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Genetic alteration of phospholipase C β3 expression modulates behavioral and cellular responses to μ opioids

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ABSTRACT **Morphine and other μ opioids regulate a number of intracellular signaling pathways, including the one mediated by phospholipase C (PLC). By studying PLC β3-deficient mice, we have established a strong link between PLC and μ opioid-mediated responses at both the behavioral and cellular levels. Mice lacking PLC β3, when compared with the wild type, exhibited up to a 10-fold decrease in the ED_{50} value for morphine in producing antinociception. The reduced ED_{50} value was unlikely a result of changes in opioid receptor number or affinity because no differences were found in whole-brain B_{max} and K_d values for μ, κ, and δ opioid receptors between wild-type and PLC β3-null mice. We also found that opioid regulation of voltage-sensitive Ca^{2+} channels in primary sensory neurons (dorsal root ganglion) was different between the two genotypes. Consistent with the behavioral findings, the specific μ agonist [D-Ala^2,(Me)Phe^4, Gly(ol)^5]enkephalin (DAMGO) induced a greater whole-cell current reduction in a greater proportion of neurons isolated from the PLC β3-null mice than from the wild type. In addition, reconstitution of recombinant PLC protein back into PLC β3-deficient dorsal root ganglion neurons reduced DAMGO responses to those of wild-type neurons. In neurons of both genotypes, activation of protein kinase C with phorbol esters markedly reduced DAMGO-mediated Ca^{2+} current reduction. These data demonstrate that PLC β3 constitutes a significant pathway involved in negative modulation of μ opioid responses, perhaps via protein kinase C, and suggests the possibility that differences in opioid sensitivity among individuals could be, in part, because of genetic factors.**

Morphine and other μ opioids are widely used analgesics. Three opioid receptors, μ, δ, and κ, have been cloned and characterized both functionally and by radioligand binding (1–4). These receptors couple to G_i and G_o proteins and, through α and βγ subunits, regulate a number of signaling pathways. These regulated pathways include inhibition of adenylyl cyclase activity (5, 6), activation of inwardly rectifying K^+ channels (7, 8), and inhibition of voltage-activated Ca^{2+} channels, predominantly of the N and P/Q types (9, 10). Recent work with cell lines has demonstrated that opioid receptors also activate phospholipase C (PLC; 11–17). Moreover, several physiological studies have implicated PLC-linked pathways in a diverse range of opioid-modulated events, such as pain regulation (18), response to brain injury (19), and opioid withdrawal (20). However, despite these data suggesting an important role for PLC-mediated pathways in opioid signal transduction, there is little direct evidence supporting a role of PLC in opioid responses.

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce two important second messengers, inositol triphosphate and diacylglycerol (21). The former increases release of Ca^{2+} from intracellular stores, and the latter activates protein kinase C (PKC). Molecular cloning and biochemical characterizations have revealed four different PLC β isoforms that are regulated by G proteins. These PLC β isoforms show different tissue expression specificity and G protein regulation profiles. PLC β1 and PLC β3 have been detected in a wide range of tissues and cell types, whereas PLC β2 has been found only in hematopoietic tissues and PLC β4 has been found only in specific neuronal tissues. All four isoforms can be regulated by the Gα subunits of the Gq class. However, only PLC β2 and PLC β3 are potently activated by Gβγ *in vitro* (22).

PLC β3 is the main isoform regulated by Gβγ in nonhematopoietic tissues including neuronal tissue (22). Thus, opioid receptor activation in neurons may, in addition to its activation of other pathways, affect PLC β3 primarily via G_i and/or G_o. Additionally, if negative feedback regulation of chemokine receptors by Gβγ-mediated PLC activation (23) is a general phenomenon, PLC β3 may play a similar role in opioid-mediated responses. To test this hypothesis, we have generated a mouse line that lacks PLC β3, and responses of the PLC β3-null mice to μ opioid receptor agonists were assessed at the behavioral and cellular levels. All of the evidence indicates that PLC β3 constitutes a pathway that is involved in inhibition of opioid-mediated responses.

