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Sodium Nitroprusside Evokes the Release of Immunoreactive Calcitonin Gene–Related Peptide and Substance P from Dorsal Horn Slices via Nitric Oxide–Dependent and Nitric Oxide–Independent Mechanisms

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The results of behavioral studies suggest that nitric oxide (NO) participates in certain spinal mechanisms that contribute to hyperalgesia. Additionally, previous studies indicate that the release of immunoreactive calcitonin gene–related peptide (iCGRP) and substance P (iSP) is increased in the dorsal horn of the spinal cord during hyperalgesia. Therefore, the aim of this study was to determine whether NO acts to enhance peptide release in the dorsal horn of rats using an *in vitro* superfusion technique. Sodium nitroprusside (SNP) was used as an NO donor. The results of this study indicate that SNP caused a dose-related, calcium-dependent increase in the release of iCGRP and iSP from dorsal horn slices of the rat spinal cord. Furthermore, pretreatment with SNP reduced the ability of capsaicin to evoke the release of either peptide, suggesting that a target for SNP exists on certain capsaicin-sensitive primary afferent terminals. In addition to increasing peptide release, SNP also caused a significant five to sixfold increase in the levels of immunoreactive guanosine 3′,5′-monophosphate (i-cGMP) in the dorsal horn. This SNP-evoked increase was significantly decreased by the guanylate cyclase inhibitor methylene blue in a dose-dependent manner. In addition, the release of iCGRP was also significantly reduced in the presence of methylene blue, although the relationship between peptide release and i-cGMP production remains unclear. Sodium nitroprusside–evoked peptide release was significantly reduced in the presence of hemoglobin (an oxide radical scavenger), suggesting that the drug effect was due to the generation of NO. However, the release of iCGRP and iSP was also evoked by sodium ferricyanide (the coproduct of SNP) and by 7-d-old, photoinactivated SNP. Taken together, these data indicate that SNP acts to evoke the release of iCGRP and iSP from primary afferent neurons in the dorsal horn. In addition, these data suggest that the SNP-evoked increase in iCGRP release may be associated with increasing tissue levels of i-cGMP. Finally, these data indicate that SNP has NO-dependent and NO-independent mechanisms of action in the dorsal horn.

[Key words: nitric oxide, sodium nitroprusside, spinal cord, substance P, calcitonin gene–related peptide, cGMP]

Hyperalgesia and central sensitization

Hyperalgesia is a condition that is characterized by decreased pain thresholds, increased pain to suprathreshold stimuli and, at times, spontaneous pain (Campbell and Meyer, 1986). While peripheral mechanisms for hyperalgesia have been established, central mechanisms are also critical for the development of hyperalgesia (Simone et al., 1991; Simone, 1992; Treede et al., 1992; Grubb et al., 1993). A major mechanism that is believed to underlie the development of hyperalgesia is termed central sensitization. In general, central sensitization is characterized by the hyperexcitability of dorsal horn neurons at the level of the spinal cord. In animals with polyarthritis (involvement of both the hindpaw and ankle), wide-dynamic-range (WDR) and nociceptive-specific neurons exhibit enhanced spontaneous firing (Menetrey and Besson, 1982). In addition, these neurons show an increased discharge response to innocuous and noxious stimulation (Schiable et al., 1987). This progressive increase in the discharge of dorsal horn neurons in response to a stimulus of constant intensity is termed "wind-up" (Davies and Watkins, 1983). Finally, dorsal horn neurons have expanded receptive fields during inflammation (Hylden et al., 1989), and therefore receive a greater afferent input when compared to dorsal horn neurons from normal animals. It has been proposed that these changes in the response properties of dorsal horn neurons are believed to be the result of synaptic plasticity and are thought to resemble the synaptic strengthening that facilitates the process of learning and memory (Meller and Gebhart, 1993).

Nitric oxide is a novel neuronal messenger

Evidence is rapidly growing in support of the role of nitric oxide (NO) as a neuronal messenger in the nervous system (Moncada et al., 1991a; Bredt and Snyder, 1992; McCall and Vallance, 1992). In the cerebellum, biochemical analyses have shown that NO, either exogenously applied or endogenously generated, activates the soluble form of guanylate cyclase, probably through a series of reduction–oxidation events where NO binds to the heme moiety of the guanylate cyclase molecule (Waldman and Murad, 1987; Southam et al., 1991). This activation of guanylate cyclase results in the generation of guanosine 3′,5′-cyclic mono-

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phosphate (cGMP). This increase in cGMP has been observed to mediate many of the effects of NO in the CNS (Garthwaite, 1991; McCall and Vallance, 1992). One major role that has been defined for NO is the mediation of learning and memory at the level of the hippocampus (Malenka et al., 1988, 1989; Chapman et al., 1992; Haley et al., 1992b).

Nitric oxide and hyperalgesia

It has recently been suggested that NO may, under certain conditions, play a role in the development of hyperalgesia. In support of this, behavioral data suggest that NMDA-evoked hyperalgesia is mediated via NO (Kitto et al., 1992; Meller et al., 1992). Intrathecal administration of NMDA produces a rapid, dose-related, thermal hyperalgesia in mice and rats (Aanonsen and Wilcox, 1987; Meller et al., 1992a), which is blocked by pretreatment with a selective NMDA antagonist, AP5 (Kolhkear et al., 1992; Meller et al., 1992a). This NMDA-induced hyperalgesia is also abolished by pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME; a competitive inhibitor of NOS) (Kitto et al., 1992; Meller et al., 1992a), methylene blue (a guanylate cyclase inhibitor) (Meller et al., 1992a), and hemoglobin (a superoxide anion scavenger) (Kitto et al., 1992). In a similar study, it was suggested that the hyperalgesia that occurs during peripheral neuropathy is dependent upon the production of NO in the spinal cord (Meller et al., 1992b). Additionally, electrophysiological studies indicate that NO has a role in prolonged chemical nociception in the rat (Haley et al., 1992a). Taken together, the results of these behavioral studies suggest that NMDA-induced hyperalgesia is mediated by an NO-cGMP–dependent pathway.

A number of pharmacological tools are now available to probe the functions of the NO-cGMP pathway, including inhibitors of NO synthesis, superoxide anion scavengers, and guanylate cyclase inhibitors, as described above. In addition, the family of nitrovasodilator drugs are NO donors. These drugs donate NO either spontaneously or enzymatically. Sodium nitroprusside (SNP) spontaneously generates NO and has been demonstrated to be a potent agent for stimulating the production of immunoreactive cGMP (i-cGMP) in cerebellar slices (Southam and Garthwaite, 1991). This drug provides a practical way to apply NO to tissues. In addition, the kinetics of NO release have been determined from SNP, and therefore, the concentration of NO formation following the administration of SNP can be calculated (Knowles et al., 1990).

