Protein Kinase C ϵ Is Required for Spinal Analgesic Synergy between Delta Opioid and Alpha-2A Adrenergic Receptor Agonist Pairs

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We recently showed that spinal synergistic interactions between δ opioid receptors (δ ORs) and α_{2A} adrenergic receptors (α_{2A} ARs) require protein kinase C (PKC). To identify which PKC isoforms contribute to analgesic synergy, we evaluated the effects of various PKC-isoform-specific peptide inhibitors on synergy between δ ORs and α_{2A} ARs using the tail flick assay of thermal nociception in mice. Only a PKC ϵ inhibitor abolished synergy between a δ OR agonist and an α_{2A} AR agonist. We tested a panel of combinations of opioid and adrenergic agonists in PKC ϵ knock-out mice and found that all four combinations of a δ OR agonist and an α_{2A} AR agonist required PKC ϵ for antinociceptive synergy. None of the combinations of a μ OR agonist with an α_2 AR agonist required PKC ϵ . Immunohistochemistry confirmed that PKC ϵ could be found in the population of peptidergic primary afferent nociceptors where δ ORs and α_{2A} ARs have been found to extensively colocalize. Immunoreactivity for PKC ϵ was found in the majority of dorsal root ganglion neurons and intensely labeled laminae I and II of the spinal cord dorsal horn. PKC ϵ is widespread in the spinal nociceptive system and in peptidergic primary afferents it appears to be specifically involved in mediating the synergistic interaction between δ ORs and α_{2A} ARs.

Introduction

Synergistic analgesic interactions between agonists of α_2 -adrenergic receptors (α_2 ARs) and opioid receptors (ORs) in rodents have been well documented (Hylden and Wilcox, 1983; Sullivan et al., 1987; Wilcox et al., 1987; Stone et al., 1997; Overland et al., 2009; Riedl et al., 2009) and polyanalgesic therapy using spinal coadministration of α_2 AR and OR agonists can reduce opioid requirements for pain management in the clinical setting (Förster and Rosenberg, 2004; Paech et al., 2004; Gregoratti et al., 2009). The α_{2A} - and α_{2C} AR subtypes, and all three OR subtypes (μ , δ , and κ) have been established as modulators of pain signaling at the spinal level (Stone et al., 1997; Fairbanks et al., 2002; Olave and Maxwell, 2002; Chakrabarti et al., 2010; Li et al., 2010). Certain α_2 AR agonists, such as clonidine (CLON) and brimonidine (BRIM), require the α_{2A} AR-subtype for spinal an-

algesic efficacy and analgesic synergy with OR agonists (Stone et al., 1997; Fairbanks and Wilcox, 1999). Anatomical studies have shown that α_{2A} ARs are extensively colocalized with δ ORs in terminals of substance-P-expressing primary afferent fibers in rodents (Overland et al., 2009; Riedl et al., 2009), making these receptors a good pair for investigation of intracellular mechanisms of spinal opioid-adrenergic analgesic synergy.

Protein kinase C (PKC) has been established as an intracellular mediator of analgesic synergy between α_{2A} ARs and δ ORs both *in vivo* and *in vitro*. Inhibition of PKC blocks *in vivo* synergy between spinally coadministered CLON and deltorphin II (DELT) in the mouse tail flick test and blocks *in vitro* CLON-DELT synergistic inhibition of depolarization-evoked calcitonin gene-related peptide (CGRP) release from rat spinal cord slices (Overland et al., 2009). To determine whether a specific isoform of PKC mediates the synergistic interaction between α_{2A} ARs and δ ORs, we evaluated the ability of isoform-specific PKC inhibitor peptides to prevent spinal BRIM-DELT synergy. Inhibitors of the α , β (Ma et al., 2006), δ (Chen et al., 2001), and ϵ (Johnson et al., 1996) isoforms of PKC were tested, but only the PKC ϵ inhibitor prevented BRIM-DELT synergy. To confirm and extend this finding, a battery of combinations of OR and AR agonists was tested for spinal analgesic synergy in mice genetically lacking PKC ϵ . In addition, the PKC ϵ activator peptide ψ εRACK (Dorn et al., 1999) was used to determine whether activation of PKC ϵ is sufficient to enhance potency of either BRIM or DELT delivered singly. Finally, expression of PKC ϵ in spinal cord and dorsal root

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Brimonidine + Deltorhin II (1:1)

