 min. (D) HEK293-MT1 cells were pretreated with vehicle (DMSO) or 10 μM gallein for 1 h, and the cells were stimulated with the indicated concentrations of melatonin for 5 min. Data were analyzed by using the Student's *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). All data shown are representative of at least three independent experiments.

(10 μM, 1 h) and GF109203x (10 μM, 1 h) led to a significant inhibition of MT1 receptor-mediated ERK1/2 activation (Figure 4D,E), suggesting that a calcium-insensitive PKC is likely to participate in G_i-coupled MT1 receptor-mediated ERK1/2 activation.

Previous studies have demonstrated that PI3K is involved in G_i-coupled receptor-mediated ERK1/2 activation.[41,43] In HEK293-MT1 cells, treatment with the PI3K inhibitor wortmannin (1 μM, 1 h) and the PI3K/PDK1 inhibitor BAG956 (10 μM, 1 h) resulted in a significant decrease in the level of melatonin-induced ERK1/2 phosphorylation (Figure 5). Collectively, it is likely that the human MT1 receptor activates the ERK1/2 signaling pathway via a PI3K/PDK1/PKC cascade.

Involvement of G_s Protein in MT1-Mediated ERK1/2 Phosphorylation. In experiments using PTX to explore the role of G_i protein in MT1-induced ERK1/2 activation, PTX treatment (50 ng/mL) resulted in an almost complete inhibition of ERK1/2 phosphorylation at later time points (≥5 min), but Western blot analysis showed only a slight, if any, difference between the PTX-treated group and the control group at 2 min (Figures 3B and 6A). This prompted us to investigate whether the G_s protein is involved in MT1 receptor-mediated signaling and ERK1/2 activation. To detect if melatonin can bind to the MT1 receptor and trigger a second

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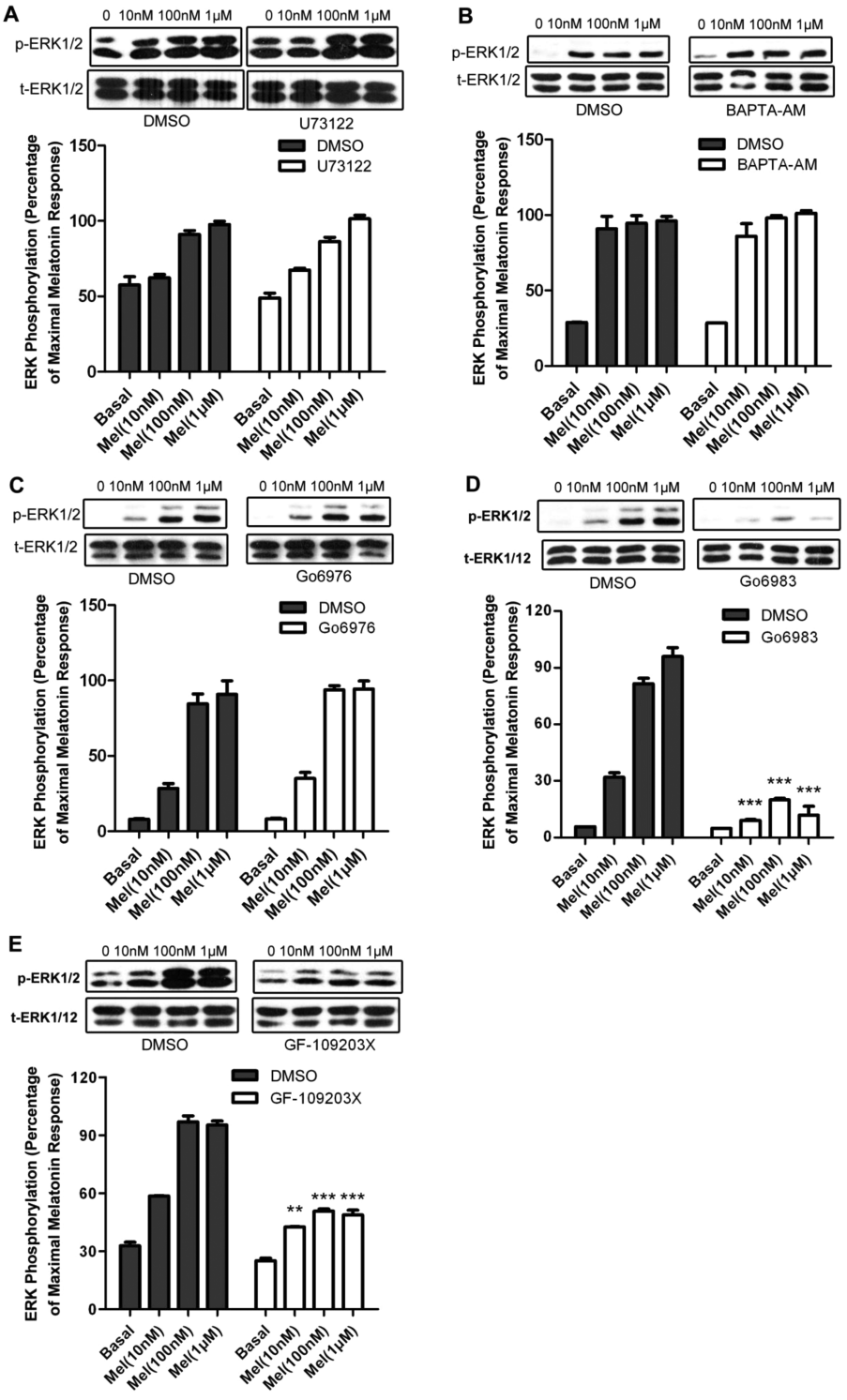

