Protein Kinase C Mediates the Synergistic Interaction Between Agonists Acting at α_2 -Adrenergic and Delta-Opioid Receptors in Spinal Cord

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Coactivation of spinal α_2 -adrenergic receptors (ARs) and opioid receptors produces antinociceptive synergy. Antinociceptive synergy between intrathecally administered α_2 AR and opioid agonists is well documented, but the mechanism underlying this synergy remains unclear. The delta-opioid receptor (DOP) and the α_{2A} ARs are coexpressed on the terminals of primary afferent fibers in the spinal cord where they may mediate this phenomenon. We evaluated the ability of the DOP-selective agonist deltorphin II (DELT), the α_2 AR agonist clonidine (CLON) or their combination to inhibit calcitonin gene-related peptide (CGRP) release from spinal cord slices. We then examined the possible underlying signaling mechanisms involved through coadministration of inhibitors of phospholipase C (PLC), protein kinase C (PKC) or protein kinase A (PKA). Potassium-evoked depolarization of spinal cord slices caused concentration-dependent release of CGRP. Coadministration of DELT and CLON inhibited the release of CGRP in a synergistic manner as confirmed statistically by isobolographic analysis. Synergy was dependent on the activation of PLC and PKC, but not PKA, whereas the effect of agonist administration alone was only dependent on PLC. The importance of these findings was confirmed *in vivo*, using a thermal nociceptive test, demonstrating the PKC dependence of CLON-DELT antinociceptive synergy in mice. That inhibition of CGRP release by the combination was maintained in the presence of tetrodotoxin in spinal cord slices suggests that synergy does not rely on interneuronal signaling and may occur within single subcellular compartments. The present study reveals a novel signaling pathway underlying the synergistic analgesic interaction between DOP and α_2 AR agonists in the spinal cord.

Introduction

Opioid analgesics remain the mainstay for treatment of moderate to severe pain states (American Pain Society, 2008). However, development of adverse side effects such as tolerance, dependence, constipation, addiction liability and opioid-induced hyperalgesia limit their utility (Angst and Clark, 2006). Many approaches have been investigated to bypass these untoward side effects, but use of multimodal analgesic techniques offers distinct advantages as combination therapies may produce analgesia at lower total drug doses. Extensive behavioral (Hylden and Wilcox, 1983; Stevens et al., 1988; Monasky et al., 1990; Ossipov et al., 1990a,b,c; Roerig et al., 1992) and electrophysiological (Sullivan et al., 1987; Wilcox et al., 1987; Omote et al., 1990) studies doc-

ument that that coactivation of α_2 -adrenergic receptors (α_2 ARs) and opioid receptors (ORs) produces synergistic interactions in spinal cord, although characterization of the mechanisms underlying this phenomenon have yet to be elucidated. Therefore, understanding the molecular mechanisms involved in the synergistic interactions of these receptors is of both clinical and theoretical importance in development of more efficacious therapies for pain management, as synergy-enabled decreases in dose may mitigate unwanted side effects.

Both α_2 ARs and ORs belong to the seven transmembrane-spanning domain G-protein-coupled receptor superfamily and share common signal transduction systems mediated primarily through inhibitory G-proteins, the activation of which inhibits pain transmission. It has been proposed that two receptor populations, acting through common signaling systems, can only synergize if they are anatomically localized to different locations within the pathway (e.g., presynaptic vs postsynaptic) (Honoré et al., 1996). In contrast, previous literature suggests that two analgesic receptor subtypes, α_{2A} ARs (Stone et al., 1998) and delta-opioid receptors (DOP) (Dado et al., 1993; Arvidsson et al., 1995; Cheng et al., 1997; Zhang et al., 1998), are extensively colocalized in terminals of capsaicin-sensitive, substance P (SP)-

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expressing primary afferent fibers in rat (Riedl et al., 2009) and that agonists acting at these receptors are able to produce analgesic synergy *in vivo* in both mouse (Stone et al., 1997) and rat (Ossipov et al., 1990c).

Given that the mechanisms underlying supra-additive receptor interactions remain unknown, we sought to determine which intracellular signaling pathways are necessary for synergy to occur between α_2 ARs and DOPs. Because of the striking correspondence of the actions and interactions between α_2 ARs and DOPs in mouse and rat, we used immunohistochemical and *in vivo* behavioral studies in mice combined with a more reduced *in vitro* spinal cord slice preparation in rats to determine whether the observed synergy between agonists acting at α_2 ARs/DOPs results from something other than multicellular interactions mediated by neuronal circuitry. We then used inhibitors of specific signaling pathways affected by the aforementioned receptor pair to elucidate the mechanisms involved in the synergistic outcome of receptor coactivation. Here, we report that coactivation of α_2 ARs and DOPs produces a synergistic interaction both *in vivo* and *in vitro*, and that this interaction takes place within the terminals of primary afferent neurons in spinal cord. Whereas the analgesic efficacy of both receptors required PLC activation, the synergistic interaction uniquely required PKC activation. These studies are the first to identify a signaling pathway underlying synergy between agonists acting at α_2 ARs and DOPs and may lead to improved understanding and increased clinical utilization of polyanalgesic therapy.

Materials and Methods

Animals. Male CD-1 ICR mice (20 ± 5 g; Harlan), male C57BL/6 mice (20 ± 5 g; Charles River) and adult male Sprague Dawley rats (300 ± 25 g; Harlan) were maintained on a 12 h light/dark cycle and food and water were available *ad libitum* to all animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota or the McGill University Animal Care and Ethics Committees.

Immunohistochemistry. Immunohistochemistry was performed as previously described (Wessendorf and Elde, 1985; Fairbanks et al., 2002; Riedl et al., 2009). In brief, male C57BL/6 mice were anesthetized with a ketamine/xylazine/acepromazine mixture and perfused transcardially with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH 6.9. Spinal cords were dissected and stored overnight in 10% sucrose at 4°C. Tissue sections were prepared using a cryostat at a thickness of 10–14 μ m, thaw-mounted onto gelatin-coated slides and stored at –20°C. Sections were incubated for 1 h at room temperature in diluent containing 1% normal donkey serum, 0.3% Triton X-100, 0.01% sodium azide and 1% bovine serum albumin in PBS. Sections were then incubated overnight at 4°C in a humid chamber with primary antisera, rinsed 3×10 min with PBS, incubated with fluorescently tagged species-specific secondary antisera (Jackson ImmunoResearch) for 1 h at room temperature, rinsed 3×10 min with PBS and coverslipped using a mixture of glycerol and PBS containing 0.1% *p*-phenylenediamine. The α_2 AR antiserum (1:1000) was prepared in rabbit against a synthetic peptide corresponding to α_2 AR_{436–450} (AFKKILCRGDRKRIV) of the rat sequence and has been previously characterized (Stone et al., 1998; Riedl et al., 2009). The rabbit DOP antiserum (1:1000) was prepared against a synthetic peptide corresponding to anti-DOP_{3–17} (1:1000; LVPSARAELQSSPLV) and has been previously characterized (Dado et al., 1993; Riedl et al., 2009). SP antibodies raised in two different species and obtained from two different sources were used in these studies and produced similar results: rat anti-SP (1:1000; Accurate Chemical) and guinea pig anti-SP (1:500; Neuromics Antibodies) and have been previously characterized (Cuello et al., 1979; Riedl et al., 2009). Images were collected using a Bio-Rad MRC 1000 confocal microscope (Bio-Rad Microscience Division) or an Olympus BX-51 equipped with a DP-71 camera and assembled in photoshop.

