Regulation of Spinal Dynorphin 1-17 Release by Endogenous Pituitary Adenylyl Cyclase-Activating Polypeptide in the Male Rat: Relevance of Excitation via Disinhibition

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ABSTRACT

Opioids inhibit release of primary afferent transmitters but it is unclear whether the converse occurs. To test the hypothesis that primary afferent transmitters influence opioid-ergic tone, we studied the functional and anatomical relationships between pituitary adenylyl cyclase-activating polypeptide (PACAP) and dynorphin 1-17 (Dyn) in spinal cord. We found that activation of the PACAP-specific receptor PAC₁ (PAC₁R) inhibited, whereas PAC₁R blockade augmented, spinal release of Dyn. It is noteworthy that in the formalin-induced pain model PAC₁R blockade (via PACAP6-38) also resulted in antinociception that was abolished by spinal κ -opioid receptor blockade. These findings indicate that Dyn release is tonically inhibited by PACAP and that blocking this inhibition, which increases the spinal release of Dyn, results in antinociception. Consistent with this conclu-

sion, we found in the spinal dorsal horn that Dyn-immunoreactive neurons 1) expressed PAC₁R and 2) were apposed by PACAP terminals. Present results, in combination with the previous demonstration that the release of spinal Dyn is tonically inhibited by opioid- and nociceptin/orphanin FQ-coupled pathways (*J Pharmacol Exp Ther* **298**:1213–1220, 2001), indicate that spinal Dyn-ergic neurons integrate multiple inhibitory inputs, the interruption of any one of which (i.e., disinhibition) is sufficient to enhance spinal Dyn release and generate antinociception. Gaining a better understanding of the role of primary afferent neurotransmitters in negatively modulating the spinal release of Dyn and the physiological use of disinhibition to increase spinal Dyn activity could suggest novel clinically useful approaches for harnessing endogenous Dyn for pain control.

Introduction

The magnitude of spinal opioid activity can determine primary afferent input to the spinal cord. For example, it is well established that morphine acts presynaptically to inhibit substance P release from primary afferent terminals (Murakami et al., 1995; Dickinson and Fleetwood-Walker, 1999). However, the extent to which primary afferents influence endogenous spinal opioid activity remains largely unknown. This could be an important consideration because bidirectional communication between endogenous opioids and pri-

mary afferents could represent a mechanism for fine-tuning the balance between nociception and antinociception.

It is noteworthy that both the endogenous opioid dynorphin 1-17 (Dyn) and some primary afferent neurotransmitters produce both nociception and antinociception. For example, whereas high doses of the neuropeptide substance P produce nociception, low doses of substance P administered to the intrathecal space act synergistically with spinal morphine to produce antinociception (Kream et al., 1993). The actions of Dyn are equally complex (see Lai et al., 2001 for review). For instance, Dyn dampens the nociceptive input that occurs after nerve injury (Xu et al., 2004), attenuates formalin-induced flinching behaviors (Ossipov et al., 1996), and elevates flinch/jump thresholds (Dawson-Basoa and Gintzler, 1996). It is noteworthy that spinal Dyn and the κ -opioid receptor (KOR) are essential for the antinociception associated with gestation and its hormonal simulation (Medina et al., 1993a,b, 1995; Dawson-Basoa and Gintzler, 1996,

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ABBREVIATIONS: PACAP, pituitary adenylyl cyclase-activating polypeptide; PAC₁R, PACAP-specific receptor PAC₁; Dyn, dynorphin 1-17; KOR, κ -opioid receptor; nor-BNI, nor-binaltorphimine; ir, immunoreactivity; CGRP, calcitonin-gene-related polypeptide; VIP, vasoactive intestinal polypeptide; ANOVA, analysis of variance; TBS, 135 mM NaCl, 25 mM Tris-HCl, 3 mM KCl, pH 7.4.

1998). These antinociceptive actions notwithstanding, intrathecally administered Dyn has also been reported to enhance sensitivity to sensory stimuli, potentiate excitatory neurotransmitter release (Long et al., 1988), and produce long-lasting hyper-responsiveness to innocuous mechanical and noxious thermal stimuli (Vanderah et al., 1996; Laughlin et al., 1997). Thus the actions of primary afferent transmitters and Dyn in the spinal cord are complex; their interactions would be expected to be equally complex and could be critical to determining the balance between nociception and antinociception.