Figure 4. Calcium-dependent PKC is not involved in MT1-mediated ERK1/2 phosphorylation. Serum-starved cells were pretreated with vehicle (DMSO) or drugs [(A) 10 μM U73122, (B) 20 μM BAPTA-AM, (C) 1 μM Go6976, (D) 10 μM Go6983, or (E) 10 μM GF109203x] for 1 h and then stimulated by the indicated concentrations of melatonin for 5 min. Cells were harvested for the detection of ERK1/2 phosphorylation. Data were analyzed by using the Student's *t* test (**$p < 0.01$; ***$p < 0.001$). All data shown are representative of at least three independent experiments.

messenger cAMP signaling pathway, HEK293-MT1 cells were transiently transfected with a reporter system consisting of the firefly luciferase coding region under the control of a minimal promoter containing the cAMP response element (CRE). As illustrated in Figure 6B, upon stimulation with melatonin, a significant dose-dependent increase in CRE-driven luciferase activity with an EC_{50} value of 5 nM was detected upon stimulation with melatonin. Intracellular accumulation of cAMP was also confirmed by a direct cAMP ELISA in HEK293-MT1 cells when they were preincubated with forskolin (10 μM). In addition, our results showed that full activation of G_s protein by pretreatment with cholera toxin (100 ng/mL) could inhibit the MT1-mediated activation of luciferase activity (Figure S2 of the Supporting Information). In contrast, melatonin exhibited a

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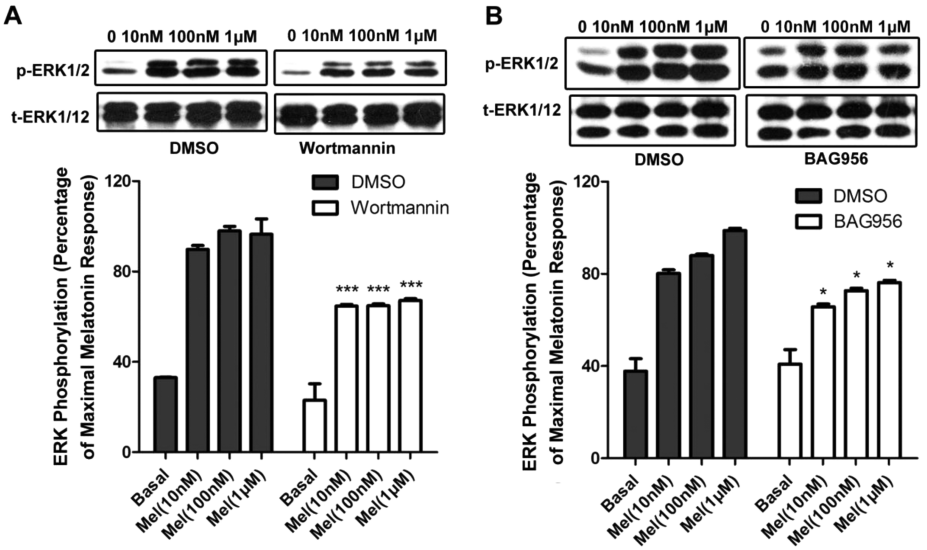

Figure 5. PI3K/PDK1/PKC pathway is involved in MT1-mediated ERK1/2 phosphorylation. Serum-starved cells were pretreated with vehicle (DMSO) or drugs [(A) 1 μM wortmannin and (B) 10 μM BAG956] for 1 h and then stimulated by the indicated concentrations of melatonin for 5 min. Cells were harvested for the detection of ERK1/2 phosphorylation. Data were analyzed by using the Student's *t* test (*$p < 0.05$; ***$p < 0.001$). All data shown are representative of at least three independent experiments.

significant inhibition of forskolin-stimulated cAMP formation in HEK293-MT2 cells (Figure 6C). These data suggest that the human MT1 receptor is likely to couple to both G_i and G_s proteins in HEK293 cells. G_s-coupled GPCRs have been shown to induce ERK1/2 phosphorylation through a cAMP/PKA-dependent pathway.[44] Therefore, we used PKA inhibitor H89 (10 μM, 1 h) to examine the role of PKA in the MT1 receptor-mediated MAPK/ERK activation. As shown in Figure 6D, H89 (10 μM, 1 h) displayed the potential to significantly inhibit melatonin-induced ERK1/2 phosphorylation at the 2 min time point. Moreover, treatment with both PTX (50 ng/mL, 16 h) and H89 (10 μM, 1 h) led to almost complete inhibition of MT1-induced ERK phosphorylation (Figure 6E).

