Involvement of Spinal Protein Kinase Cγ in the Attenuation of Opioid μ-Receptor-Mediated G-Protein Activation after Chronic Intrathecal Administration of [D-Ala^2,*N*-MePhe^4,Gly-Ol^5]Enkephalin

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The present study was designed to investigate the role of a protein kinase C (PKC) isoform in the uncoupling of the μ-opioid receptor from G-proteins after repeated intrathecal injection of a selective μ-receptor agonist, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), in the spinal cord of mice. The activation of G-proteins by opioids was measured by monitoring the guanosine-5′-*o*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding. Mice were injected intrathecally with saline or DAMGO once a day for 1–7 d. At 24 hr after every injection the spinal cord membranes were prepared for the assay. The enhanced [^{35}S]GTPγS binding by μ-agonists DAMGO, endomorphin-1, or endomorphin-2 was attenuated clearly in spinal cord membranes obtained from mice that were treated intrathecally with DAMGO for 5 and 7 d, but not for 1 or 3 d. By contrast, no change in levels of [^{35}S]GTPγS binding induced by the δ-receptor agonist SNC-80 or κ-receptor agonist U-50,488H was noted in membranes obtained from mice that were treated with DAMGO. Concomitant intrathecal administration of a specific PKC inhibitor Ro-32-0432 with DAMGO blocked the attenuation of DAMGO-induced G-protein activation that was caused by chronic DAMGO treatment. Western blotting analysis showed that chronic DAMGO treatment increased the levels of PKCγ, but not PKCα, PKCβI, and PKCβII isoforms, in spinal cord membranes. Furthermore, mice lacking PKCγ failed to exhibit the desensitization of the DAMGO-stimulated [^{35}S]GTPγS binding after repeated DAMGO injection. These findings indicate that repeated intrathecal administration of DAMGO may activate the PKCγ isoform and in turn cause a desensitization of μ-receptor-mediated G-protein activation in the mouse spinal cord.

Key words: μ-opioid receptor; protein kinase C; phosphorylation; tolerance; G-protein; spinal cord

The opioid agonists modulate a number of physiological processes including pain, reward, stress, and immune responses via the stimulation of various opioid receptors (Mansour et al., 1988). One of the major opioid receptor types, the μ-opioid receptor, was cloned in 1993 and classified as a G-protein-coupled receptor (Chen et al., 1993). Opioids mainly inhibit cyclic AMP formation, close voltage-sensitive Ca^{2+} channels, and open K^+ channels via the stimulation of $G_{i/o}$ proteins (Childers, 1991). Over the past few years opioids, including μ-opioids, also have been shown to activate the phosphoinositide-signaling cascade in a variety of cells and neural tissues (Chen and Huang, 1991; Mangoura and Dawson, 1993; Smart et al., 1995; Ueda et al., 1995).

The receptor-coupled hydrolysis of membranal phosphoinositides, particularly phosphatidyl inositol 4,5-bisphosphate (PIP_2), yields two intracellular second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates protein kinase C (PKC), and IP_3 mobilizes Ca^{2+} after binding with cytoplasmic IP_3 receptors (Berridge, 1987; Fisher et al., 1992, 1993). These processes appear to be an important part of the signal transduction mechanism for controlling the various cellular events in the CNS.

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PKC is a key regulatory enzyme that modulates both presynaptic and postsynaptic neuronal function, synthesis and release of neurotransmitters, and the regulation of receptors. PKC has expanded into a family of closely related protein, which can be subdivided and classified on the basis of certain structural and biochemical similarities. Several PKC isoforms, especially conventional PKCs (cPKCs) including α, βI, βII, and γ that are Ca^{2+}-dependent and activated by both phosphatidylserine (PtdSer) and DAG, have been identified in neurons of the spinal cord; in each case immunocytochemistry has shown that they are concentrated in the superficial laminae of the dorsal horn (Malmberg et al., 1997; Martin et al., 1999).

We previously reported that activation of PKC in the spinal cord is implicated in the development of spinal antinociceptive tolerance to μ-opioid receptor agonists in mice (Narita et al., 1995). The μ-opioid receptor contains several potential phosphorylation sites (Chen et al., 1993; Kaufman et al., 1995). It has been hypothesized that activated PKC directly phosphorylates the opioid receptor and subsequently induces the uncoupling of opioid receptors from G-proteins (Pei et al., 1995). However, there is little or no direct evidence to support the contention that repeated stimulation of μ-opioid receptors by opioid μ-agonists produces the PKC-dependent uncoupling of μ-opioid receptors from G-proteins. In the present study we therefore investigated whether repeated intrathecal injection of a highly selective μ-opioid receptor agonist, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO), causes any changes in the increase of μ-opioid

receptor-stimulated guanosine-5′-*o*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding and the levels of membrane-located cPKC isoforms in the mouse spinal cord. We also investigated whether repeated intrathecal injections of DAMGO could affect the DAMGO-stimulated [^{35}S]GTPγS binding in mice lacking the PKCγ isoform.