MATERIALS AND METHODS

Generation of PLC β3-Null Mice. A 10-kb genomic DNA, isolated from a 129SV agouti mouse strain library containing two exons of the PLC β3 gene, was used to make the gene-targeting construct. The two exons encoded residues 368–460, which are located in the middle of the catalytic domain of the enzyme. Part of the exons was replaced with a neomycin-resistance gene in the gene-targeting construct (Fig. 1). The gene-targeting construct was transfected into embryonic stem (ES) cells by electroporation. After selection with Geneticin, four ES cell clones were obtained, in which one of the PLC β3 genes was disrupted as identified by both PCR and Southern analysis. Two of the ES cell clones were microinjected into blastocysts, and four chimeras were generated. These chimeras then were backcrossed with CD1 mice to produce heterozygotes. Finally, interbreeding of heterozygous siblings yielded animals (F_1) homozygous for the desired mutation, i.e., mice lacking PLC β3. The F_1 animals were crossed to produce F_2 homozygotes. The wild-type littermates

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Abbreviations: DRG, dorsal root ganglion; PLC, phospholipase C; PKC, protein kinase C; ICV, intracerebroventricular; DAMGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; PMA, phorbol 12-myristate 13-acetate.

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also were bred in parallel. Animals from F_1 to F_4 of both lines derived from the two ES clones were used in the study. Animals were maintained under a specific pathogen–free environment.

Intracellular Ca^{2+} Measurement. Muscle cells were cultured from aorta of 6- to 10-week-old adult mice, as described (24). Cells were grown at 37°C in DMEM supplemented with 10% FBS. Calcium measurements were performed as described previously (25). All experiments were performed in 15 mM Hepes-buffered Hanks' balanced salt solution (HBSS) with 1.26 mM Ca^{2+} at a maintained temperature of 37°C. Data were collected by using IMAGE-1 software (Universal Imaging, Media, PA) and analyzed and graphed by using Microsoft EXCEL.

Opioid Binding to Murine Brain Membranes. Whole brains were dissected from PLC β3 wild-type and PLC β3-null mice; the cerebellum was removed and membranes were prepared as described previously (26). Saturation-binding assays were performed with the opioid receptor-selective radioligands [^{3}H]DAMGO (μ), [^{3}H]naltrindole (δ), and [^{3}H]U69,593 (κ) as described (27), by using concentrations ranging from 0.025 to 3.2 nM (DAMGO is [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin). Incubation times were 60 min ([^{3}H]DAMGO and [^{3}H]U69,593) or 3 hr ([^{3}H]naltrindole). Nonspecific binding was measured by inclusion of 10 μM naloxone.

Mouse Tail-Flick Assay. The tail-flick test on 8- to 12-week-old mice was carried out by using a 55°C water bath as described (27). Unless otherwise stated, control latencies were determined, after which mice received i.p. or intracerebroventricular (ICV) doses of morphine sulfate with the antinociceptive effect measured 20 min later. In the receptor selectivity studies, the κ-selective antagonist nor-BNI or the δ-selective antagonist ICI 174,864 was given with the morphine sulfate. β-FNA, the μ-selective antagonist, was injected 24 hr before the agonist. If the mouse failed to display a tail flick in 15 sec, the animal received a maximal antinociceptive score of 100%. Antinociception was calculated according to the following formula: % antinociception = 100 × [(test latency − control latency)/(15 − control latency)].

Electrophysiology. Primary cultures were prepared from mice (4–6 weeks old) as described previously (28). Compounds were applied to the cell under study by using pressure ejection (6–10 kPa) from separate, blunt-tipped (i.d. of 12–15 μm) glass micropipettes or from a gravity-fed, vacuum-removed microperfusion system ("U-tube"). The currents were evoked and recorded by using an Axopatch 1B patch-clamp amplifier and PCLAMP 6.0 or 7.0 software (Axon Instruments, Foster City, CA) just before termination of a 15-sec drug application (28). Cells were bathed in a solution containing 5.0 mM $CaCl_2$, 67 mM choline chloride, 0.8 mM $MgCl_2$, 100 mM tetraethylammonium chloride, 5.6 mM glucose, 5.3 mM KCl, and 10 mM Hepes (pH 7.3–7.4, 325–340 mOsM). Glass recording patch pipettes were filled with recording solution consisting of the following: 140 mM $CsMeSO_3$, 10 mM Hepes, 5 mM EGTA, 5 mM ATP-Mg^{2+}, and 0.1 mM GTP-Na^+, pH 7.35, and an osmolarity of 300–310 mOsM (all compounds from Sigma). For the microdialysis of PLC β2, prepared as described (29), the patch pipettes were filled with a modified internal recording solution. In addition to the PLC β2 ($\approx$10 nM), $CaCl_2$ (1.67 mM) and a reduced concentration of EGTA (3 mM) were included to establish a free $[Ca^{2+}]_i$ of $\approx$100 nM to maintain PLC activity. BSA (1 mg/ml) was also added to prevent the protein from adhering to the patch pipette. The tip of the recording pipette first was filled with the standard internal recording buffer and then "back-filled" with the experimental solution.