Evidence that the NSXXNPXXY motif located in the region of transmembrane 7 (TM7) is critical for receptor activation is accumulating,[45,46] and a change in this motif has been reported to impair G protein selectivity.[47] Therefore, this TM7 region was selected for further construction of a series of mutants, and the MT1-C289F mutant was found to have a dramatically reduced G_s-coupling ability, while the G_i coupling ability is not impaired. We, then, used this mutant MT1-C289F to confirm the involvement of the G_s/cAMP/PKA signaling pathway in the MT1 receptor-mediated ERK1/2 activation at the early time point of 2 min. As shown in Figure 7A and 7B, this mutant was found to inhibit forskolin-stimulated cAMP levels and luciferase activity. Further results demonstrated that PTX pretreatment led to an almost complete inhibition in melatonin-induced ERK1/2 phosphorylation at 2 min (Figure 7C), while treatment with H89 (10 μM, 1 h) led to no significant inhibition of the melatonin-induced ERK1/2 phosphorylation at the time point of 2 min (Figure 7D). Taken together, these findings indicate that the G_s/cAMP/PKA signaling pathway is likely involved in the MT1 receptor-induced ERK1/2 activation at early time point (2 min) at least in HEK293 cells.

Arrestins and EGFR Transactivation Are Not Involved in MT1-Mediated ERK1/2 Activation. We next determined the role of β-arrestins and EGFR transactivation in MT1-mediated ERK1/2 activation. In a β-arrestin-GFP recruitment assay, only β-arrestin2-GFP was recruited into the cell membrane when the MT1 receptor was activated by melatonin, suggesting that β-arrestin2, but not β-arrestin1, is involved in agonist-mediated MT1 receptor internalization (Figure 8A). Therefore, the β-arrestin2-V54D mutant, a dominant negative construct,[48] was constructed and transfected into HEK293-MT1 cells. The mutant β-arrestin2-V54D has been suggested to interact normally with the receptors but to fail to mediate subsequent interactions with other proteins required for receptor internalization[48] and has been widely used to inhibit β-arrestin2-mediated receptor internalization. As shown in Figure 8B and Figure S3A of the Supporting Information, overexpression of β-arrestin2-V54D exhibited no effect on MT1 receptor-mediated ERK1/2 activation, whereas overexpression of wide-type β-arrestin2 led to a significant decrease in the level of ERK1/2 phosphorylation. Moreover, using embryonic fibroblast (MEF) cells derived from a β-arrestin2-deficient mouse, we demonstrated that MT1 could activate ERK1/2 in a pattern similar to that in normal MEF cells (Figure S3B of the Supporting Information).

To further assess whether EGFR transactivation is involved in MT1-mediated ERK1/2 activation, HEK293-MT1 cells were treated with AG1478, an EGFR-specific tyrosine kinase inhibitor, for 1 h before being exposed to melatonin. As shown in Figure 8C, our result demonstrated that AG1478 did not attenuate melatonin-induced ERK1/2 activation. These results suggest that both β-arrestins and EGFR transactivation are not involved in the regulation of MT1-mediated ERK1/2 activation.

DISCUSSION

It is generally accepted that many GPCRs regulate diverse processes ranging from proliferation and differentiation to apoptosis via crosstalk with mitogen-activated protein kinase (MAPK) pathways.[28] Melatonin has been found to decrease the level of GnRH by regulating mitogenic responses via MAPK signaling.[30,31] Recent studies showed that the MAPK/ERK pathway represents a major signaling cascade mediated by melatonin during oxidative stress.[49,50] Using UVB-stressed