MATERIALS AND METHODS

Animals. Male CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) and PKCγ knock-out mice (The Jackson Laboratory, Bar Harbor, MA), which were maintained on C57BL/6 and 129Sv mixed genetic backgrounds as described previously (Abeliovich et al., 1993), were used. Animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12 hr light/dark cycle.

[^{35}S]GTPγS binding assay. The spinal cord was homogenized in ice-cold Tris-Mg^{2+} buffer containing (in mM) 50 Tris-HCl, pH 7.4, 5 $MgCl_2$, and 1 EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 48,000 × *g* at 4°C for 10 min. The pellets were resuspended in [^{35}S]GTPγS binding assay buffer containing (in mM) 50 Tris-HCl, pH 7.4, 5 $MgCl_2$, 1 EGTA, and 100 NaCl and recentrifuged at 48,000 × *g* at 4°C for 10 min. The final pellets were resuspended in assay buffer as membranous fractions for the [^{35}S]GTPγS binding. The reaction was initiated by the addition of membrane suspension (3–8 μg of protein for each assay as determined by the method of Bradford, 1976) into the assay buffer with the opioid receptor agonists, 30 μM guanosine-5′-diphosphate (GDP), and 50 pM [^{35}S]GTPγS (1000 Ci/mmol; Amersham, Arlington Heights, IL). The suspensions were incubated at 25°C for 2 hr, and the reaction was terminated by filtering through Whatman GF/B glass filters, which had been soaked previously in a soaking buffer of 50 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ at 4°C for 2 hr, using a Brandel cell harvester (model M-24; Brandel, Gaithersburg, MD). Then the filters were washed three times with 5 ml of an ice-cold Tris-HCl buffer, pH 7.4, and transferred to scintillation counting vials containing scintillation cocktail, 0.5 ml of Soluene-350 (Packard Instrument, Meriden, CT), and 4 ml of Hionic Fluor (Packard Instrument). The radioactivity in the samples was determined with a liquid scintillation analyzer (model 1600CA, Packard Instrument). Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS.

Western blotting. The spinal cord was removed quickly after decapitation of mice and homogenized in ice-cold buffer A containing (in mM) 20 Tris-HCl, pH 7.5, 2 EDTA, 0.5 EGTA, and 1 phenylmethylsulfonyl fluoride plus 25 μg/ml leupeptin, 0.1 mg/ml aprotinin, and 0.32 M sucrose. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the supernatant was ultracentrifuged at 100,000 × *g* for 30 min at 4°C. The resulting supernatant was retained as the cytosolic fraction. The pellets were washed with buffer B (buffer A without sucrose) and homogenated in buffer B with 1% Triton X-100. After incubation for 45 min, soluble fractions were obtained by ultracentrifugation at 100,000 × *g* for 30 min and then were retained as membranous fractions for Western blotting. An aliquot of tissue sample was diluted with an equal volume of 2× electrophoresis sample buffer (Protein Gel Loading Dye-2X, Amresco, Solon, OH) containing 2% SDS and 10% glycerol with 0.2 M dithiothreitol. Proteins (5–20 μg/lane as determined by the method of Bradford, 1976) were separated by size on 4–20% SDS-polyacrylamide gradient gel by using the buffer system of Laemmli (1970) and were transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection of PKC isozymes the membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk (Bio-Rad Laboratories, Hercules, CA) for 1 hr more at room temperature with agitation. The membrane was incubated with primary antibody diluted in TBS [PKCα, PKCβI, PKCβII, and PKCγ at ratios of 1:4000 (α), 1:3000 (βI and βII), 1:1000 (γ); Santa Cruz Biotechnology, Santa Cruz, CA] containing 5% nonfat dried milk overnight at 4°C. Then the membrane was washed twice for 5 min and twice for 10 min in Triton X-TBS (TTBS) containing TBS and 0.05% Triton X-100, followed by 2 hr of incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) diluted 1:10,000 in TBS containing 5% nonfat dried milk. After this incubation the membranes were washed twice for 5 min and then three times for 10 min in TTBS. The antigen–antibody peroxidase complex was detected finally by enhanced chemiluminescence (Pierce, Rockford, IL) according to the manufacturer's instructions and visualized by exposure to Amersham Hyperfilm (Amersham Life Sciences, Arlington Heights, IL).