Drug preparation and administration. Drugs used were clonidine (CLON), chelerythrine, U73122 [1-[6-[(11 β)-3-methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione], idazoxan, H89 [N-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline], tetrodotoxin (TTX) (all from Sigma), deltorphin II (DELT) (Tocris Bioscience), and naltrindole (gift from Dr. Philip Portoghesi, University of Minnesota, Minneapolis, MN). All drugs for behavioral experiments were dissolved in 0.9% saline and administered intrathecally (i.t.) in a volume of 5 μ l according to the method of Hylden and Wilcox (1980) as modified by Wigdor and Wilcox (1987) in conscious mice. For spinal cord neuropeptide release experiments, U73122 was dissolved in ethanol and diluted in HEPES buffer. All other drugs were dissolved in dH₂O and diluted in HEPES buffer. Control experiments with HEPES (shown) and HEPES with ethanol (data not shown) demonstrated that diluted ethanol had no effect on either basal or K⁺-stimulated CGRP release.

Antinociception. Thermal nociceptive responsiveness was assessed using the warm water (52.5°C) tail-immersion assay, as described previously (Janssen et al., 1963). Briefly, mice were gently wrapped in a soft cloth such that their tails were exposed, and three-quarters of the length of the tail was dipped into the warm water. Tail-flick latencies were obtained before drug application to establish a baseline response. Opioid and adrenergic receptor agonists were injected i.t. as 5 and 10 min pretreatments, respectively. The opioid receptor antagonist was injected concomitant with agonist injection and the adrenergic receptor antagonist was injected i.t. as a 10 min pretreatment before adrenergic receptor agonist injection. PLC and PKC antagonists were injected i.t. as 1 h pretreatments before latency determination, whereas PKA antagonist was injected i.t. as a 30 min pretreatment. A maximum cutoff of 12 s was set to avoid tissue damage. The results were then expressed as a percentage of the maximum possible effect (%MPE) according to the equation: % MPE = $\frac{\text{Postdrug latency} - \text{Predrug latency}}{\text{Cutoff} - \text{Predrug latency}} \times 100$.

Neuropeptide release from spinal cord slices. For determination of calcitonin gene-related peptide (CGRP) release from spinal cord slices, adult male Sprague Dawley rats (275–325 g) were used. Animals were anesthetized with isoflurane and quickly decapitated. Spinal cords were removed by hydraulic extrusion and placed in ice-cold, oxygenated HEPES buffer containing (in mM): 120 NaCl, 5.4 KCl, 0.8 MgCl₂, 1.8 CaCl₂, 20 HEPES, 0.01 glycine, 15 glucose, and commercial protease inhibitor cocktail (Roche). Two centimeter segments of the lumbar enlargement were removed, divided midsagittally, and chopped into 0.3 $\times$ 0.3 mm pieces (McIlwain tissue chopper), and the halves were placed into separate 1 ml perfusion chambers. The tissue was perfused at a flow rate of 0.35–0.4 ml/min in HEPES buffer maintained at 37°C, aerated with 95% O₂–5% CO₂ and pH adjusted to 7.4. The tissue was allowed a perfusion equilibration period of 30 min to stabilize peptide release and then collected for 6 min periods in 12 $\times$ 75 mm glass test tubes. Basal release was assessed by perfusing the tissue with HEPES buffer for 6 min. After this period, peptide release was evoked by perfusing the tissue for an additional 6 min with HEPES buffer containing 60 mM K⁺. In release inhibition experiments, tissue was perfused for 6 min with HEPES buffer containing DELT, CLON, or the combination in a 1:1 concentration ratio before the K⁺ stimulation. When PLC inhibitor, PKC inhibitor, PKA inhibitor, or TTX were used, these compounds were present in the superfusate throughout the entire experiment. Immunoreactive CGRP in the collected samples was assayed by enzyme-linked immunosorbent assay (ELISA) (SPI Bio, Catalog No. 589001). No difference in either basal or K⁺-evoked CGRP release was observed from slices made from either whole cord or cord separated to exclude the ventral horn (data not shown).

Electrophysiological recording. Adult male Sprague Dawley rats (275–325 g) were anesthetized with isoflurane and quickly decapitated. Spinal cords were removed by hydraulic extrusion and placed in ice-cold, oxygenated artificial CSF (aCSF) with the following composition (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH₂PO₄, 1.3 MgSO₄, 2.5 CaCl₂, 26.2 NaHCO₃, and 11 glucose. The lumbar enlargement was cut into 1 cm sections, from which dorsal horizontal slices with a thickness of ~500 μ m were taken using a vibratome while the spinal cord was immersed in aCSF. The slicing solution also contained 10 mM kynurenic acid to maintain viability. After recovery, slices were superfused with normal aCSF (22–23°C) containing 100 μ M picrotoxin. Field EPSPs (fEPSPs) were evoked every

10 s using a suction electrode placed on the dorsal root entry zone, and recorded using a glass electrode filled with aCSF and placed in the ipsilateral superficial dorsal horn 2–5 mm rostral or caudal of the stimulating electrode. Data were digitized at 5 kHz using a Multiclamp 700A amplifier (Molecular Devices), and analyzed using custom software (Igor Pro, Wavemetrics). fEPSP amplitude following application of TTX (0.1 or 1 μ M) is expressed as the percentage of baseline amplitude recorded for 5 min before TTX application.

Data analysis. The ED₅₀ (nanomoles, *in vivo*) or EC₅₀ (nanomolar, *in vitro*) values and 95% confidence intervals (CIs) of both CLON and DELT were calculated using the graded dose–response curve method of Tallarida and Murray (Tallarida, 1992). A minimum of three doses or concentrations were used for each drug or drug combination. In some instances (e.g., multiple doses or concentrations with efficacies approaching 0 or 100%), only the linear portion of the dose/concentration–response curve was included in the ED₅₀/EC₅₀ value calculation. To determine differences in agonist potency between groups, nonoverlapping 95% CIs were considered to represent statistically significant differences. In the experiments testing for synergistic interactions, dose/concentration–response curves, ED₅₀/EC₅₀ values, and 95% CIs were first generated for CLON and DELT administered alone as described above. CLON and DELT were then coadministered at a constant dose/concentration ratio based on the potency ratio of the two drugs given separately. For example, if CLON had an ED₅₀ or EC₅₀ value of 1 nmol or nM (*in vivo* and *in vitro*, respectively) and DELT had an ED₅₀ or EC₅₀ value of 1 nmol or nM, the agents were coadministered in a 1:1 ratio and a third dose/concentration–response curve was generated for the combination treatment.

