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Role of mPKCI, a Novel μ-Opioid Receptor Interactive Protein, in Receptor Desensitization, Phosphorylation, and Morphine-Induced Analgesia

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ABSTRACT

The human μ-opioid receptor (HμOR) is a G-protein coupled receptor that mediates analgesia, euphoria and other important central and peripheral neurological functions. In this study, we found in a yeast two-hybrid screen that a protein kinase C-interacting protein (PKCI) specifically interacts with the C terminus of HμOR. The interaction of PKCI with HμOR was recapitulated in Chinese hamster ovary cells that express the full-length HμOR and PKCI proteins. The affinity of HμOR for an opioid ligand and its ability to mediate the activation of a G-protein were not altered by their interaction. However, the association of PKCI with HμOR reduced agonist-induced inhibition of adenylyl cyclase and suppressed HμOR desensitization partially at the G protein level and completely at the adenylyl cyclase level. Furthermore, PMA-induced, but not DAMGO-induced, HμOR phosphorylation was partially inhibited by the coexpression of PKCI, suggesting that PKCI exerts a selective regulatory effect on HμOR signaling. This effect was specific to the μ-opioid receptor because δ-opioid receptor desensitization was unaffected by PKCI. In addition, behavioral studies revealed that both basal and morphine-induced analgesia were significantly enhanced in the mutant mice that lacked expression of PKCI gene, and these mice developed a greater extent of tolerance to morphine analgesia. Taken together, these results suggest that PKCI functions as a negative regulator in HμOR desensitization, phosphorylation, and in mediating morphine analgesia.

Morphine, a major component of opium, has a wide range of effects in the body, but the most important one is the relief of pain. Early efforts to understand the endogenous targets for opiate drugs led to the discovery of three main classes of opioid receptors: μ- (MOR), δ-, and κ-opioid receptors. The pharmacological actions of opiates and recent studies of opioid receptor-deficient mouse models (Kieffer and Gaveriaux-Ruff, 2002) indicate that MOR represents the major molecular target for morphine analgesia and opioid addiction.

Many processes can regulate the functions of opioid receptors. Similar to other G protein-coupled receptors (GPCRs), opioid receptors generally undergo rapid desensitization, within seconds to minutes, after being activated by a ligand; this desensitization is thought to be mediated by ligand-dependent phosphorylation of the receptors followed by association of the phosphorylated receptors with arrestins. This sequence of events causes functional uncoupling of the receptors from their cognate heterotrimeric G proteins and promotes the rapid endocytosis of receptors (Lefkowitz, 1998). Receptor desensitization is crucially important in opioid pharmacology because this phenomenon has been associated with the development of tolerance to and dependence on opioid agents. In addition to receptor phosphorylation and endocytosis, other activation-dependent opioid receptor regulatory processes that have been implicated in the development of morphine tolerance and dependence include receptor down-regulation and mitogen activated protein kinase activation (Law et al., 1984; Trapaidze et al., 1996; Yu et al., 1997; Polakiewicz et al., 1998).

Agonist-induced phosphorylation has been clearly demonstrated (Arden et al., 1995; Pei et al., 1995; Zhang et al., 1996; Appleyard et al., 1997; Li et al., 2002) for all three opioid

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ABBREVIATIONS: MOR, μ-opioid receptors; GRK, G protein-coupled receptor kinase; PKC, protein kinase C; PKCI, protein kinase C-interacting protein; GPCRs, G protein-coupled receptors; HμOR, human μ-opioid receptor; HμCHO, Chinese hamster ovary cells stably expressing human μ-opioid receptor; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; PMA, phorbol 12-myristate 13-acetate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; MPE, maximum possible effect; mPKCI, murine protein kinase C-interacting protein.

receptors. It is generally accepted that phosphorylation of opioid receptors could initiate the desensitization process and promote internalization. In fact, multiple protein kinases have been implicated in opioid receptor phosphorylation. Thus far, three groups of protein kinases are reported to be involved: 1) members of the G protein-coupled receptor kinase (GRK) family (Pei et al., 1995; Yu et al., 1997; Lefkowitz, 1998; Zhang et al., 1998), 2) second messenger-activated kinases, such as protein kinases A and C (PKC) (Zhang et al., 1996; Wang and Sadee, 2000), and 3) tyrosine kinases (Appleyard et al., 2000; Kramer et al., 2000). However, the specific kinases in the brain that actually participate in opioid receptor phosphorylation are not known with certainty.

GPCR internalization is thought to occur after the receptors are phosphorylated by a GRK. In this process, receptors are endocytosed into an intracellular compartment in which specific phosphatases remove the phosphates and allow the dephosphorylated receptor to return to the cell surface. On the other hand, the receptors are targeted to lysosomes for degradation, which leads to receptor down-regulation (Ferguson et al., 1996; Krupnick and Benovic, 1998; Finn and Whistler, 2001). Although internalization has been thought to contribute directly to functional desensitization of opioid signaling by rapidly reducing the number of receptors present at the cell surface, it has been proposed that internalization also mediates receptor resensitization (Koch et al., 1998; Law et al., 2000). As a regulatory process, internalization is also dependent on the properties of the opioid ligand. Etorphine and many other opioids elicit rapid MOR desensitization and internalization, but morphine does not cause internalization (Keith et al., 1996). Recent studies on MOR revealed that administration of DAMGO markedly enhanced the analgesia after continuous administration of morphine (He et al., 2002). These results imply that internalization of MOR may provide protection against the development of tolerance (He et al., 2002). Phosphorylation of the MOR receptor seems important for the initiation of internalization (Whistler et al., 2001); however, the dynamic relationship between receptor phosphorylation and receptor internalization, desensitization, and down-regulation remains to be determined.

