Substance P Release in the Rat Periaqueductal Gray and Preoptic Anterior Hypothalamus after Noxious Cold Stimulation: Effect of Selective Mu and Kappa Opioid Agonists

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ABSTRACT

Intracerebral microdialysis was used to measure changes in the extracellular level of substance P (SP) released from the periaqueductal gray (PAG) and the preoptic anterior hypothalamus (POAH) of freely moving Sprague-Dawley rats after noxious cold stimulation. Artificial cerebrospinal fluid was perfused into the dialysis probe in the PAG or POAH and samples were collected every 30 min for 4 hr. SP-like immunoreactivity in the samples was measured by radioimmunoassay. In the PAG, SP base-line release was 0.43 ± 0.08 fmol/fraction. SP release was increased to 1.3 ± 0.4 fmol/fraction during the first collection period after noxious cold. Pretreatment with the selective mu opioid receptor agonist PL017 (0.8–3.4 nmol) or the kappa opioid receptor agonist dynorphin A1–17 (4.6–9.2 nmol), administered into the PAG by microinjection, produced dose-related inhibition of the cold-evoked SP release. Naloxone (10 mg/kg s.c.) administration 10 min before these opioid agonists reduced the inhibition of SP release. In the POAH, SP base-line release was 0.45 ± 0.06 fmol/fraction and noxious cold did not cause any significant change in SP release. Microdialysis of SP (271 fmol–271 pmol/μl/min, for 30 min) into the PAG, but not the POAH, induced dose-related analgesia (35–68% MPA) in the cold-water tail-flick test. However, microdialysis of SP into the POAH or PAG failed to induce any significant change in body temperature. These data suggest that 1) SP released from the PAG acts as a neuromediator to transmit nociceptive information; 2) opioid receptor agonists can suppress this information by inhibiting SP release; 3) SP evoked by noxious cold may have a role in triggering the antinociceptive function of the PAG; and 4) SP does not appear to act as a neuromodulator for thermoregulatory responses in the POAH.

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ABBREVIATIONS: aCSF, artificial cerebrospinal fluid; CNS, central nervous system; CWT, cold water tail-flick test; Dyn, dynorphin A1–17; i.c.v., intracerebroventricular; MPA, maximum possible analgesia; Nal, naloxone; PAG, periaqueductal gray; PL017, Tyr-Pro-N-MePhe-o-Pro-NH₂; POAH, preoptic anterior hypothalamus; RIA, radioimmunoassay; SP, substance P; Tb, body temperature.
elevation of somatostatin release (Tiseo et al., 1990). The SP increase could be inhibited by i.c.v. pretreatment with Dyn. It is not known, however, if noxious cold can also induce an increase of SP in the brain. SP release from brain regions in vivo is detectable by a combination of intracerebral microdialysis and RIA methods (Brodin et al., 1983; Lindefors et al., 1986, 1987).

In the present study, with use of intracerebral microdialysis and RIA, we investigated SP release from the PAG after noxious cold-water stimulation with or without pretreatment with the mu opioid receptor agonist PL017 or the kappa opioid receptor agonist Dyn. We reported previously that both drugs produce analgesic effects when dialyzed into the PAG in the CWT (Xin et al., 1993). For comparison, we also investigated whether noxious cold could induce any change in SP release level from another brain region, i.e., the POAH, which is a primary site for Tb regulation and contains SP nerve terminals and receptors (Panula et al., 1984). Finally, SP was perfused into the PAG and POAH to examine its effects on analgesia and Tb.

**Methods**

**Animals.** Male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, PA) weighing 250–300 g were used in this study. They were housed three per cage for at least 1 week before surgery and were fed laboratory chow and tap water ad libitum. The temperature of the room was 22 ± 2°C and a 12-hr light/12-hr dark cycle was used.