It was previously demonstrated that the release of immunoreactive calcitonin gene–related peptide (iCGRP) and substance P (iSP) is increased in the dorsal horn of the spinal cord in various species during hyperalgesia (Oku et al., 1987a,b; Nanayama et al., 1989; Schiable et al., 1990; Garry and Hargreaves, 1992). In addition, both CGRP and SP have previously been implicated as having a role in nociceptive processing at the level of the spinal cord (Bossut et al., 1988; Kawamura et al., 1989; Tsai et al., 1989; Otsuka and Yangisawa, 1990; Coderre and Melzack, 1991; Murray et al., 1991; Sun and Larson, 1991; Galeazza et al., 1992; Satoh et al., 1992; Sluka et al., 1992). As a result, we have hypothesized that the enhanced release of iCGRP and iSP from the dorsal horn may be a biochemical marker for the development of hyperalgesia (Garry and Hargreaves, 1992).

Therefore, the aim of this study was to determine whether NO facilitates hyperalgesia at the level of the spinal cord by enhancing the release of peptides in the dorsal horn. To achieve this, we measured the release of iCGRP and iSP from the spinal dorsal horn of the rat in response to SNP using an *in vitro* superfusion technique. We also measured the levels of i-cGMP in dorsal horn slices following stimulation with SNP. Finally, we evaluated whether the effects of SNP on peptide release and i-cGMP levels were selectively due to the spontaneous generation of NO.

Materials and Methods

Spinal cord superfusion and measurement of iCGRP and iSP. Male Sprague–Dawley rats (175–199 gm, Harlan Company, Madison, WI) were housed in our animal care facility for 1 week following delivery with 12 hr light/dark cycles and received food and water ad lib. All procedures were approved by the ACUC at the University of Minnesota. On the day of the experiment, rats were decapitated and the spinal cords were removed by hydraulic extrusion using ice-cold Krebs buffer. The lumbar enlargement was isolated and the ventral half of the spinal cord was removed and discarded. The dorsal half of the spinal cord was placed on a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomshall, UK) and 200 μm cubes were prepared. The tissue was placed into 1 cc chambers, which were pumped (0.5 ml/min) with oxygenated Krebs buffer [NaCl (135 mM), KCl (3.5 mM), MgCl (1 mM), NaH_2PO_4 (1 mM), $NaHCO_3$ (20 mM), $CaCl_2$ (2.5 mM), bovine serum albumin (0.1%), dextrose (3.3 mM), bacitracin (3%), ascorbic acid (0.1 mM), thiorphan (0.01 mM) pH 7.4 at 37°C]. Fractions were collected every 3 min throughout the experiment into tubes containing 100 μl of 1 M MES (Sigma Chemical Co., St. Louis, MO), which maintained the pH of the fractions at 6.1. Each fraction was divided for radioimmunoassay (RIA) of iCGRP and iSP. The CGRP antiserum (MI-C2, kindly provided by Dr. M. Iadarola) binds near the C-terminal end of CGRP but does not cross-react with cholecystokinin, neuropeptide Y, or other peptides with similar C-terminal residues such as FMRFamide peptides. The CGRP antiserum detected amylin but only at concentrations that were 3 orders of magnitude in excess of the minimal detectable concentration of CGRP. The SP antiserum (SP-1, kindly provided by Dr. S. Leeman) binds near the C-terminal sequence of the peptide, and has no cross-reactivity with 100 pmol of substance K, physalaemin, met-enkephalin, or eledoisin. Under nonequilibrium conditions, the minimum detection limit for these assays is approximately 1 fmol/tube, with 50% displacements at 10–20 fmol/tube. The inter- and intraassay coefficients of variations are less than 6% and 12%, respectively. All drugs used in these experiments were evaluated for possible interference in the RIAs. The antibody–antigen complex was separated from the free tracer using an immunomagnetic precipitation technique (Advanced Magnetics Inc., Cambridge, MA).

Drugs. Sodium nitroprusside (SNP), hemoglobin, and methylene blue, were purchased from Sigma Chemical Co. (St. Louis, MO). Capsaicin and sodium ferricyanide were purchased from Fluka (Ronkonkoma, NY).

Effects of SNP on spontaneous and capsaicin-evoked peptide release. Following an equilibration period in Kreb's buffer (36 min), the tissue was superfused with SNP [1–100 mM (n = 4–6/dose)] for 21 min to evaluate the effect of SNP on peptide release. Based on the studies of Knowles et al. (1990), these doses of SNP generate 1–100 μM NO/min.

To determine whether the effects of sodium nitroprusside were calcium dependent, we evaluated peptide release following superfusion with the highest dose of SNP in the absence of calcium. Following a 36 min equilibration period, the tissues were superfused with SNP (100 mM; 21 min stimulation period) in the presence (n = 5) or absence (n = 5) of calcium. The Ca^{2+}-free Kreb's buffer also contained 10 mM EGTA to chelate endogenous calcium.

To determine whether SNP activated a subclass of small-diameter afferent fibers in the dorsal horn, we evaluated the effect of SNP pretreatment on capsaicin-evoked release of iCGRP and iSP as previously described (Garry and Hargreaves, 1992). Following the equilibration period, dorsal horn slices were superfused with either (1) capsaicin (10 μM, n = 6) for 6 min, (2) SNP (3–100 mM, n = 4–6/dose) for 21 min, or (3) SNP (3–100 mM, n = 4–6/dose) for 21 min prior to and during a stimulation with capsaicin (10 μM, 6 min). Capsaicin selectively excites a subclass of small-diameter primary afferent terminals (C- and some Aδ-type fibers) (Fitzgerald, 1983).

Effect of a guanylate cyclase inhibitor on peptide release and i-cGMP levels. In order to determine whether SNP-evoked effects on peptide

release could be attributed to the activation of guanylate cyclase, we attempted to block the effects of SNP on peptide release by superfusing the tissue with methylene blue [a known guanylate cyclase inhibitor (Waldman and Murad, 1987)]. Following an equilibration period, tissues were superfused with either (1) Kreb's buffer only (n = 7), (2) methylene blue only (10 μM, n = 8) for 21 min, (3) SNP only (30 or 100 mM, n = 8–14/dose) for 12 min, or (4) methylene blue (10 or 100 μM, n = 8–14/dose) for 9 min prior to and during a stimulation with SNP (30 and 100 mM) for 12 min. Following the superfusion experiment, the tissue levels of i-cGMP were assessed. Tissues were removed from the chamber and cGMP extraction was performed by boiling the tissue for 3 min in a 50 mM Tris, 4 mM EGTA buffer (pH 7.6) as has been previously described (Southam and Garthwaite, 1991). Following boiling, the tissue was sonicated and centrifuged and the supernatants were acetylated and assayed for immunoreactive cGMP (i-cGMP) using a commercially available RIA (Peninsula Laboratories, Belmont, CA).

Evaluation of the NO-dependent and-independent mechanisms of SNP. To determine whether the effects of SNP were mediated by the generation of NO, we attempted to block the effects of SNP on peptide release and i-cGMP by pretreating the tissue with hemoglobin. Nitric oxide binds to the heme moieties of guanylate cyclase and hemoglobin. Therefore, in the presence of excess hemoglobin, extracellular nitric oxide is bound, reducing the levels of NO that are available to bind to guanylate cyclase (Moncada et al., 1991b). Following an equilibration period, tissues were superfused with either (1) Kreb's buffer only (n = 8), (2) SNP (100 mM, n = 8) for 18 min, or (3) hemoglobin (3 or 10 mg/ml, n = 10–12/dose) prior to and during a stimulation with SNP (100 mM; 18 min). Following this superfusion experiment, the tissue from each chamber was prepared for assessment of i-cGMP as described above.