DAMGO, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, phorbol 12-myristate 13-acetate (PMA), and 4-α-PMA were purchased from Research Biochemicals (Natick, MA). PMA and 4-α-PMA were reconstituted in DMSO and then diluted into bath solution. The final DMSO concentration (0.1%) had no effect on Ca^{2+} current magnitudes. Pretreatment with either compound was for 30 min at a concentration of 100 nM. After washing of cultures, DAMGO responses were obtained within 40 min.

RESULTS

Generation of PLC β3-Deficient Mice and Determination of PLC Signaling. The PLC β3 gene was disrupted as shown in Fig. 1 *A* and *B*. The X and Y boxes represent conserved amino acid sequence motifs and comprise the catalytic domain (22). The exon of the PLC β3 gene, encoding part of the X box, was disrupted by the insertion of a neomycin-resistance gene-expression unit. Mice homozygous for disrupted PLC β3 genes were generated as described (30, 31); homozygosity was detected by PCR with the primers shown. The genomic structure of the gene was confirmed by Southern analysis (data not shown). The null mutation also was verified by Western analysis; no PLC β3 protein was detected with a PLC β3-specific antiserum in homogenates of dorsal root ganglion (DRG) neurons from homozygous PLC β3-deficient mice (Fig. 1*C*).

To test whether PLC β3 mediates opioid receptor-induced PLC activation in a nontransfection system, we established primary cultures of aortic muscle cells from wild-type and PLC β3-null mice (24). DAMGO (1 μM) elicited naloxone-sensitive increases in intracellular Ca^{2+} in the cells derived from wild-type animals (Fig. 2 *A* and *C*), whereas DAMGO showed no effect in cells lacking PLC β3. The PLC β3 deficiency did not affect the response to ligands that are known to activate the Gαq pathway (Fig. 2 *B* and *C*). These results clearly indicate that PLC β3 can be regulated by the μ opioid receptor in primary cultured cells and that this action likely is mediated by the G$\beta\gamma$ pathway.

Analgesia and Opioid Receptor Analysis. Having established that μ opioids can regulate PLC, we investigated whether ablation of PLC β3-mediated pathways affected μ-mediated analgesia. Both wild-type and PLC β3-deficient mice were tested in the 55°C warm-water tail-flick assay 30 min

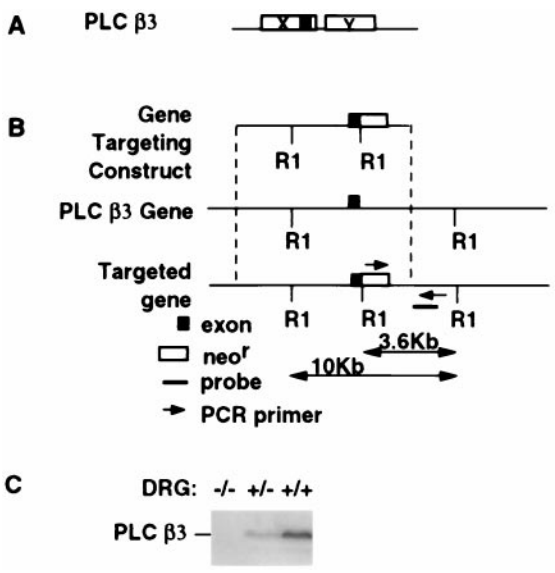

FIG. 1. Generation of PLC β3-null mice. (*A*) Schematic representation of a PLC molecule. The X and Y boxes comprise the catalytic domain. The solid box represents the exon manipulated in the gene-targeting construct. (*B*) Gene-targeting construct and the homologous recombination process. (*C*) Western analysis of DRG neurons isolated from the mice (+/+, wild type; +/−, heterozygous; −/−, homozygous) with a PLC β3-specific antibody.