Figure 1. Selective inhibition of PKC ϵ , but not PKC α , PKC β , or PKC δ , abolishes spinal analgesic synergy between BRIM and DELT. **A**, Dose-response curves for DELT (●), BRIM (■), and 1:1 combinations of both drugs with a pretreatment of one of the following: saline (□), PKC α/β inhibitor (FARKGALRQ; ▼), PKC δ inhibitor (SFNSYELGSL; △), PKC ϵ inhibitor (EAVSLKPT; ◇), or scrambled PKC ϵ inhibitor (LSEKPAV; ◆). All pretreatments were administered 30 min before BRIM and DELT. Error bars for each data point represent SEM. **B–F**, Isobolograms showing DELT dose and ED₅₀ (■) on the x-axis, BRIM dose and ED₅₀ (●) on the y-axis, theoretical additive ED₅₀ for a 1:1 combination (○ on line of theoretical additivity), and observed combined ED₅₀ (●). Error bars represent 95% confidence limits. **B**, Isobologram for BRIM and DELT after saline pretreatment illustrating a synergistic interaction. **C**, Isobologram for BRIM and DELT after pretreatment with a PKC α/β inhibitor (6 nmol, i.t.) illustrating a synergistic interaction. **D**, Isobologram for DELT and BRIM after pretreatment with a PKC δ inhibitor (6 nmol, i.t.) illustrating a synergistic interaction. **E**, Isobologram for DELT and BRIM after pretreatment with a PKC ϵ inhibitor (6 nmol, i.t.) illustrating an additive interaction. **F**, Isobologram for DELT and BRIM after pretreatment with a scrambled PKC ϵ inhibitor (6 nmol, i.t.) illustrating a synergistic interaction. **F**, Isobologram for DELT and BRIM after pretreatment with a scrambled PKC ϵ inhibitor (6 nmol, i.t.) illustrating a synergistic interaction. ED₅₀ values for dose-response curves and *p* values for interactions can be found in Table 1.

ganglia (DRG) was evaluated using immunohistochemistry to determine whether PKC ϵ is expressed in spinal terminals of peptidergic nociceptors, where analgesic synergy between α_2 ARs and δ ORs is thought to occur.

Materials and Methods

Animals. Adult C57BL/6J PKC ϵ wild-type (PKC ϵ -WT), knock-out (PKC ϵ -KO), and heterozygous mice of both sexes (20 $\pm$ 5 g) used for all experiments were bred from pairs of hybrid (50% C57BL/6J, 50% 129S4)

mice heterozygous for the mutant PKC ϵ gene (Khasar et al., 1999) and were maintained on a 12 h light/dark cycle with food and water available *ad libitum* to all animals. Heterozygous mice were used only for the PKC-isoform-specific peptide inhibitor experiments to conserve WT and KO mice and to make efficient use of all mice bred. All experiments were approved by the institutional animal care and use committee of the University of Minnesota.

Drug preparation and administration. Agonists used were DELT (Tocris Bioscience), SNC80, BRIM (UK 14304), CLON, morphine sulfate (MOR), and endomorphin II (ENDO) (all from Sigma). The peptide activator of PKC ϵ (ψ ERACK; HDAPIGYD; Dorn et al., 1999) and isoform-specific peptide inhibitors of PKC including the PKC α/β inhibitor FARKGALRQ (Ma et al., 2006), the PKC δ inhibitor SFNSYELGSL (Chen et al., 2001), and the PKC ϵ inhibitor EAVSLKPT (Johnson et al., 1996), were purchased from Biomartik. The scrambled PKC ϵ inhibitor (LSETKPAV) was from Anaspec. Peptide inhibitors or activators were administered 30 min before agonist administration and were preceded by an injection of 5 μ l of distilled water to produce hypoosmotic shock and to facilitate passage of peptides through cell membranes (Khasar et al., 1999; Aley et al., 2000). BRIM was dissolved in a solution of 80% acetone, 6% DMSO, and normal saline to form a stock solution. SNC80 was dissolved with an equimolar amount of tartaric acid in saline. All other drug stocks were prepared in normal saline. All drugs were diluted from stock solution into sterile 0.9% saline and injected intrathecally in a volume of 5 μ l in awake mice as described previously (Hylden and Wilcox, 1980).