Little is known about the biochemical and molecular mechanisms responsible for functional regulation of opioid receptors, including which protein(s) directly interact with MOR and play a role in fine-tuning control of these regulatory processes. Therefore, the present study was designed to identify and functionally characterize novel intracellular protein(s) that participate in the cellular regulation of MOR signaling. Our results provide the first evidence that a novel protein PKCI interacts with MOR and that this interaction leads to the suppression of MOR desensitization and PKC-related MOR phosphorylation. In addition, from in vivo studies, we demonstrate that the analgesic effect of morphine, as well as the extent of tolerance, is greatly enhanced in mutant mice that lack expression of the PKCI gene.

Materials and Methods

Yeast Two-Hybrid Screening. The cDNA corresponding to the C-terminal tail (the last 65 amino acids, from 338 to 403) of mouse MOR was amplified by PCR and subcloned into the EcoRI and PstI sites of the pGBT9 yeast vector containing the GAL4 DNA-binding domain. This bait plasmid (pGBT9- MOR) was then transformed into yeast strain CG1945. Yeast two-hybrid screening was performed using mouse brain cDNA library (provided by Dr. Lin Mei, University of Alabama, Birmingham, AL) constructed in pACT2 vector containing the GAL4 activation domain. Positive clones were selected on plates lacking leucine, tryptophan, and histidine with 5 mM 3-aminotriazole and by colony-lift filter assay for β-galactosidase activity. The His^+ and $LacZ^+$ positive colonies were restreaked, and retested for their His and LacZ phenotypes for three times. After that, the pACT2-cDNA of positive clones was rescued in *Escherichia coli* KC8, and was cotransformed in yeast with pGBT9- MOR, or pLAM5′, which was an unrelated protein fused to the DNA-binding domain to eliminate false positives and to confirm two-hybrid interactions. Vector pGBT9 and pATC2 with no insert were used in control experiments. Thereafter, the cDNA inserts from positive clones were sequenced.

Cell Culture and Transient Transfection. Chinese hamster ovary cells that stably express full-length HμOR (HμCHO) were cultured in Ham's F-12 nutrient mixture containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml geneticin and incubated at 37°C with 5% CO_2. For transient transfection, the HμCHO cells were plated onto 150-mm-diameter dishes and grown to 50% confluence. Cells then were transfected with ~10 μg of murine protein kinase C-interacting protein (mPKCI)/pcDNA3 or mPKCI-*Myc*-*His*/pcDNA3.1 plasmid using LipofectAMINE after the instruction of manufacturer (Invitrogen, Carlsbad, CA). The efficiency of LipofectAMINE-mediated PKCI transfection was approximately 30 to 40%, estimated based on transfection rate of GFP plasmid control. The pcDNA3.1 plasmid with no insert was used as control. Experiments were performed 2 to 3 days after transfection.

Coimmunoprecipitation and Phosphorylation of the MOR. Transfected cells were washed twice with ice-cold phosphate-buffered saline (PBS), and lysed in $RIPA^+$ buffer (1% Nonidet P-40, 0.5% disodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1 μM okadaic acid, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A in PBS) for 1 h on ice. The lysate was centrifuged at 150,000*g* for 15 min at 4°C. Recovered supernatant was first incubated with 25 mg of protein A/Sepharose CL-4B beads (Amersham Biosciences, Piscataway, NJ) for 1 h to clarify any nonspecific binding to protein A from the lysate. At the same time, 3 μl of rabbit serum containing anti-HμOR polyclonal antibody (Svingos et al., 1996) was preincubated with 25 mg of protein A/Sepharose CL-4B beads for 1 h. The supernatant of the lysate was then added to the antibody-bound protein A beads, and the mixture was incubated for 2 h at 4°C with continuous rotating. Immunoprecipitates were washed three times with $RIPA^+$ buffer, mixed with 1× SDS sample buffer (4% SDS, 25 mM Tris-HCl, pH 6.8, 5% glycerol, 0.5% 2-mercaptoethanol, and 0.005% bromphenol blue), and analyzed by Western blotting using monoclonal anti-*Myc* antibody (Invitrogen).

For receptor phosphorylation, HμCHO and HμCHO-mPKCI cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium, and incubated at 37°C for 2 h with 300 μCi/ml of [^{32}P]orthophosphate (8500 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) in phosphate-free Dulbecco's modified Eagle's medium. Metabolically labeled cells were then exposed to 1 μM DAMGO or PMA for 10 min. Cells were cooled to 4°C by washing with ice-cold PBS. Proteins were extracted for 1 h at 4°C with 0.8 ml of $RIPA^+$ buffer and subjected to immunoprecipitation as described above. The immunoprecipitated proteins were then dissociated from the beads by extraction with 60 μl of SDS sample buffer and separated on 8% SDS-PAGE gels. The phosphorylated proteins were identified by autoradiography using Hyperfilm-MP (Amersham Biosciences) with intensifying screens. Densities for the bands of interest were quantified using a PhosphorImager and ImageQuant software (Amersham Biosciences) and were normalized to the amounts

of extracted cell protein. Phosphorylation differences between MORs under different treatments were analyzed by the Student's *t* test.