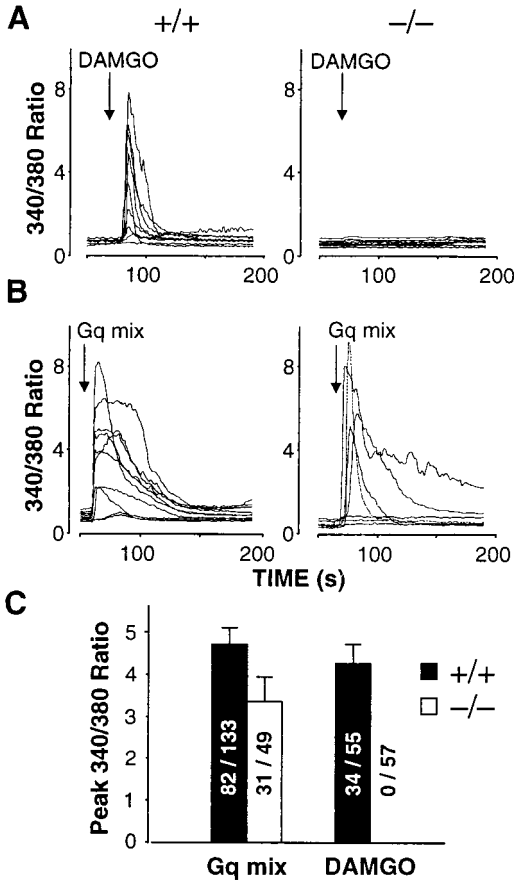

FIG. 2. DAMGO induces increases in intracellular Ca^{2+} through PLC β3. Primary cultured muscle cells from wild-type (+/+) and transgenic (−/−) mice were loaded with fura-2. At the time shown, they were stimulated with 1 μM DAMGO (*A*) or the Gq mix (50 nM bradykinin, 50 nM bombesin, and 10 nM endothelin), a mixture of agonists that activate receptors tightly coupled to Gq/11 (*B*). Traces are from individual cells and are typical of results obtained in seven experiments. Calcium responses from all experiments are summarized in *C*. The ratios of responding cells to total tested cells are shown within or above bars. (Bars = mean ± SEM of the fold increase in peak 340/380 fluorescence ratio in cells that responded.)

after administration of morphine. Animals showed no significant difference in control latency response in the tail flick test before morphine administration (3.01 ± 0.48 sec and 3.03 ± 0.36 sec, respectively, for wild-type and PLC β3-null mice; mean ± SEM). Morphine produced dose-dependent antinociception (up to 2 hr) in the mouse tail-flick test after i.p. administration in both sets of mice. However, the PLC β3-null mice demonstrated a 5-fold-increased sensitivity to morphine compared with the wild-type mice [ED_{50} values and 95% confidence limits for morphine of 2.58 (2.02–3.29) mg/kg and 12.8 (9.48–17.3) mg/kg, respectively] (Fig. 3*A*). It is noteworthy that the antinociceptive curves of wild-type mice overlapped those in CD-1 mice and were parallel with those in the PLC β3-null mice (not shown). Thus, interbreeding between CD-1 and 129 strains of mice did not significantly change antinociceptive responses, suggesting that the difference in antinociceptive ED_{50} values may not be attributed to genetic background variation resulting from interbreeding. The increased sensitivity to morphine was more pronounced after ICV administration of morphine. A 10-fold leftward shift in the dose-response curve of morphine in the PLC β3-null mice was observed compared with the wild-type mice [ED_{50} values and 95% confidence limits = 0.03 (0.02–0.07) nmol for the transgenic mice and 0.26 (0.14–0.51) nmol for the wild type] (Fig. 3*B*).

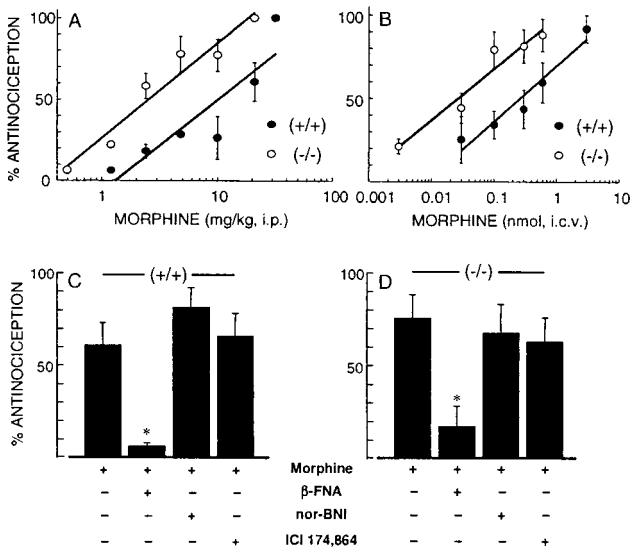

FIG. 3. Antinociceptive effect of morphine (*A* and *B*). Wild-type (●, +/+) or mutant (○, −/−) mice were administered different doses of morphine i.p. (*A*) or ICV (*B*). Receptor selectivity of antinociceptive effect (*C* and *D*). Morphine was administered ICV to wild-type (*C*) or transgenic (*D*) mice with or without pretreatment with ICV β-FNA (20 nmol, −24 hr) or coadministered either the κ-selective antagonist, nor-BNI (3 nmol), or the δ-selective antagonist, ICI 174,864 (4 nmol). Antinociception was measured in the mouse tail-flick assay. Data are presented as the mean percentage antinociception ± SEM. Six to 10 mice were used to obtain each point. *, Significantly different from morphine, $P < 0.01$.