Behavioral measures. Thermal nociceptive responsiveness was assessed using the warm water (52.5°C) tail immersion assay as described previously (Janssen et al., 1963). Briefly, each animal was gently held wrapped in a cloth and the tail dipped into a controlled temperature water bath. Withdrawal latency was recorded as the amount of time that passed before a rapid movement of the tail and was not allowed to exceed 12 s. A baseline latency was recorded before drug administration and subsequent latencies were recorded 7 min after each dose immediately before the next dose. Each agonist or combination was administered sequentially approximately every 7 min in increasing doses to generate a cumulative dose-response curve, each mouse receiving no less than three and no more than four doses (Shin and Eisenach, 2003). Each dose-response curve was generated, with an $n = 4$ –6 as indicated in each table or figure legend. For DELT, two dose-response curves were obtained on separate days to confirm the potency and efficacy of this drug in the PKC ϵ mutant mice. These curves did not differ significantly in potency or efficacy and were combined for a total $n = 12$. This curve was then reused in the analysis for each combination tested. This allowed us to minimize the number of animals necessary to complete this study. Data are represented as percent maximum possible effect (%MPE) values, which were determined using the following equation:

$$100 \times (\text{test-baseline}) / (\text{maximum-baseline})$$

Immunohistochemistry. All animal tissues were fixed by intracardial perfusion as described previously (Vulchanova et al., 1998). Tissue sections were cut at 14 μ m thickness and thaw mounted onto ProbeOn slides. Tissue sections were incubated for 1 h at room temperature in permeabilization buffer (TBS containing 0.2% Triton X-100, 0.2% Tween 20) and then incubated overnight at room temperature in primary antisera diluted in blocking buffer (TBS containing 0.2% Tween 20, 0.2% casein). Primary antibodies used were as follows: rabbit anti-PKC ϵ (1:2000; Santa Cruz Biotechnology); guinea pig anti-substance P (SP; 1:1000; Neuromics); mouse anti-CGRP (1:2000; Research Biochemicals International); mouse anti-PKC ϵ (1:300; Santa Cruz Biotechnology); rabbit anti- δ OR (Dado et al., 1993; raised against amino acids 103–120 of δ OR); and rabbit anti- α_2 AR (Stone et al., 1998). After rinsing with TBS, sections were incubated for 1 h at room temperature with Cy3- or Cy5-conjugated secondary antisera (1:500; Jackson ImmunoResearch). Sections were rinsed again in TBS, dehydrated through a series of increasing ethanol concentrations followed by xylenes, and coverslipped using DPX mounting medium. Tissues were visualized using an Olympus FluoView 1000 BX2 upright confocal microscope and images were processed for color, contrast, and brightness using Adobe Photoshop.

Data analysis. The ED₅₀, in nanomoles with 95% confidence limits, of all agonists and combinations were calculated using the graded dose-

Table 1. Interactions between BRIM and DELT in the presence or absence of PKC isoform-specific peptide inhibitors

Drug or pretreatment	ED ₅₀ , nmol (95% CL)	Outcome	<i>p</i> value
BRIM	1.44 ($\pm$ 0.41)		
DELT	3.43 ($\pm$ 0.41)		
BRIM + DELT (1:1)			
Theoretical additive	1.96 ($\pm$ 0.47)		
Saline	0.44 ($\pm$ 0.15)	Synergistic	5.6*10 ^{−10}
PKC α/β inhibitor	0.38 ($\pm$ 0.14)	Synergistic	1.8*10 ^{−9}
PKC δ inhibitor	0.38 ($\pm$ 0.11)	Synergistic	1.3*10 ^{−9}
PKC ϵ inhibitor	3.28 ($\pm$ 1.74)	Additive	0.15
PKC ϵ scramble	0.46 ($\pm$ 0.13)	Synergistic	1.6*10 ^{−9}

All pretreatments were administered 30 min prior to BRIM and DELT, and inhibitors were given as a single 6 nmol dose. A *p* value <0.05 indicates a synergistic interaction. *n* for each group ranged from 4 to 6. Statistical analyses for all interactions were performed using JFlashCalc software (M. Ossipov, University of Arizona).

CL, confidence limit.

response curve method of Tallarida and Murray (1987). Dose ratios for drug combinations were estimated based on comparison of ED₅₀ values and dose-response curves and were chosen to approximate equi-effective doses. Isobolographic analyses were performed using the numerical method (Tallarida et al., 1989; Ossipov et al., 1997). Theoretical additive and observed combination ED₅₀ values were compared statistically via the Student's *t* test with the JFlashCalc Pharmacological Calculations Program software package generously provided by Dr. Michael Ossipov (Department of Pharmacology, University of Arizona College of Medicine, Tucson, AZ). For all isobolograms, error bars for theoretical additive and observed combination ED₅₀ values represent the vector sum of vertical and horizontal confidence limits.