[^{35}S]GTPγS Binding Assay. HμCHO or HμCHO-mPKCI cells were pretreated for 20 min with or without 5 μM DAMGO in the culture medium and then harvested in Tris/EGTA/$MgCl_2$ (50 mM Tris-HCl, 10 mM EGTA, 5 mM $MgCl_2$, pH 7.4) and were dispersed by agitation. After centrifugation at 500*g*, the cell pellet was suspended in ice-cold Tris/EGTA/$MgCl_2$ and homogenized with a Wheaton homogenizer. The homogenate was centrifuged at 15,200*g* at 4°C for 10 min, resuspended in membrane buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% BSA, pH 7.4). Cell membranes (30 μg of protein) were preincubated at room temperature for 1 h with the appropriate concentrations of DAMGO (10^{-10}~10^{-5} M), then further incubated with 10 μM GDP (Sigma), in assay buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA) containing 80 pM [^{35}S]GTPγS (1250 Ci/mmol; PerkinElmer Life and Analytical Sciences) for 30 min at 30°C. Basal binding was assessed in the presence of 10 μM GDP and absence of DAMGO. Nonspecific binding was measured in the presence of 20 μM unlabeled GTPγS. The reaction was terminated by rapid filtration under vacuum through Brandel GF/B glass filters, followed by three washes with 3 ml of ice-cold 10 mM Tris-HCl, pH 7.4. Bound radioactivity was determined with the use of an LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Data are reported as mean ± S.E. of at least three separate experiments, each of which was performed in triplicate. Net-stimulated [^{35}S]GTPγS binding is defined as stimulated binding minus basal binding. Percentage stimulation is defined as (net stimulated binding/basal binding) × 100%. EC_{50} values were calculated by nonlinear regression analysis of concentration-effect curves with Prism.

Determination of Adenylyl Cyclase Activity. HμCHO and HμCHO-mPKCI cells were treated with or without 5 μM DAMGO in medium for 20 min. Cell membranes were collected by homogenizing the cells with buffer containing 2 mM Tris-HCl, pH 7.4, and 2 mM EGTA and centrifuged twice at 10,000*g* for 10min. Cell membranes corresponding to 30 μg of protein were added to the assay tubes. Then they were incubated with 20 μM forskolin and varying concentrations of DAMGO in the assay buffer: 80 mM Tris-HCl, pH 7.4, 10 mM theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, and 0.01 mM GTP for 12 min at 30°C, then was boiled for 2 min to terminate the reaction. [^{3}H]cAMP ([2,8-^{3}H]; 36.2 Ci/mmol, PerkinElmer Life and Analytical Sciences) at a final concentration of 4 nM in citrate-phosphate buffer, pH 5.0, and the cAMP binding proteins prepared from bovine adrenal glands were added to each sample, then were incubated on ice for 90 min. The radioactivity of supernatant containing bound cAMP, isolated using charcoal and centrifugation, was measured by liquid scintillation counting and converted to picomoles of cAMP by comparison to a standard curve. Dose-response curves were obtained by nonlinear regression analysis using Prism.

Chronic Morphine Treatment and the Hot-Plate Test. The derivation of mutant mice that have a homozygous deletion of the mPKCI gene (*mPKCI*$^{-/-}$), or so-called knockout mice, mice that have a heterozygous deletion of this gene (*mPKCI*$^{+/-}$), and corresponding wild-type mice (*mPKCI*$^{+/+}$), has been described previously (Su et al., 2003). *mPKCI*$^{+/+}$, *mPKCI*$^{+/-}$, and *mPKCI*$^{-/-}$ mice used in the present study were 2-month old male mice weighing between 20 and 30 g, and more than 96% of their genetic background is from the 129 strain mice. Experiments were conducted with an approved animal protocol from School of Pharmacy, University of Maryland Baltimore Animal Care and Use Committee. Mice were treated daily (between 2:00 PM and 3:00 PM) with morphine (10 mg/kg, s.c.) and antinociception was assessed by the hot-plate test 30 min later each day for 7 days. In each antinociception assay, nociceptive latencies were assessed as the response time to the hot plate (55°C). The response was defined by the animal forepaw licking. Thirty seconds was the cut-off time to avoid tissue damage. Data were reported as mean ± S.E. of the maximum possible effect (% MPE), which was determined by using the following calculation: 100% × [(drug response time − basal response time)/(30 − basal response time)] = % MPE. Dose-response curves were determined using a cumulative dosing scheme on days 1 and 7. In brief, mice were treated with 5, 10, and 20 mg/kg morphine on day 1. On day 7, mice were cumulatively treated with 10, 20, 40, and 80 mg/kg of morphine. The time interval between injections is about 35 min. ED_{50} was calculated by nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA).

Results

Identification of mPKCI That Specifically Interacts with the C Terminus of MOR. In this study, we searched, using the yeast two-hybrid system, for proteins that might play a role in MOR signaling. We used the C terminus of the MOR coding region as the bait (see *Experimental Procedures*) and screened a mouse whole-brain cDNA library. Using this approach, one clone was found to be strongly positive for activation of histidine biosynthesis as well as activation of the lacZ reporter. Sequence analysis revealed that the cDNA from this clone encoded a previously identified gene called *mPKCI* (Klein et al., 1998) with an unknown function (Fig. 1). We further examined the association between HμOR and mPKCI in mammalian cells using HμCHO. There is no endogenous PKCI expression in CHO cells (assessed by Western blot; data not shown), and the Myc-tagged mPKCI was transiently expressed in HμCHO cells. The mPKCI was immunoprecipitated with anti-MOR antiserum under basal or DAMGO-stimulated conditions. The mPKCI protein was visualized as a band with a size of 15 kDa on Western blot using a monoclonal anti-Myc antibody under both conditions (basal or DAMGO stimulated), but this protein was not present in the sample that lacked the polyclonal anti-MOR antiserum (Fig. 2), indicating that the interaction of HμOR with mPKCI was recapitulated in mammalian cells.