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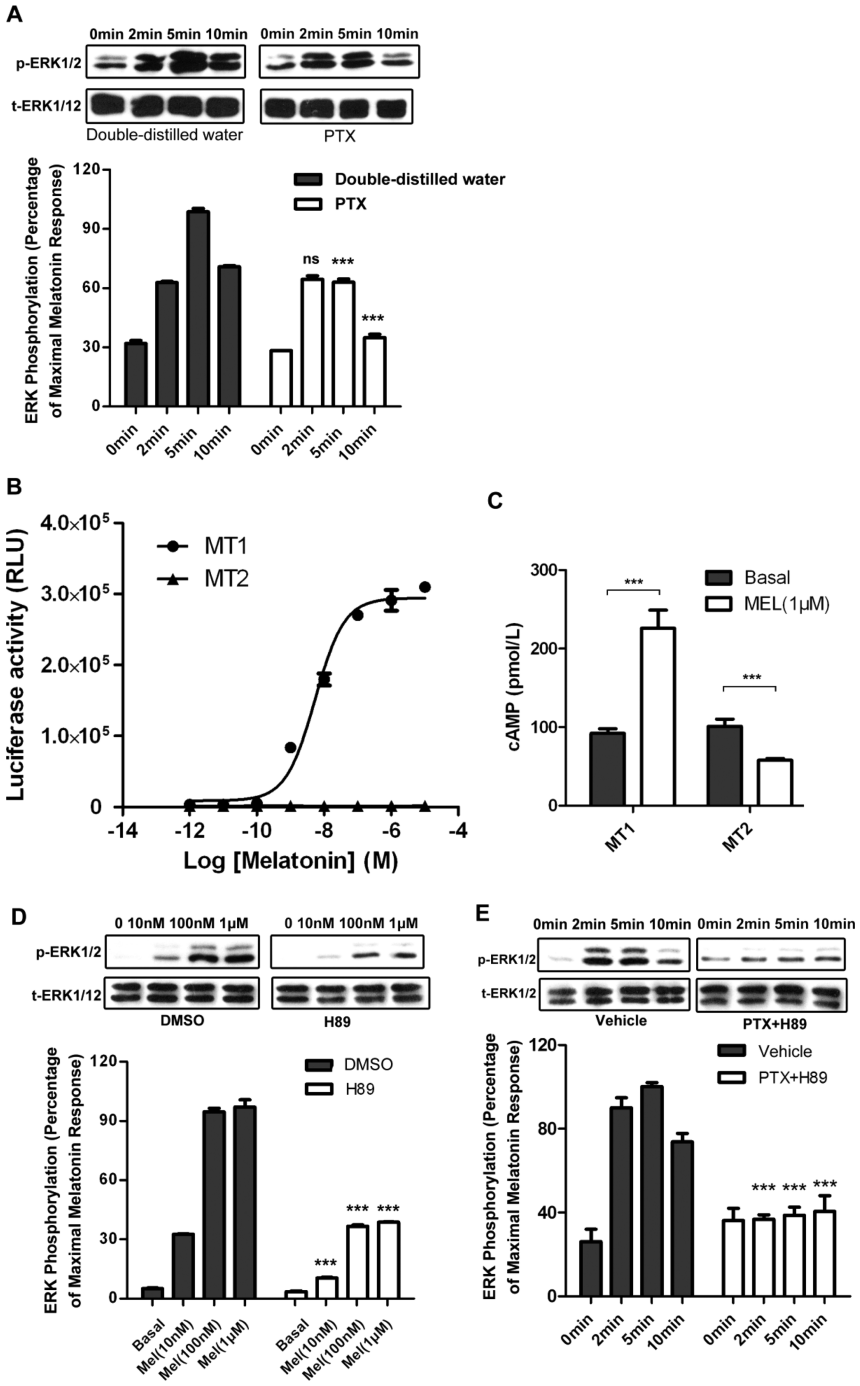

Figure 6. G_s protein is involved in MT1-mediated ERK1/2 phosphorylation. (A) Serum-starved HEK293-MT1 cells were pretreated with vehicle (doubly distilled water) or 50 ng/mL PTX for 16 h, and the cells were stimulated with 1 μM melatonin for the indicated periods of time. (B) HEK293 cells were transiently transfected with pCRE-luc and MT1 or MT2 and were reseeded in 96-well plates after 24 h. After an additional 24 h, cells were stimulated with the indicated concentrations of melatonin for 4 h, and then cells were lysed for the detection of firefly luciferase activity. (C) HEK293 cells were transiently transfected with MT1 or MT2 and reseeded in 24-well plates after 24 h. After an additional 24 h, cells were pretreated with 10 μM forskolin for 15 min and then stimulated with 1 μM melatonin for an additional 15 min. Cells were lysed for the detection of the cAMP level. (D) Serum-starved HEK293-MT1 cells were pretreated with vehicle (DMSO) or 10 μM H89 for 1 h, and then the cells were stimulated with the indicated concentration of melatonin for 2 min. (E) Serum-starved HEK293-MT1 cells were pretreated with vehicle (DMSO and doubly distilled water) or PTX (50 ng/mL, 16 h) combined with H89 (10 μM, 1 h), and then the cells were stimulated with 1 μM melatonin for the indicated periods of time. Data were analyzed by using the Student's t test (***$p < 0.001$). All data shown are representative of at least three independent experiments.