To evaluate further the contribution of NO to the SNP-evoked effect, we superfused dorsal horn slices with SNP that had been prepared 1 week in advance of the experimental day. SNP was dissolved in sterile Kreb's buffer, and stored at room temperature under ultraviolet light for 1 week. Based on the known kinetics of this drug and the half-life of NO (Knowles et al., 1990), we calculated that no nitric oxide should be present in the preparation when it has been in solution for 7 d (i.e, a time equal to 20 half-lives of SNP dissolution). Following an equilibration period, tissues were superfused with either (1) Kreb's buffer (n = 8), (2) fresh SNP (100 mM, n = 6) for 18 min, or (3) 1-week-old SNP (100 mM, n = 7) for 18 min. Following this superfusion experiment, the tissue from each chamber was prepared for assessment of i-cGMP as described above.

The products of SNP breakdown are sodium ferricyanide and NO (Southam and Garthwaite, 1991). Therefore, we further evaluated the selectivity of SNP by determining the effect of sodium ferricyanide on peptide release and i-cGMP levels. Following an equilibration period, tissues were superfused with either (1) normal Kreb's buffer (n = 8), (2) SNP (100 mM, n = 4–6), or (3) sodium ferricyanide (100 mM, n = 4–6) for 18 min. Following this superfusion experiment, the tissue from each chamber was prepared for assessment of i-cGMP as described above.

Statistics. All superfusion data is presented as a sum score (mean ± SEM), determined by calculating total peptide release over consecutive fractions. A_{50} values were calculated using PHARM/PCS Software (Microcomputer Specialists). The A_{50} value is defined as the concentration of a drug that produces a 50% effect of a graded response; in this case, the A_{50} dose is the concentration of SNP that evokes 50% of maximal secretion of either iSP or iCGRP. In these studies, maximal secretion of iCGRP or iSP is defined by the release of each peptide in the presence of 10 μM capsaicin. The results from the i-cGMP assays are presented as pmol/gm wet weight (mean ± SEM). One-way and multiway ANOVAs were performed to analyze experiments according to experimental design. Post hoc tests were used to determine the source of statistical differences between various groups (Duncan's or Tukey's) or relative to the control group (Dunnett's). When the experimental design only included two groups, a Student's t test was performed to evaluate whether differences between groups were statistically significant. The level of significance was $p < 0.05$.

Results

Effects of SNP on spontaneous and capsaicin-evoked peptide release

Basal levels of release of iCGRP (Fig. 1*A*; 3412 ± 247 fmol/gm/9 min) and iSP (Fig. 1*B*; 578 ± 93 fmol/gm/9 min) were detectable. Sodium nitroprusside caused a dose-related increase in the release of iCGRP (Fig. 1*A*) and iSP (Fig. 1*B*) from dorsal horn slices. At the highest dose examined, SNP caused a 5.4-fold increase in the release of iCGRP and a 4.9-fold increase in the release of iSP, as compared to basal levels. The A_{50} value of SNP-evoked release of iCGRP was 30.7 ± 1.2 mM. The A_{50} value of SNP-evoked release of iSP was 30.3 ± 3.98 mM.

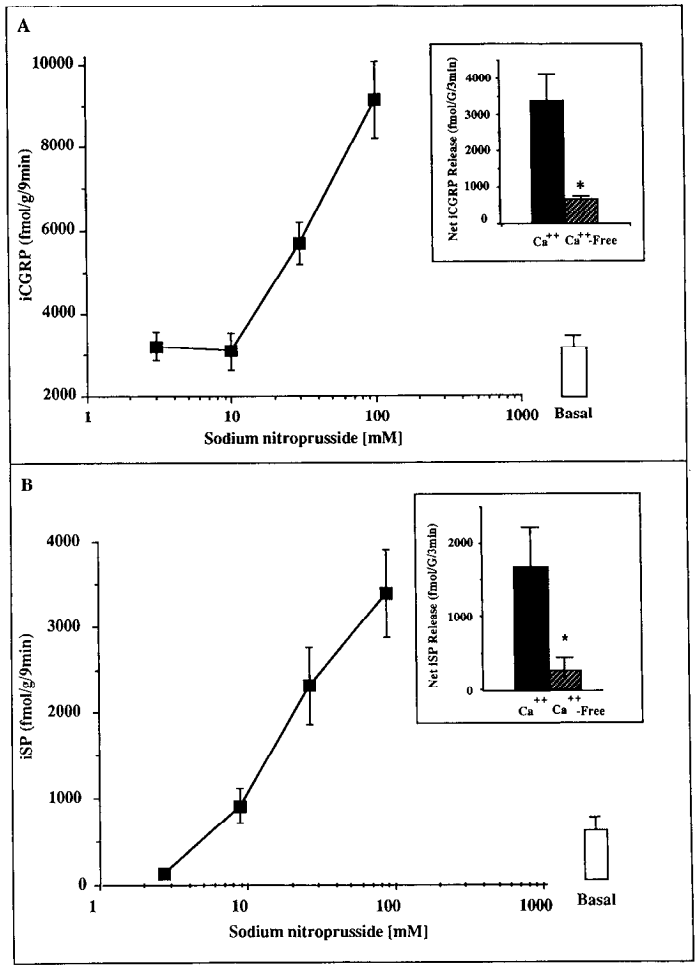

Figure 1. The dose–response curve for the effect of SNP on the release of iCGRP (*A*) and iSP (*B*) from rat dorsal horn slices. Peptide release is expressed as a sum of three fractions immediately following a treatment. The *open bar* represents the basal release of peptide in the presence of Kreb's buffer only. Following superfusion with Kreb's buffer, the tissues were superfused with SNP [1–100 mM (n = 4–6/dose)]. *Insets* illustrate the calcium dependence of the SNP-evoked peptide release (100 mM SNP). The *solid bars* illustrate peptide release in the presence of calcium (n = 5) and the *hatched bars* represent peptide release in the absence of calcium (n = 5). Peptide release is expressed as net release, which is calculated by subtracting the basal release from the SNP-evoked release. *, $p < 0.05$.

To evaluate the possibility that SNP-evoked release of iCGRP and iSP was due to a nonexocytotic mechanism, we determined the ability of SNP to evoke peptide release in the absence of calcium. Dorsal horn slices that were superfused in the absence of calcium showed a significant reduction in the SNP-evoked release of iCGRP (Fig. 1*A*, inset) and iSP (Fig. 1*B*, inset) when compared to the SNP-evoked release of peptides in the presence of calcium ($p < 0.05$).