Characterizing the Effect of mPKCI on the Function of MOR. To determine whether the interaction of mPKCI

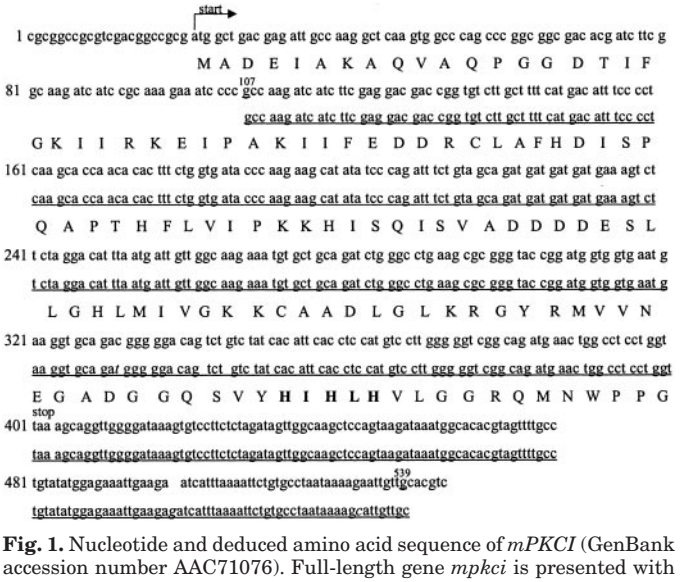

Fig. 1. Nucleotide and deduced amino acid sequence of *mPKCI* (GenBank accession number AAC71076). Full-length gene *mpkci* is presented with 80 nucleotides each line. Start and stop codons are labeled. Deduced amino acids are represented by single-letter codes. The characteristic motif of HIT protein family, HIHLH, is shown in bold. The sequence of the cDNA insert of the positive clone is also aligned, 99% matched the nucleotides from 107 to 539 of *mpkci*. The number of the start and end nucleotide of the cDNA insert is labeled. Mismatched nucleotides are shown in italic.

with HμOR would lead to changes in receptor pharmacology, we examined receptor ligand binding, G protein coupling, and inhibition of adenylyl cyclase activity. We found that the binding properties of MOR with [^{3}H]diprenorphine in cells expressing both HμOR and mPKCI (B_{max}, 0.54 ± 0.02 pmol/mg; K_d, 0.32 ± 0.05 nM) were similar to that when only the receptor was expressed (B_{max}, 0.50 ± 0.03 pmol/mg; K_d, 0.30 ± 0.03 nM). In a GTPγS binding assay, DAMGO, a μ-agonist, showed a similar G protein activation efficacy in both HμCHO and HμCHO–mPKCI cells with maximal response of 224 ± 0.8% and an EC_{50} of 9.3 ± 0.8 nM in the HμCHO and maximal response of 182 ± 4.8% and EC_{50} of 10.5 ± 1.3 nM in the HμCHO–mPKCI cells. After pretreatment with 5 μM DAMGO for 20 min, receptor-mediated GTP binding upon re-challenge with agonist was suppressed in both cell types, a phenomenon termed "acute desensitization". However, the level of HμOR desensitization was moderately attenuated in cells coexpressing HμOR and mPKCI (Table 1). DAMGO preincubation induced only a 3.5-fold shift in EC_{50} in the HμCHO–mPKCI cells, whereas it produced a 7.4-fold shift in the HμCHO cells. This effect seemed specific to MOR because DOR desensitization was unaffected by mPKCI (data not shown). The influence of HμOR-mPKCI association on receptor signaling and desensitization was also examined at the level of a downstream effector (i.e., the production of cAMP, measured by adenylyl cyclase assay). The ability of HμOR to inhibit adenylyl cyclase activity seemed to be reduced in the presence of mPKCI, and MOR desensitization was not observed in HμCHO plus mPKCI cells after pretreatment of DAMGO (Table2). These results revealed that the HμOR-mPKCI interaction altered the receptor-mediated inhibition of adenylyl cyclases activity and abolished DAMGO-induced desensitization.

Both HμOR-mPKCI and HμOR expressing cells displayed similar basal levels of MOR phosphorylation. However, the receptor phosphorylation induced under different circumstances was differentially affected by mPKCI. Thus, the PMA-induced MOR phosphorylation was reduced by 50% in HμOR-mPKCI cells compared with the cells expressing only MOR. In contrast, DAMGO-stimulated phosphorylation was unchanged by the presence of mPKCI (Fig. 3).

Enhancement of Morphine Analgesia in Mice Lacking the mPKCI Gene. Our in vitro studies suggested that mPKCI could serve as a negative regulator of MOR phosphorylation and desensitization. Because phosphorylation and desensitization are thought to play important roles in the development of tolerance in vivo, we conducted behavioral studies on mPKCI gene knockout mice to enhance our understanding of the role of mPKCI in regulating the in vivo function of MOR. The mutant mice were viable, fertile, and showed no overt anatomical deficit as reported previously (Su et al., 2003). To determine whether morphine-induced analgesia was affected in mPKCI knockout mice, the dose-response effect as well as the time course of morphine analgesia was examined in the knockout and wild-type mice using a hot-plate assay. We found that the *mPKCI*$^{-/-}$ mice displayed a moderate but statistically significant elevation of basal threshold compared with that of *mPKCI*$^{+/+}$ mice, with values of 11.8 ± 0.6 s (n = 15) for *mPKCI*$^{-/-}$, and 9.5 ± 0.6 s (n = 15) for the *mPKCI*$^{+/+}$ ($p < 0.01$) respectively. However, there was no difference in the basal threshold between heterozygotes (*mPKCI*$^{+/-}$) and wild-type littermates ($p > 0.05$, data not shown). Compared with the wild-type mice, mPKCI knockout mice (*mPKCI*$^{-/-}$) also displayed significantly enhanced analgesia at relatively low doses of morphine, 5 and 10 mg/kg ($p < 0.01$), but not at a high dose, 20 mg/kg ($p > 0.05$; Fig. 4*A*). There was no enhancement of morphine-induced analgesia in heterozygous mPKCI$^{+/-}$ mice (data not shown).