The receptor selectivity of morphine after ICV administration was tested in both wild-type and PLC β3-null mice with selective opioid receptor antagonists to ensure that the analgesic effects of morphine were associated with the μ opioid receptor. Concentrations of the antagonists were chosen to guarantee inhibition of the specific opioid receptor of interest. Nor-BNI was used at a dose of 3 nmol, which has been shown to inhibit only antinociception mediated by κ receptors (32). Similarly, a dose of 4 nmol ICI 174,864 was chosen for its δ receptor antagonistic selectivity (33). The μ-selective, irreversible antagonist, β-FNA, was administered as a single, 20-nmol ICV injection 24 hr before testing, a time and dose established previously as having a specific action at μ, but not δ or κ, receptors (26, 34). The pretreatment of both wild-type and PLC β3-null mice with β-FNA blocked the antinociceptive effect of morphine, whereas coadministration of either nor-BNI or ICI 174,864 with morphine had no significant effect on morphine-mediated antinociception (Fig. 3 *C* and *D*). These results demonstrate that morphine produced its antinociceptive effect through the μ receptor in these mice.

The change in morphine efficacy *in vivo* could have been the result of changes in the number or affinity of opioid receptors. To test this, we performed saturation-binding assays with selective radioligands for each opioid receptor type, using brain membranes prepared from wild-type and PLC β3-null mice. The maximal binding of [^{3}H]DAMGO (μ), [^{3}H]naltrindole (δ), and [^{3}H]U69,593 (κ) to brain membranes was not significantly different between the wild-type and PLC β3-null mice (Table 1). Moreover, there were no differences between the wild-type and PLC β3-null mice in the affinities of the μ, δ, or κ opioid receptors for their selective radioligands. Thus, the differences in the antinociceptive effect of morphine observed between the wild-type and PLC β3-null mice were unlikely because of changes in opioid receptor number or affinity.

Cellular Opioid Responses. We wished to establish a cellular basis for the striking behavioral findings noted above. Because

Table 1. Comparison among the number and affinity of μ, δ, and κ opioid receptors in wild-type (+/+) and transgenic mice lacking PLC β3 (−/−)

	K_d, nM		B_{max}, fmol/mg of protein	
PLC β3	+/+	−/−	+/+	−/−
[^{3}H]DAMGO (μ)	0.49 ± 0.06	0.51 ± 0.01	120 ± 11	110 ± 8.5
[^{3}H]Naltrindole (δ)	0.55 ± 0.02	0.54 ± 0.07	86 ± 0.07	76 ± 1.8
[^{3}H]U69,593 (κ)	0.36 ± 0.04	0.59 ± 0.19	13 ± 0.83	18 ± 3.3

Murine brain membranes were prepared and used in opioid-binding assays. [^{3}]DAMGO, [^{3}H]naltrindole, and [^{3}H]U69,593 saturation binding at concentrations ranging from 0.025 to 3.2 nM was measured in 50 mM Tris·HCl, pH 7.5, at 25° as described in *Materials and Methods*. Data are expressed as mean ± SEM for at least three experiments performed in triplicate.

there were no changes in opioid receptors, we sought to determine whether there was a change in μ opioid signaling "efficacy." To do this, we studied the regulation of voltage-dependent Ca^{2+} channel activity in primary sensory (DRG) neurons, the first link in nociceptive pathways. The whole-cell variation of the patch-clamp technique was used to record and compare whole-cell Ca^{2+} currents of DRG neurons from wild-type and PLC β3-null mice recorded in the absence and presence of DAMGO. Application of DAMGO to isolated DRG neurons resulted in a reversible reduction in Ca^{2+} currents evoked by 100-msec steps to +10 mV from a holding potential of −80 mV. Neurons were considered unresponsive if DAMGO application resulted in <10% inhibition of peak current magnitude at the highest concentration tested, typically 3 μM. The observed reductions in peak whole-cell Ca^{2+} current in the presence of DAMGO were mediated by μ opioid receptors because an equimolar concentration of the μ-selective antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (3 μM) was found to block completely the DAMGO effect in DRG neurons from wild-type and PLC β3-null mice (data not shown). DAMGO (100, 300, and 3,000 nM) produced a concentration-dependent reduction in Ca^{2+} current magnitude in both wild-type and PLC β3-deficient neurons. The DAMGO-induced current reductions, for wild-type and PLC β3-deficient neurons, respectively, were: 100 nM, 15.9 ± 2.5% and 18.9 ± 1.9%; 300 nM, 23.9 ± 3.5% and 33.5 ± 2.0%; and 3,000 nM, 29.0 ± 3.4% and 38.3 ± 1.9%. For the two higher concentrations, the differences between wild-type and transgenic cells reached statistical significance ($P = 0.02$, two-tailed t test; Fig. 4). In a separate experiment, 30 nM DAMGO did not elicit a definable μ opioid-mediated reduction in peak Ca^{2+} current in any cell tested for either PLC β3 genotype (n = 6–12, data not shown).