**Microdialysis probe preparation.** The microdialysis probes used in this study were constructed in a concentric shape consisting of a 1.5-mm length of hollow tubular dialysis membrane (acrylonitrile-sodium methallyl sulfonate, 40-kdalton molecular weight cutoff, 230 μm internal diameter, 300 μm outside diameter, Hospal, Uden, The Netherlands) with the tip sealed by epoxy resin and the other end mounted to a 26-gauge thin-wall stainless steel cannula (15-mm length, Small Parts, Inc., Miami Lakes, FL). A 22-mm length of thin polymide-coated fused silica tubing (75 μm internal diameter, 140 μm outside diameter, Polymicro Technologies, Phoenix, AZ) as an inlet was threaded through the stainless steel cannula into the dialysis membrane, leaving approximately 0.4 mm free between the end of the silica tubing and the epoxy at the tip of the dialysis membrane. Another 22-mm length of fused silica tubing as an outlet was also threaded through the stainless steel cannula into the upper end of the dialysis membrane. The upper end of the stainless steel cannula was sealed by epoxy after both inlet and outlet silica tubing were inserted. For administration of opioid agonists, a 30-mm length of fused silica tubing was attached to the outside of the stainless steel cannula to form a side catheter, with its tip near the tip of the dialysis membrane. The upper ends of inlet, outlet and side catheter were each connected with a length of PE-10 tubing. A schematic drawing of the probe with attached catheter is shown in figure 1.

**Surgery and probe implantation.** Rats were anesthetized with ketamine and the stylet was replaced by a PET-10 tubing was threaded through the tether and connected with the output of the swivel and the input cannula of the dialysis probe. The input of the swivel was then connected to a 1-ml tuberculin microdialysis probe, so that its dialysis membrane tip protruded exactly 1.5 mm beyond the guide and was located within the PAG or POAH. aCSF (see "Materials") was injected into the side catheter (0.2 μl) from its inlet to avoid the injection of air into the brain during the experiment, after which the inlet was sealed.

**Materials.** The following drugs were tested: mu opioid receptor agonist PL017 (Tyr-Pro-N-MePhe-o-Pro-NH₂); kappa opioid receptor agonist Dyn (both from Multiple Peptide Systems, San Diego, CA); naloxone (Nal, DuPont Merck Pharmaceutical Co., Wilmington, DE), and SP (Peninsula Laboratories, Inc., Belmont, CA). aCSF (composition in mM: NaCl, 125; KCl, 2.5; NaH₂PO₄, 0.5; Na₂HPO₄, 5; CaCl₂, 1.2; MgCl₂, 1; ascorbic acid, 0.1; and bovine serum albumen, 0.025%, pH 7.4) was the vehicle and control solution. SP antiserum was prepared in rabbits and was generously provided by Dr. Susan E. Leeman of Boston University. The final dilution of the antiserum used was 1:180,000. Half-maximal displacement was 2 fmol/250 μl. Cross-reactivities of this antiserum were such that no measurable displacement was observed at 100 pM with substance K, met-enkephalin, somatostatin or physalaemin. Isotope [125I]Tyr³-SP was purchased from Dupont New England Nuclear Research Products (Boston, MA). The second antiserum (goat anti-rabbit γ-globulin, used for separation of anti-SP-bound tracer from free tracer, was purchased from Calbiochem Corporation (La Jolla, CA).