In the time-course study, a single dose of 10 mg/kg morphine elicited analgesia in the wild-type mice, which declined 90 min after injection and was absent in 3 h. The pattern and duration of morphine-induced analgesia in the mPKCI knockout mice was similar to that of the wild-type mice, except that the knockout mice showed an overall enhancement of analgesia, especially at the initial period after morphine administration. Therefore, the subsequent declining rate of analgesia was more rapid in mPKCI knockout mice than that in the wild-type mice, as shown with steeper recovering slope (Fig. 4B)

Development of Morphine-Induced Tolerance in mPKCI$^{-/-}$ Mice. In wild-type mice, 10 mg/kg morphine produced acute analgesia. With chronic morphine exposure, wild-type mice developed tolerance to morphine-induced analgesia. Thus, as shown in Fig. 5A, the analgesic response, assessed by % MPE, gradually decreased after repeated morphine administration (10 mg/kg, s.c., once per day) and almost disappeared on the seventh day. The *mPKCI*$^{-/-}$ mice developed tolerance as well. The time-course response curves for both genotypes were parallel (Fig. 5A), although the mice displayed different levels of analgesia among the genotypes.

The extent of tolerance was further determined by comparing the dose-response curve between the wild-type and mPKCI knockout mice (Fig. 5, B and C). With long-term morphine exposure (i.e., 7 days) there was a rightward shift of the dose-response curve in both *mPKCI*$^{+/+}$ and *mPKCI*$^{-/-}$ mice compared with their control groups, indicating the development of chronic tolerance. Furthermore, this rightward shift of EC_{50} in the *mPKCI*$^{-/-}$ mice was 19 times greater than that in *mPKCI*$^{+/+}$ mice (Table 3). Therefore, the extent of tolerance development in *mPKCI*$^{-/-}$ mice to morphine was greater than that in *mPKCI*$^{+/+}$ mice as measured by comparing the EC_{50} rightward shift. Nevertheless the analgesic effect in *mPKCI*$^{-/-}$ mice was still higher than that of wild type at the end of 7 days' treatment (Fig. 5A) because of their initial high level of analgesia.

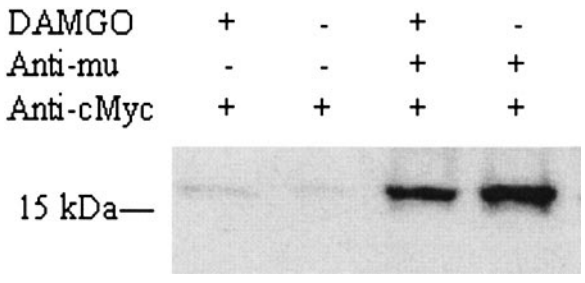

Fig. 2. μOR interacted with mPKCI in CHO cells. HμCHO cells transiently transfected with mPKCI were treated with or without 1 μM DAMGO. Receptors were immunoprecipitated with anti-HμOR antiserum and subsequent immunoblotting with antibodies directed against cMyc tag of mPKCI. Molecular size standards, which were derived from prestained markers, were electrophoresed in adjacent lanes. The expected size of mPKCI was 15 kDa.

Discussion

Mutagenesis analyses for several GPCRs have indicated that the third cytoplasmic loop and C termini are most likely involved in the coupling of receptor-G protein complexes and in functional regulation of these receptors (Benovic et al., 1988; Dohlman et al., 1991; Eason et al., 1995). Our previous studies showed that a C-terminally truncated MOR completely lost DAMGO-induced desensitization and 60% of receptor phosphorylation (Deng et al., 2000). Therefore, in the present study, the C terminus of MOR was used as bait in the yeast-two hybrid screening system to search for a protein that interacts with MOR. Using this strategy, we identified the mPKCI protein.

PKCI is a member of the HIT family of proteins that contain a His*X*His*X*His motif, in which *X* is a hydrophobic amino acid (Klein et al., 1998). PKCI was originally identified as a bovine protein that could inhibit the in vitro phospholipid and Ca^{2+}-dependent kinase activity of bovine brain PKC. However, subsequent studies have questioned the physiological relevance of bovine PKCI as an inhibitor of PKC and the interaction between PKCI and PKC (Walsh and Dzwonczyk, 1985; Fraser and Walsh, 1991; Klein et al., 1998). In addition, a structure-based analysis using X-ray crystallography and in vitro enzyme assays suggest that the HIT proteins function as nucleotidyl hydrolases or transferases (Lima et al., 1997). Recent studies indicate that PKCI knockout mice display increased susceptibility to carcinogenicity, suggesting that PKCI may normally play a tumor suppressor role (Su et al., 2003). Nevertheless, the definitive function(s) of the PKCI protein are not known. The present

TABLE 1
Effect of DAMGO pretreatment on DAMGO-stimulated [^{35}S] GTPγS binding in HμOR and HμOR-mPKCI expressing cells

	HμCHO		HμCHO-mPKCI	
	Maximal Response	EC_{50}	Maximal Response	EC_{50}
	%	*nM*	%	*nM*
Without pretreatment of DAMGO	224.0 ± 6.2	9.3 ± 0.8	182.0 ± 4.8	10.5 ± 0.8
With pretreatment of DAMGO	168.0 ± 4.2$^{\#}$	68.9 ± 0.8$^{\#\#}$	141.0 ± 2.9$^{\#}$	36.8 ± 0.8$^{\#*}$

$^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$, compared with that without DAMGO pretreatment.
* $p < 0.05$, compared with that in HμCHO cells.

TABLE 2
Effect of DAMGO pretreatment on HμOR-mediated inhibition of forskolin-stimulated adenylyl cyclase activity in cells expressing HμOR alone or with mPKCI

	HμCHO		HμCHO-mPKCI	
	Maximal Response	EC_{50}	Maximal Response	EC_{50}
	%	*nM*	%	*nM*
Without pretreatment of DAMGO	68.9 ± 2.9	2.6 ± 0.9	69.1 ± 2.3	10.2 ± 0.8**
With pretreatment of DAMGO	55.7 ± 0.3$^{\#}$	9.2 ± 0.6$^{\#\#}$	68.5 ± 0.4*	13.4 ± 0.6

$^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$, compared with that without DAMGO pretreatment.
* $p < 0.05$; ** $p < 0.01$, compared with that in HμCHO cells.