Table 1. Opioid-induced increase in [^{35}S]GTPγS binding to spinal cord membranes of mice

Treatment	% Stimulation (% of control)
10 μM DAMGO (μ-agonist)	89.2 ± 3.5
10 μM DAMGO + 10 μM CTOP (μ-antagonist)	−1.6 ± 3.2*
10 μM Endomorphin-1 (μ-agonist)	64.8 ± 3.6
10 μM Endomorphin-1 + 10 μM CTOP	5.7 ± 1.9*
10 μM Endomorphin-2 (μ-agonist)	68.9 ± 3.4
10 μM Endomorphin-2 + 10 μM CTOP	−2.3 ± 0.2*
10 μM DPDPE (δ-agonist)	18.1 ± 2.4
10 μM DPDPE + 0.1 μM NTI (δ-antagonist)	0.4 ± 1.2*
10 μM [D-Ala^2]deltorphin II (δ-agonist)	38.0 ± 6.0
10 μM [D-Ala^2]deltorphin II + 1 μM NTI	−0.4 ± 2.7*
10 μM SNC-80 (δ-agonist)	30.4 ± 4.3
10 μM SNC-80 + 1 μM NTI	1.5 ± 4.5*
10 μM U-50,488H (κ-agonist)	15.3 ± 1.2
10 μM U-50,488H + 0.1 μM nor-BNI (κ-antagonist)	3.5 ± 2.7*
10 μM U-69,593 (κ-agonist)	16.1 ± 2.0
10 μM U-69,593 + 0.1 μM nor-BNI	−0.6 ± 3.5*

The reaction was initiated by the addition of membrane suspension obtained from the mouse spinal cord into the assay buffer with 10 μM opioid, 30 μM GDP, and 50 pM [^{35}S]GTPγS. Data represent the mean ± SEM for 3–17 samples. The basal [^{35}S]GTPγS binding in mice treated with saline for 5 d was 29.2 ± 1.2 fmol/mg protein. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. *$p < 0.05$ versus agonist alone. *F* values of the one-way ANOVA in DAMGO, endomorphin-1, endomorphin-2, DPDPE, [D-Ala^2]deltorphin II, SNC-80, U-50,488H, and U-69,593 is $F_{(1,18)} = 120.608$, $F_{(1,10)} = 93.215$, $F_{(1,7)} = 238.911$, $F_{(1,10)} = 19.470$, $F_{(1,10)} = 14.072$, $F_{(1,4)} = 32.297$, $F_{(1,4)} = 32.937$, and $F_{(1,4)} = 32.667$, respectively.

Intrathecal injection. Intrathecal administration was performed by following the method described by Hylden and Wilcox (1980), using a 25 μl Hamilton syringe with a 30 gauge needle. Injection volumes were 5 μl for intrathecal injection.

Drugs. The drugs used were DAMGO (Bachem California, Torrance, CA); endomorphin-1 (Tocris Cookson, Ballwin, MO); endomorphin-2 (Tocris Cookson); D-Phe-Cys-Tyr-D-Try-Orn-Thr-Pen-Thr-NH_2 (CTOP; Bachem California); [D-Pen^{2,5}]enkephalin (DPDPE; Bachem California); [D-Ala^2]deltorphin II (Molecular Research Laboratories, Durham, NC); SNC-80 (Tocris Cookson); naltrindole (NTI; Research Biochemicals, Natick, MA); U-50,488H (Research Biochemicals); U-69,593 (Research Biochemicals); nor-BNI (Research Biochemicals); Ro-32-0432 (Calbiochem-Novabiochem, San Diego, CA); GTPγS (Research Biochemicals); and GDP (Sigma, St. Louis, MO).

Statistical analysis. The data are expressed as the mean ± SEM. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test (comparison with a control group) or Newman–Keuls test (comparisons between multiple groups).

RESULTS

Effects of chronic intrathecal treatment with DAMGO on increases of the [^{35}S]GTPγS binding induced by μ-, δ-, and κ-opioid agonists in the spinal cord

Under these conditions groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 1–7 d. At 24 hr after the last injection of each group the spinal cord membranes were prepared for each assay. As shown in Table 1, the μ-opioid receptor agonists DAMGO, endomorphin-1, and endomorphin-2, each at 10 μM, produced marked increases in [^{35}S]GTPγS binding to spinal cord membranes obtained from mice treated intrathecally with saline for 5 d. To determine whether these increases of the [^{35}S]GTPγS binding were mediated by the stimulation of μ-opioid receptors, we studied the effects of the μ-opioid receptor antagonist CTOP on μ-agonist-