The increased sensitivity of the PLC β3-deficient cells to μ opioid-mediated regulation of voltage-dependent Ca^{2+} channels also was evident in the proportion of cells responding to DAMGO at each concentration. Under control conditions, using DRG neurons resected from caudal spinal segments, which have a higher proportion of opioid-responsive cells, ≈80% of cultured DRG neurons responded to 3 μM DAMGO. Although the response rate was similar for each PLC β3 genotype in the presence of 100 nM DAMGO, a significantly greater proportion of the PLC β3-null DRG neurons than the wild-type cells responded at both the 300 nM and 3 μM concentrations ($P < 0.05$, χ^2 test; Fig. 4*B*). At the highest concentration, the response rate in wild-type neurons was 85%, whereas 100% of PLC β3-deficient neurons had a definable response.

To confirm that the differences in μ opioid modulation of voltage-dependent Ca^{2+} channels between the two genotypes were dependent on the presence or absence of the PLC enzyme, we determined the effect of intracellular dialysis of an excess of purified recombinant PLC. PLC β2, a hematopoietic

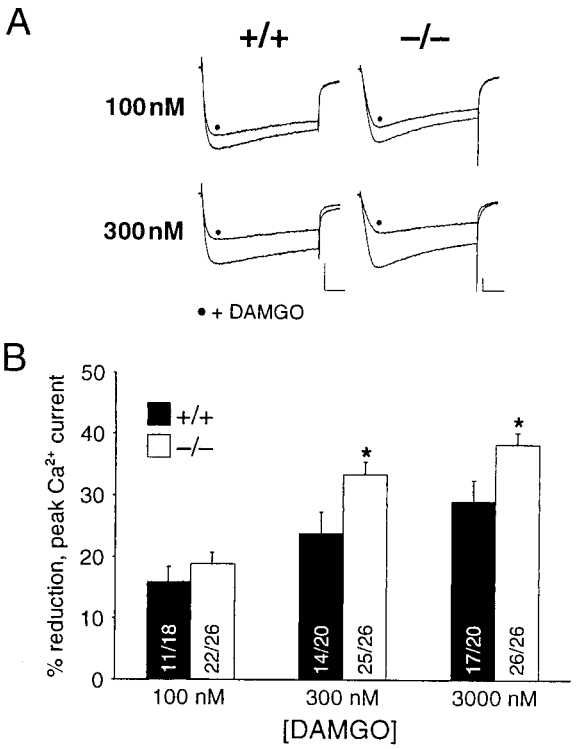

FIG. 4. μ Opioid-mediated regulation of voltage-dependent calcium channels in DRG neurons. (*A*) Whole-cell calcium currents were evoked by 100-msec steps to +10 mV from V_h = −80 mV just before and after the addition of 100 nM and 300 nM DAMGO (●). Calibration bars: 1 nA, vertical; 20 msec, horizontal. (*B*) Concentration–response relationship of DAMGO-mediated inhibition of Ca^{2+} current in wild-type (+/+) and PLC β3-null (−/−) DRG neurons, plotted as percent reduction in peak whole-cell current magnitude. The data from responding cells are presented as mean ± SEM. The ratios within each column represent the proportion of neurons in which the DAMGO-mediated reduction of peak whole-cell current was ≥10% of the control current out of the total number of cells tested. Data were compared by using Student's two-tailed t test (*, $P \leq 0.02$).