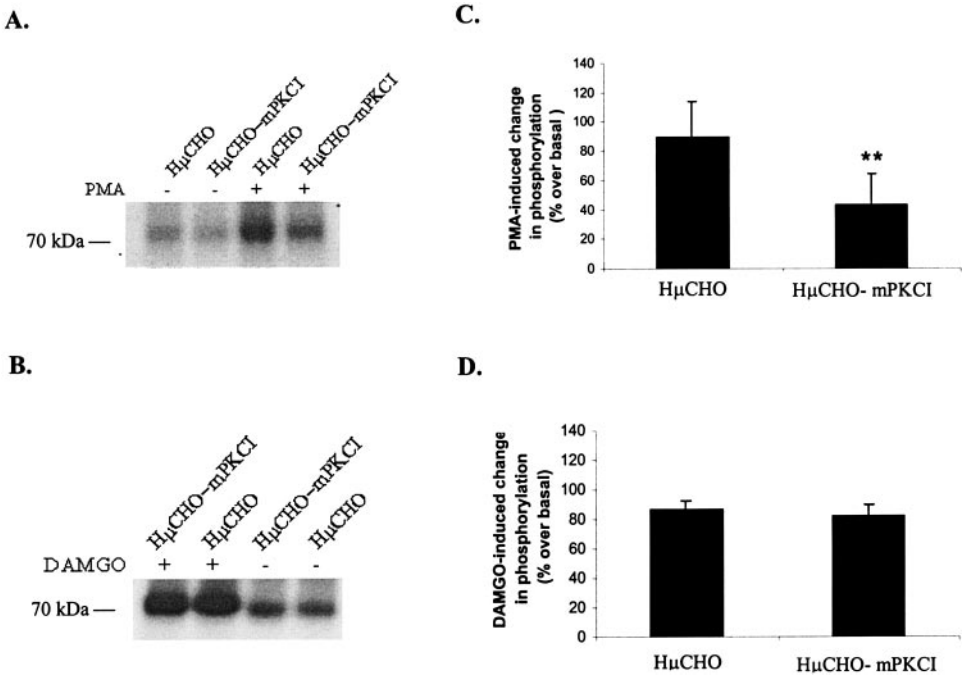

Fig. 3. PMA-induced, not DAMGO-induced HμOR phosphorylation was reduced by mPKCI. A, HμCHO and HμCHO-mPKCI cell preparations were treated in the presence or absence of 1 μM PMA. B, HμCHO and HμCHO-mPKCI cell preparations were incubated with or without 1 μM DAMGO. Cell extracts were immunoprecipitated with anti-HμOR antiserum, and the radiolabeled proteins were identified by autoradiography. Molecular size standards, which were derived from prestained markers, were electrophoresed in adjacent lanes. The expected size of phosphorylated HμOR was 70 KDa. PMA-induced (C) and DAMGO-induced (D) changes in phosphorylation density were presented as the percentage over the basal phosphorylation (without drug treatment). The density of receptor phosphorylation was quantitatively analyzed by PhosphorImager. The drug-induced change in phosphorylation density was calculated following the formula: (density of drug-induced phosphorylation − density of basal phosphorylation)/(density of basal phosphorylation) × 100%. **, $p < 0.01$ versus HμCHO cells (Student's *t* test).

study provides the first evidence that mPKCI can inhibit PKC-related phosphorylation of MOR. Although it is unclear whether the inhibition of MOR phosphorylation in mPKCI-expressing cells is caused by the direct inhibition of PKC activity or exerted through an indirect effect, our results suggest that PKCI could play an important role in the regulation of neurotransmitter receptors, in addition to its potential role in tumor suppression.

Previous evidence for the expression of mPKCI in mouse brain tissues, at both mRNA and protein levels (Klein et al., 1998), provide additional support for PKCI's potential involvement with MOR and/or other neurotransmitter receptors. However, studies on the intracellular localization of PKCI in normal human mammary epithelial cells and human breast cancer cells revealed that PKCI was present mainly in the nucleus with less amounts in the cytoplasm (Klein et al., 1998), but this was not studied in neuronal cells. Therefore, the intracellular location of PKCI in neuronal cells remains to be determined.

It is generally accepted that phosphorylation of opioid receptors can initiate desensitization and promote internalization. Agonist-induced phosphorylation, usually mediated through GRK, is insensitive to pretreatments with the PKC inhibitor staurosporine, whereas phorbol ester-induced MOR phosphorylation could be inhibited by staurosporine, indicating that multiple kinases and pathways are involved in MOR