In the present study, we investigated the relationship between Dyn and pituitary adenylyl cyclase-activating polypeptide (PACAP). Like substance P, PACAP is a primary afferent neurotransmitter (Moller et al., 1993) that has both pronociceptive and antinociceptive actions. PACAP sensitizes dorsal horn neurons to formalin and *N*-methyl-D-aspartate receptor-mediated nociceptive responses (Dickinson et al., 1997) and modulates nociceptive behaviors associated with inflammatory and neuropathic pain states (Davis-Taber et al., 2008). It is noteworthy that the intrathecal application of PACAP not only results in nociceptive behaviors but also in antinociceptive effects (Shimizu et al., 2004), analogous to substance P. We selected PACAP for this study not only because of its relevance to nociception but also because its spinal cord levels have been reported to increase in parallel with Dyn (Chery-Croze et al., 1986; Medina et al., 1993b; Papka et al., 2006), which could indicate functional interactions between them. Specifically, we were interested in investigating the capacity of PACAP to modulate spinal Dyn release and thereby affect nociception.

The present article demonstrates the functional interconnectivity among spinal PACAP, the PACAP-specific receptor PAC₁ (PAC₁R), and Dyn. We also demonstrate anatomical relationships among PACAP, PAC₁R, and Dyn that could mediate their demonstrated functional interactions. Our findings indicate that disinhibition of spinal Dyn release by PAC₁R blockade could underlie the ability of PACAP6-38 to elicit antinociception. In addition, results underscore the importance of excitation via disinhibition to the modulation of spinal Dyn-ergic tone.

Materials and Methods

Experimental Animals. Experiments used male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY; 150–300 g), which were maintained in an approved controlled environment on a 12-h light/dark cycle. Food and water were available ad libitum. All experimental procedures were reviewed and approved by the Animal Care and Use Committees of the State University of New York Downstate Medical Center and University of Minnesota.

In Vivo Perfusion of Spinal Intrathecal Space. The intrathecal space was perfused by using the push-pull method. Two PE-10 catheters (8.5-cm inflow and 7.0-cm outflow) were introduced into the spinal subarachnoid space via the atlanto-occipital membrane under sodium pentobarbital anesthesia (50 mg/kg i.p.). The longer of the two cannulae was inserted to the middle of the lumbar enlargement, and the shorter one was inserted on the caudal portion of the thoracic spinal cord. The intrathecal space was perfused with Krebs-Ringer buffer (138 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 11 mM NaHCO₃, 1 mM NaH₂PO₄, and 11 mM glucose) prewarmed to 37°C. The outflow tubing was placed on ice to cool the perfusate. The spinal cord was equilibrated with the perfusion medium for 20 min; this medium was discarded. Thereafter, four 10-min perfusate sam-

ples were collected from each animal for quantification of Dyn release: two before (for basal release) and two subsequent to the intrathecal application of either PACAP, PACAP6-38, or a combination. After the initial 20-min equilibration period, the basal rate of spinal Dyn release did not significantly vary over the ensuing 70-min period of intrathecal perfusion. Therefore, release of Dyn in the presence of a treatment was compared with the basal release immediately preceding it (Fig. 1).

Dyn Competitive Peptide Enzyme Immunoassay. The content of Dyn in intrathecal perfusate was quantified by using a competitive enzyme immunoassay performed as instructed in the kit (Peninsula Laboratories, Belmont, CA). The anti-Dyn antibody used is highly selective for Dyn 1-17; it does not recognize either Dyn 1-13, Dyn 1-8, α -NeoEndorphin, β -endorphin, Dyn B, or Leu-enkephalin (see Gintzler et al., 2008 for further characterization). A standard curve (0.5–32 pg/assay well) in which the percentage of inhibition of binding was plotted against the log concentration of unlabeled Dyn in the reaction well was generated in each assay. Biotinylated-Dyn (6 pg/well; Peninsula Laboratories) was used as tracer. Plates were counted with an Envision 2102 Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). Values of experimental samples were calculated from the standard curve using the forecast function of Excel (Microsoft, Redmond, WA). All standard and experimental samples were run in duplicate.