We next evaluated the effect of SNP pretreatment on capsa-

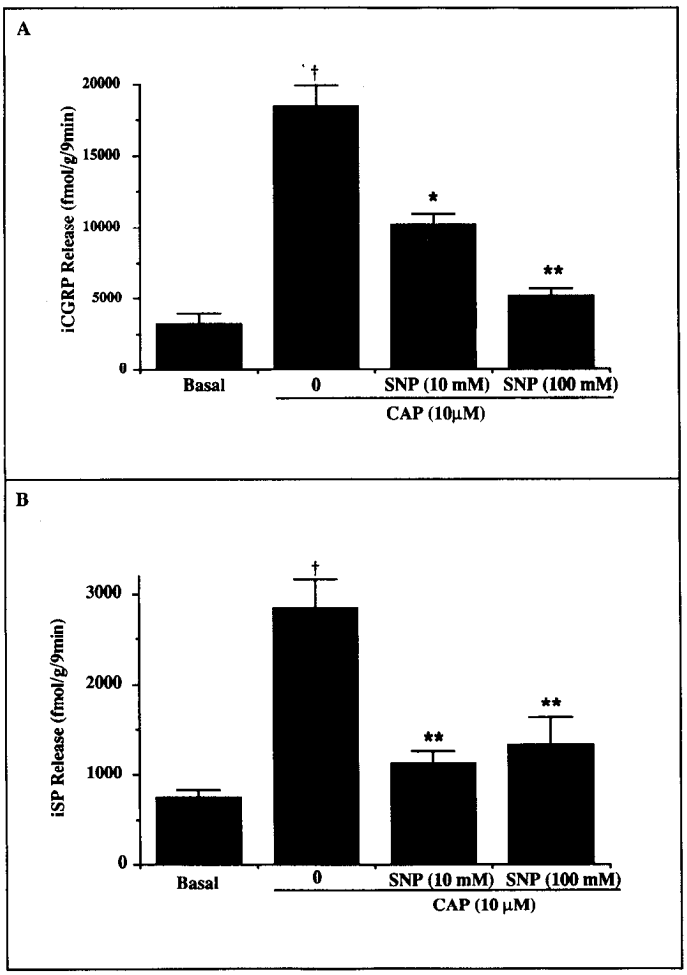

Figure 2. The effect of SNP pretreatment on the release of iCGRP (*A*) and iSP (*B*) from dorsal horn slices. Peptide release is expressed as a sum of three fractions immediately following a treatment. The basal release (spontaneous peptide release in response to Kreb's buffer only) is the mean basal release of all the treatment groups that were not significantly different. Following superfusion with Kreb's buffer, tissues were superfused with either (1) capsaicin (*CAP*) ($n = 6$) or (2) SNP prior to and during capsaicin stimulation ($n = 4$–6/dose). †, $p < 0.01$ versus basal release. *, $p < 0.05$; **, $p < 0.01$; versus CAP treatment alone.

icin-evoked release of iCGRP and iSP from dorsal horn slices. Figure 2*A* illustrates that 10 μM capsaicin was efficacious in increasing the release of iCGRP over basal levels ($p < 0.01$). However, administration of SNP prior to and during the capsaicin pulse significantly reduces the ability of capsaicin to evoke the release of iCGRP from dorsal horn slices [$F(3,27) = 35.97$, $p < 0.01$]. Similarly, Figure 2*B* illustrates that 10 μM capsaicin was efficacious in increasing the release of iSP over basal levels ($p < 0.01$). SNP pretreatment also decreases the ability of capsaicin to evoke the release of iSP from dorsal horn slices [$F(3,27) = 19.97$, $p < 0.05$].

Does SNP alter the levels of i-cGMP in the dorsal horn of the spinal cord?

Figure 3 illustrates that administration of SNP causes a significant increase in the levels of i-cGMP in the dorsal horn of the spinal cord [$F(2,27) = 99.02$, $p < 0.01$]. While we observed no detectable increase in the levels of i-cGMP following stimulation with 30 mM SNP, i-cGMP levels were increased approximately

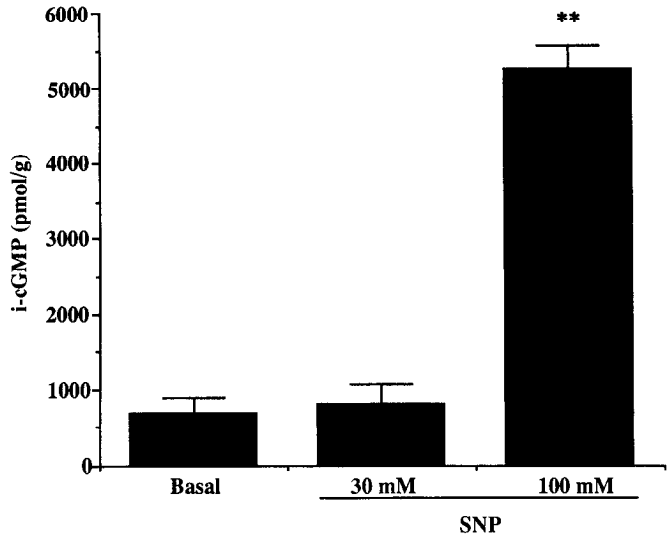

Figure 3. The effect of SNP on tissues levels of i-cGMP in dorsal horn slices. The bar labeled *Basal* represents the levels of i-cGMP in the presence of Kreb's buffer only. Tissues were superfused with either (1) Kreb's buffer ($n = 6$) or (2) SNP (30 or 100 mM, $n = 6$–8/dose). **, $p < 0.01$ versus basal levels.

10-fold over basal levels following stimulation with 100 mM SNP.

Are the SNP-evoked effects on peptide release related to its effect on i-cGMP?

In order to evaluate whether the effect of SNP on peptide release was related to the SNP-evoked increase in i-cGMP, we determined the effect of methylene blue on the level of i-cGMP and peptide release in dorsal horn slices. Figure 4 illustrates that 100 mM SNP was efficacious in increasing the tissue levels of i-cGMP in the dorsal horn. Importantly, this SNP-evoked increase in the level of i-cGMP was reduced by methylene blue in a dose-related fashion [$F(5,32) - 93.63$, $p < 0.001$]. The SNP effect on tissue levels of i-cGMP is completely blocked by pretreatment with methylene blue at concentrations of 10 and 30 mM. Methylene blue alone, at any concentration, had no effect on the level of i-cGMP.

Figure 5*A* demonstrates that methylene blue, at concentrations greater than 10 μM, also produces a dose-related blockade of the SNP-evoked release of iCGRP from dorsal horn slices [$F(5,53) = 10.61$, $p < 0.01$], although, the 10 μM dose produced a significant increase in iCGRP release when compared to the effect of SNP alone ($p < 0.01$). When the dose of methylene blue was increased to 10 and 30 mM, however, there was a significant suppression of SNP-evoked iCGRP release ($p < 0.01$).

Figure 5*B* illustrates that 10 μM methylene blue was not effective in reducing the release of iSP. We superfused the tissues with higher concentrations of methylene blue; however, control experiments indicated that these higher concentrations of methylene blue significantly reduced the ability of the SP antibody to bind to its antigen in our RIA. Therefore, the amount of iSP that was released, in the presence of methylene blue, could not be analyzed with accuracy by our RIA method.

Are the effects of SNP selectively due to the generation of NO?