Results

Selective inhibition of PKC ϵ prevents analgesic synergy between BRIM and DELT

To determine whether a specific isoform of PKC is necessary for spinal analgesic synergy between an α_2 AR agonist and a δ OR agonist, we evaluated whether intrathecal pretreatment with various isoform-specific peptide inhibitors of PKC could prevent synergy between spinally delivered BRIM and DELT in the warm-water tail immersion assay. Isobolographic analysis revealed that pretreatment with 6 nmol of either a PKC α/β dual isoform inhibitor (FARKGALRQ; Ma et al., 2006) or a PKC δ inhibitor (SFNSYELGSL; Chen et al., 2001) had no effect on analgesic potency or efficacy of BRIM and DELT coadministered in approximately equi-effective doses; however, pretreatment with a PKC ϵ inhibitor (EAVSLKPT; Johnson et al., 1996) significantly reduced potency of the BRIM-DELT combination such that the interaction was no longer synergistic (Fig. 1, Table 1). In addition, a scrambled version of the PKC ϵ inhibitor (LSETKPAV) had no effect on potency of codelivered BRIM and DELT (Fig. 1, Table 1). These results indicate that of the four isoforms evaluated, only the ϵ isoform of PKC is involved in spinal analgesic synergy between agonist combinations acting at α_2 ARs and δ ORs. Numerical details for all statistical analyses are reported in Table 1.

Spinal analgesic synergy between δ OR and α_2 AR agonists requires PKC ϵ

To confirm that PKC ϵ plays a role in spinal analgesic synergy between α_2 ARs and δ ORs, various α_2 AR and δ OR agonists were administered intrathecally to PKC ϵ -KO and PKC ϵ -WT littermates and the effect of each single drug and each drug combination was evaluated using the warm-water tail immersion assay. Each of the δ OR agonists, DELT and SNC80, was coadministered intrathecally in approximately equi-effective doses with each of the α_2 AR agonists CLON and BRIM. All agonists produced comparable antinociception in both

Table 2. δ OR and α_2 AR agonist combinations in PKCε-WT and PKCε-KO mice

	ED ₅₀ , nmol (95% CL)					
Genotype	Agonists		Observed combined	Theoretical additive	Outcome (<i>p</i> value)	Dose ratio
	DELT	BRIM				
PKCε-WT	3.11 (± 0.67)	1.43 (± 0.48)	0.49 (± 0.30)	1.96 (± 0.47)	Synergistic (1.9*10 ^{−6}) Additive (0.49)	1:1
PKCε-KO	3.68 (± 0.75)	1.53 (± 0.56)	1.83 (± 0.82)	2.16 (± 0.57)		
	DELT	CLON				
PKCε-WT	3.11 (± 0.67)	3.46 (± 1.09)	2.00 (± 1.00)	3.28 (± 0.63)	Synergistic (0.03)	1:1
PKCε-KO	3.68 (± 0.75)	2.98 (± 1.20)	2.61 (± 1.49)	3.29 (± 0.80)	Additive (0.40)	
	SNC80	BRIM				
PKCε-WT	49.30 (± 6.31)	1.43 (± 0.48)	4.16 (± 1.75)	23.69 (± 4.50)	Synergistic (8.0*10 ^{−11})	1:30
PKCε-KO	49.50 (± 5.42)	1.53 (± 0.56)	41.73 (± 16.54)	24.62 (± 4.82)	Subadditive (0.045)	
	SNC80	CLON				
PKCε-WT	49.30 (± 6.31)	3.46 (± 1.09)	14.69 (± 4.69)	26.98 (± 4.40)	Synergistic (2.5*10 ^{−4})	1:15
PKCε-KO	49.50 (± 5.42)	2.98 (± 1.20)	18.76 (± 6.08)	25.06 (± 5.34)	Additive (0.11)	

Each δ OR agonist (DELT, SNC80) was delivered in combination with an α_2 AR agonist (BRIM, CLON). A *p* value <0.05 indicates an interaction significantly different from the theoretical additive, either synergistic or subadditive as indicated. Dose ratios are listed with adrenergic value first. *n* = 6 for all groups except DELT, for which *n* = 12. Statistical analyses for all interactions were performed using JFlashCalc software (M. Ossipov, University of Arizona). CL, confidence limit.

Figure 2. BRIM and DELT require PKCε for spinal analgesic synergy. **A, C**, Dose-response curves for BRIM (■), DELT (●), and a 1:1 combination of the two (□/○) in PKCε-WT (**A**) and PKCε-KO (**C**) mice. Error bars for each data point represent SEM. **B, D**, Isobolograms showing DELT dose and ED₅₀ (●) on the x-axis, BRIM dose and ED₅₀ (■) on the y-axis, theoretical additive ED₅₀ for a 1:1 combination (○ on line of theoretical additivity), and observed combined ED₅₀ (●). Error bars represent 95% confidence limits. **B**, BRIM and DELT produce analgesic synergy at the spinal level in PKCε-WT mice. **D**, The combination of BRIM and DELT is additive in PKCε-KO mice. ED₅₀ values for dose-response curves and *p* values for interactions can be found in Table 2.