Tissue Preparation for Immunohistochemistry. Rats were deeply anesthetized with a mixture of ketamine (68 mg/kg), xylazine (4.6 mg/kg), and acepromazine (0.9 mg/kg). Rats were perfused through the ascending aorta with 100 ml of ice-cold oxygenated calcium-free Tyrode's buffer (115 mM NaCl, 5 mM KCl, 2 mM MgCl₂ · 6H₂O, 400 μ M MgSO₄ · H₂O, 3 mM glucose, 25 mM NaHCO₃, pH 7.2) followed by 500 ml of buffered formaldehyde freshly prepared from paraformaldehyde (4% w/v formaldehyde, 75 mM KH₂PO₄, 85 mM Na₂HPO₄ · 7H₂O, pH 6.9). After fixation, spinal cord segments were identified and placed in 5% buffered sucrose overnight at 4°C. Spinal segments were mounted for sectioning in Tissue-Tek OCT mounting medium (VWR, West Chester, PA). Tissue was quickly frozen, and sections were cut at a nominal thickness of 10 μ m on a cryostat (Bright Instruments, Huntington, UK), thawed onto Fisherfinest Capillary-Gap slides (Thermo Fisher Scientific, Waltham, MA) and stored at –20°C until used. Sections were rinsed in TBS (135 mM NaCl, 25 mM Tris-HCl, 3 mM KCl, pH 7.4) and then permeabilized in TBS containing 0.2% Triton X-100 and 0.2% Tween 20. The sections were then incubated overnight at room temperature with a 1:30,000 dilution in TBS containing 0.2% Tween 20 of guinea pig anti-preprodynorphin 235-248 (Preprodynorphin 235-248 is a cryptic portion of the DYN precursor that serves as a marker peptide for Dyn-ergic neurons) (Arvidsson et al., 1995). Other antibodies added to the anti-preprodynorphin solution for coincubation included mouse anti-PACAP (Mab-JHH1; gift of Jens Hannibal, University of Copenhagen, Copenhagen, Denmark) and rabbit anti-PAC₁R (Schulz et al., 2004). To label plasma membranes, we used mouse anti-Na⁺-K⁺ ATPase, (α 5, raised against the α subunit of the chicken Na⁺-K⁺ ATPase, raised by D. M. Fambrough and obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City, IA). To label peptidergic primary afferent fibers, we used goat anti-calcitonin-gene related polypeptide (CGRP), gift of Dr. Hunter Heath (Mayo Clinic, Rochester, MN). The sections were washed for 2 h at room temperature with three changes of TBS and coincubated with 3 μ g/ml donkey anti-guinea pig IgG conjugated to Cy2, 3 μ g/ml donkey anti-mouse IgG conjugated to Cy5, and 3 μ g/ml donkey anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in TBS-Tween diluent. The sections were washed in three changes of TBS, rinsed in H₂O, dehydrated in increasing concentrations of ethanol (50–100%), and cleared in xylene. The slides were mounted with cover slips using DPX mountant (Fluka Chemical Corp., Ronkonkoma, NY).

Specificity of Antisera. The specificity of the PACAP and PAC₁R antisera used in immunohistochemistry studies using absorption controls was tested by adding 10 μ g of the peptide against which the antiserum was raised to 1 ml of the diluted antiserum. In both cases, the resulting labeling was substantially reduced or abolished. Specificity of the antibody used to visualize the anti- α subunit of Na⁺-K⁺ ATPase (used as a marker of the plasma membrane) was examined by Western analysis using polyacrylamide gel electrophoresis (4–12% polyacrylamide, NuPAGE; Invitrogen, Carlsbad, CA) of rat spinal cord membrane (5 μ g/lane). A single band of ~97,000 kDa was detected (predicted molecular mass ~112 kDa), consistent with previous studies (Peterson and Hokin, 1981).

Imaging. Images were collected by using either a conventional fluorescence microscope (Olympus BX-50; Olympus, Tokyo, Japan) with a Scion CCD camera or a confocal microscope (Olympus Fluoview 1000) equipped with laser lines at 488, 543, and/or 633 nm. High-magnification confocal images were collected with a 60 $\times$, 1.4 numerical aperture, oil immersion objective. All images are of coronal sections. Images were collected with 12-bit intensity depth and were converted to 8-bit grayscale images with ImageJ (<http://rsb.info.nih.gov/ij/>). To avoid automatic rescaling, we used the brightness/contrast "set" command to specify an intensity range of 0 to 4095. Digital images were adjusted for publication (e.g., sharpened, resized, merged, and adjusted for brightness and/or contrast) with ImageJ or Photoshop (Adobe, San Jose, CA). Control and experimental images were manipulated identically.

Quantification of PAC₁R and Dyn Coexpression. We counted the number of Dyn-immunoreactive (ir) neuronal profiles that were double-labeled for PAC₁R-ir in an arbitrary sample of Dyn-ir neurons taken from three male rats. To be counted as PAC₁R-positive, a Dyn-ir neuron had to have at least three PAC₁R-ir puncta within 3 μ m of each other and within the area of the Dyn-ir labeling. Labeling was typically examined on a single optical section. However, if a cell seemed not to be double-labeled, we confirmed that assessment by examining adjacent optical sections.