Isobolographic analysis. Isobolographic analysis is the accepted standard for quantitative evaluation of drug interactions (Tallarida, 1992). Dose/concentration–response curves were first constructed for CLON and DELT administered separately and the ED₅₀/EC₅₀ values were calculated and then used to determine an equieffective dose/concentration ratio between the two as described above. The interaction between the two drugs was tested by comparing the theoretical additive ED₅₀/EC₅₀ value for the combination based on the dose/concentration–response curves of each drug administered separately and the observed experimental ED₅₀/EC₅₀ value of the combination using a *t* test. These values are based on the total dose of both drugs. An interaction is considered synergistic if the experimental ED₅₀/EC₅₀ value is significantly less (*p* < 0.05) than the calculated theoretical additive ED₅₀/EC₅₀ values.

Isobolographic analysis allows for graphical representation of drug interactions (see Figs. 2B, D, 4B). This representation depicts the ED₅₀/EC₅₀ value of each agent as the *x*- or *y*-intercept. For example, Figure 2B represents the ED₅₀ value of CLON as the *y*-intercept and the ED₅₀ of DELT as the *x*-intercept. The line connecting these two points is the theoretical additive line and depicts the dose combinations expected to yield 50% efficacy if the drug interaction is strictly additive. The theoretical additive ED₅₀ value and its confidence interval are determined mathematically and plotted spanning this line. The observed ED₅₀ value for the combination is plotted at the corresponding *x*, *y* coordinates along with its 95% confidence interval for comparison to the theoretical additive ED₅₀ value. The same comparisons are made for EC₅₀ values.

The magnitude of drug synergism can also be expressed in terms of an Interaction Index (γ) (Tallarida, 2002). The index is defined by the equation: $a/A + b/B = \gamma$, where *A* and *B* are the doses/concentrations of drugs *A* and *B* administered separately that give a specified level of effect and (*a*, *b*) is the combination dose/concentration that produces this same level of effect (the ED₅₀/EC₅₀ values are commonly used for this calculation). In the absence of a drug interaction, $\gamma = 1$. If the interaction is synergistic, $\gamma < 1$. The interaction index is used here as a quantitative

Figure 1. Colocalization of α_2 AR and DOP with SP in mouse spinal cord. **A–C**, Representative images of the dorsal horn of mouse spinal cord double-labeled with DOP (**A**, magenta) and SP (**B**, green) antisera. When images **A** and **B** are digitally merged (**C**), instances of colocalization appear as white. **D–F**, Representative images of mouse spinal cord double-labeled with α_2 AR (**D**, magenta) and SP (**E**, green) antisera. When images **D** and **E** are digitally merged (**F**), instances of colocalization appear white. The extensive colocalization observed between both α_2 AR and DOP with SP suggests that α_2 AR and DOP colocalize on SP-containing fibers in the mouse spinal cord. This extensive colocalization, already well characterized in rats (Riedl et al., 2009), appears to generalize to mice.

measure to characterize the magnitude of synergism by the CLON–DELT combination between treatment groups.

All dose–response and concentration–response and isobolographic analyses were performed with the FlashCalc 4.5.3 pharmacological statistics software package generously supplied by Dr. Michael Ossipov (Department of Pharmacology, University of Arizona College of Medicine, Tucson, AZ).

Results

Colocalization of α_2 AR, DOP, and SP immunoreactivity in the dorsal horn of mouse spinal cord

Previous reports have demonstrated that both α_2 AR and DOP are expressed in the peptidergic population of primary afferent sensory neurons in rat (Dado et al., 1993; Arvidsson et al., 1995; Stone et al., 1998; Zhang et al., 1998; Riedl et al., 2009). However, anatomical characterization of α_2 AR and DOP has not been fully investigated in mice. Therefore, we double-labeled mouse spinal cord sections with antibodies directed against α_2 AR, DOP, and the neuropeptide SP (Fig. 1). On merging of the digital images of sections, double-labeled elements appear white (Fig. 1C,F). Colocalization of α_2 AR immunoreactivity (α_2 AR-IR) and SP-IR was observed with the rabbit-derived α_2 AR and both rat- and guinea pig-derived SP antibodies obtained from independent sources (Fig. 1D–F; data not shown). Similarly, rabbit-derived anti-DOP labeling colocalized with both the rat- and the guinea pig-derived SP antibodies (Fig. 1A–C; data not shown). The independent colocalization of α_2 AR-IR and DOP-IR with multiple SP antibodies is entirely consistent with anatomical localization in rat and strongly suggests that the colocalization observed is not artifactual. The extensive colocalization observed between both α_2 AR and DOP with SP suggests that α_2 AR and DOP colocalize in SP-containing fibers in the mouse spinal cord and may be positioned to mediate the antinociceptive effects of spinally delivered agonists for these receptors.

Intrathecal CLON–DELT: Behavioral antinociceptive synergy

Intrathecal administration of either CLON or DELT produced dose-dependent antinociception at 10 and 5 min postinjection, respectively (Fig. 2A); these pretreatment times were chosen to match the time of peak effect of each agent given alone (data not shown). Comparison of the respective ED₅₀ values revealed a potency ratio between CLON and DELT of ~1:1. Coadministra-

Figure 2. Coadministration of DELT and CLON is synergistic in the tail flick test. **A**, Nociceptive thermal responses were challenged by intrathecal administration of DELT, CLON, and their combination. DELT (filled triangles) and CLON (filled circles) inhibited the behavior in a dose-dependent manner with similar potency and efficacy. When both DELT and CLON were coadministered at a constant dose ratio of 1:1 (open circles), the resulting potency was ~ 30 -fold higher than either drug given alone, suggesting that the interaction was synergistic. Error bars represent mean $\pm$ SEM for each dose point ($n = 6$ animals/dose). **B**, Isobolographic analysis of the data in Figure 1A. The y -intercept represents the CLON ED₅₀ (5 nmol; 95% CI = 3.6–6.4), and the x -intercept represents the DELT ED₅₀ (3.9 nmol; 95% CI = 3.2–4.5) when each was administered alone. The heavy line connecting the intercepts is the theoretical additive line and the open circle represents the theoretical additive combined ED₅₀. Coordinates for drug combinations falling below this line and outside the confidence limits indicate a synergistic interaction. When the two compounds were coadministered at a 1:1 dose ratio, the resultant ED₅₀ (closed circle) (0.17 nmol; 95% CI = 0.11–0.23) of DELT in the presence of CLON fell well below the additive line, indicating that the interaction was synergistic. Error bars parallel to each axis represent the lower 95% CI for each compound given alone. The error bars on the combined dose points represent the upper and lower 95% CIs. **C**, Coadministration of DELT and CLON show additivity in the presence of the PKC inhibitor chelerythrine. Nociceptive thermal responses were challenged by intrathecal administration of DELT, CLON, and their combination in the presence of an inhibitor of PKC. DELT (filled triangles) and CLON (filled circles) inhibited the responses in a dose-dependent manner with similar potency and efficacy. Coadministration of DELT and CLON at a 1:1 dose ratio (open circles) was ~ 1.9 -fold more potent than either drug given alone, compared with ~ 30 -fold potency shift in the absence of chelerythrine (see Fig. 1A). Error bars represent mean $\pm$ SEM for each dose point ($n = 6$ animals/dose). **D**, Isobolographic analysis was applied to the data in Figure 1C. The y -intercept represents the ED₅₀ (7.7 nmol; 95% CI = 6.1–9.3) for CLON, and the x -intercept represents the ED₅₀ (7.9 nmol; 95% CI = 6.8–9.0) for DELT when each was administered alone. Coadministration at a 1:1 dose ratio resulted in an ED₅₀ (closed circle) (4.1 nmol; 95% CI = 3.2–5.0) for DELT in the presence of CLON that fell on the theoretical additive line, indicating a strictly additive interaction in the presence of the PKC inhibitor.