**Microdialysis in vivo experiments.** On the test day, the rat was placed into an individual plastic cage where it could move freely in an environmental room kept at 21 ± 0.3°C and 52 ± 2% relative humidity. A light-weight stainless steel spring tether (30.5 cm, M105, Instech Laboratories, Inc., Plymouth Meeting, PA) was connected to the screw captured in the head block by inserting the tether blade into the slot of the screw. A small tubular nut locked the tether in place. The upper end of the tether was attached to the clamp of a single-channel swivel (series 375, Instech Laboratories, Inc., Plymouth Meeting, PA) which was held on a lever arm. A length of PE-10 tubing was threaded through the tether and connected with the output of the swivel and the input cannula of the dialysis probe. The input of the swivel was then connected to a 1-ml tuberculin
syringe (Becton Dickinson & Co., Rutherford, NJ) clamped to a perfusion pump (Harvard Apparatus, Inc., South Natick, MA). The output of the probe was connected with a length of PE-10 tubing fed into a 1.5-m polyethylene collection tube which was mounted in a tube holder clipped on the tether just over the rat’s head. aCSF was continually perfused into the dialysis probe at a rate of 4 μl/min. After at least a 1-hr perfusion for equilibrium, dialysate samples were collected every 30 min for 240 min. Samples were frozen immediately after collection and stored at −70°C until assayed. The first three samples were collected as base-line for SP. Thenoxious cold stimulus was performed by placing the rat’s tail into a circulating cold water bath (model 9500, Fisher Scientific, Pittsburgh, PA) until the animal flicked its tail or reached cutoff time (20 sec/min), in a 1:1 mix of ethylene glycol/water maintained at −10°C. The cold stimulus was given five times during a 5-min test period. In some cases, a water bath of 25°C was also used as a control. The mu opioid receptor agonist PL017 (0.8–3.4 nmol), the kappa opioid receptor agonist Dyn (2.3–9.2 nmol) or aCSF was microinjected in a volume of 0.5 μl into the brain region through the side catheter attached to the dialysis probe 5 min before the noxious cold stimulus. In two groups, Nal (10 mg/kg) was administered s.c. 10 min before microinjection of PL017 or Dyn into the PAG.

**RIA.** In each RIA, standard curves were constructed for SP by use of known amounts of synthetic standards ranging from 0.1 to 24 fmol. The assay was carried out directly in the tubes used for collection of the dialysate fractions during microdialysis. The dialysate fractions (120 μl) and the SP standards in aCSF (120 μl) were reincubated with rabbit antiserum against SP (80 μl) for 24 hr at 4°C. After addition of [125I]Tyrothricin-SP (200–5000 cpm in 80 μl; approximate specific activity 81 TBq/mmol = 1480 μCi/μg), all samples were further incubated for 24 hr at 4°C. To separate bound from free tracer, the second antiserum was added to each assay tube at the end of incubation as recommended by the manufacturer. All assay tubes were vortexed briefly and centrifuged for 20 min at 700 × g by a refrigerated SUREspin centrifuge at 4°C. The supernatant was carefully decanted and the precipitate containing antibody-bound [125I]Tyrothricin-SP was counted in a gamma counter (NE-1600, Nuclear Enterprises, Scotland, UK). Levels of SP were then calculated by linear regression analysis derived from logarithmic transformation of data determined for known amounts of synthetic SP. Separate samples without standards and additional samples also without antibodies were incubated simultaneously to measure maximal tracer binding and unspecified binding, respectively. The detection limit of this assay was 0.2 fmol/250 μl.

**Microdialysis in vitro.** In vitro recovery of SP through the acrylonitrile membrane was determined by placing microdialysis probes in aCSF solution (200 μl, at 37°C bath) containing 1 nM synthetic SP. The probes were continuously perfused at a flow rate of 4 μl/min. After equilibrium, three 120-μl samples of perfusate were collected and assayed for SP by RIA as described above. The SP-containing aCSF solution was also assayed. The recovery of the probe membrane was determined by calculating the percentage of the SP concentration in the perfusate versus that in the medium solution. The in vitro recovery of this dialysis membrane for SP was 1.9 ± 0.6% (mean ± S.E.M., n = 6). The percentage of the amount of SP that may bind to the probe material or tubing was 0.2 ± 0.12% (mean ± S.E.M., n = 5). This was estimated by the difference between the SP amount in the input solution and the sum of SP amount in the both perfusate and medium solutions.