Assessment of Nociceptive Responsiveness and Intrathecal Administration of Drugs. After a 30-min acclimation period to individual observation cages, 50 μ l of a 5% formalin solution was injected subcutaneously into the plantar surface of the right hind paw, and the rats were then returned to the clear observation cages. Rats were observed for periods of time corresponding to phase 1 (0–10 min) and phase 2 (30–50 min) of the formalin test. Nociceptive events, defined as lifting the injected paw, were recorded during the initial 3 min of phase 1 and a representative 10-min period of phase 2 (30–35 and 40–45 min after intraplantar administration).

All drugs were administered in 5 to 10 μ l over a 60-s period to the subarachnoid space of the lumbar spinal cord via a permanent indwelling intrathecal cannula. Experiments commenced 5 days after surgery. Complete delivery was insured by flushing the cannula with an additional 10 μ l of saline. The competitive PAC₁R antagonist PACAP 6-38 (Murakami et al., 1995; Dickinson and Fleetwood-Walker, 1999) was administered 10 min (12 nmol based on previous reports) (Dickinson et al., 1997; Davis-Taber et al., 2008) before formalin injection. The κ -opioid antagonist nor-binaltorphimine (nor-BNI) was administered 18 to 24 h before formalin, because

reported interactions between nor-BNI and spinal μ -opioid receptors (Guirmand et al., 1994) are no longer observed 18 to 24 h after intrathecal nor-BNI application (Dawson-Basoa and Gintzler, 1996). Intraplantar formalin-induced nociceptive behavior was compared among groups [intrathecal saline (control), intrathecal PACAP6-38, intrathecal nor-BNI, intrathecal PACAP6-38 plus nor-BNI]. No animal was used to determine responsiveness to more than one concentration of a single drug.

Data Analysis. A two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test was used to analyze the effects on Dyn release of treatment, time, and the interactions between treatment and time. One-way ANOVA was used to analyze nociceptive responsiveness and Dyn release within or between groups.

Results

Modulation of Spinal Dyn Release by PACAP and PAC₁R Antagonism. The basal rate of spinal Dyn release (i.e., release before drug treatment) did not differ among groups that received PACAP (7.94 $\pm$ 0.8 fmol/10 min; n = 8), PACAP6-38 (8.47 $\pm$ 0.68 fmol/10 min; n = 12), or a combination (7.22 $\pm$ 0.88 fmol/10 min; n = 8). Based on the dose-effect relationship for PACAP to inhibit spinal Dyn release (Fig. 1A), 1 nmol was selected for further study. One-way (Fig. 1) ANOVA within-group analysis revealed a significant treatment effect for both PACAP (1 nmol; $F_{2,14}$ = 11.15; p = 0.0013) and PACAP6-38 (10 nmol; $F_{2,10}$ = 5.09; p = 0.029; Fig. 1B). For 10 to 20 min and 30 to 40 min after the spinal administration of PACAP, Dyn release was reduced to 6.55 $\pm$ 0.59 and 6.46 $\pm$ 1 fmol, respectively (p < 0.01 for both time points; n = 8), a reduction of approximately 26%. Strikingly, PACAP6-38, a highly selective PAC₁R antagonist, significantly increased (~38%) spinal Dyn release 30 to 40 min after its intrathecal application (p < 0.05; n = 12). This suggested that spinal Dyn release is under substantial tonic inhibition by spinal PAC₁R. We tested this hypothesis by examining the effect on Dyn release of vasoactive intestinal polypeptide (VIP), which is active at the VPAC₁ and VPAC₂ receptors but not at the PAC₁R (Harmar et al., 1998). Intrathecal VIP (1 or 10 nmol) did not inhibit spinal Dyn release (n = 3 for 1 nmol dose; n = 7 for 10 nmol). Moreover, the combined intrathecal application of PACAP and PACAP6-38 resulted in the enhancement of spinal Dyn release that was indistinguishable from that produced by PACAP6-38 alone, indicating that PAC₁R mediates the PACAP-induced inhibition of Dyn release.