U937 cells, the MAPK/ERK pathway has been demonstrated to be involved in both constitutive and stress-induced melatonin signaling to promote mitochondrial protection and cell survival.[51] Collectively, these data suggest that the MAPK/ERK pathway is likely to contribute to the functional pleiotropism of melatonin. In addition, although the well-established therapeutic applications of melatonin are still confined to circadian rhythm sleep disorders and depression,

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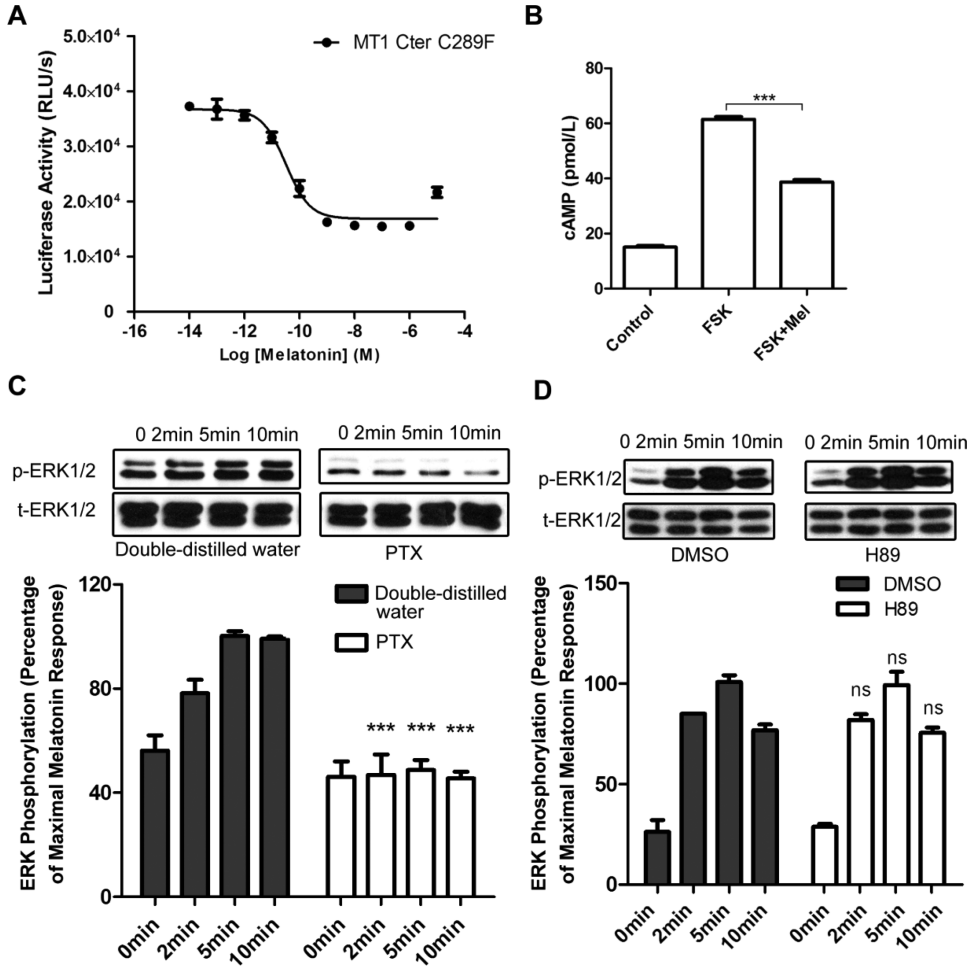

Figure 7. G_i protein is involved in MT1-mediated ERK1/2 phosphorylation. (A) HEK293 cells were transiently transfected with pCRE-luc and MT1-C289F and reseeded in 96-well plates after 24 h. After an additional 24 h, cells were stimulated with the indicated concentrations of melatonin for 4 h, and then cells were lysed for the detection of firefly luciferase activity. (B) HEK293 cells were transiently transfected with MT1-C289F and reseeded in 24-well plates after 24 h. After an additional 24 h, cells were pretreated with 10 μM forskolin for 15 min and then stimulated with 1 μM melatonin for an additional 15 min. Cells were lysed for the detection of the cAMP level. (C) HEK293 cells were transiently transfected with MT1-C289F. Serum-starved cells were pretreated with vehicle (doubly distilled water) or 50 ng/mL PTX for 16 h, and the cells were stimulated with 1 μM melatonin for the indicated periods of time. (D) HEK293 cells were transiently transfected with MT1-C289F. Serum-starved cells were pretreated with vehicle (DMSO) or 10 μM H89 for 1 h, and the cells were stimulated with 1 μM melatonin for the indicated periods of time. Data were analyzed by using the Student's t test (***$p < 0.001$). All data shown are representative of at least three independent experiments.

seasonal affective disorder, migraines, and cluster headaches, accumulating evidence suggests more therapeutic potentials for the treatment of sexual dysfunction, gallbladder stones, obesity, and even cancer.[52] Therefore, a detailed characterization of the signaling pathways that trigger ERK1/2 activation through the melatonin MT1 receptor is essential for fully understanding its physiological and pathological roles. In this study, HEK293 cells, which are commonly used in the characterization of GPCRs, were used to assess signaling pathways involved in MT1 receptor-mediated ERK1/2 activation. Although a study by Chan et al. showed that HEK293 cells endogenously express the MT1 receptor,[53] HEK293 cells have been widely used to investigate MT1 receptor-mediated signaling, including cAMP formation, Ca^{2+} mobilization, and ERK1/2 phosphorylation.[19,54,55] By using stably or transiently transfected HEK293 cells combined with the addition of different kinase inhibitors, we showed that the MT1 receptor signals to the ERK1/2 pathway via G_i- and G_s-dependent dual pathways in response to the stimulation of melatonin.