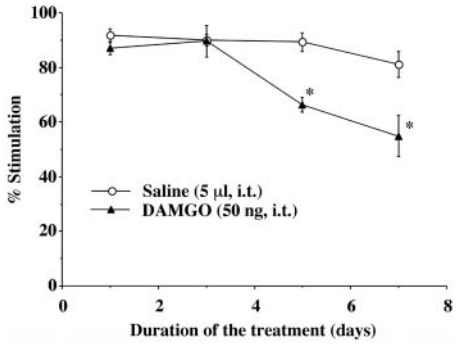

Figure 1. Time course effect of repeated intrathecal injection of DAMGO on the DAMGO-induced increase in [^{35}S]GTPγS binding to spinal cord membranes. Groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 1–7 d. At 24 hr after every injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without DAMGO (10 μM). Data points represent the mean ± SEM for 4–17 independent samples. The basal [^{35}S]GTPγS binding in mice treated with saline for 1, 3, 5, and 7 d was 31.2 ± 3.6, 31.8 ± 1.1, 29.2 ± 1.2, and 30.3 ± 1.9 fmol/mg protein, respectively. On the contrary, the basal [^{35}S]GTPγS binding in mice treated with DAMGO for 1, 3, 5, and 7 d was 33.2 ± 1.2, 33.2 ± 7.9, 28.6 ± 1.3, and 31.1 ± 1.5 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. $^*p < 0.05$ versus saline-treated group. F values of one-way ANOVA in 5 and 7 d treatment are $F_{(1,29)} = 27.659$ and $F_{(1,6)} = 11.381$, respectively.

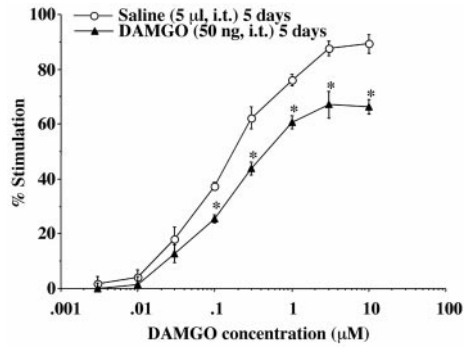

Figure 2. Concentration–response curve for the DAMGO-induced increase in [^{35}S]GTPγS binding to spinal cord membranes obtained from chronically saline- or DAMGO-treated mice. Groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 5 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without DAMGO (0.001–10 μM). Data points represent the mean ± SEM for 3–17 independent samples. The basal [^{35}S]GTPγS binding in mice treated with saline and DAMGO for 5 d was 29.2 ± 1.2 and 28.6 ± 1.3 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. $^*p < 0.05$ versus saline-treated group. F values of one-way ANOVA in 0.1, 0.3, 1, 3, and 10 μM DAMGO are $F_{(1,25)} = 32.158$, $F_{(1,13)} = 17.020$, $F_{(1,25)} = 25.531$, $F_{(1,13)} = 11.379$, and $F_{(1,29)} = 27.659$, respectively.

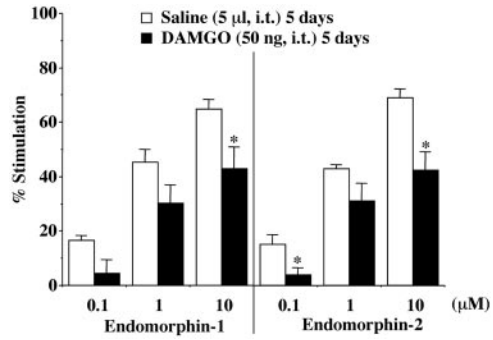

Figure 3. Effect of repeated intrathecal injection of DAMGO on the endomorphin-induced increase in [^{35}S]GTPγS binding to spinal cord membranes. Groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 5 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without endomorphin-1 and endomorphin-2 (0.1–10 μM). Data represent the mean ± SEM for three to nine independent samples. The basal [^{35}S]GTPγS binding in mice treated with saline and DAMGO for 5 d was 29.2 ± 1.2 and 28.6 ± 1.3 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. $^*p < 0.05$ versus saline-treated group. F values of one-way ANOVA in 10 μM endomorphin-1, 0.1 μM endomorphin-2, and 10 μM endomorphin-2 are $F_{(1,13)} = 9.135$, $F_{(1,4)} = 9.351$, and $F_{(1,10)} = 14.037$, respectively.