tion of the drugs (CLON at 10 min and DELT at 5 min) at a constant dose ratio equal to the potency ratio (1:1) yielded a third antinociceptive dose–response curve shown in Figure 2A. This combination dose–response curve is expressed in terms of the doses of CLON (0.01, 0.1, 1, 3) given in the presence of the same doses of DELT (0.01, 0.1, 1, 3) as opposed to total drug (e.g., 0.02, 0.2, 2, 6) to facilitate visual appreciation of the potency shifts of each drug in the presence of the other. The potency of each drug was increased ~ 30 -fold in the presence of the other, suggesting that the interaction was synergistic. The dose–response data from Figure 2A are represented graphically as an isobologram in Figure 2B, which shows that the ED₅₀ value of the combination (closed circle) is significantly lower than the theoretical additive ED₅₀ value (open circle). This interaction was confirmed as synergistic by statistical comparison (t test) between the observed combined ED₅₀ value and the theoretical additive ED₅₀ value. The interaction index, γ , was 0.04; this small γ value indicates that the synergistic interaction between CLON and DELT is of a high magnitude.

Inhibition of PKC completely and selectively reverses CLON–DELTA synergistic inhibition of nociceptive responses in the tail flick test

To assess the signaling mechanisms mediating the observed behavioral antinociceptive synergy between α_2 AR and DOP ago-

nists, we evaluated the effect of intrathecally coadministered CLON and DELT in the tail flick assay when mice were pretreated with selective inhibitors of PLC, PKC and PKA (U73122, 3 nmol, i.t.; chelerythrine, 1 nmol, i.t.; and H89, 6 nmol, i.t., respectively). PKC inhibition was selected because of its activation downstream of diacylglycerol produced and Ca^{2+} mobilized by PLC, previous behavioral work (Roerig, 1998) and recent evidence in trigeminal nociceptors showing that application of the inflammatory mediator bradykinin (BK) rapidly induces functional DOP competence through a PKC-dependent signaling mechanism (Patwardhan et al., 2005). PKA inhibition was chosen as a negative control to show specificity of the PKC effect.

After administration of the PKC inhibitor, CLON (50 min) and DELT (55 min) were administered separately or coadministered at a constant dose ratio equal to the potency ratio (1:1), and three antinociceptive dose–response curves were generated (Fig. 2C). The dose–response data from Figure 2C and the resulting isobologram (Fig. 2D) show that the ED₅₀ value of the combination did not differ significantly from the theoretical additive ED₅₀. This interaction was confirmed as additive by statistical comparison (t test) between the observed combined ED₅₀ value and the theoretical additive ED₅₀ value. The interaction index, γ , was 1.05, indicating an absence of a supra-additive drug interaction in the presence of the PKC inhibitor. Although pretreatment with PKC inhibitor completely abolished the synergy between CLON and DELT, it did not significantly change the relative potency of the agonists given separately.

In contrast to the PKC inhibitor, pretreatment with U73122 (a PLC inhibitor) (Fig. 3A, dark bars) reversed or reduced the inhibitory effects of high-efficacy doses of both CLON and DELT as well as a synergistic combination dose. This result permits speculation that PLC-mediated vesicle translocation to the plasma membrane contributes to both the inhibitory effects of single agonist administration and their synergistic interaction, whereas PKC is unique to the synergistic interaction. Inhibition of PKA with H89 (Fig. 3B, vertical striped bars) had no effect on CLON administered alone or the synergistic CLON–DELTA combination, indicating that PKA, unlike PKC, is not required for CLON–DELTA synergy. Pretreatment with H89 consistently reduced the inhibitory effect of DELT administered alone (Fig. 3B).

It is known that there is a low basal level of adrenergic tone in the form of norepinephrine release from descending noradrenergic neurons that terminate in the dorsal horn of the spinal cord (Pertovaara, 2006). Therefore, we hypothesized that the observed inhibitory effect of DELT in the tail flick assay was partially mediated through α_2 -adrenergic receptor activation by endogenous NE. PKA has been implicated in modulation of neurotransmitter

release dynamics from nerve terminals (Trudeau et al., 1996); thus, inhibiting PKA with H89 could be interfering with the aforementioned descending noradrenergic tone, thus reducing the inhibitory effect of DELT administration.

To test this hypothesis, the effects of CLON, DELT, and the CLON-DELT combination were challenged with i.t. pretreatment of the α_2 AR antagonist, idazoxan (10 nmol, i.t.) (Fig. 3C, downward diagonal stripes). As expected, i.t. pretreatment with idazoxan completely reversed the inhibitory effect of CLON administered alone. Pretreatment with idazoxan also reduced the inhibitory effect of the CLON-DELT combination. In support of the hypothesized ongoing noradrenergic tone, i.t. pretreatment with idazoxan also reduced the inhibitory effect of DELT, supporting a role for endogenous α_2 AR activation in the effect of DELT administration alone. Activation of α_2 ARs by endogenous NE has previously been reported to be involved in opioid receptor-mediated antinociception, as mice lacking functional α_2 ARs or wild-type mice treated with idazoxan showed decreased morphine potency in inhibiting nocifensive responses to i.t. administration of SP (Stone et al., 1997). We also challenged both agonists administered separately and in combination with the DOP antagonist, naltrindole (8.8 nmol, i.t.) (Fig. 3D, upward diagonal stripes). The inhibitory effects of both DELT alone and the CLON-DELT combination were reduced with DOP antagonist pretreatment. However, i.t. pretreatment with the DOP antagonist had no effect on CLON administered alone. These data suggest that, although the effect of CLON administered alone *in vivo* is mediated solely through α_2 ARs, the effect of i.t. DELT administration is primarily mediated through DOP but partially mediated through α_2 AR activation by endogenous NE.