As has been demonstrated by some studies using native tissues and mammalian cell lines such as HEK293 and CHO cells, the MT1 receptor was primarily coupled to the cAMP signaling cascade via a PTX-sensitive G_i protein upon being exposed to an agonist,[15−18,56] leading to the inhibition of PKA activity and CREB phosphorylation.[57] To determine the role of the G_i protein-dependent pathway in MT1 receptor-mediated ERK1/2 activation, HEK293 cells stably expressing the MT1 receptor exhibited a time-dependent activation of ERK1/2 in response to melatonin, peaking at approximately 5 min and returning to basal levels at 30 min. This MT1 receptor-mediated ERK1/2 phosphorylation was predominantly inhibited by preincubation with PTX (50 ng/mL, 16 h), indicating that G_i proteins are essentially involved in MT1-mediated ERK1/2 phosphorylation. It is generally accepted that upon activation, G_i-coupled GPCRs exert their action through the inhibition of adenylate cyclase activity directly via G_i protein, but they also exhibit the potential to inhibit certain adenylate cyclase isoforms, to recruit G protein-coupled receptor kinase 2 (GRK2) and Src, and to activate PLCβ and

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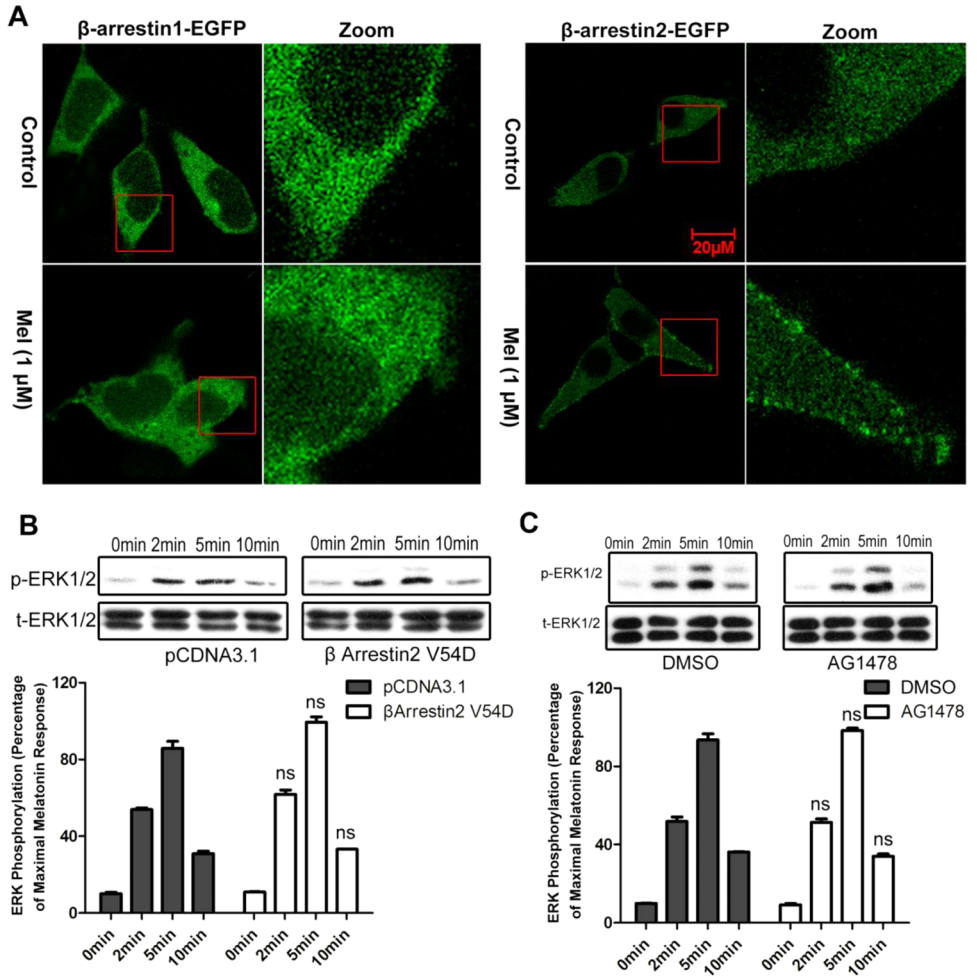