Hemoglobin. To evaluate whether the SNP-evoked effects were selective to the generation of a superoxide anion, the effects of

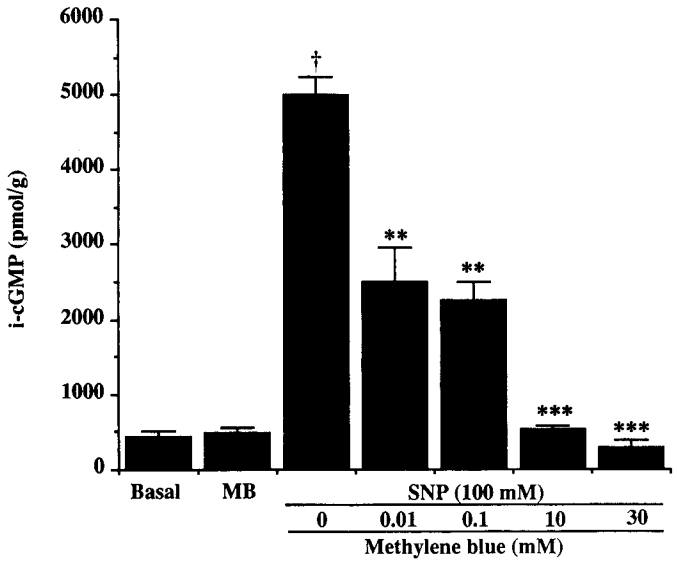

Figure 4. The effect of methylene blue (*MB*) on the SNP-evoked increase in i-cGMP levels in dorsal horn slices. The basal level refers to i-cGMP levels in the presence of Kreb's buffer only. MB represents mean i-cGMP levels in the presence of methylene blue (0.01–30 mM; no significant difference between doses). Following superfusion with Kreb's buffer, tissue slices were superfused with either (1) Kreb's only ($n = 7$), (2) methylene blue (0.01–30 mM, $n = 6$–8/dose), or (3) methylene blue (0.01–30 mM) prior to and during superfusion with SNP (100 mM) ($n = 8$–14/dose). †, $p < 0.01$ versus basal levels. **, $p < 0.005$; ***, $p < 0.01$; versus SNP treatment.

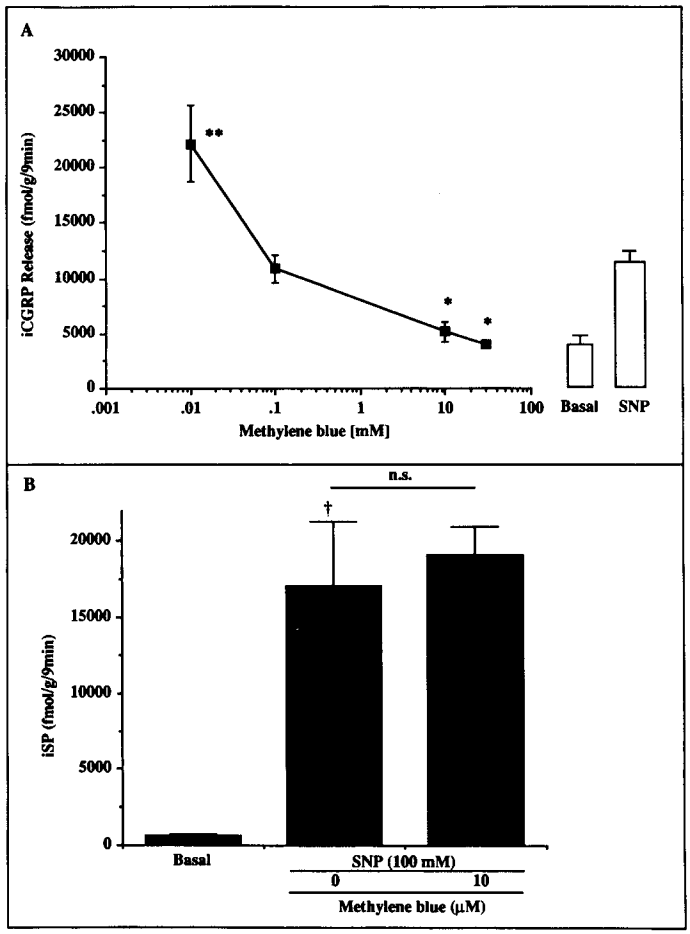

Figure 5. The effect of methylene blue on the SNP-evoked increases in the release of iCGRP (*A*) and iSP (*B*) from dorsal horn slices of rat spinal cord. Basal release represents the mean spontaneous peptide release in response to Kreb's buffer only. Following superfusion with Kreb's buffer, tissue slices were superfused with either (1) Kreb's buffer only ($n = 7$), (2) methylene blue only (0.01–30 mM) ($n = 6$–8/dose), (3) SNP only (100 mM) ($n = 7$), or (4) methylene blue (0.01–30 mM) prior to and during SNP (100 mM) stimulation ($n = 8$–14/dose). †, $p < 0.001$ versus basal release. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; versus SNP treatment.

hemoglobin on SNP-evoked peptide release were evaluated. Figure 6*A* illustrates that hemoglobin significantly reduced the SNP-evoked release of iCGRP at 3 and 10 mg/ml [$F(2,28) = 6.75$, $p < 0.01$]. Hemoglobin alone significantly increased spontaneous iCGRP release at 10 mg/ml [$F(2,33) = 3.94$, $p < 0.029$]. Similarly, Figure 6*B* illustrates that hemoglobin significantly reduced the SNP-evoked release of iSP at 3 and 10 mg/ml [$F(2,29) = 5.09$, $p < 0.01$]. There was no significant difference in the basal levels of iSP release between any of the groups [$F(2,32) = 1.44$, $p =$ NS]. In contrast to the release of CGRP, hemoglobin alone had no significant effect on the release of iSP at any concentration.

To evaluate whether the SNP-evoked i-cGMP increase was due to the generation of a superoxide anion from SNP, we evaluated the ability of hemoglobin to block the SNP-evoked increase in i-cGMP. Figure 7 illustrates that both doses of hemoglobin significantly reduced the ability of SNP to increase i-cGMP levels in the dorsal horn [$F(3,48) = 92.59$, $p < 0.01$].

Photoinactivated SNP. To evaluate further whether the SNP-evoked effects were dependent upon the spontaneous release of NO from SNP, we evaluated the effect of 1-week-old SNP on the release of iCGRP and iSP and on the level of i-cGMP in the dorsal horn. Figure 8*A* illustrates that both fresh and 7-d-old SNP produced a significant increase in the release of iCGRP release when compared to basal release [$F(1,13) = 119.68$, $p < 0.01$]. In addition, there was no significant difference in the amount of peptide released when the tissue was treated with fresh SNP or week-old SNP [$F(1,13) = 1.38$, $p =$ NS]. Similarly, Figure 8*B* illustrates that both fresh and week-old SNP caused a significant increase in the release of iSP when compared to basal release [$F(1,9) = 361.70$, $p < 0.01$]. In contrast to the release of iCGRP, the magnitude of the response evoked with week-old SNP was significantly greater than the response evoked with fresh SNP [$F(1,13) = 13.28$, $p < 0.01$].