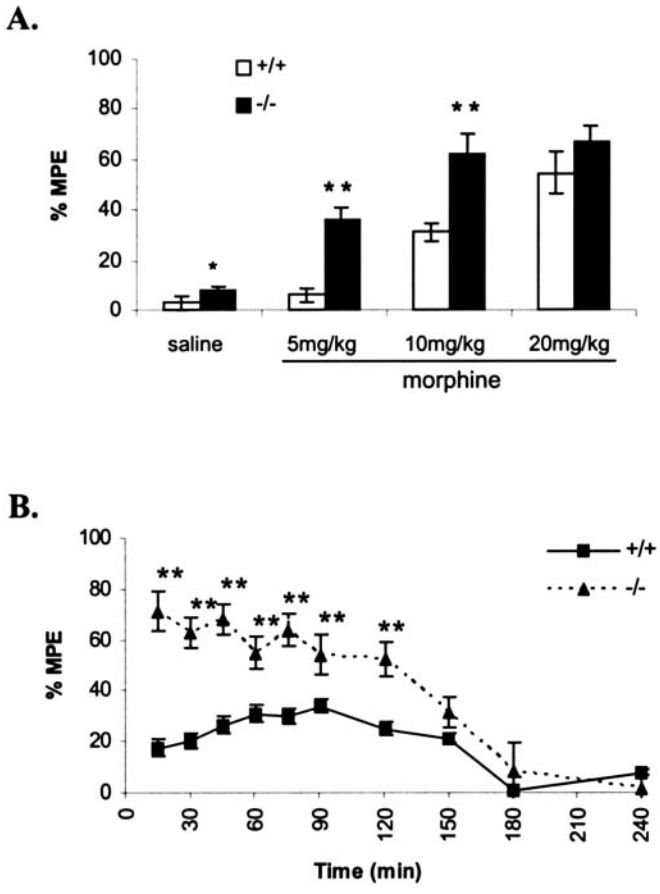

Fig. 4. Hot-plate responses. A, dose-response: morphine induced augmented analgesia in $mPKCI^{-/-}$ mice at low and intermediate doses. $mPKCI^{+/+}$ (n = 13, open) and $mPKCI^{-/-}$ (n = 9, black) were injected with saline and cumulative morphine, at indicated dose, and then hot-plate response was assessed 30 min after injection. B, time course: morphine produced elevated but not prolonged analgesia in $mPKCI^{-/-}$ mice over a 4-h period. Hot-plate responses were recorded 15, 30, 45, 60, 75, 90, 120, 150, 180 and 240 min after a single 10 mg/kg morphine injection in $mPKCI^{+/+}$ (n = 13, ■) and $mPKCI^{-/-}$ (n = 9, ▲) mice. Data were presented as mean ± S.E. of each group of mice. **, $p < 0.01$ versus $mPKCI^{+/+}$ (Student's t test).

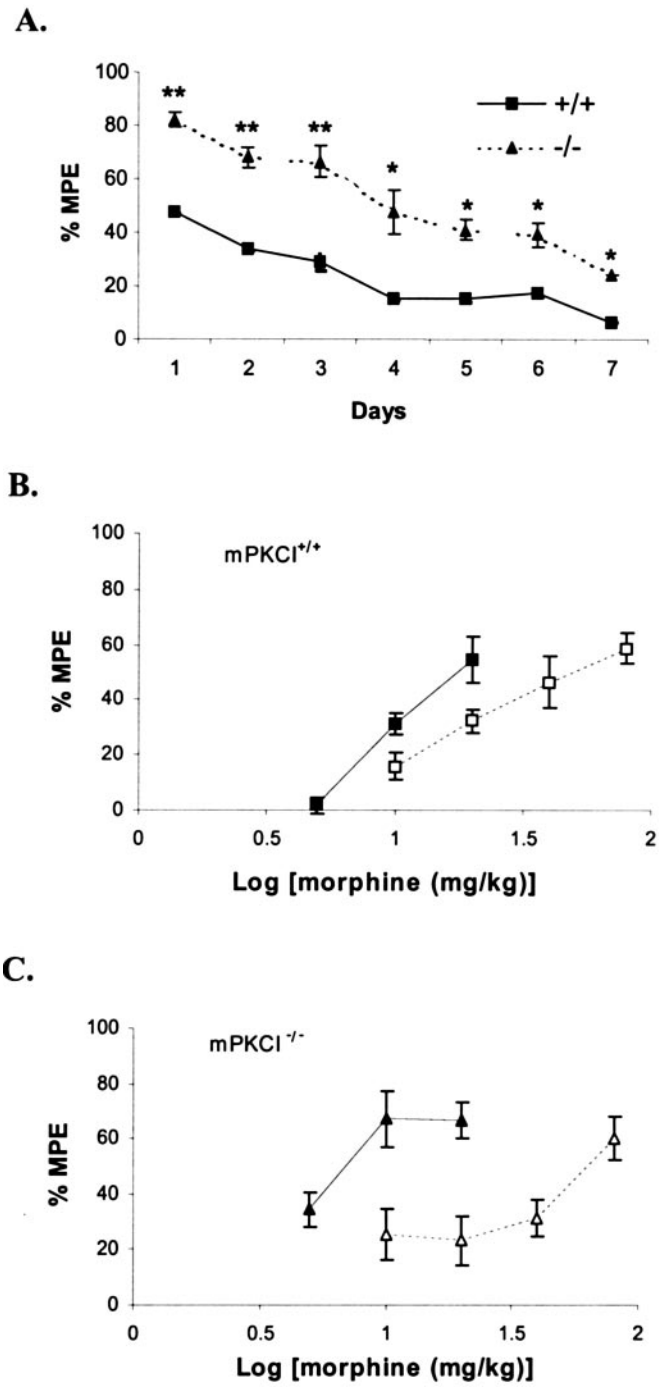

Fig. 5. Chronic tolerance A, time course of tolerance development $mPKCI^{+/+}$ (■, n = 17) and $mPKCI^{-/-}$ (▲, n = 9) mice were treated daily with morphine (10 mg/kg, s.c.) for 7 days, and hot-plate latencies were recorded 30 min after the injection on the days indicated. B, dose-response curve shift in $mPKCI^{+/+}$ mice (n = 13, ■, day 1; □, day 7); C, dose-response curve shift in $mPKCI^{-/-}$ mice (n = 9; ▲, day 1; △, day 7). Dose-response curves were determined using a cumulative dosing scheme on days 1 (5, 10, 20 mg/kg of morphine) and 7 (10, 20, 40, 80 mg/kg) in mice, as described under *Materials and Methods*. ED_{50} was determined by nonlinear regression analysis using GraphPad Prism software. Data were presented as the mean ± S.E.

phosphorylation, under varying circumstances. Our finding that mPKCI inhibited PMA-induced, but not DAMGO-induced phosphorylation, suggests that mPKCI exerts differential regulatory activities on the function of MOR. According to the DNA sequence analysis of MOR receptor (Wang et al., 1993), there are consensus sites for PKC phosphorylation in the intracellular loops and proximal region of the C terminus of the receptor; several serine and threonine residues located in the distal region of the C terminus have been suggested as the recognition sites for GRK (Deng et al., 2000). Therefore, it is possible that PKCI could interact with PKC sites without affecting GRK sites because they are located in distinct domains of the receptor.