**Analgesia testing.** Separate experiments were conducted to investigate the analgesic effect of SP microdialyzed into the PAG or POAH of the rat. The microdialysis system used to deliver SP into the PAG or POAH was similar to that described above (see “Microdialysis in Vivo Experiments”) without the sample collecting and microinjecting procedures. At the beginning, aCSF was continuously perfused into the brain regions at 4 μl/min. The CWT was used to assess the analgesic effects of SP according to standard procedures in our laboratory (Tiseo et al., 1988). Animals were held firmly over the water bath (~10°C) and their tails submerging approximately halfway into the solution. The nociceptive threshold was taken as the latency until the rat removed or flicked its tail. Three latencies were measured: 60, 30 and 0 min before drug perfusion. For each animal, the first reading was discarded to minimize variability, and the remaining two were averaged to determine the base-line latency. After the predrug latency measurements, the input solution was switched to the SP-containing aCSF (27.1 fmol–271 pmol/μl) and was continuously perfused at 4 μl/min for 30 min. The latency to tail-flick was tested at the end of SP perfusion and 15, 30, 60 and 120 min after that. If an animal did not respond within 60 sec, the trial was terminated and a maximum latency of 60 sec was recorded. The analgesic effect of SP treatment was calculated for each rat as follows: percent maximum possible analgesia (% MPA) = [(postdrug latency − base-line latency)/(60-base-line latency)] × 100. In some cases, Nal in a dose of 10 mg/kg was injected s.c. 10 min before the CWT to investigate the effect of Nal injection alone on the tail-flick response. Testing was also carried out after pretreatment with Nal (10 mg/kg s.c.) 10 min before the low dose (27.1 fmol/μl) or high dose (271 pmol/μl) of SP perfusion.

**Tb response testing.** Effects on Tb of SP microdialyzed into the PAG or POAH were also investigated in separate experiments. The microdialysis procedure was the same as that used in the analgesia testing. Tb measurements were made according to standard procedures in our laboratory (Geller et al., 1986). After a 1-hr acclimation period, a thermistor probe (YSI series 400, Yellow Springs Instrument Co., Inc., Yellow Springs, OH) was lubricated and inserted approximately 7 cm into the rectum. The rat was lightly hand-held by the tail, but was otherwise free to move. Tb measurements were read from a digital thermometer (model 49 TA, YSI). The first three measurements were taken at 30-min intervals. To allow for adaptation to the procedure, the first reading was discarded and the subsequent two averaged to establish a base line. After the third measurement, the input solution was switched to SP-containing aCSF and perfused at 4 μl/min for 30 min. Tb was measured at the end of SP perfusion and 15, 30, 45, 60, 90 and 120 min later.

**Verification of dialysis probe placement.** At the end of the experiments, rats were placed for at least 10 min in a bucket containing dry ice within a metal mesh basket. The animals were almost instantaneously anesthetized by the carbon dioxide, rapidly asphyxiated and cooled. Their brains were excised and coronally cut; the visible track of the microdialysis probe was checked. In some cases, bromophenol blue (0.2%) was microdialyzed into the PAG or POAH for 30 min after the experiment ended. The brains were removed, rapidly frozen and stored at ~70°C. A block of tissue containing the track of the probe and the stain of the dialyzed dye was cut and checked (fig. 2). Data from rats in which the probes were not located within the PAG or POAH were not included in the results.

Fig. 2. Photographs of coronal sections of rat’s brain showing spread of bromophenol blue after 30 min dialysis into the PAG (A) and POAH (B). Arrows point to the edge of the dye stain. Horizontal bar = 1 mm in length. Ac, anterior commissure; aq, aqueductus; oc, optic chiasm; pag, periaqueductal gray; poah, preoptic anterior hypothalambus.
Data presentation and statistical analysis. The results for SP are reported as mean ± S.E.M. values of the measured dialysate concentrations. The results for the analgesic effect of SP are reported as % MPA (mean ± S.E.M.). The results for the Tb effect of SP are expressed as Tb changes (ΔT, mean ± S.E.M.) from base line. The changes of cold-evoked SP release in the groups pretreated with opioid agonists and Nal are expressed as the percentage of cold-evoked SP in the group without pretreatment. In the experiment that examined the effects of pretreatment with opioid agonists and Nal on cold-evoked SP release, data were evaluated by a one-way analysis of variance followed by a post hoc Fisher’s test. Other experimental data were analyzed by a repeated-measures analysis of variance model, in which factor 1 was treatment and factor 2 was time. The 5% level of probability was accepted as statistically significant.