stimulated [^{35}S]GTPγS binding. The increase of [^{35}S]GTPγS binding by DAMGO, endomorphin-1, or endomorphin-2 was blocked completely by coincubation with 10 μM CTOP (Table 1). The incubation of CTOP alone had no effect on the basal [^{35}S]GTPγS binding level (data not shown). Figure 1 shows the changes in the 10 μM DAMGO-induced increase in [^{35}S]GTPγS binding to spinal cord membranes after the daily intrathecal treatments with saline or DAMGO. The increased [^{35}S]GTPγS binding induced by DAMGO (10 μM) to spinal cord membranes was not affected by intrathecal treatment with DAMGO (50 ng) for 1–3 d but was attenuated significantly in mice treated for 5 and 7 d. The concentration–response curve and also the maximal increase for the DAMGO-induced increases in [^{35}S]GTPγS binding to spinal cord membranes were attenuated significantly in mice treated intrathecally with DAMGO for 5 d as compared with saline-treated mice (Fig. 2). Chronic intrathecal treatment with DAMGO for 5 d also significantly reduced the levels of [^{35}S]GTPγS binding to spinal cord membranes induced by either endomorphin-1 or endomorphin-2 (Fig. 3).

The δ-opioid receptor agonists DPDPE, [D-Ala^2]deltorphin II, and SNC-80 produced robust stimulation of [^{35}S]GTPγS binding at 10 μM in mice treated intrathecally with saline for 5 d (Table 1), These effects were blocked completely by coincubation with the specific δ-opioid receptor antagonist NTI (Table 1). Incubation of the κ-opioid receptor agonist U-50,488 H or U-69,593 increased the [^{35}S]GTPγS binding to the spinal cord membranes obtained from mice treated intrathecally with saline for 5 d (Table 1). The increase of [^{35}S]GTPγS binding induced by a κ-agonist was abolished by the κ-opioid receptor antagonist nor-BNI (Table 1). Under these conditions the increases of the [^{35}S]GTPγS binding stimulated by δ- and κ-opioid receptor agonists were not affected by chronic intrathecal treatment with DAMGO (Fig. 4). The levels of [^{35}S]GTPγS binding stimulated by DPDPE, [D-Ala^2]deltorphin II, SNC-80, U-50,488H, or U-69,593 at 10 μM in mice treated with DAMGO for 5 d were similar to those found in mice treated with saline for 5 d.

The role of PKC in the development of μ-opioid receptor desensitization induced by chronic treatment with DAMGO

Then the role of PKC in the development of μ-opioid receptor desensitization was investigated. Groups of mice were treated

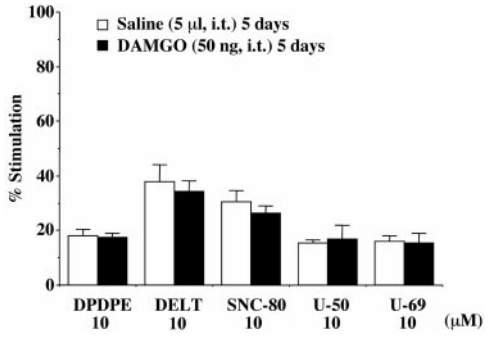

Figure 4. Effect of repeated intrathecal injection of DAMGO on the δ-opioid receptor agonists DPDPE-, [D-Ala^2]deltorphin II-, and SNC-80- or κ-opioid receptor agonists U-50,488H- and U-69,593-induced increases in [^{35}S]GTPγS binding to spinal cord membranes. Groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 5 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without 10 μM DPDPE, [D-Ala^2]deltorphin II (*DELT*), SNC-80, U-50,488H (*U-50*), and U-69,593 (*U-69*). Data represent the mean ± SEM for three to nine independent samples. The basal [^{35}S]GTPγS binding in mice treated with saline and DAMGO for 5 d was 29.2 ± 1.2 and 28.6 ± 1.3 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test.