However, it is noted that DAMGO-induced desensitization was partially suppressed by the MOR and mPKCI interaction at the G-protein level and then amplified to complete loss at the adenylyl cyclase level. A possible explanation to this paradox is that DAMGO-induced desensitization is the consequence of receptor phosphorylation caused by multiple protein kinases including GRK, PKC, and tyrosine kinases, as discussed above. Alteration in the activity of any of these kinases with respect to receptor phosphorylation (in this case, PKC) could lead to a change in receptor desensitization. There is also a possibility that mPKCI interference with receptor desensitization involves different mechanisms that are not related to receptor phosphorylation.

The striking findings in our studies were that the PKCI gene knockout mice displayed an elevation of basal threshold for pain, a higher analgesic efficacy to morphine, and a greater extent of tolerance than those of the wild-type mice. It is our observation that the difference in analgesia between the knockout mice and wild type is in the lower dose range. In the literature, it has been demonstrated that morphine-induced analgesia was dose-dependently increased in MOR knockout mice when injected with morphine greater than 10 mg/kg in both tail-flick and hot-plate assays, suggesting that additional components (e.g., other opioid receptor subtypes), might be involved in morphine-induced analgesia at high dose (Sora et al., 1997). This would explain why the effect of mPKCI, a specific interactive protein with MOR, is less observable in higher doses of morphine in which morphine-elicited analgesia might be mediated more than one type of receptors.

The effects of a higher analgesic efficacy and a greater extent of tolerance were clearly different from what had been previously observed in a study of β-arrestin gene knockout mice, which revealed a stronger morphine analgesic effect and a lower tolerance development pattern (Bohn et al., 1999, 2000). The differing efforts of PKCI and β-arrestin on morphine-mediated behavioral changes in mice is not surprising because these two proteins seem to be involved in distinct signal transduction pathways: β-arrestin-mediated GRK-phosphorylated receptor trafficking and PKCI attenuation of PKC-induced receptor phosphorylation. The overall manifestation of tolerance obviously involves the interplay of various neural circuits and the regulation of numerous cellular signaling components, in addition to the opioid receptors. For example, there is evidence that activation of PKC might play an integral part in the development of morphine antinociceptive tolerance (Inoue and Ueda, 2000; Zeitz et al., 2001). Thus, PKC-γ mutant mice displayed reduced development of tolerance to the analgesic effects of morphine. In the present study, mPKCI was shown to inhibit PKC-mediated phosphorylation of MOR in vitro. If this holds true for the in vivo situation, this inhibition of PKC could be alleviated in the mPKCI knockout mice, and the resulting increase in PKC activity might result in augmented development of tolerance. In future studies, it will be of interest to determine whether there are other behavioral changes in response to morphine administration, especially in the development of dependence, in the PKCI knockout mice. The differing efforts of PKCI and β-arrestin on morphine-mediated analgesia and tolerance in mice also suggest that the analgesic effect and development of tolerance effect may be regulated by different mechanisms. The enhancement in analgesia may lead to a decrease or increase of tolerance development depending on which signal transduction pathways are involved.

TABLE 3
Effect of chronic morphine exposure on morphine ED_{50} in mice

	ED_{50} (Day 1)	ED_{50} (Day 7)	ED_{50} Shift
$MPKCI^{+/+}$	14.6 ± 3.2	30.5 ± 1.2#	2.1
$MPKCI^{-/-}$	3.8 ± 3.5*	151.1 ± 11.0## **	39.8**

# $p < 0.05$; ## $p < 0.01$, compared with that in day 1.
* $p < 0.05$; ** $p < 0.01$, compared with $mPKCI^{+/+}$ mice.

The precise mechanisms underlying the behavioral changes in the mPKCI knockout mice remain to be determined. The results of the study suggest that mPKCI might be involved with several events that can alter receptor function, such as desensitization and PKC-induced receptor phosphorylation. Thus, it is possible that lack of expression of mPKCI causes the receptor to become more susceptible to desensitization and thus leads to the development of a greater extent of tolerance. On the other hand, however, desensitization and PKC-induced MOR phosphorylation may not be the mechanism for the underlying behavioral change observed in mPKCI knockout mice, because these cellular responses observed under DAMGO may not be exactly the same as those underlying the behavioral changes induced by morphine. Although both of them are μ-agonists, the roles of DAMGO and morphine in MOR mediated cellular signaling are varied.

Although our cellular studies demonstrated that mPKCI reduced MOR's ability to inhibit adenylyl cyclase and phosphorylation, they did not explain why PKCI knockout mice show increased analgesia. We have hypothesized that the interaction of mPKCI and MOR may not only affect receptor phosphorylation but also alter ion gating and intracellular Ca^{2+} disposition, which are two well established MOR-mediated neuronal functions that relate to the mechanism of opioid analgesia (Williams et al., 1982). The interaction of mPKCI and MOR presumably has an inhibitory effect on ion gating, the inhibition would be removed in the knockout mice, and that could lead to a better function of ion channels and increase of morphine analgesia. Future testing of mPKCI effect on MOR-mediated ion channels, particularly voltage-gated Ca^{2+} and K^{+} channels, would provide insightful information on the mechanism of mPKCI activity in morphine analgesia.

In summary, the present study indicates that the mPKCI protein, which we identified by yeast two-hybrid screening, specifically interacts with the C terminus of MOR. This interaction leads to attenuation of receptor desensitization and inhibition of PKC-induced receptor phosphorylation. Furthermore, a deficiency in the expression of mPKCI in mice

can significantly enhance both basal and morphine-induced analgesia and causes a greater extent of tolerance to morphine than in wild-type mice.

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