Sodium ferricyanide. Since SNP releases sodium ferricyanide, as well as NO, we evaluated the ability of sodium ferricyanide to evoke the release of peptides and alter the level of i-cGMP in the dorsal horn. Sodium ferricyanide caused a significant 7.2-fold increase in the release of iCGRP when compared to basal levels (Fig. 9*A*; $p < 0.005$). The magnitude of iCGRP release evoked by sodium ferricyanide was comparable to the release evoked by 100 mM SNP. Sodium ferricyanide caused a 2.01-fold increase in iSP release from dorsal horn slices (Fig. 9*B*; $p < 0.05$). This release was not significantly different from that produced by 100 mM SNP [$F(2,14) = 2.12$, $p =$ NS]. In contrast to our results with SNP, hemoglobin did not reduce the sodium ferricyanide-evoked release of iCGRP or iSP (data not shown).

Figure 10 illustrates that both week-old SNP and sodium ferricyanide cause a significant increase in the levels of i-cGMP when compared to basal levels [$F(9,48) = 92.59$, $p < 0.05$]. Additionally, the levels of i-cGMP production that were evoked

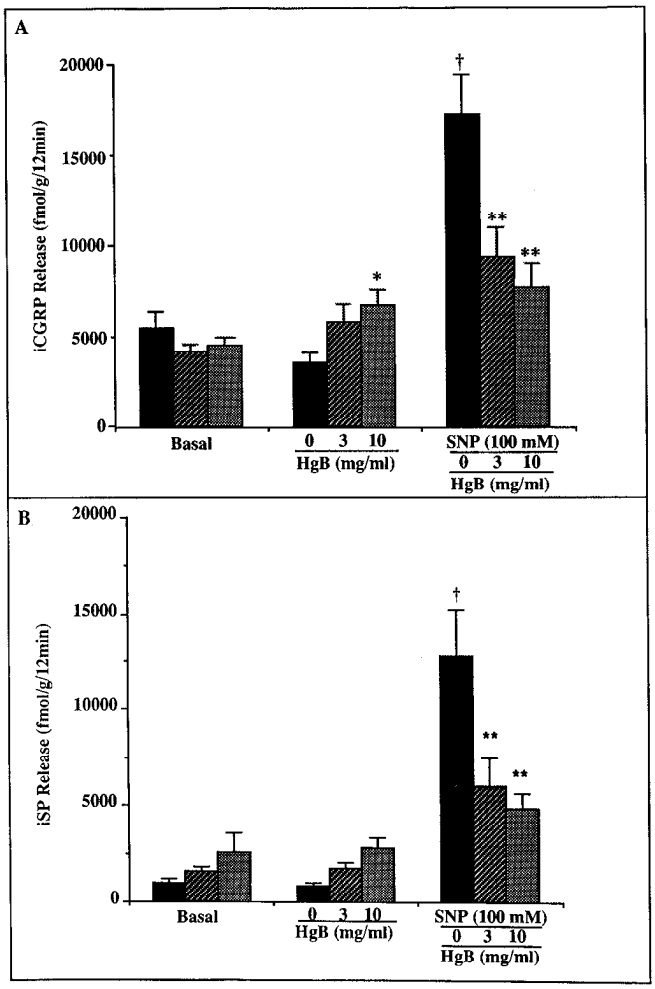

Figure 6. The effect of hemoglobin (*HgB*) on SNP evoked release of iCGRP (*A*) and iSP (*B*). The bars labeled *Basal* represent the spontaneous peptide release in response to Kreb's buffer only. The bars labeled *HgB* represent the peptide release in response to hemoglobin alone. Following superfusion with Kreb's buffer, tissues were superfused with either (1) Kreb's buffer (n = 8), (2) SNP (100 mM, n = 8), or (3) hemoglobin (3 or 10 mg/ml, n = 10–12/dose) prior to and during SNP (100 mM) treatment. †, significant difference ($p < 0.001$) when compared to basal release. *, $p < 0.05$; **, $p < 0.01$; versus SNP treatment.

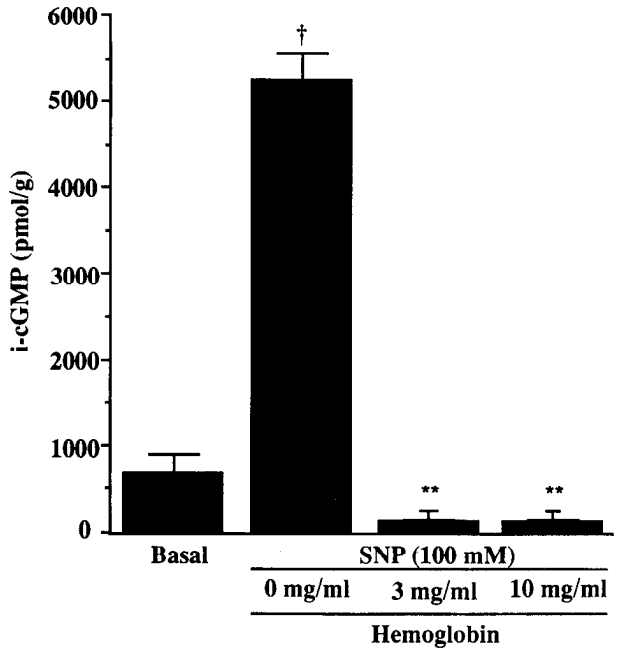

Figure 7. The effect of hemoglobin (*HgB*) on SNP-induced i-cGMP levels. The bar labeled *Basal* represents the levels of i-cGMP in the presence of Kreb's buffer only. Following superfusion with Kreb's buffer, tissues were superfused with either (1) Kreb's buffer (n = 8), (2) SNP (100 mM, n = 8), or (3) hemoglobin (3 or 10 mg/ml, n = 10–12/dose) prior to and during SNP (100 mM) treatment. †, significant difference ($p < 0.01$) versus basal levels. **, $p < 0.01$ versus SNP treatment.

by week-old SNP and SF were not significantly different when compared to each other. However, 100 mM SNP evoked a significantly greater increase in the tissue levels of i-cGMP when compared to either week-old SNP or sodium ferricyanide ($p < 0.01$).

Discussion

SNP is a potent stimulus for the release of neuropeptides and the production of cGMP

These data demonstrate that sodium nitroprusside is a potent stimulus for evoking the release of iCGRP and iSP from rat dorsal horn slices *in vitro*. We have demonstrated that SNP increases the release of iCGRP and iSP from spinal cord slices in a dose-related calcium-dependent manner. To our knowledge, this is the first demonstration that an NO donor evokes the release of neuropeptides in the CNS. In addition to increasing peptide release, SNP was effective in increasing the levels of i-cGMP in dorsal horn slices. This observation is consistent with previous studies on cerebellar slices where SNP and other NO donors stimulate increases in the levels of i-cGMP (Southam and Garthwaite, 1991). We did not discern in the present studies, however, whether SNP acts to increase peptide release and i-cGMP levels or whether it acts to inhibit the degradation of these compounds. The calcium dependence of the peptide release, however, supports the theory that SNP does increase peptide release. Regardless of the mechanism, these data demonstrate that SNP is a potent compound that increases the tissue levels of i-cGMP in dorsal horn slices and increases the levels of iCGRP and iSP in the superfusates that were collected from slices of the dorsal horn.