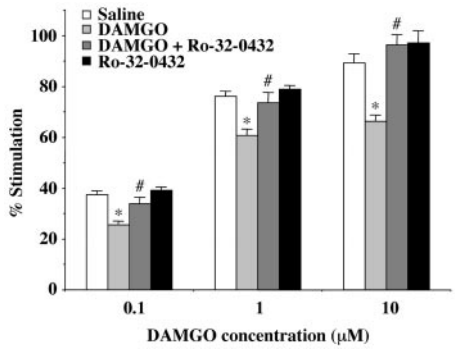

Figure 5. Blockade of PKC inhibitor on the decreased level of the DAMGO-stimulated [^{35}S]GTPγS binding induced by repeated intrathecal injection of DAMGO. Groups of mice were treated intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) in the absence or presence of the specific PKC inhibitor Ro-32-0432 (250 ng/mouse) once a day for 5 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without DAMGO (0.1–10 μM). Each *column* represents the mean ± SEM for 3–17 samples. The basal [^{35}S]GTPγS binding in mice treated with saline alone, DAMGO alone, DAMGO combined with Ro-32-0432, and Ro-32-0432 alone for 5 d was 29.2 ± 1.2, 28.6 ± 1.3, 29.8 ± 3.1, and 30.1 ± 1.8 fmol/mg protein, respectively. The statistical significance of differences among the groups was assessed with a one-way ANOVA, followed by Newman–Keuls test. $^{*}p < 0.05$ versus saline-treated group; $^{\#}p < 0.05$ versus DAMGO-treated group. *F* values of one-way ANOVA in 0.1, 1, and 10 μM DAMGO are $F_{(3,35)} = 11.096$, $F_{(3,35)} = 8.486$, and $F_{(3,39)} = 16.199$, respectively.

intrathecally with DAMGO in the absence or presence of a specific PKC inhibitor, Ro-32-0432 (250 ng/mouse). As shown in eFigure 5, treatment of Ro-32-0432 coadministered with DAMGO completely blocked the decrease of the DAMGO-stimulated [^{35}S]GTPγS binding induced by repeated DAMGO injection for 5 d. Treatment with Ro-32-0432 alone had no effect on the DAMGO-stimulated [^{35}S]GTPγS binding (Fig. 5).

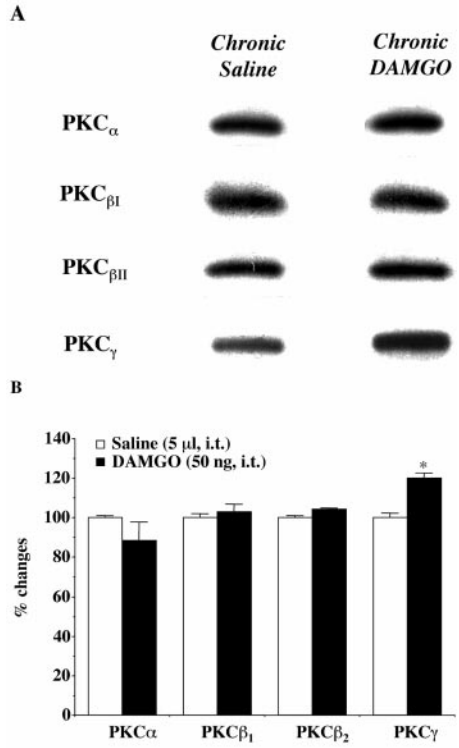

Figure 6. The levels of cPKC isoforms in spinal cord membranes after repeated intrathecal injections of DAMGO. Groups of mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 5 d. At 24 hr after the last injection the spinal cord membranes were prepared for immunoblotting. *A*, Representative Western blot of PKCα, PKCβI, PKCβII, and PKCγ isoform proteins. *B*, Changes in the membrane-located protein levels of cPKC isoforms in spinal cord membranes after repeated intrathecal injections of DAMGO. Each *column* represents the mean ± SEM for four samples. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. $^{*}p < 0.05$ versus saline-treated group. The *F* value of one-way ANOVA in the PKCγ isoform is $F_{(1,6)} = 46.531$.

Identification of cPKC isoforms involved in μ-opioid receptor desensitization induced by chronic treatment with DAMGO

The levels of cPKC isoforms in membranes of the spinal cord after the repeated injection of DAMGO were analyzed quantitatively by Western blot analysis. Groups of mice were treated intrathecally with DAMGO (50 ng/mouse) or saline for 5 d; on day 6 spinal cord membranes were prepared for the assay. Immunoreactivity of the PKCγ isoform was enhanced significantly by DAMGO treatment as compared with that of saline treatment, whereas the other three cPKC isoforms, PKCα, PKCβI, and PKCβII, were not altered (Fig. 6*A*,*B*).

Lack of μ-opioid receptor desensitization induced by chronic treatment with DAMGO in PKCγ knock-out mice

To investigate further the role of the PKCγ isoform in the process of chronic DAMGO-induced desensitization, we next investigated whether repeated intrathecal injections of DAMGO caused no effect on the DAMGO-stimulated [^{35}S]GTPγS binding in mice lacking the PKCγ isoform. Immunoblot analysis showed that no PKCγ protein could be detected in both membranous and cytosolic fractions of the spinal cord obtained from PKCγ knock-out mice (Fig. 7*A*). Immunoreactivities of the other three cPKC isoforms, PKCα, PKCβI, and PKCβII, in mice lacking the PKCγ