Figure 8. β-Arrestin2 and EGFR are not involved in MT1-mediated ERK1/2 phosphorylation. (A) The activated MT1 receptor could recruit the β-arrestin2, not β-arrestin1. HEK293 cells were transiently cotransfected with FLAG-MT1 and β-arrestin2-EGFP or β-arrestin1-EGFP. Cells were stimulated with 1 μM melatonin for 5 min, and then cells were fixed and visualized under a confocal microscope. (B) HEK293 cells were cotransfected with FLAG-MT1 and functional deficiency mutant β-arrestin2-V54D. Serum-starved cells were stimulated with 100 nM melatonin for the indicated period of time. Cells were harvested for the detection of ERK1/2 phosphorylation. (C) Serum-starved cells were pretreated with 10 μM AG1478 for 1 h and then stimulated with 100 nM melatonin for the indicated period of time. Cells were harvested for the detection of ERK1/2 phosphorylation. Data were analyzed by using the Student's t test. All data shown are representative of at least three independent experiments.

PLCε via $G_{\beta\gamma}$ subunits released from PTX-sensitive G_i.[35,58,59] The $\alpha 2_A$ adrenergic, M2 muscarinic acetylcholine, D2 dopamine, and A1 adenosine receptors were found to activate the ERK1/2 pathway largely by $G_{\beta\gamma}$ subunits released from PTX-sensitive G proteins in COS-7 cells.[60,61] Previous studies showed the indirect involvement of $G_{\beta\gamma}$ subunits in the activation of PLC-dependent signal transduction cascades caused by the MT1 receptor.[15,21] Using $G_{\beta\gamma}$ inhibitor gallein (10 μM, 1 h) or overexpression of the Gα subunit of transducin, a $G_{\beta\gamma}$ scavenger, the $G_{\beta\gamma}$ subunits released from PTX-sensitive G protein were demonstrated to be involved in MT1 receptor-mediated ERK1/2 phosphorylation. Taken together, it seems likely that, upon MT1 receptor activation, $G_{\beta\gamma}$ subunits that dissociated from the activated G_i protein may interact with phospholipase C, resulting in diacylglycerol-mediated increases in PKC activity. PKC in turn activates raf-1 by stimulating the formation of active Ras−Raf-1 complexes, leading to activation of the ERK1/2 signaling cascade.[62]

In addition to coupling to the PTX-sensitive G_i protein, activated human MT1 receptors were demonstrated to functionally couple to PTX-insensitive $G_{q/11}$ proteins.[19,23] However, melatonin-induced Ca^{2+} mobilization was observed only in the ovine pars tuberalis and model cell lines.[18,19] In our preliminary experiment, Ca^{2+} mobilization was hardly detected in HEK293 cells that were stably or transiently transfected with the MT1 receptor in response to melatonin (Figure S1 of the Supporting Information). Furthermore, the MT1 receptor-mediated ERK1/2 phosphorylation was not blocked by treatment with PLC inhibitor U73122, intracellular calcium chelator BAPTA-AM, or calcium-sensitive PKC inhibitor go6976, but broad spectrum PKC inhibitors Go6983 and GF109203x and PI3K inhibitor wortmannin and PI3K/PDK1 inhibitor BAG956 were found to effectively inhibit MT1 receptor-induced ERK1/2 activation. Previous observations suggest that PTX-sensitive G protein-coupled P2Y receptors in human umbilical vein endothelial cells activate the MAPK/ERK pathway through a PI3K-dependent mechanism, in which PDK1 and PKCζ are two key components within the signal cascade.[63] In HEK293 cells, PI3K/PDK1 has been found to activate calcium-independent PKC isotypes PKCζ and PKCδ.[64] It has been reported that some membrane receptors could activate MEK/ERK through the PI3K/PDK1/PKCζ path-

dx.doi.org/10.1021/bi500092e | Biochemistry 2014, 53, 2827−2839

way.[63,65] Recently, the Rac-GEF P-Rex1 was reported to be involved in PI3K-regulated MEK/ERK activation in breast cancer.[66] Taken together, these results suggest that activated MT1 receptors trigger the MAPK/ERK pathway through a $G_{\beta\gamma}$-initiated PI3K/PDK1/PKCζ cascade, and G_q protein is not likely to play a part in MT1 receptor-induced ERK1/2 phosphorylation at least in our system using HEK293 cells. However, further studies are necessary to clarify the mechanism to link the calcium-independent PKC to MT1 receptor-mediated MEK/ERK activation.