Sites of SNP action within the dorsal horn of the spinal cord

Our results indicate that SNP is acting on a pool of capsaicin-sensitive primary afferent neurons. This hypothesis is supported by the observation that SNP pretreatment significantly reduced the ability of capsaicin to evoke peptide release. In the dorsal horn preparation, the release of iSP could be the result of activation of primary afferent neurons, interneurons, or descending tracts (Hökfelt et al., 1975; Linderoth et al., 1992). In contrast, the release of iCGRP in the dorsal horn can only result from the stimulation of primary afferent neurons (Chung et al., 1988). The present results, however, do not identify the actual mechanism by which SNP inhibits capsaicin-evoked peptide release. It is possible that SNP blocks the nonselective cation channel activated by capsaicin in certain populations of primary afferent neurons (Takaki et al., 1991). Alternatively, SNP may act to desensitize certain primary afferent neurons to capsaicin stimulation or deplete stores of peptides in capsaicin-sensitive neurons. It is also possible that SNP interferes with the binding of capsaicin on primary afferent neurons. Although further stud-

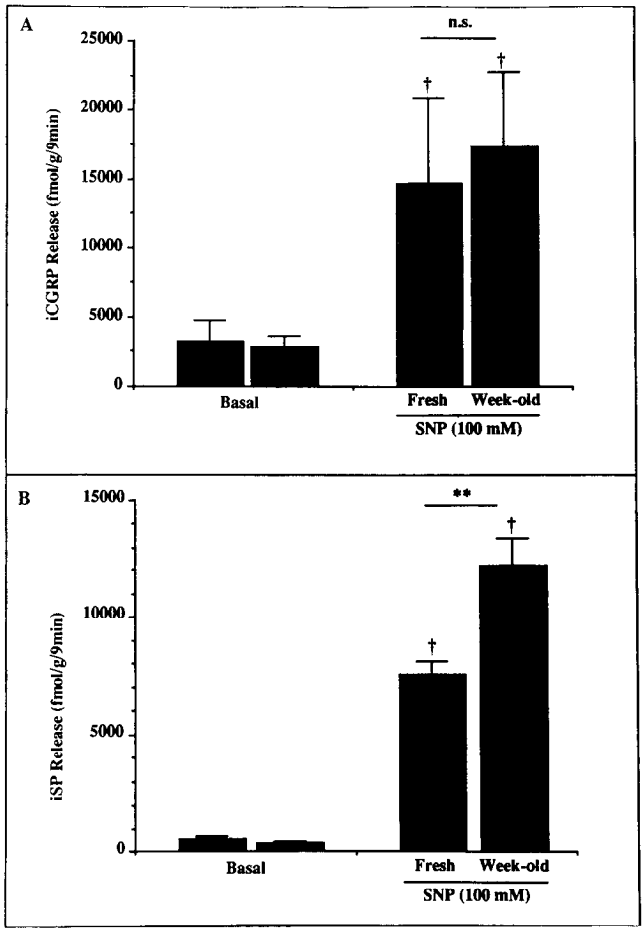

Figure 8. The effect of 7-d-old SNP on the release of iCGRP (*A*) and iSP (*B*) from dorsal horn slices. The bar labeled *Basal* (peptide release in response to Kreb's buffer only) represents the mean peptide release from both treatment groups (no significant difference between groups). Following superfusion with Kreb's buffer, tissue slices were superfused with either (1) Kreb's buffer ($n = 8$), (2) fresh SNP (100 mM, $n = 6$), or (3) 7-d-old SNP (100 mM, $n = 7$). †, $p < 0.01$ versus basal release. **, $p < 0.01$ (fresh vs week-old SNP).

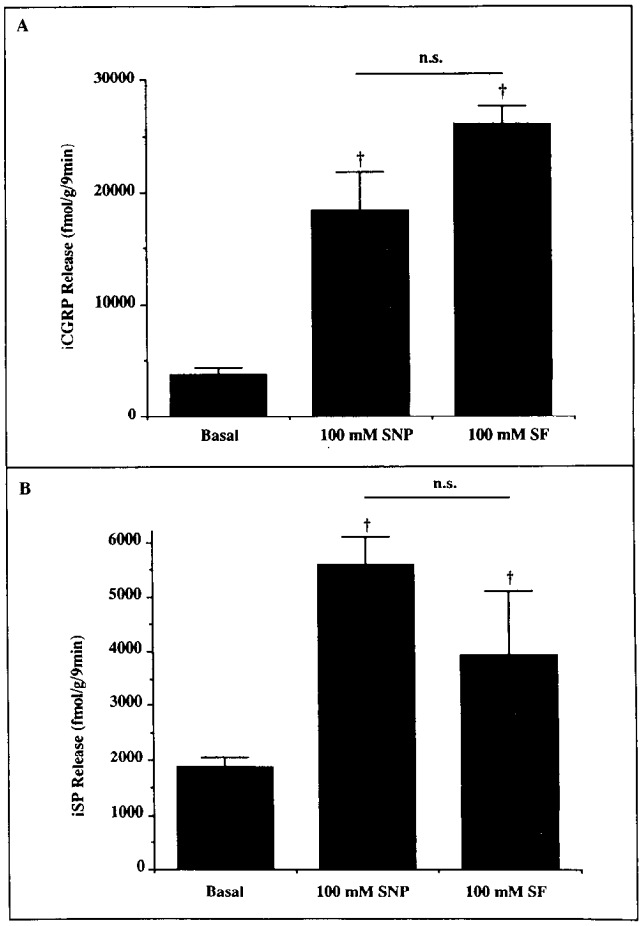

Figure 9. The effect of sodium ferricyanide (*SF*) on the release of iCGRP (*A*) and iSP (*B*) from dorsal horn slices. The bar labeled *Basal* (peptide release in response to Kreb's buffer only) represents the mean peptide release from both treatment groups (no significant difference between groups). Following superfusion with Kreb's buffer, tissue slices were superfused with either Kreb's buffer ($n = 8$), (2) SNP (100 mM, $n = 4$–6), or (3) sodium ferricyanide (100 mM, $n = 4$–6). †, $p < 0.01$ versus basal release.

ies will be required to elucidate the precise mechanism(s) by which SNP pretreatment inhibits capsaicin-evoked peptide release, the present results indicate that SNP and capsaicin activate an overlapping pool of primary afferent neurons. Additionally, the similarity in A_{50} values for SNP-evoked iCGRP and iSP release suggests that SNP is acting on neurons with similar response properties to chemical stimuli. These results suggest that iCGRP and iSP may be simultaneously released from the same neuron.