PACAP Immunoreactivity in the Dorsal Horn. Consistent with earlier reports (Papka et al., 2006), PACAP-ir varicosities were observed throughout the dorsal horn, being

Fig. 1. PAC₁R regulates Dyn release from the spinal cord. A, PACAP dose-dependently inhibits spinal Dyn release. B, activation and blockade of spinal PAC₁R inhibits and stimulates, respectively, spinal Dyn release. The spinal intrathecal space was perfused at 5 μ l/min using the push-pull method as described (Materials and Methods). Each spinal perfusate sample was collected over a 10-min period, and the content of Dyn was quantified by using a competitive peptide enzyme immunoassay. Drugs were administered via the inflow spinal catheter. Data are expressed as femtomoles of Dyn per 10-min period. PACAP produced a significant inhibition of Dyn release that was eliminated by the PAC₁R blocker PACAP6-38. PACAP6-38, in the absence of an exogenous PAC₁R agonist, substantially increased Dyn release, indicating its tonic inhibition by endogenous PACAP.

Fig. 2. PACAP-ir occurs in primary afferents and apposes Dyn-ir neurons. A and B, low-magnification images of PACAP-ir (A) and its absorption control (B). PACAP-ir was dramatically reduced by addition of PACAP peptide to the primary antibody solution. C–F, PACAP-ir frequently apposes Dyn-ir somata and is expressed in CGRP-ir varicosities. C, Dyn-ir somata (asterisks). D, PACAP-ir varicosities. E, CGRP-ir varicosities. F, Merged image showing the relationship between the different substances. Arrows mark PACAP-ir varicosities in which CGRP is expressed and that are apposed to Dyn-ir somata. Arrowheads indicate other PACAP-ir varicosities that do not appose Dyn-ir somata but express CGRP-ir. Circles indicate PACAP-ir terminals that do not express CGRP-ir. To allow more labeling to be visible, the images are projections of three optical sections taken at 0.3- μ m intervals. However, the same pattern of double-labeling and apposition was observed in each of the optical sections. Expression of CGRP-ir by PACAP-ir varicosities indicates that they are primary afferents. Bar in B applies only to A and B; bar in C applies to C–F.

most common in substantia gelatinosa and the marginal zone (Fig. 2A). PACAP-ir was frequently found double-labeled by CGRP-ir, indicating that PACAP-ir is expressed in primary afferent fibers (Fig. 2, C–F). These fibers often apposed Dyn-ir cell somata, suggesting that they interacted with a receptor on Dyn neurons.

Expression of PAC₁R on Dyn Somata. PAC₁R, which is highly selective for PACAP (Harmar et al., 1998), was also expressed most strongly in the superficial dorsal horn (Fig. 3, A and B). Within the superficial dorsal horn, the distribution of PACAP overlapped with that of PAC₁R (compare Figs. 2A and 3A). PAC₁R-ir frequently surrounded the perimeters of cells including Dyn-ir neurons (Fig. 3C). Moreover, PAC₁R-ir lay alongside immunoreactivity for the plasma membrane marker Na⁺-K⁺ ATPase (Fig. 3, D–G), consistent with PAC₁R being expressed in the cell membrane. PAC₁R-ir was expressed by most, if not all, Dyn-ir neurons examined. In a sample taken from three rats (L6 segment, one section per rat; 16–19 Dyn-ir profiles evaluated from each rat), 51 of 53 Dyn-ir neuronal profiles were double-labeled for PAC₁R-ir (96%). PACAP-ir varicosities frequently apposed Dyn-ir neurons (Fig. 2, C–F), including PAC₁R-expressing Dyn-ir neurons (Fig. 4, A–D), consistent with the direct modulation of Dyn-ergic activity by PACAP.

Antinociception Produced by Intrathecal PACAP6-38 Is Mediated via Spinal KOR. The intrathecal application of PACAP6-38 has been reported to attenuate nociceptive responsiveness in a variety of assays (Dickinson et al., 1997; Davis-Taber et al., 2008). Based on the observed apposition of PACAP terminals onto Dyn somata and the ability of intrathecal PACAP6-38 to enhance release of Dyn from the spinal cord, we hypothesized that spinal Dyn, at least in part, mediates analgesic effects of intrathecal PACAP6-38. We tested

this hypothesis by determining the effect of spinal KOR blockade on the spinal antinociception produced by PACAP6-38. Consistent with a previous report (Davis-Taber et al., 2008), intrathecal PACAP6-38 (12 nmol) reduced by 41 and 44% during phase 1 and phase 2, respectively, the number of paw lifts elicited by the intraplantar injection of formalin (50 μ l, 5%) (Fig. 5). Strikingly, the antinociception produced by intrathecal PACAP6-38 was abolished by KOR blockade (one-way ANOVA; $F_{2,6} = 11.84$; $p < 0.01$), which by itself did not alter nociceptive responsiveness to intraplantar formalin (data not shown).