CLON-DELT: Synergistic inhibition of K^+ -stimulated CGRP release from spinal cord slices

To determine whether the antinociceptive interaction observed *in vivo* was attributable to activation of receptors at the level of primary afferent terminals, we determined the ability of α_2 AR and DOP agonists to inhibit K^+ -stimulated release of the neuropeptide CGRP *in vitro* using the spinal cord slice preparation. Measurement of CGRP release was chosen because, although K^+ stimulation causes depolarization of all cells in the slice preparation, CGRP in the spinal cord is exclusively released by primary afferent terminals (Franco-Cereceda et al., 1987; Plenderleith et al., 1990). Stimulation of spinal cord slices with 60 mM K^+ significantly increased the concentration of immunoreactive CGRP (iCGRP) in the superfusate from 44.7 ± 5.2 pg/ml (basal level) to 221 ± 44.4 pg/ml (data not shown). This increase was inhibited in a concentration-dependent manner by pretreatment with either the α_2 AR agonist CLON (closed circles) or the DOP agonist

Figure 3. Effect of the PLC inhibitor U73122, the PKA inhibitor H89, the α_2 AR antagonist idazoxan, and the DOP antagonist naltrindole on the ability of CLON, DELT, and the CLON-DELT combination to inhibit nociceptive thermal responses in the tail flick test. High-efficacy doses of CLON and DELT as well as the low-dose CLON-DELT combination were tested under control conditions (light bars) and in the presence of U73122 (3 nmol i.t., dark bars), H89 (6 nmol i.t., vertical striped bars), idazoxan (10 nmol i.t., downward diagonal stripes), and naltrindole (8.8 nmol i.t., upward diagonal stripes). **A**, The upstream inhibitor of PLC, U73122, abolished the inhibitory effects of the agonists administered alone ($76.0 \pm 3.9\%$ vs $4.6 \pm 1.6\%$ inhibition for CLON and $78.1 \pm 5.7\%$ vs $25.7 \pm 3.7\%$ inhibition for DELT in the presence of U73122) as well as the synergistic effect of their low-dose combination ($69.6 \pm 3.3\%$ vs $17.0 \pm 7.1\%$ inhibition in the presence of U73122). **B**, Pretreatment with the PKA inhibitor, H89, had no effect on either CLON administered alone ($74.1 \pm 5.1\%$ vs $64.9 \pm 3.0\%$ inhibition in the presence of H89) or the low-dose combination ($82.5 \pm 4.5\%$ vs $72.9 \pm 3.8\%$ inhibition in the presence of H89). Pretreatment with H89 reduced the effect of DELT administered alone ($88.4 \pm 4.2\%$ vs $40.8 \pm 6.2\%$ inhibition for DELT in the presence of H89). **C**, Pretreatment with the α_2 AR antagonist, idazoxan, completely reversed the effect of CLON administered alone ($75.7 \pm 6.3\%$ vs $0.2 \pm 1.5\%$ inhibition in the presence of idazoxan), and reduced the effects of both DELT alone ($87.9 \pm 4.4\%$ vs $37.3 \pm 4.5\%$ inhibition in the presence of idazoxan) and the CLON-DELT combination ($95.4 \pm 2.6\%$ vs $53.0 \pm 4.4\%$ inhibition in the presence of idazoxan). **D**, Pretreatment with the DOP antagonist, naltrindole, had no effect on CLON administered alone ($74.2 \pm 4.5\%$ vs $64.7 \pm 4.4\%$ inhibition in the presence of naltrindole), but reduced the inhibitory effect of DELT administered alone ($85.3 \pm 5.9\%$ vs $22.1 \pm 2.4\%$ inhibition in the presence of naltrindole). Pretreatment with naltrindole also reduced the effect of the CLON-DELT combination ($92.3 \pm 4.0\%$ vs $44.6 \pm 3.7\%$ inhibition in the presence of naltrindole). Error bars represent mean $\pm$ SEM ($n = 6$ animals/dose). * $p < 0.05$; Student's *t* test.

DELT (closed squares) (Fig. 4A). To determine whether a synergistic interaction exists between these receptors in this preparation, slices were superfused with both drugs in combination. The resultant concentration–response curve (Fig. 4A, open squares and open triangles) shows the effect of fixed-ratio combinations of the two agents administered simultaneously. The potency of each drug was increased ~ 30 -fold in the presence of the other, similar to results obtained *in vivo*, suggesting that the interaction is synergistic. The concentration–response data from Figure 4A are represented graphically as an isobologram in Figure 4B, which shows that the EC_{50} of the combination (closed circle) is significantly lower than the theoretical additive EC_{50} (open circle). Statistical comparison (*t* test) between the observed combined EC_{50} value and the theoretical additive EC_{50} value demonstrates that this interaction is synergistic. The interaction index, γ , for the combination was 0.06, indicating a substantial synergistic interaction between α_2 AR and DOP agonists at the level of primary afferent terminals. Pretreatment of spinal cord slices with the nonselective OR antagonist naloxone (1 μ M) or the α_2 AR antagonist idazoxan (1 μ M) abolished the inhibitory action of DELT and CLON, respectively, confirming that the observed effects were OR- and α_2 AR-mediated (data not shown).

Figure 4. Coadministration of DELT and CLON inhibits K^+ -evoked release of CGRP from spinal cord slices in a synergistic manner. **A**, K^+ -evoked release of CGRP was challenged by administration of DELT, CLON and their combination. DELT (filled squares) and CLON (filled circles) inhibited the release of CGRP in a concentration-dependent manner with similar potency and efficacy. Coadministration of DELT and CLON at constant concentration ratio of 1:1 (open squares and open triangles) was ~30-fold more potent than either drug given alone, suggesting that the interaction was synergistic. Error bars represent mean $\pm$ SEM for each concentration point ($n = 3$ –9 samples/concentration). **B**, Isobolographic analysis of the data in Figure 3A. The y-intercept represents the CLON EC_{50} (1.9 nM; 95% CI = 0.5–3.3), and the x-intercept represents the DELT EC_{50} (2.3 nM; 95% CI = 0.5–4.2) when each was administered alone. The heavy line connecting the intercepts is the theoretical additive line and the open circle represents the theoretical additive combined EC_{50} . Coordinates for drug combinations falling below this line and outside the confidence limits indicate a synergistic interaction. Coadministration of CLON and DELT at a 1:1 concentration ratio resulted in an EC_{50} (0.06; 95% CI = 0.01–0.1) of DELT in the presence of CLON that fell well below the additive line, indicating that the interaction was synergistic. Error bars parallel to each axis represent the lower 95% CI for each compound given alone. The error bars on the combined concentration points represent the upper and lower 95% CIs.