Table 3. μ OR and α_2 AR agonist combinations in PKC ϵ -WT and PKC ϵ -KO mice

Genotype	ED ₅₀ , nmol (95% CL)		Observed combined	Theoretical additive	Outcome (<i>p</i> value)	Dose ratio
	MOR	BRIM				
PKC ϵ -WT	2.17 ($\pm$ 0.66)	1.43 ($\pm$ 0.48)	0.72 ($\pm$ 0.32)	1.72 ($\pm$ 0.41)	Synergistic (2.4×10^{-4})	1:1
PKC ϵ -KO	2.33 ($\pm$ 0.81)	1.53 ($\pm$ 0.56)	0.75 ($\pm$ 0.30)	1.85 ($\pm$ 0.48)	Synergistic (2.1×10^{-4})	
	MOR	CLON				
PKC ϵ -WT	2.17 ($\pm$ 0.66)	3.46 ($\pm$ 1.09)	0.35 ($\pm$ 0.07)	2.67 ($\pm$ 0.59)	Synergistic (2.1×10^{-10})	1:1
PKC ϵ -KO	2.33 ($\pm$ 0.81)	2.98 ($\pm$ 1.20)	0.41 ($\pm$ 0.10)	2.61 ($\pm$ 0.68)	Synergistic (3.3×10^{-8})	
	ENDO	BRIM				
PKC ϵ -WT	1.24 ($\pm$ 0.58)	1.43 ($\pm$ 0.48)	0.14 ($\pm$ 0.04)	1.33 ($\pm$ 0.40)	Synergistic (1.7×10^{-7})	1:1
PKC ϵ -KO	1.16 ($\pm$ 0.55)	1.53 ($\pm$ 0.56)	0.24 ($\pm$ 0.05)	1.32 ($\pm$ 0.41)	Synergistic (2.1×10^{-6})	
	ENDO	CLON				
PKC ϵ -WT	1.24 ($\pm$ 0.58)	3.46 ($\pm$ 1.09)	0.74 ($\pm$ 0.13)	2.39 ($\pm$ 0.66)	Synergistic (7.7×10^{-6})	1:3
PKC ϵ -KO	1.16 ($\pm$ 0.55)	2.98 ($\pm$ 1.20)	0.81 ($\pm$ 0.18)	2.33 ($\pm$ 0.72)	Synergistic (1.7×10^{-4})	

Each μ OR agonist (MOR, ENDO) was delivered in combination with an α_2 AR agonist (BRIM, CLON). A *p* value <0.05 indicates an interaction significantly different from the theoretical additive. Dose ratios are listed with adrenergic value first. *n* = 6 for all groups. Statistical analyses for all interactions were performed using JFlashCalc software (M. Ossipov, University of Arizona).

CL, confidence limit.

PKC ϵ -WT and PKC ϵ -KO mice when administered alone (Table 2). All four combinations of an α_2 AR agonist and a δ OR agonist produced analgesic synergy in PKC ϵ -WT mice; however, none of these combinations synergized in PKC ϵ -KO mice (Table 2). Dose-response curves and isobolograms for the BRIM + DELT (1:1) combination are shown in Figure 2 as representative examples of the synergistic interaction between the α_2 AR and δ OR agonist combinations in WT but not in PKC ϵ -KO mice. These data indicate that spinal analgesic synergy between α_2 ARs and δ ORs requires PKC ϵ . Numerical details for all statistical analyses are reported in Table 2.

Analgesic synergy between μ OR and α_2 AR agonists does not require PKC ϵ

To determine whether PKC ϵ is necessary for analgesic synergy between α_2 ARs and μ ORs, each of the μ OR agonists, MOR and ENDO, was coadministered intrathecally in approximately equieffective doses with CLON or BRIM and tested using the warm-water tail immersion assay. Both MOR and ENDO produced antinociception that was comparable between PKC ϵ -WT and PKC ϵ -KO mice (Table 3). All four combinations of α_2 AR and μ OR agonists produced analgesic synergy in both PKC ϵ -WT and PKC ϵ -KO mice (Table 3). Dose-response curves and isobolograms for the BRIM + MOR (1:1) combination shown in Figure 3 indicate that PKC ϵ is not required for synergy between α_2 ARs and μ ORs. These data suggest that synergistic interactions between α_2 ARs and μ ORs occur through different mechanisms than those mediating synergy between α_2 ARs and δ ORs and are independent of PKC ϵ . Numerical details for all statistical analyses are included in Table 3.