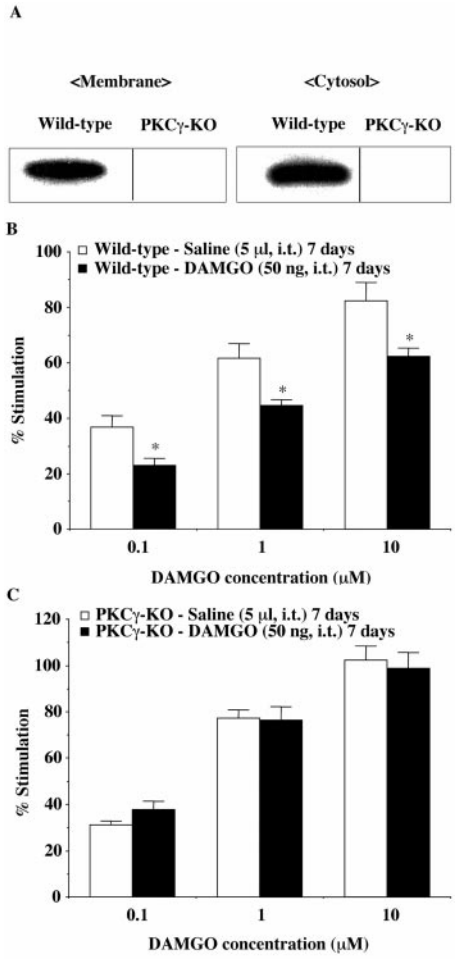

Figure 7. *A*, Immunoblot analysis of protein levels of membranous or cytosolic fractions of PKCγ in the spinal cord obtained from wild-type and PKCγ knock-out (*KO*) mice. *B*, Effect of repeated intrathecal injections of DAMGO on the DAMGO-induced increase in [^{35}S]GTPγS binding to spinal cord membranes from wild-type mice. Groups of wild-type mice were injected intrathecally with saline (5 μl/mouse) or DAMGO (50 ng/mouse) once a day for 7 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without DAMGO (0.1–10 μM). Data represent the mean ± SEM for 11 independent samples. The basal [^{35}S]GTPγS binding in wild-type mice treated with saline and DAMGO for 7 d was 31.1 ± 2.8 and 27.5 ± 1.2 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test. $^{*}p < 0.05$ versus saline-treated group. *F* values of one-way ANOVA in 0.1, 1, and 10 μM DAMGO are $F_{(1,20)} = 5.954$, $F_{(1,20)} = 10.640$, and $F_{(1,20)} = 8.244$, respectively. *C*, No development of the μ-opioid receptor-mediated downregulation by repeated DAMGO injection in mice lacking the PKCγ isoform. Groups of PKCγ knock-out mice were injected intrathecally with saline (5 ml/mouse) or DAMGO (50 ng/mouse) once a day for 7 d. At 24 hr after the last injection the spinal cord membranes were prepared for each assay. The assay was conducted with or without DAMGO (0.1–10 μM). Data represent the mean ± SEM for 11 independent samples. The basal [^{35}S]GTPγS binding in PKCγ knock-out mice treated with saline and DAMGO for 7 d was 26.7 ± 2.1 and 29.1 ± 3.2 fmol/mg protein, respectively. The statistical significance of differences between the groups was assessed with a one-way ANOVA, followed by Dunnett's test.

isoform were detectable to the same degree as the wild type (data not shown), confirming the specificity of these mutant mice. As shown in Figure 7*B*, repeated intrathecal treatment with DAMGO once a day for 7 d consecutively produced a 20.0% reduction in the [^{35}S]GTPγS binding stimulated by DAMGO at 10 μM to spinal membranes of wild-type mice ($p < 0.05$ vs saline pretreatment). However, the same chronic treatment with DAMGO did not affect significantly the increases of the DAMGO-stimulated [^{35}S]GTPγS binding in mice lacking the PKCγ isoform (Fig. 7*C*).

DISCUSSION

Chronic daily intrathecal DAMGO treatment attenuates the increases of [^{35}S]GTPγS bindings stimulated by μ-opioid, but not δ- or κ-opioid, agonists in the mouse spinal cord

We found in the present study that mice repeatedly intrathecally injected with a selective μ-opioid receptor agonist DAMGO showed the time-dependent desensitization of a μ-opioidergic system to activate G-proteins in the spinal cord. This desensitization was selective to μ-, but not to δ- and κ-, opioidergic systems to activate G-proteins. These results indicate that chronic intrathecal injection of μ-opioids uncouples the spinal μ-opioid receptor from G-proteins, resulting in the desensitization of a μ-opioidergic system in the mouse spinal cord.