Results

SP base-line release from the PAG and POAH. The average basal release of SP from the PAG was 0.43 ± 0.08 fmol/fraction during the 3-hr collection period (table 1). In the POAH, SP base-line release was 0.45 ± 0.06 fmol/fraction during the 3-hr collection period.

SP release from the PAG after noxious cold stimulation. SP release level was significantly increased to 1.3 ± 0.4 fmol/fraction during the collection period of 30 min after noxious cold stimulation and then returned to base-line level during the next collection period (fig. 3, top). In contrast, 25°C water did not produce any significant change in SP release from the PAG.

SP release from the POAH after noxious cold stimulation. Neither noxious cold nor 25°C water stimuli evoked any significant change in the level of SP released from the POAH (fig. 3, bottom).

Effects of pretreatment with PL017 in the PAG on SP release evoked by noxious cold. Although aCSF microinjection into the PAG did not affect the SP increase evoked by cold, PL017 microinjected into the PAG significantly reduced the SP release evoked by noxious cold in a dose-dependent manner (fig. 4, top). PL017, in a dose of 0.8 nmol, reduced the cold-evoked SP increase to an average 0.48 fmol/fraction increase over base-line, a 42% reduction compared with that in the aCSF-pretreatment group (0.84 fmol/fraction equals 100%). PL017, in dose of 1.7 nmol, reduced that SP increase 66% and 3.4 nmol, 89%, compared with that in the aCSF-pretreatment group. These doses of PL017 were capable of producing analgesia (40% MPA–85% MPA) in the CWT, as reported previously (Tiseo et al., 1988; Xin et al., 1993). Dyn, in a dose of 2.3 nmol, did not induce significant changes in cold-evoked SP increase.

Effect of Nal on the inhibition of cold-evoked SP release caused by PL017 and Dyn. Nal (10 mg/kg) administered s.c. 10 min before PL017 or Dyn microinjection reduced the inhibition of cold-evoked SP increase by these opioid agonists. The 89% reduction of SP increase caused by PL017 (3.4 nmol) was decreased to a 7% reduction when Nal was given before PL017 (fig. 4, top). The 87% reduction of SP increase caused by Dyn (9.2 nmol) was decreased to only 6% when Nal was given before Dyn (fig. 4, bottom).

Effects of SP microdialyzed into the PAG and POAH on the latency to tail-flick. The lowest dose (27.1 fmol/µl) of SP microdialyzed into the PAG produced a slight but not significant increase in latency to tail-flick in the CWT (fig. 5). After a 30-min perfusion at this dose, the amount of SP delivered into the PAG can be estimated at as much as 65 fmol, which is the mean amount of extracellular SP evoked by cold, according to the in vitro recovery rate of the probe for SP. However, higher doses of SP microdialyzed into the PAG produced a dose-related analgesia. SP in a dose of 271 fmol/µl fraction increase over base line, respectively, a 36 to 87% reduction compared with that in the aCSF-pretreatment group. These doses of Dyn were capable of producing analgesia (28% MPA–70% MPA) in the CWT, as reported previously (Tiseo et al., 1988; Xin et al., 1993).