Discussion

PACAP is present in primary afferent fibers projecting to the dorsal horn (Moller et al., 1993) and is a transmitter in nociceptive C-fibers (Moller et al., 1993; Xu and Wiesenfeld-Hallin, 1996; Dickinson and Fleetwood-Walker, 1999). The pronociceptive actions of PACAP are most likely mediated via PAC₁R based on the ability of the PAC₁R-selective antagonist PACAP6-38 to decrease nociception (Ohsawa et al., 2002; Davis-Taber et al., 2008). Nevertheless, the functional and spatial relationships of the spinal PACAP/PAC₁R system to endogenous mediators of spinal opioid antinociception and the mediators of the antinociceptive actions of spinal PAC₁R blockade remain largely unknown.

Modulation of Spinal Dyn Release by PACAP/PAC₁R. Individually both PACAP and PACAP6-38 (a PAC₁R antagonist) had profound influences on the rate of spinal Dyn release albeit in opposite directions: PACAP inhibited Dyn release, whereas PACAP6-38 enhanced it. The ability of intrathecal PACAP to inhibit spinal Dyn release suggests that Dyn release is inhibited by primary afferents containing

Fig. 3. PAC₁R-ir occurs in or near the cell membranes of Dyn-ir dorsal horn neurons. A and B, low-magnification images of PAC₁R-ir (A) and its corresponding absorption control (B). PAC₁R-ir was abolished by the addition of immunizing peptide to the primary antibody solution. C, PAC₁R-ir (green; arrows) was found around the perimeters of Dyn-ir neuronal somata (red) in the spinal dorsal horn. D–G, PAC₁R-ir was found surrounding Dyn-ir neurons adjacent to the plasma membrane marker Na⁺-K⁺ ATPase. A single optical section stained for Dyn (blue), PAC₁R (red), and Na⁺-K⁺ ATPase (green). D, Na⁺-K⁺ ATPase-ir. E, PAC₁R-ir. F, Dyn-ir. G, merged image showing the relationships between the Dyn, Na⁺-K⁺ ATPase, and PAC₁R. A Dyn-ir neuron (asterisk) is surrounded by PAC₁R-ir (arrowheads) and Na⁺-K⁺ ATPase (arrows). PAC₁R-ir and Na⁺-K⁺ ATPase are found in similar distributions around the perimeter of the Dyn-ir soma, suggesting that both occur in the plasma membrane. Scale bar in A applies to A and B; scale bar in C applies only to C; scale bar in G applies to D to G.

PACAP. This inference was substantiated by demonstrating that blockade of spinal PAC₁R was sufficient to significantly enhance spinal Dyn release, indicating that Dyn release is tonically inhibited by endogenous PACAP. The presence of tonic PACAP-mediated inhibition of Dyn-release could explain the relatively modest magnitude of inhibition observed after the intrathecal application of PACAP.

Multiple observations indicate the importance of PAC₁R to PACAP regulation of spinal Dyn release: 1) PACAP6-38, which is 100- and 10-fold more selective for PAC₁R than for VPAC₁ and VPAC₂ receptors, respectively, augmented the release of spinal Dyn. 2) VIP, which activates VPAC₁ and VPAC₂ receptors (Shivers et al., 1991) but not PAC₁R (Harmar et al., 1998) did not inhibit spinal Dyn release (data not shown). 3) The concomitant intrathecal application of PACAP6-38 and PACAP completely abolishes the ability of PACAP to inhibit Dyn release. In the aggregate, these data indicate that PAC₁R is the receptor that mediates the inhibition of spinal Dyn release by endogenous PACAP.

Relationship of Dyn to PACAP and PAC₁R in Spinal Cord. The present study also found that CGRP-ir-expressing

spinal PACAP-ir terminals apposed Dyn-ir somata in the superficial dorsal horn. This indicates that PACAP-containing primary afferent terminals synapse onto Dyn neurons. In addition, as would be expected of a membrane receptor, PAC₁R-ir was found along side Na⁺-K⁺ ATPase-ir and was frequently expressed in a circumferential pattern around Dyn-ir somata, suggesting that PAC₁R is located in the plasma membrane. The presence of PAC₁R in Dyn-ergic somatic membranes, which were frequently apposed by PACAP-ir varicosities, suggests that spinal Dyn-ergic neurons can be directly modulated by PACAP *in vivo*. This, in combination with the ability of the PAC₁R antagonist PACAP6-38 to stimulate the release of spinal Dyn, strongly suggests that *in vivo* spinal Dyn is subject to ongoing direct negative modulation by PACAP.