Activation of PKC ϵ does not alter potency or efficacy of BRIM or DELT delivered singly.

To determine whether activation of PKC ϵ is sufficient to enhance potency of α_2 AR or δ OR agonists given singly, we preceded BRIM or DELT dosing with a pretreatment of the PKC ϵ activator ψ ERACK (Dorn et al., 1999). As expected, intrathecal delivery of ψ ERACK caused significant hyperalgesia (Joseph and Levine, 2010). This hyperalgesia was dose dependent (data not shown) and peaked at 30 min after delivery (Fig. 4). Pretreatment with ψ ERACK had no effect on the potency or efficacy of BRIM or DELT (Fig. 4), indicating that activation of PKC ϵ is not sufficient to enhance potency of these agonists when they are delivered alone.

Immunohistochemical evaluation of localization of PKC ϵ in spinal cord and dorsal root ganglion

To determine whether PKC ϵ is located in peptidergic primary afferent nociceptors, where spinal analgesic synergy between δ ORs and

α_2 ARs is thought to occur, sections of spinal cord and lumbar DRG were colabeled with antibodies directed against PKC ϵ and each of the following: SP, CGRP, α_2 AR, and δ OR. Similar to previous reports (Khasar et al., 1999; Hucho et al., 2005; Wu et al., 2012), PKC ϵ -immunoreactivity (ir) was observed in a large majority of DRG neurons (Fig. 5), as well as in laminae I and II of the superficial spinal cord dorsal horn (Fig. 6). As expected, PKC ϵ -ir was not present in spinal cord or DRG of PKC ϵ -KO mice (data not shown). In DRG, a subpopulation of CGRP- and SP-expressing neurons also displayed PKC ϵ -ir (Fig. 5). In superficial dorsal horn, PKC ϵ -ir was found in a portion of puncta displaying CGRP-ir or SP-ir (Fig. 6). In addition, we found that PKC ϵ -ir partially colocalized with both α_2 AR-ir and δ OR-ir in the superficial dorsal horn (Fig. 6). This pattern of labeling indicates that PKC ϵ is found in the location consistent with its role in mediating synergistic interactions between α_2 ARs and δ ORs. Although the pattern of labeling in superficial dorsal horn appears typical of that associated with incoming primary afferent terminals, closer examination at higher magnification revealed that PKC ϵ -ir can also be found in neuronal cell bodies intrinsic to laminae I and II of the spinal cord, as evidenced by colocalization of PKC ϵ -ir with ir for the neuronal label NeuN (data not shown). These results indicate that PKC ϵ is expressed by multiple types of neurons involved in nociceptive processing at the spinal level.

Discussion

The present study demonstrates that PKC ϵ is necessary for synergistic analgesic interactions between α_2 AR and δ OR agonists at the spinal level *in vivo*. In contrast, other combinations that produce spinal analgesic synergy, such as those involving α_2 ARs and μ ORs, do not require PKC ϵ . Because previous reports have placed α_2 ARs and δ ORs on the spinal terminals of peptidergic primary afferent neurons (Riedl et al., 2009) and shown that α_2 AR and δ OR agonists synergistically inhibit CGRP release from spinal cord slices (Overland et al., 2009) and spinal synaptosomes (Riedl et al., 2009), our behavioral data are consistent with the observations that PKC ϵ colocalizes with α_2 ARs, δ ORs, SP, and CGRP. Immunolabeling of δ ORs and subsequent identification of δ OR localization within either peptidergic or nonpeptidergic subpopulations of sensory neurons remains controversial. Considering this controversy, we believe that the functional evidence linking δ OR agonists to inhibition of peptide release from primary afferents (Bao et al., 2003; Kondo et al., 2005; Wang et al., 2010; Beaudry et al., 2011) and the demonstrated involvement of PKC in the synergistic inhibition of CGRP release by coadministered α_2 AR and δ OR agonists in spinal cord

Figure 3. BRIM and MOR do not require PKC ϵ for spinal analgesic synergy. **A, C**, Dose-response curves for BRIM (■), MOR (●), and a 1:1 combination of the two (□/○) in PKC ϵ -WT (**A**) and -KO (**C**) mice. Error bars for each data point represent SEM. **B, D**, Isobolograms showing MOR dose and ED₅₀ (■) on the x-axis, BRIM dose and ED₅₀ (■) on the y-axis, theoretical additive ED₅₀ for a 1:1 combination (○ on line of theoretical additivity), and observed combined ED₅₀ (●). Error bars represent 95% confidence limits. **B**, BRIM and MOR produce analgesic synergy at the spinal level in PKC ϵ -WT mice. **D**, The combination of BRIM and MOR remains synergistic in PKC ϵ -KO mice. ED₅₀ values for dose-response curves and *p* values for interactions can be found in Table 3.