Inhibition of PKC completely and selectively reverses CLON-DELT synergistic inhibition of CGRP release from spinal cord slices

To determine whether the reduction of antinociceptive synergy with PKC inhibition observed *in vivo* generalized to the level of primary afferent terminals, we tested whether the PKC inhibitor could similarly affect the synergistic interaction between CLON and DELT in reducing inhibition of K^+ -stimulated release of the neuropeptide CGRP *in vitro* using the spinal cord slice preparation. To determine whether there are differential signaling mechanisms mediating the effects of these agonists administered alone and in the synergistic combination, we challenged the ability of CLON, DELT and the combination to inhibit K^+ -stimulated CGRP release from rat spinal cord slices with selective inhibitors of PLC, PKC and PKA (Fig. 5). High-efficacy concentrations of CLON and DELT were tested under control conditions (light bars) and in the presence of the PKA inhibitor H89 (1 μ M), the PLC inhibitor U73122 (10 μ M), or the PKC inhibitor chelerythrine (2.5 μ M) (vertical striped bars, dark bars, and horizontal striped bars, respectively). Only the PLC inhibitor significantly reversed the inhibitory effects of CLON and DELT when administered alone, consistent with behavioral results. Furthermore, neither inhibition of PKC nor inhibition of PKA with H89 administration reversed the effect of either CLON or DELT administered alone *in vitro*.

When the effect of inhibitors of PLC, PKC and PKA on DELT-CLON synergy were evaluated, inhibition of both PLC and PKC, but not PKA, blocked the synergistic interaction. These data suggest that, although the effect of both agonists administered separately and together requires activation of the PLC pathway, only the synergistic effect of both agonists coadministered relies on activation of PKC. PKC, but not PKA dependence in the spinal cord slice preparation is consistent with the *in vivo* data in Figures 2 and 3.

CLON-DELT synergy is maintained in the presence of tetrodotoxin

The similar results reported in (Riedl et al., 2009) for greater-than-additive inhibition of CGRP release from spinal cord synaptosomes suggests that this interaction takes place in subcellular compartments co-containing the receptors. To determine whether the synergistic interaction between the two agonists requires colocalization within subcellular compartments rather than through interneuronal circuitry, we evaluated whether CLON-DELT synergy in the spinal cord slice preparation is maintained in the presence of the sodium channel blocker, TTX, which inhibits neural transmission. We chose a concentration of 1 μ M TTX based on (1) a literature survey of rat (Murasu and Randic, 1983; Ryu et al., 1988; Yoshimura and Jessell, 1989; Yoshimura and Jessell, 1990) and mouse (Han et al., 2007) studies, and (2) a positive control experiment showing that 1 μ M TTX, but not 0.1 μ M, completely blocked fEPSPs in rat spinal cord slices ($4.9 \pm 5.9\%$ response in the presence of TTX vs baseline) (Fig. 6A). The presence of TTX (1 μ M) in the superfusate throughout the experiment

did not affect the synergistic inhibition of the CLON-DELT combination (0.1 nM, 1:1 concentration ratio) of K^+ -evoked CGRP release from spinal cord slices ($72.7 \pm 10.4\%$ vs $60.9 \pm 8.9\%$ inhibition in the presence of TTX) (Fig. 6B). Together, these results support and extend recent findings in spinal cord synaptosomes (Riedl et al., 2009) that the observed synergy between agonists acting at these two receptors occurs within single subcellular compartments (i.e., the terminals of primary afferent nociceptive fibers in the dorsal horn of the spinal cord).

CLON-DELT combination causes significant CGRP release in the absence of K^+ stimulation

Since DOP agonist-induced CGRP release from cultured DRG neurons has been shown to correlate with functional DOP insertion into the plasma membrane (Bao et al., 2003), we sought to determine whether the low-concentration CLON-DELT combination could also cause CGRP release in the absence of K^+ stimulation (Fig. 7). Spinal cord slices were superfused with CLON, DELT, and the CLON-DELT combination (10 nM, 10 nM and 0.1 nM, respectively) to test for CGRP release in the absence of K^+ depolarization. Stimulation of spinal cord slices with CLON (10 nM) failed to cause significant release of CGRP. In agreement with (Bao et al., 2003), however, stimulation of spinal cord slices with DELT (10 nM) caused significant release of CGRP. Stimulation of spinal cord slices with a 100-fold lower concentration of the CLON-DELT combination also caused significant release of CGRP in the absence of K^+ stimulation. These data suggest that the CLON-DELT combination may act synergistically at DOP and α_2 AR to externalize large dense-core vesicles containing CGRP, and thus may act to insert functional receptors into the membrane in the same manner as DOP agonist alone.

Figure 5. Effect of the PKC inhibitor chelerythrine, the PLC inhibitor U73122 and the PKA inhibitor H89 on the ability of CLON, DELT, and the CLON-DELT combination to inhibit CGRP release from spinal cord slices. (Note: ordinate represents percentage inhibition of release; values near 0 indicate blockade of release inhibition.) High-efficacy concentrations of CLON and DELT, as well as the low-concentration CLON-DELT combination were tested under control conditions (light bars) and in the presence of H89 (1 μ M, vertical striped bar), U73122 (10 μ M, dark bars), or chelerythrine (2.5 μ M, horizontal striped bars). Chelerythrine did not significantly affect the ability of the individual agonists to inhibit CGRP release ($113 \pm 7.6\%$ vs $62.3 \pm 19.3\%$ inhibition for CLON in the presence of chelerythrine and $87.4 \pm 10.4\%$ vs $61.5 \pm 16.2\%$ inhibition for DELT in the presence chelerythrine); however, chelerythrine abolished the synergistic effect of the low-concentration combination ($61.5 \pm 8.2\%$ vs $-15.0 \pm 29.5\%$ inhibition in the presence of chelerythrine). In contrast, U73122 blocked the inhibition of release by either agent alone ($113 \pm 7.6\%$ vs $-3.1 \pm 2.5\%$ inhibition for CLON in the presence of U73122 and $87.4 \pm 10.4\%$ vs $36.7 \pm 21.0\%$ inhibition for DELT in the presence U73122) as well as the synergistic inhibition of release ($66.6 \pm 7.5\%$ vs $-25.3 \pm 25.8\%$ inhibition in the presence of U73122), indicating that PLC activation is required for inhibition of release. H89 did not significantly affect the ability of the individual agonists to inhibit CGRP release ($113 \pm 7.6\%$ vs $77.9 \pm 18.9\%$ inhibition for CLON in the presence of H89 and $87.4 \pm 10.4\%$ vs $68.9 \pm 23.6\%$ inhibition for DELT in the presence H89). Treatment with H89 also had no effect on CLON-DELT synergism ($66.6 \pm 7.5\%$ vs $64.8 \pm 10.0\%$ inhibition in the presence of H89), supporting the specific requirement of PKC activation for synergy. Error bars represent mean $\pm$ SEM ($n = 3-8$ samples/group). * $p < 0.05$; Student's t test.