cell-specific isoform, exhibits the same substrate specificity and G protein regulation profile as PLC β3, but has the advantage of having a greater constitutive activity (22). The inclusion of PLC β2 protein (10 nM) in the recording pipette significantly reduced DAMGO-elicited (300 nM) suppression of Ca^{2+} current magnitude in PLC β3-deficient DRG neurons (without PLC β2, 44.7 ± 2.6%, n = 9; with PLC β2, 32.6 ± 2.8%, n = 20; P = 0.01, two-tailed t test; Fig. 5*A*). In addition, the magnitude of current reduction in PLC β3-deficient cells with recombinant PLC β2 protein was similar to that seen in wild-type neurons, either without or with PLC β2 (30.6 ± 5.3%, n = 9, and 34.0 ± 2.6%, n = 9, respectively). The current reductions by DAMGO in this experiment were equivalent to those in prior experiments. The ability of PLC β2 dialyzed through patch pipettes to restore wild-type-like responses in PLC β3-null DRG neurons suggests that the differences between wild-type and PLC β3-null mice in their responses to μ opioids were a direct result of the lack of PLC β3.

The mechanism by which alteration of PLC activity effected a change in opioid responses also was investigated. Our recording conditions "clamped" the intracellular Ca^{2+} concentration (with EGTA), suggesting against the possibility that the inositol trisphosphate pathway was involved. We postulated that PKC was the mediating pathway: if an absence of PLC β3 and an associated decrease in PKC activity resulted in increased opioid responses, then increased PLC or PKC activity would result in decreased opioid responses. To test this hypothesis, DRG neurons of both genotypes were tested for

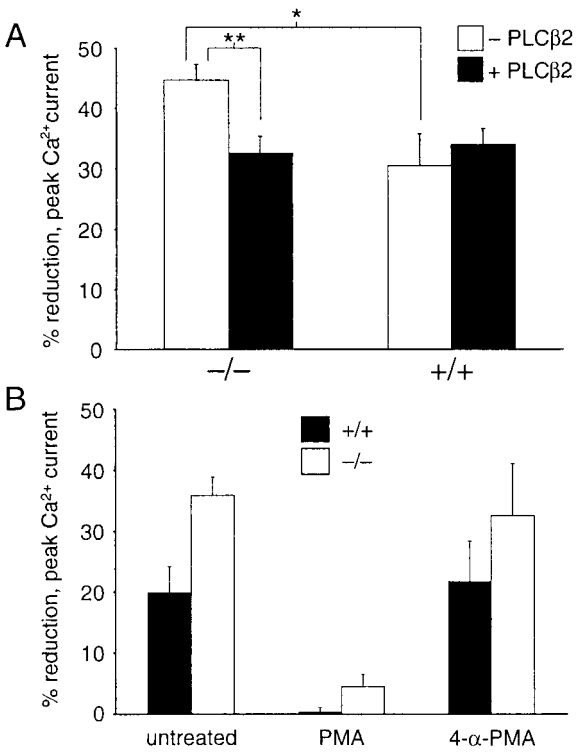

FIG. 5. Effects of PLC and PKC activation on μ opioid-mediated regulation of voltage-dependent calcium channels in DRG neurons. (*A*) The effect of added PLC β2 ($\approx$10 nM) on DAMGO-mediated (300 nM) inhibition of peak whole-cell Ca^{2+} current was measured in DRGs from wild-type (+/+) and transgenic (−/−) mice. Values are mean percent reduction in peak whole-cell current magnitude ± SEM. Data were compared by using Student's two-tailed *t* test (*, $P \leq 0.03$; **, $P \leq 0.01$). Cells in which there was $\geq$10% "rundown" or "run-up" of the peak whole-cell current over the course of the recording were excluded from the analysis. (*B*) Ca^{2+} currents were recorded in the absence and presence of 300 nM DAMGO, and the mean peak current reductions (%) were plotted. Neurons either were untreated or treated for 30 min with either PMA (100 nM) or 4-α-PMA (100 nM). The difference between untreated wild-type and transgenic neurons in this experiment is statistically significant ($P \leq 0.01$), as is the difference between untreated and PMA-treated neurons for either genotype ($P \leq 0.001$). There was no difference between genotypes in the PMA-treated group, nor was there a significant effect of 4-α-PMA in either genotype.

DAMGO responses after no pretreatment or after a 30-min pretreatment with either the PKC activator PMA (100 nM) or the inactive phorbol 4-α-PMA (100 nM). Ca^{2+} current magnitudes were similar in untreated and treated cells from either genotype and were similar to current magnitudes in earlier experiments. By contrast, pretreatment with PMA (Fig. 5*B*) virtually eliminated DAMGO-induced (300 nM) regulation of Ca^{2+} currents. This occurred in both wild-type neurons (untreated, 19.9 ± 4.2%, $n = 9$; PMA, 0.3 ± 0.8%, $n = 7$) and transgenic neurons (untreated, 35.8 ± 3.0%, $n = 7$; PMA, 4.5 ± 2.0%, $n = 9$). Pretreatment with the inactive 4-α-PMA had no effect on DAMGO-induced Ca^{2+} current reductions in either wild-type (21.7 ± 6.6%, $n = 5$) or transgenic neurons (32.5 ± 8.6%, $n = 4$).