(Overland et al., 2009) strongly support colocalization of δ ORs and α_{2A} ARs in peptidergic primary afferents. Factoring in the observed colocalization of PKC ϵ with SP and CGRP in dorsal horn presented here strengthens our inference that PKC ϵ mediates the synergistic interaction between α_{2A} ARs and δ ORs within peptidergic terminals.

One caveat common to most *in vivo* pharmacological studies, including this one, is that agonists often have activity at receptors other than their primary target. The fact that four synergistic combinations thought to involve primarily α_{2A} ARs and δ ORs (CLON-DELT, BRIM-DELT, CLON-SNC80, BRIM-SNC80) all required PKC ϵ strengthens the evidence that these two receptor subtypes are responsible for analgesic synergy between these compounds. Although opioid-adrenergic analgesic synergy is well documented at the spinal level, there has been little investigation into the mechanisms underlying these interactions. The present study indicates that different combinations of an OR and an AR agonist can produce analgesic synergy through different mechanisms. The combination of: (1) the colocalization of α_{2A} ARs and δ ORs on peptidergic primary afferent nociceptors (Stone et al., 1998; Overland et al., 2009; Riedl et al., 2009), (2) the

present result that PKC ϵ can also be found in these neurons, and (3) the observation that four different combinations of α_{2A} AR and δ OR agonists all required PKC ϵ for spinal analgesic synergy suggests that synergy between these receptors occurs in primary afferent terminals. Several studies indicate that signaling mechanisms initiated by agonist binding at α_{2A} ARs and/or δ ORs could lead to activation of PKC ϵ . Agonist binding at either α_{2A} ARs or δ ORs induces release of intracellular calcium stores via G_{α_i} -associated $G\beta/\gamma$ activation of phospholipase C (PLC) (Dorn et al., 1997; Yoon et al., 1999). Therefore, these receptors could produce activation of PKC ϵ through increased production of diacylglycerol via the same pathway. In fact, a link has been established between G_i signaling and activation of PKC ϵ specifically in primary afferent neurons (Dina et al., 2009). It is also possible that α_{2A} ARs and/or δ ORs signal to PKC ϵ through the more typical G_{α_q} to PLC pathway. Delta ORs have been shown to couple to several G-proteins, including G_{α_q} (Standifer et al., 1996; Sánchez-Blázquez and Garzon, 1998) and $G_{\alpha_{16}}$ (Lee et al., 1998; Chan et al., 2003), both of which signal to PLC. Alternatively, it is possible that only baseline activity of PKC ϵ is needed for the observed interaction between receptors, rather than receptor-mediated activation. Although either

Figure 4. Activation of PKC ϵ by intrathecal $\Psi\epsilon$ RACK does not significantly alter potency or efficacy of BRIM or DELT. **A**, Time course of tail-flick latencies after intrathecal delivery of $\Psi\epsilon$ RACK (30 nmol, i.t.) revealed significant hyperalgesia 30 min after injection (*significantly different from baseline, $p < 0.01$, one-way ANOVA with Bonferroni *post hoc* analysis). **B**, Pretreatment with $\Psi\epsilon$ RACK (30 nmol, 30 min) did not change potency or efficacy of DELT. **C**, Pretreatment with $\Psi\epsilon$ RACK (30 nmol, 30 min) did not change potency or efficacy of BRIM.

Figure 5. Colocalization of immunoreactivity (ir) for PKC ϵ and the neuropeptides SP and CGRP in lumbar DRG. PKC ϵ -ir is shown in red in the left panels (**A**, **D**), with either SP-ir (**B**) or CGRP-ir (**E**) in green in the middle panels and overlaid images in the right panels illustrating that some neurons expressing PKC ϵ also express SP or CGRP (**C**, **F**). **A–C**, Colabeling for PKC ϵ -ir and SP-ir. **D–F**, Colabeling for PKC ϵ -ir and CGRP-ir.

α_{2A} ARs or δ ORs may activate PKC ϵ , it is clear from our data showing similar efficacy of AR and OR agonists delivered individually in WT versus PKC ϵ -KO mice that PKC ϵ is not involved in the antinociceptive activity of these drugs when administered alone.