The precise mechanisms through which PACAP directly inhibits spinal Dyn release remain to be established. PAC₁R is coupled to at least two different signal transduction pathways, stimulation of adenylyl cyclase and cAMP production via G_s (Ohtaki et al., 1998) and stimulation of phospholipase C and inositol trisphosphate production (Rawlings et al.,

Fig. 4. PACAP terminals (red) appose Dyn neurons (blue) expressing PAC₁R-ir (green). A–D, images of a single optical section in the superficial dorsal horn. A, Dyn-ir cell soma (asterisk). B, PAC1R-ir in the same microscopic field as A. PAC1R-ir surrounds the Dyn-ir cell (arrows). C, PACAP-ir in the same field as A and B. A PACAP-ir terminal is apposed to the Dyn-ir cell soma seen in A (arrow-head). D, a merged image showing the relationships of Dyn-ir, PAC₁R-ir, and PACAP-ir to each other. PAC₁R-ir is found adjacent to the PACAP-ir apposition as well as on other sites around the cell. E, lower-magnification image of Dyn-ir in the superficial dorsal horn. The cell imaged in A–D is outlined by the box. Bar in D applies to A–D; bar in E applies only to E.

1994), presumably G_q -mediated (Van Rampelbergh et al., 1997). Although activation of both signal transduction pathways has been shown to mediate excitation (Rawlings et al., 1994), it should be noted that G_s activation (via PAC₁R) can inhibit calcium currents via the generation of $G_{\beta\gamma}$ (Kamaishi et al., 2004), and phospholipase C activation can result in presynaptic inhibition of transmitter release (Edelbauer et al., 2005). Thus, activation of PAC₁R-coupled signaling could directly mediate inhibition of Dyn release in addition to its known excitatory actions.

Spinal KOR Mediates the Antinociception Produced by Spinal PAC₁R Blockade. To determine the relevance of the spinal Dyn/KOR pathway to the previously reported PACAP6-38-induced antinociception, we determined the ability of spinal KOR blockade to abolish it. Intrathecal nor-BNI eliminated the antinociceptive effects of intrathecal PACAP6-38. This strongly suggests that KOR activation, presumably via spinal Dyn release, underlies the antinociception that results from spinal PAC₁R antagonism.

The well characterized pronociceptive actions of Dyn notwithstanding (see Introduction), there are numerous reports of antinociceptive actions of Dyn that are manifest under acute and chronic pain conditions (Dawson-Basoa and Gint-

zler, 1996; Ossipov et al., 1996; Xu et al., 2004). Dyn-mediated antinociception is physiological as well as pharmacological: the antinociception associated with gestation and its hormonal stimulation depends on the release of spinal Dyn and the activation of spinal KOR (Dawson-Basoa and Gintzler, 1998). Thus Dyn-mediated antinociception is not a spurious action but an important component of its pleiotropic character.

Bidirectional Interactions between Primary Afferent Transmitters and Spinal Opioid-ergic Neurons. A major mechanism underlying antinociception produced by opioids is their ability to inhibit the release of primary afferent transmitters (Jessell and Iversen, 1977; Yaksh et al., 1980). This notwithstanding, our findings indicate that interactions between primary afferent transmitters and spinal Dyn-ergic neurons are not unidirectional. The ability of intrathecal PACAP to inhibit spinal Dyn release demonstrates the capacity of a primary afferent transmitter to negatively modulate the release of an endogenous opioid, which could contribute to the nociception it produces. This mechanism would operate in parallel with the well established ability of primary afferent transmitters to directly excite nociceptive relay neurons.

Fig. 5. Spinal KOR mediates the analgesic effects of blocking spinal PAC₁R. Nociceptive behavior elicited by intraplantar formalin (50 μ l, 5%) was quantified and compared among control rats (treated with intraplantar formalin and intrathecal saline) and rats that had received intraplantar formalin and either intrathecal PACAP6-38 (12 nmol) or intrathecal PACAP6-38 plus nor-BNI (26 nmol). nor-BNI (or its vehicle) and PACAP6-38 (or its vehicle) were administered 18 h and 10 min, respectively, before formalin administration. Data are expressed as mean $\pm$ S.E.M. Intrathecal treatment with nor-BNI abolished the antinociception produced by intrathecal PACAP6-38, indicating its mediation by spinal KOR. $n = 10, 5$, and 5 , respectively, for control rats, rats receiving PACAP6-38, and rats receiving PACAP6-38 after an overnight treatment with intrathecal nor-BNI.