DISCUSSION

We present evidence that a PLC isoform plays a significant role in morphine-mediated analgesia and also have provided evidence for a correlative cellular mechanism. The data suggest that PLC β3 is a significant negative modulatory pathway for opioid responses.

Our results show that μ opioids can couple to PLC β3 in aortic muscle cells. PLC β3 was the sole isoform that mediated G$\beta\gamma$-induced PLC activation in this cell type. This result is consistent with the observations that G$\beta\gamma$ could not activate PLC β1 in cotransfected COS-7 cells (35–37) and could only weakly activate PLC β1 in reconstitution assays by using purified proteins (38, 39). It is interesting to note that Gq-mediated PLC activation was hardly affected by the PLC β3 deficiency despite the fact that PLC β3 also can be activated by Gq *in vitro* (31, 39).

The coupling of opioid receptors to PLC β3 in neurons may provide a mechanism by which PLC modulates opioid responses at a cellular and behavioral level. The observation that mice lacking PLC β3 are more sensitive to morphine in the antinociceptive tests indicates that PLC activation does not directly mediate the analgesic effect of morphine. This conclusion is consistent with the current views on the mechanisms for opioid-mediated analgesia, which probably is mediated by the regulation of Ca^{2+} or K^+ channels, but also demonstrates the importance of the PLC system in the modulation of opioid-signaling pathways (40–42). Mice lacking PLC β3 exhibited the hypersensitive phenotype not only at the behavioral level but also in DRG neurons. Additionally, the wild-type-like response can be reconstituted by perfusing purified PLC β proteins into PLC β3-deficient DRG neurons. This reversal of the phenotype indicates that the phenotype may result more likely from the lack of the PLC protein rather than any developmental change. Thus, we believe that the PLC β3 pathway probably is involved in a cellular mechanism that inhibits opioid-mediated responses—if not in all cells, at least in DRG neurons.

The greater efficacy of opioid signal transduction in the absence of PLC β3 suggests that this enzyme may suppress opioid signaling by modification of opioid-dependent signaling components. Because PKC activation markedly reduced opioid responses, our results are consistent with the idea that the PLC–PKC pathway strongly modulates opioid-signaling efficacy. Regulation could be effected at the level of the opioid receptor or its G protein(s) or at the level of the targeted protein, in this case, a Ca^{2+} channel. Opioid receptors can be phosphorylated by protein kinases including PKC (43, 44) and calmodulin kinase II (43, 45, 46), which can be stimulated by PLC activation. Additionally, G protein α subunits can be phosphorylated by PKC (47, 48) and, thus, may be the targets of this modulatory pathway, although this has not been demonstrated directly in neurons. Finally, the ion channels regulated by opioid receptors also can be phosphorylated by these kinases (44, 46, 49–54), producing changes that could account for a reduction in opioid-induced regulation, for example, by reducing G protein interactions or by inactivating DAMGO-targeted channels. Against this last possibility is the finding that current magnitudes were similar between the wild-type and PLC β3-null neurons and that pretreatment with PMA did not alter current magnitudes. DRG neurons isolated from the PLC β3-null mice may provide a means for the future study of these mechanisms and of the cellular correlates of opioid-induced analgesia.

In summary, we have shown that mice lacking PLC β3 have increased behavioral and cellular responses to μ agonists, strongly supporting the notion that PLC modulates opioid agonist efficacy. Moreover, our studies in DRG neurons suggest the possible involvement of PKC, although our experiments cannot eliminate the possibility that other PLC-dependent signaling pathways also are involved. Additional experiments will be required to determine unequivocally whether this is the case. Our results suggest further that differences in the PLC β3 genes could account for varying responses to opioids among individuals and suggest new directions for research into tolerant and addictive states.

Note Added in Proof. Wang *et al*. (55) have reported that a targeted disruption in the PLC β3 gene causes embryonic lethality. Wang *et al*. used a construct that deleted exons 11–17 of the PLC β3 gene, whereas the construct used in the present studies partially deleted one exon. The differences between their construct and ours may account for the absence of lethality in our animals. Further investigation will be required to resolve fully these differences.

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