The effectors that PKC ϵ acts upon to produce the synergistic interaction between α_{2A} ARs and δ ORs remain unknown. The

Figure 6. Immunoreactivity (ir) for PKC ϵ in superficial dorsal horn of lumbar spinal cord relative to SP-ir, CGRP-ir, δ OR-ir, or α_{2A} AR-ir. PKC ϵ -ir is shown in red in the left panels, with SP-ir (**A**, **B**), CGRP-ir (**C**, **D**), α_{2A} AR-ir (**E**, **F**), or δ OR-ir (**G**, **H**), in green in the middle panels and overlaid images in the right panels. **A**, Low-magnification images of superficial dorsal horn illustrating the pattern of PKC ϵ -ir and SP-ir in this tissue section. **B**, High-magnification images of lamina I/II showing in yellow that PKC ϵ -ir and SP-ir partially colocalize in spinal terminals of primary afferent neurons. **C**, Low-magnification images of PKC ϵ -ir and CGRP-ir in superficial dorsal horn. **D**, High-magnification images of lamina I/II showing in yellow that PKC ϵ -ir and CGRP-ir partially colocalize in spinal terminals of primary afferent neurons. **E**, Low-magnification images of PKC ϵ -ir and α_{2A} AR-ir in superficial dorsal horn. **F**, High-magnification images of lamina I/II showing in yellow that PKC ϵ -ir and α_{2A} AR-ir partially colocalize in spinal terminals. **G**, Low-magnification images of PKC ϵ -ir and δ OR-ir in superficial dorsal horn. **H**, High-magnification images of lamina I/II showing in yellow that PKC ϵ -ir and δ OR-ir partially colocalize in spinal terminals.

failure of the PKC ϵ activator ψ εRACK to increase the potency of either agonist given alone suggests that simple receptor phosphorylation by PKC ϵ is not the only event necessary to increase agonist potency. Nonetheless, one mechanism by which PKC ϵ may exert its effects is through phosphorylation of α_2 ARs or δ ORs themselves. Modification of these receptors may induce greater functional capability by increasing activity with inhibitory G-proteins, making them more responsive to agonists or altering the way they interact with each other. Indeed, there is evidence that PKC can induce functional competence of δ ORs trigeminal ganglion cultures directly (Patwardhan et al., 2005), as well as *in vivo* in the periphery (Rowan et al., 2009), although it is unknown which isoform of PKC is responsible for the observed effects. Additional evidence suggests that lack of the ϵ isoform of PKC decreases stimulus-induced release of CGRP (Sweitzer et al., 2004). Because primary afferent plasma membrane δ OR expression is thought to be limited at a basal state and augmented by trafficking of large dense-core vesicles under certain stimulus conditions (Zhao et al., 2011), including δ OR agonist exposure (Bao et al., 2003; Overland et al., 2009), loss of PKC ϵ may result in decreased surface and/or functional availability of δ ORs. Further studies are needed to determine what specific role, if any, PKC ϵ plays in the modulation of trafficking and function of δ ORs or α_2 ARs.

Because PKC ϵ is found in the majority of DRG neurons, it is not surprising that it is expressed in several subpopulations of sensory neurons (Hucho et al., 2005; Ferrari et al., 2010; Joseph and Levine, 2010). Studies by Ferrari et al. (2010) and Joseph and Levine (2010) have shown that PKC ϵ mediates hyperalgesic priming in the peripheral terminals of isolectin-B4-binding neurons. The present study indicates a different role for PKC ϵ in DRG by showing that in the central terminals of some peptidergic afferents, it can be involved in enhanced antinociception upon activation of the appropriate receptor combination. We also report that PKC ϵ can be found not only in primary afferent neurons, but also intrinsic spinal neurons located in the superficial dorsal horn (laminae I and II). The complexity of the involvement of PKC ϵ in nociceptive processing is becoming increasingly apparent because it clearly plays multiple roles across several neuronal subtypes at both the peripheral and spinal levels.

The data presented here show that synergistic analgesic interactions between adrenergic and opioid agonists at the spinal level occur through different mechanisms depending on the anatomical relationship between the targeted receptor subtypes. Specifically, synergistic interactions between agonist combinations acting at α_2 ARs and δ ORs require PKC ϵ and likely occur in the spinal terminals of peptidergic nociceptors. Conversely, synergistic interactions between agonists acting at receptors thought to be located on different neurons, such as α_2 ARs and μ ORs, do not require PKC ϵ . PKC ϵ is widespread in the spinal nociceptive system and has multiple known pro-nociceptive actions. In contrast, the present study shows that coactivation of α_2 ARs and δ ORs leads to involvement of PKC ϵ in antinociceptive synergy. Our results have revealed a new role for PKC ϵ in spinal nociceptive processing and furthered our understanding of synergistic analgesic interactions between α_2 ARs and ORs at the spinal level.

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