Correlation between peptide release and the level of i-cGMP in the dorsal horn

Our data suggest that there is a positive association between increasing levels of i-cGMP in the dorsal horn and increasing the release of iCGRP from slices of the dorsal horn (since our RIA for iSP would not perform in the presence of higher concentrations of methylene blue, we are limiting this conclusion only to the release of iCGRP). For example, 100 mM SNP stimulates increases in the release of iCGRP and in the level of i-cGMP in the dorsal horn. Moreover, certain doses of methylene blue are efficacious in decreasing the SNP-evoked release of iCGRP *and* in reducing the SNP-evoked increase in the level of i-cGMP in the dorsal horn. Finally, doses of hemoglobin that are efficacious in reducing SNP-evoked peptide release completely abolish the SNP-evoked increase in the level of i-cGMP in the dorsal horn. Our data indicate, however, that the strength of the association between increasing levels of i-cGMP and increased release of iCGRP in the dorsal horn is equivocal, since there are instances when an association between these two factors is not apparent. For example, 30 mM is the A_{50} concentration for the effect of SNP on the release of iCGRP and iSP from the dorsal horn. In contrast, 30 mM SNP has no effect on the basal level of iCGMP in the dorsal horn. Additionally, when 10 μM methylene blue is administered to the dorsal horn slices, there is a reduction in the SNP-evoked increase in the level of i-cGMP, yet the release of iCGRP is significantly increased. Finally, photoinactivated SNP and sodium ferricyanide were equally potent in their ability to increase the release of iCGRP and iSP when compared to fresh SNP, while neither of these compounds was as potent as fresh SNP in their ability to increase the level of i-cGMP in the dorsal horn. Taken together, these data suggest that there is a positive association between increased release of iCGRP and increasing levels of i-cGMP in the dorsal horn;

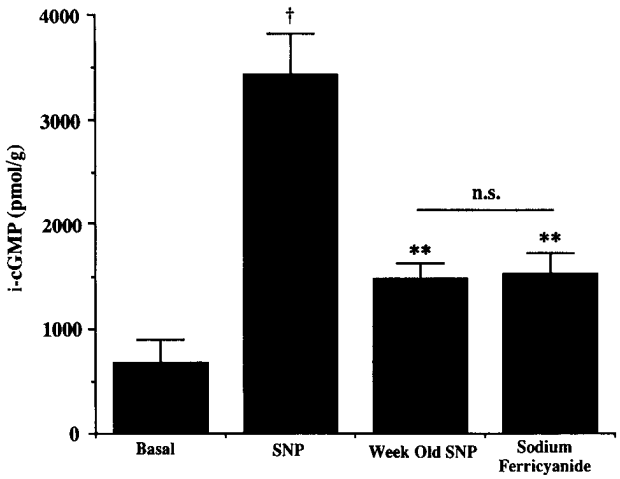

Figure 10. The effect of 7-d-old SNP and sodium ferricyanide on the tissue levels of i-cGMP in the dorsal horn. The bar labeled *Basal* (i-cGMP levels in response to Kreb's buffer only) represents the mean levels from all treatment groups (no significant difference between groups). Following superfusion with Kreb's buffer, tissue slices were superfused with either (1) Kreb's buffer (n = 16), (2) SNP (100 mM, n = 10–12), (3) 7-d-old SNP (100 mM, n = 7), or (4) sodium ferricyanide (100 mM, n = 6). †, significant difference ($p < 0.01$) when compared to basal levels. **, $p < 0.01$ when compared to basal levels.

however, the strength of this association remains to be elucidated.

SNP, NO, and hyperalgesia

The present data demonstrate that SNP acts to evoke the release of iCGRP and iSP from dorsal horn slices. These data are consistent with previous studies that demonstrate that SNP causes hyperalgesia following intrathecal administration in mice (Kitto et al., 1992) and with the hypothesis that the increased release of these peptides may serve as biochemical markers for hyperalgesia (Garry and Hargreaves, 1992). Therefore, these data suggest that the release of iCGRP, iSP, or other related cofactors may be required for the development and/or maintainence of SNP-evoked hyperalgesia. In addition, the fact that SNP increases the production of i-cGMP in the dorsal horn is consistent with previous behavioral studies suggesting that an NO-cGMP pathway is involved in the maintainence of hyperalgesia (Kitto et al., 1992; Meller et al., 1992a,b; Meller and Gebhart, 1993).

Although a source of endogenous NO has not been defined in these studies, immunohistochemical studies have localized the NO-synthesizing enzyme to the superficial dorsal horn (Bredt et al., 1991). Since NO can readily diffuse to its site of action, it is conceivable that an endogenous source of NO is available to act upon primary afferent neurons. Therefore, it is possible that an endogenous NO system exists in the dorsal horn, which may play a role in the development or maintainence of nociception and hyperalgesia.

SNP has NO-dependent and -independent effects

NO-dependent effects of SNP. It has previously been demonstrated that the NO scavenger hemoglobin blocks SNP-evoked hyperalgesia (Kitto et al., 1992). Our data support these behavioral findings, since hemoglobin reduced the ability of SNP to evoke peptide release [we did observe that hemoglobin (10 mg/ml) caused a slight increase in the basal release of iCGRP, although this concentration of hemoglobin significantly reduced the SNP-evoked increase in the release of iCGRP]. In addition, we observed that hemoglobin was efficacious in suppressing SNP-evoked increases in i-cGMP levels. These data suggest that *one* mechanism of action of SNP is dependent upon the release of NO from the drug. While hemoglobin scavenges NO, however, it has also been demonstrated to scavenge carbon monoxide, which is also generated in neural tissue and regulates cGMP (Verma et al., 1993). However, carbon monoxide is not formed by SNP dissolution, suggesting that the effects of hemoglobin are most likely due to the scavenging of NO.

NO-independent effects of SNP. Upon further evaluation of the NO-dependent activity of SNP, however, we observed that SNP has effects that cannot be attributed to the release of NO by the drug. In support of this conclusion, we observed that both sodium ferricyanide and a solution of SNP that has been photolytically inactivated can still evoke peptide release and stimulate the production of i-cGMP in dorsal horn slices. In fact, photoinactivated SNP was more potent in evoking the release of iSP than was fresh SNP. Interestingly, inactivated SNP and sodium ferricyanide were equipotent in stimulating the production of i-cGMP. These data suggest that the ability of the inactivated SNP to increase i-cGMP levels was due to its sodium ferricyanide content. However, both of these agents were less efficacious in stimulating the production of i-cGMP than was a fresh solution of SNP, suggesting that NO-dependent and NO-independent factors contribute to the effect of SNP on the levels of i-cGMP in the dorsal horn. Recent studies in cerebellar slices support the observation that SNP has NO-independent activity (Benz et al., 1992; Stout and Woodward, 1992). From these results, we conclude that there are NO-dependent and an NO-independent pathways that facilitate the observed increases in i-cGMP levels and neuropeptide release in the dorsal horn.

In summary, we conclude that SNP is a potent stimulus for evoking the release of iCGRP and iSP from dorsal horn slices. Furthermore, our results suggest that SNP is acting on a pool of capsaicin-sensitive primary afferent neurons. Sodium nitroprusside is also a potent stimulus for evoking increases in i-cGMP in the dorsal horn. Our data suggest that there is an association between i-cGMP levels and iCGRP release in the dorsal horn, although the strength of this association remains undetermined. In addition, these data demonstrate that SNP has NO-dependent and NO-independent mechanisms of action in the dorsal horn for increasing tissue levels of i-cGMP and evoking the release of iCGRP and iSP.

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