Possible Relevance of Disinhibition to the Activation of Spinal Dyn-ergic Activity. We previously demonstrated that the release of spinal Dyn is subject to tonic inhibition by spinal δ -opioid- and nociceptin/orphanin FQ receptor-coupled pathways (Gupta et al., 2001). Moreover, the loss of this inhibition, e.g., during the hormonal simulation of pregnancy, results in augmented spinal Dyn release (Gupta et al., 2001) and spinal antinociception. These observations in combination with the present results underscore that 1) spinal Dyn-ergic neurons are a point of integration of multiple inhibitory inputs, many of which, (e.g., PACAP and nociceptin) carry nociceptive information and 2) the interruption of those inputs is sufficient to enhance spinal Dyn release and generate antinociception.

The prominence of tonic inhibition of spinal Dyn neurons suggests that disinhibition is likely to be a key mechanism for increasing spinal Dyn-ergic tone. There seem to be several advantages of disinhibition over direct excitation. First, utilization of disinhibition to enhance Dyn-ergic activity is less likely to interfere with long-lasting changes in excitatory synaptic activity (e.g., long-term potentiation) than would the utilization of (for instance, glutamatergic) excitatory inputs to drive Dyn-ergic neurons. Second, the maximum increase in Dyn release resulting from disinhibition is self-limited, being determined by the degree to which the neuron had been inhibited. A third possible advantage is that the default state would be antinociceptive: if a directly excitatory input were damaged, the result would be lowered Dyn release (nociception). In contrast, damage to the inhibitory inputs through which disinhibition occurs would result in increased Dyn release (antinociception). Finally, the consequences of disinhibition can be more precisely regulated than those of direct excitation because they depend on whether the

inhibition is primarily somatic or dendritic. Dendritic disinhibition would allow very subtle, discretionary, and broadly distributed excitation by increasing the efficiency of information transmission from the dendrites to the soma. In contrast, disinhibition of the soma would increase the membrane voltage time constant, which could result in greater activity by maximizing the temporal summation of excitatory inputs. This study has established PACAP appositions onto the somata of spinal Dyn-ergic neurons. However, given the sparse dendritic labeling of Dyn neurons, we cannot eliminate the possibility that PACAP terminals also appose Dyn dendrites. Consequently, disinhibition of Dyn-ergic neurons could enable not only a greater augmentation of Dyn-ergic activity but, if PACAP/Dyn dendritic appositions are present, could also allow a more selective and defined control of Dyn release than would be the case for direct excitation.

Implications of the Active Suppression of Spinal Dyn-ergic Activity. The demonstrated activation of spinal Dyn-ergic neurons by disinhibition suggests that they function as a high-gain antinociceptive system that is kept in reserve. Given numerous reports of the inability of opioid receptor blockade to lower nociceptive response thresholds and induce nociception (El-Sobky et al., 1976; Grevert and Goldstein, 1978; Robertson et al., 2008; Younger et al., 2009), it is widely accepted that endogenous opioid-ergic neurons are normally quiescent. The current study provides an expanded understanding of basal opioid-ergic activity. The ability of intrathecal PACAP6-38 as well as naloxone (Gupta et al., 2001) to significantly enhance the release of spinal Dyn indicates that Dyn release is actively suppressed. Thus the lack of opioid-ergic tone invariably found in the absence of perturbing stimuli (Gintzler, 1980) can result from active inhibitory processes and intrinsically low firing rates. The active suppression of Dyn release suggests that spinal Dyn-ergic neurons are not engaged under normal circumstances but instead are held in abeyance, awaiting physiological demand. A better understanding of the ability of primary afferent transmitters to inhibit spinal Dyn-ergic tone and the use of disinhibition to increase it could point the way to developing new approaches for harnessing endogenous Dyn for the clinical management of pain.

Authorship Contributions

Participated in research design: Liu, Wessendorf, and Gintzler.
Conducted experiments: Liu, Schnell, and Wessendorf.
Contributed new reagents or analytic tools: Schulz.
Performed data analysis: Liu, Wessendorf, and Gintzler.
Wrote or contributed to the writing of the manuscript: Liu, Schnell, Wessendorf, and Gintzler.
Other: Gintzler acquired funding for the research.

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