Figure 6. CLON-DELT combination synergy in the spinal cord slice preparation is maintained in the presence of the sodium channel blocker, TTX. **A**, Positive control for the efficacy of 1 μ M TTX to eliminate interneuronal signaling: TTX completely blocked evoked fEPSPs in rat spinal cord slice preparations [$4.9 \pm 5.9\%$ response in the presence of TTX (dark bar) vs baseline (light bar)]. **A** (Inset), Representative traces of evoked fEPSPs in the presence or absence of TTX (0.1 or 1 μ M). **B**, The inhibitory action of a synergistic CLON-DELT combination was challenged by the addition of TTX (1 μ M) to the superfusate. TTX did not alter the synergistic inhibition of K^+ -evoked (60 mM) CGRP release invoked by the CLON-DELT combination (0.1 nM, 1:1 concentration ratio) ($72.7 \pm 10.4\%$ vs $60.9 \pm 8.9\%$ inhibition in the presence of TTX), supporting that synergy between α_2 ARs and DOPs does not rely on multicellular circuitry. Error bars represent mean $\pm$ SEM ($n = 3-4$ slices or samples/group). * $p < 0.05$ compared with baseline; Student's t test.

Discussion

The results from this study indicate that the synergistic interaction between agonists acting at α_2 ARs and DOPs can occur at the level of primary afferent terminals to inhibit release of nociceptive neuropeptides from spinal cord slices and that this inhibition

Figure 7. Low-concentration CLON-DELT combination causes significant CGRP release in the spinal cord slice preparation in the absence of K^+ stimulation. The ability of CLON, DELT, and the CLON-DELT combination to cause release of CGRP in spinal cord slices was investigated in the absence of K^+ depolarization. CLON did not significantly increase CGRP levels above baseline levels [46.3 ± 5.9 pg/ml (basal level) vs 37.9 ± 8.5 pg/ml (CLON, 10 nM)]. In contrast, both DELT [46.3 ± 5.9 pg/ml (basal level) vs 79.2 ± 14.3 pg/ml (DELT, 10 nM)] and a 100-fold lower concentration of the CLON-DELT combination [46.3 ± 5.9 pg/ml (basal level) vs 128.5 ± 35.9 pg/ml (CLON-DELT, 0.1 nM)] were able to stimulate significant CGRP release without K^+ stimulation, suggesting that the CLON-DELT combination may act synergistically at DOPs and α_2 ARs to externalize large dense-core vesicles. Error bars represent mean $\pm$ SEM ($n = 3-11$ samples/group). * $p < 0.05$ compared with HEPES; Student's t test.

translates to synergistic antinociception *in vivo*. Whereas PLC activation is required for both antinociception and inhibition of neuropeptide release by α_2 AR and DOP agonists given singly or together, PKC activation is specifically required for the synergistic interaction between coadministered agonists. In contrast, PKA is not involved in the effects of α_2 AR and DOP agonists administered separately or in combination, reinforcing the unique ability of PKC to mediate α_2 AR/DOP synergy. That the synergistic interaction observed *in vitro* is maintained in the presence of TTX indicates that α_2 AR/DOP synergy can take place in single subcellular compartments in the absence of multicellular circuitry. These *in vivo* and *in vitro* results confirm and extend a recent report showing that these receptors inhibit neuropeptide release in a greater-than-additive manner from spinal cord synaptosomes (Riedl et al., 2009).

Behavioral and *in vitro* synergy between agonists acting at α_2 AR and DOP

Synergistic interactions between classes of analgesic agonists have been frequently reported in the literature, although the mechanisms underlying this phenomenon remain to be fully defined. It has been suggested, for example, that synergy will be observed between agonists acting at the following receptor pairs: δ -opioid/ α_2 A-adrenergic (Stone et al., 1997), δ -opioid/ α_2 C-adrenergic (Fairbanks et al., 2002), μ -opioid/ α_2 A-adrenergic (Stone et al., 1997) and μ -opioid/ α_2 C-adrenergic (Fairbanks et al., 2000). We and others have previously demonstrated that both α_2 ARs and DOPs are localized on the terminals of capsaicin-sensitive, SP-expressing primary afferent neurons in the dorsal horn of the spinal cord in rat (Dado et al., 1993; Arvidsson et al., 1995; Stone et al., 1998; Zhang et al., 1998) where they are highly colocalized (Riedl et al., 2009). The present study demonstrates that these two receptors colocalize identically in mouse, underscoring the congruence between species. The localization of the α_2 AAR subtype together with previous studies showing that the effect of CLON is eliminated in mice lacking functional α_2 AARs (Stone et al., 1997) suggests that the CLON effect seen in the CLON-DELT

Figure 8. Proposed mechanism of CLON-DELT analgesic synergy localized to primary afferent terminals in the dorsal horn of the spinal cord. **A**, Model of DOP agonist-induced DOP insertion coinciding with neuropeptide release (adapted from Bao et al., 2003). Activation of DOPs through administration of DELT causes activation of PLC (presumably through G_i), thereby increasing intracellular Ca^{2+} concentrations via IP₃ receptors. This spike in Ca^{2+} mediates exocytosis of LDCVs, thus releasing neuropeptides and inserting intracellular “reserve” DOPs to the plasma membrane. **B**, Proposed model of CLON-DELT synergy mediated by PKC through coactivation of α₂ARs and DOPs. Coactivation of α₂ARs and DOPs causes neuropeptide release at a 100-fold lower concentration than DELT administration alone via the same mechanism as **A**. In contrast to DOP agonist-induced DOP insertion, however, coactivation of α₂ARs and DOPs causes activation of PKC through increased levels of DAG. Activation of PKC, in turn, mediates the synergistic interaction of α₂ARs and DOPs. One of several hypotheses for this mechanism is that the phosphorylation target(s) of PKC allow enhanced G_{i/o} coupling of both the α₂AR and DOP (yellow stars). An alternative hypothesis is that activation of PKC favors the formation of α₂AR/DOP heterodimers with an enhanced inhibitory mode of action.

synergistic interaction is mediated through this particular α₂AR subtype. Because α_{2A}ARs and DOPs colocalize in the terminals of primary afferent neurons (Riedl et al., 2009), we sought to use selective agonists for the α₂AR and DOP in both a behavioral model and a more reduced spinal cord slice preparation to determine whether the synergistic interaction between these agonists to colocalized receptors could take place within a single subcellular compartment. This study shows that the selective α₂AR and DOP agonists CLON and DELT are each able to dose-dependently inhibit nociceptive responses when administered i.t. in the tail flick assay and to synergize in producing this antinociceptive effect. These results confirm previous findings using a different α₂AR agonist (brimonidine, also known as UK-14,304) (Stone et al., 1997). Furthermore, this interaction appears to take place within the terminals of primary afferent neurons: CLON and DELT inhibit K⁺-evoked release of CGRP in a greater-than-additive manner from both spinal cord synaptosomes (Riedl et al., 2009) and superfused spinal cord slices (this study). That this synergy is maintained in the presence of the sodium channel blocker, TTX, further indicates that this interaction does not rely on multineuronal circuitry.