PKC in the spinal cord is involved in μ-opioid receptor desensitization induced by chronic treatment with DAMGO

Tolerance to opioid analgesia is a major drawback in clinical use of these analgesics. It has been proposed that chronic adaptive molecular mechanisms in opioid tolerance involve some protein kinases, including protein kinase A (PKA), PKC, and G-protein-coupled receptor kinase. We have reported previously that intrathecal pretreatment with a membrane-permeable PKC inhibitor, calphostin C, but not with a specific PKA inhibitor, blocks the development of the antinociceptive tolerance to intrathecally administered DAMGO in mice (Narita et al., 1995). We also found that the activation of PKC by phorbol esters attenuates either the opioid-induced antinociceptive effect or the G-protein activation (Narita et al., 1996, 1997). These findings indicate that activated PKC is involved in the process of the development of opioid tolerance.

Treatment of a specific PKC inhibitor Ro-32-0432 coadministered with DAMGO into the spinal cord completely blocked the desensitization of spinal μ-opioidergic systems induced by chronic DAMGO treatment. Considering that the activation of PKC induces the phosphorylation of several membrane proteins, the receptor itself seems to be one of the good targets. Indeed, the opioid receptors can be phosphorylated by PKC (Pei et al., 1995). It is therefore most likely that the activation of PKC by chronic treatment with μ-opioids may lead to the phosphorylation of membrane-bound μ-opioid receptors and in turn causes the uncoupling of μ-opioid receptors from G-proteins after chronic treatment with μ-opioids in the spinal cord.

Identification of the PKCγ isoform involved in μ-opioid receptor desensitization induced by chronic treatment with DAMGO

Recent cloning studies revealed that the PKC family consists of at least 12 isoforms possessing distinct differences in structure, substrate requirement, expression, and localization that therefore may underlie diverse physiological functions (Way et al., 2000). In the present study we thus examined whether repeated intrathecal administration of DAMGO for 5 d could alter the levels of membrane-bound PKC isoforms, especially cPKCs, in the mouse spinal cord. In spinal cord membranes obtained from mice injected repeatedly with DAMGO for 5 d, the upregulation of the

PKCγ isoform in the spinal cord was clearly noted. Three other cPKC isoforms, PKCα, PKCβI, and PKCβII, were not altered significantly after repeated administration of DAMGO. The results are consistent with the previous finding that chronic spinal administration of morphine caused a significant increase in levels of PKCγ in spinal cord membranes of rats (Mao et al., 1995). These data raise the possibility that the PKCγ isoform dominantly modulates an autoinhibition of spinal μ-opioidergic systems.

The PKCγ isoform has been identified in neurons of the brain and spinal cord. A recent study in mice lacking the PKCγ isoform has shown that the PKCγ isoform may be critical for synaptic plasticity such as neuropathic pain (Malmberg et al., 1997). The neuropathic pain after partial nerve ligation results in a somatotopically organized upregulation of PKCγ in the superficial dorsal horn and its translocation from the cytoplasm and nucleus toward the plasma membrane of immunoreactive neurons (Mayer et al., 1995). The present study with PKCγ knock-out mice revealed that repeated spinal administration of μ-agonist in mice lacking the PKCγ isoform gene failed to cause any desensitization of G-protein activation by the μ-agonist. Taking together with the present results of the immunoblotting, the PKCγ isoform is likely to be one of the most important factors to modulate the homologous desensitization of spinal μ-opioidergic systems. Recent studies with dual immunofluorescence labeling showed that PKCγ was present in only 5% of cells expressing μ-opioid receptors in rat spinal cord (Polgar et al., 1999). If this is also the case in the mouse, one wonders how μ-opioid receptors in the spinal cord could be phosphorylated directly by PKCγ. The results obtained from this study would suggest the possibility that chronic treatment with DAMGO may cause an increase of the expression of PKCγ in μ-opioid receptor-containing cells.

Because it has been well recognized that there is no cross-tolerance to opioid-induced analgesia/antinociception among opioid receptor types, the lack of cross-talk between μ- and δ- or κ-opioidergic systems on the desensitization of opioid-induced G-protein activation after repeated treatment with μ-agonists was expected. However, it should be pointed out that either δ- or κ-opioid receptors possess the phosphorylation sites for PKC (Mollereau et al., 1994; Yasuda et al., 1998). Although the exact mechanism is unclear at this time, it is possible that the remaining PKC isoforms activated by chronic treatment with a δ- or κ-opioid receptor agonist provide sufficient kinase activity to regulate δ-and κ-opioidergic systems.

In conclusion, the present results indicate a potential role for PKC, especially the PKCγ isoform, in the process of uncoupling of the spinal μ-opioid receptor from G-proteins in mice chronically treated intrathecally with a specific μ-opioid receptor agonist, DAMGO. We propose that PKCγ may be activated after the repeated administration of μ-opioids and may play an important role in the development of μ-opioid receptor-mediated tolerance.

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