To our surprise, PTX pretreatment exhibited only a slight impact, if any, on melatonin (1 μM)-induced ERK1/2 phosphorylation at 2 min in HEK293 cells expressing the human melatonin MT1 receptor. We, then, postulated that G_s protein is likely involved in the MT1 receptor-mediated ERK1/2 signaling pathway. Employing the cAMP response element (CRE)-driven luciferase reporter system, a dose-dependent increase in CRE-driven luciferase activity was measured in response to melatonin in HEK293 cells expressing MT1. MT1 receptor-induced intracellular cAMP accumulation was confirmed by an ELISA-based direct cAMP assay, suggesting that G_i protein as well as G_s protein is involved in MT1 receptor-induced signaling cascades in HEK293 cells that have been transfected with MT1. In cultured myometrial cells, a modest increase in the basal level of cAMP production was observed in response to a nonphysiological (1 μM) melatonin concentration.[67] Recent studies confirmed that a G_s-mediated signaling cascade is involved in the regulation of the antiproliferation of prostate epithelial cells through the MT1 receptor.[25,26] In this study, to identify the role of PKA in MT1-mediated ERK1/2 activation, HEK293 cells stably expressing the MT1 receptor were pretreated with or without PKA inhibitor H89 (10 μM) for 1 h and were then incubated with varying concentrations of melatonin. H89 treatment caused a significant decrease in the level of melatonin-induced ERK1/2 phosphorylation. Moreover, we constructed a site-directed mutant, MT1-C289F, which induces a G_i-dependent signaling cascade. In contrast to the wild-type MT1 receptor, PTX treatment resulted in a significant attenuation of MT1-C289F-induced ERK1/2 activation at 2 min in response to melatonin, whereas PKA inhibitor H89 showed no effect on MT1-C289F-mediated ERK1/2 phosphorylation. It seems likely that upon exposure to melatonin, the MT1-receptor triggers ERK1/2 activation at early time points ($\leq$2 min) via the G_s/cAMP/PKA pathway in HEK293 cells. Moreover, the MT1 receptor has been found to functionally couple to G_s in cancerous cells.[25,26] Therefore, it is possible that coupling of G_s to ERK1/2 pathway may be involved in the MT1 receptor-mediated antiproliferative action of melatonin on human cancerous cells.

It is well-known that the EGFR tyrosine kinase has emerged as an important transducer in the GPCR-mediated MAPK/ERK cascade, a process termed transactivation.[28] Previous studies suggest that GPCR-induced EGFR transactivation is mediated by the release of HB-EGF, a precursor form of the EGFR ligands, which is generated by the activation of matrix metalloproteinases. Melatonin has been found to positively or negatively affect the growth of different cancer cell lines via EGFR/MAPK mitogenic signaling.[68,69] Picinato et al. demonstrated that melatonin induces the production of insulin growth factor (IGF) and promotes insulin receptor tyrosine phosphorylation followed by the activation of PI3K/AKT and MEK/ERK pathways in the pancreas and INS-1 cells.[29] Niacin receptor GPR109A has been found to signal to the ERK1/2 signaling pathway via the platelet-derived growth factor receptor (PDGFR) in CHO-K1 cells.[36] However, in the study presented here, melatonin-mediated ERK1/2 activation was not blocked by the EGFR inhibitor in HEK293 cells stably expressing the MT1 receptor. In addition, β-arrestins not only serve as scaffolds to initiate clathrin-dependent internalization but also function as signal transducers for many GPCRs to mediate ERK1/2 activation. Bondi et al. reported that in MT1-CHO cells, β-arrestin2 was required for clathrin-mediated MT1 receptor endocytosis and for the formation of the β-arrestin2−MEK1/2−ERK1/2 complex.[70] We confirmed that the endocytosis of MT1 in HEK293 cells could recruit β-arrestin2. However, when we overexpressed the functionally deficient β-arrestin2 mutant β-arrestin2-V54D in HEK293-MT1 cells, MT1-induced ERK1/2 phosphorylation was not impaired, indicating that β-arrestins were not involved in ERK1/2 phosphorylation in HEK293-MT1 cells. These discrepancies may be explained by cell type-specific signaling mechanisms.

In summary, this study provides a detailed delineation of the melatonin-mediated activation of ERK1/2 in HEK293 cells that are transfected with melatonin receptor MT1. On the basis of our data, we propose that, upon exposure to melatonin, activated MT1 receptors primarily coupled to G_i proteins and the dissociation of $\beta\gamma$ subunits from $G\alpha_i$ led to the activation of the PI3K/PDK1/PKC pathway, which is further involved in ERK1/2 phosphorylation, at later time points ($\geq$5 min), whereas MT1 could also activate ERK1/2 through a G_s/cAMP/PKA pathway at early time points ($\leq$2 min). However, additional studies will be necessary to further clarify the role of the ERK1/2 pathway in MT1 receptor-modulated physiological functions.

■ ASSOCIATED CONTENT

Supporting Information

Description of the method and a figure depicting calcium mobilization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the Ministry of Science and Technology (Grants 2012CB910402 and 2012AA020303-05) and the National Natural Science Foundation of China (Grant 81173106).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ms. Aiping Shao and Mr. Hanmin Chen for their technical assistance and equipment usage.

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