Signaling mechanisms mediating α₂AR and DOP synergy in the spinal cord

Because we observed that the analgesic synergy between agonists acting at α₂ARs and DOPs in spinal cord occurs within the terminals of primary afferent neurons, we sought to address the signaling mechanisms involved in this interaction. In small dorsal root ganglion (DRG) neurons, DOPs are known to predominantly localize to the cytoplasm and have been shown through immunofluorescence (Dado et al., 1993; Arvidsson et al., 1995), electron microscopy (Cheng et al., 1995; Zhang et al., 1998) and

biochemical evidence (Wang et al., 2008) to often associate with the membrane of large dense-core vesicles that contain neuropeptides (e.g., CGRP, SP), with only a limited number of DOPs distributed in the plasma membrane. Because, under basal conditions, DOPs are mainly localized to the cytoplasm, it has been suggested that the majority are “reserve” receptors that are then targeted to and inserted in the plasma membrane in response to physiological changes (Zhang et al., 1998; Cahill et al., 2001a,b; Bao et al., 2003; Cahill et al., 2003; Gendron et al., 2006). Activation of DOPs through agonist binding has been shown to trigger a slow but long-lasting exocytosis of large dense core vesicles (Wang et al., 2008), leading to an increase in cell surface area, insertion of functional DOPs, and CGRP release in a PLC- and Ca^{2+} -dependent manner, presumably through activation of G_q (Bao et al., 2003). It is also known that activation of either the DOP (Yoon et al., 1999) or α_{2A}AR (Dorn et al., 1997) can mobilize IP₃-sensitive Ca^{2+} stores through a signal transduction pathway that involves activation of PLC by G_{βγ} subunits released from agonist-induced dissociation of the G_i heterotrimer. That DOPs and α₂ARs share common

signaling pathways through similar G-proteins and can both mobilize intracellular Ca^{2+} through activation of PLC suggests that trafficking mechanisms for α₂ARs in primary afferent terminals are similar to those of DOPs.

Whereas GPCR-mediated activation of PLC and subsequent release of Ca^{2+} from internal stores is often associated with receptors coupling to stimulatory G-proteins (e.g., G_s and G_q), α₂ARs and DOPs preferentially couple through inhibitory G-proteins. However, evidence exists in the literature suggesting that ORs can couple not only to G_i/G_o, but to a variety of G-proteins. For instance, opioids can produce analgesia through activation of PLA₂ and have been shown to act through various G-proteins to activate phospholipase C (PLC), thereby mobilizing Ca^{2+} , activating PKC and enhancing presynaptic voltage-gated, ATP-gated and Ca^{2+} -gated K⁺-channel activity (for review, see Aantaa et al., 1995; Connor and Christie, 1999; Millan, 1999, 2002; Law et al., 2000).

In support of the mechanism of DOP agonist-induced receptor externalization via PLC (Bao et al., 2003), the results from this study show that the effect of α₂AR and DOP agonists administered separately or in combination is blocked by an inhibitor of PLC both behaviorally and *in vitro*. This outcome suggests that agonist-driven externalization via PLC is involved in the analgesic effects of CLON, DELT, and the CLON-DELT combination. Therefore, the possibility exists that each agonist also promotes the externalization of the other receptor, presenting an opportunity for mutual enhancement of receptor number. This mechanism is further supported by the present results showing that administration of the CLON-DELT combination results in significant release of CGRP (thus enabling/signaling externalization of “reserve” receptors) from spinal cord slices in the absence of K⁺ stimulation at a 100-fold lower concentration than is needed to produce release with agonist alone.

In contrast to PLC, however, downstream activation of PKC seems to be uniquely involved in the synergistic interaction between CLON and DELT. We have shown that inhibition of PKC completely blocked the synergistic combination, but did not significantly blunt the action of either agonist alone in either the tail flick test or inhibition of K^+ -evoked CGRP release from spinal cord slices. This involvement of PKC in the synergistic interaction is consistent with the role of PKC in enhancing DOP “competence” (Patwardhan et al., 2005). Furthermore, the failure of PKA inhibition to block the synergistic effect underscores the specificity of PKC’s involvement. These data are also consistent with previous antinociception results indicating that PKC, but not PKA, activity regulates the synergistic interaction between morphine and CLON in inhibiting nocifensive responses to i.t. administration of SP (Wei and Roerig, 1998), suggesting that PKC may mediate multiple opioid and α_2 AR subtype interactions in the spinal cord.

In the proposed model (Fig. 8), we postulate that agonist-induced receptor insertion via PLC is necessary for the spinal analgesic effects of CLON and DELT by allowing “reserve” receptors to be trafficked to the plasma membrane. We further speculate that, when α_2 ARs and DOPs are coactivated in the primary afferent terminal, PKC is activated, presumably through increased levels of diacylglycerol (DAG) downstream of PLC, is translocated to the plasma membrane and functions to mediate/facilitate the synergistic interaction.

One of several possible explanations for the differential signaling following receptor coactivation is the formation of heterodimeric complexes between α_2 ARs and DOPs. The emergence of novel pharmacological properties from heterodimer activation distinct from either component receptor alone has been previously investigated for dopamine receptors (Rashid et al., 2007). In the case of ORs, *in vitro* evidence indicates synergistic binding and coupling potentiation of coactivated κ -ORs and DOPs (Jordan and Devi, 1999) and conformational changes via crosstalk that modulate receptor function between α_2 ARs and μ -ORs (Villardaga et al., 2008). Furthermore, α_2 AR-DOP synergy is lost in mice lacking functional α_{2A} ARs (Stone et al., 1997), whereas it is retained in μ -OR KO mice (Guo et al., 2003), supporting the specific involvement of DOPs in the synergistic interaction. Together these data demonstrate that α_2 AR-DOP synergy is dependent on the presence of both receptors, but, to this point, α_2 AR-DOP heterodimer formation *in vivo* is purely theoretical. An alternative hypothesis is that the phosphorylation target(s) of PKC allow enhanced $G_{i/o}$ coupling of both the α_2 AR and DOP. Evidence for a specific role for PKC is a first step in elucidating the pathways involved in α_2 AR/DOP synergy, but further work is needed to determine the contribution of downstream targets of PKC.

Conclusion

Synergy is important in clinical pain management as much lower doses of each drug can be administered to produce analgesia, thus reducing unwanted side effects and improving treatment outcomes. These results provide strong evidence that synergy between analgesic agonists acting at anatomically colocalized receptor populations in spinal cord can occur, and that this interaction is not dependent on multineuronal circuitry. In the case of α_2 AR and DOP agonist combinations, the synergistic interaction appears to be mediated through the activation of PKC. The phosphorylation target(s) of PKC mediating the enhanced potency remain unknown, as does the mechanism of enhancement. Identifying the molecular basis of spinal analgesic synergy may contribute to

improved therapeutic strategies to control chronic pain and understand mechanisms of chronic pain induction.

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