Table 1

<table>
<thead>
<tr>
<th>Collection Period</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
<th>150 min</th>
<th>180 min</th>
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<tr>
<td>PAG (n = 4)</td>
<td>0.56 ± 0.08</td>
<td>0.37 ± 0.09</td>
<td>0.46 ± 0.10</td>
<td>0.38 ± 0.12</td>
<td>0.41 ± 0.10</td>
<td>0.39 ± 0.08</td>
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<tr>
<td>POAH (n = 3)</td>
<td>0.54 ± 0.09</td>
<td>0.42 ± 0.11</td>
<td>0.31 ± 0.12</td>
<td>0.36 ± 0.13</td>
<td>0.53 ± 0.08</td>
<td>0.41 ± 0.12</td>
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Fig. 3. (Top panel) Changes of SP release level (mean ± S.E.M.) in the PAG after noxious cold (−10°C, solid column, n = 6) or 25°C water (open column, n = 5) stimulation to the rat’s tail. (Bottom panel) Changes of SP release level (mean ± S.E.M.) in the POAH after noxious cold (solid column, n = 5) or 25°C water (open column, n = 4) stimulation to the rat’s tail. Arrow represents the time when the stimulation starts. *P < .05, compared with 25°C water stimulation group.
which is 10 times higher than the lowest dose, induced 30% MPA at 30 min after the end of SP perfusion; and SP, in a dose of 27.1 pmol/μl, induced 41% MPA at 15 min. SP, in doses of 271 pmol/μl, produced 35% MPA at the end of the perfusion and 65% MPA 15 min later. The analgesic effect lasted until 30 min after the end of perfusion (38% MPA).

However, SP, in doses which could produce analgesia when given into the PAG, did not induce any significant change in the tail-flick latency when it was microdialyzed into the POAH (fig. 6).

Effects of SP microdialyzed into the POAH on Tb. The same doses of SP (27.1–271 pmol/μl) that produced analgesia in the PAG failed to produce any significant change in Tb when microdialyzed into the PAG and POAH (fig. 8).

Discussion

It has been reported by our laboratory and others that SP release could be evoked from the spinal cord by noxious cold (Tiseo et al., 1990) or noxious mechanical (Kuraishi et al., 1983, 1985) stimuli. The increased SP level is selective for noxious cold and mechanical stimuli as there is no such effect on the cold-evoked SP increase in the PAG. The average increased SP level (0.84 fmol/fraction) evoked by the cold over base-line release (0.43 fmol/fraction) in the non-pretreatment group is taken as 100%. The average increased SP level in each pretreatment group is compared with that in the non-pretreatment group. $n = 4$ in each group. *P < .05.
increase during noxious heat (Kuraishi et al., 1985; Tiseo et al., 1990). The PAG is one of the brain regions that receives a major projection arising from the spinal cord lamina I pain-responsive neurons (Hylden et al., 1986). One study with retrograde labeling and in situ hybridization histochemistry demonstrated that 61% of the spinomesencephalic projection neurons (part of which distribute to the PAG) in lamina I of the spinal cord express preprotachykinin mRNA (which encodes tachykinin peptides, including SP), and this expression is significantly increased (80%) after peripheral inflammation, which indicates that SP plays an important role in nociception in ascending CNS pathways (Noguchi and Ruda, 1992). Other studies showed that the PAG also contains extensive SP-positive nerve terminals as well as cell bodies (Hökfelt et al., 1977; Moss and Basbaum 1983) and moderate-to-high concentrations of SP receptors (Quirion et al., 1983). In addition, reports indicated that tissue levels of SP in the PAG can be changed after administration of drugs (Brodin et al., 1983). The PAG is one of the brain regions that receives nociceptive input through ascending CNS pathways (Noguchi and Ruda, 1992). Other studies showed that the PAG also contains extensive SP-positive nerve terminals as well as cell bodies (Hökfelt et al., 1977; Moss and Basbaum 1983) and moderate-to-high concentrations of SP receptors (Quirion et al., 1983). In addition, reports indicated that tissue levels of SP in the PAG can be changed after administration of drugs (Brodin et al., 1983). Increased SP release within the PAG during cold stimulation may represent a nociceptive signal arising from the spinal cord to this region. This idea is supported by the present observation that there is an immediate increase of SP release (the first 30-min collection) from the PAG after noxious cold water, but not 25°C water, stimulation. On the basis of the present results, however, we could not determine whether the increased SP in the PAG was released by the nerve terminals projecting directly from the spinal cord or by the local SP-containing cells within the PAG which were secondarily excited by the spinal cord ascending projection.

Our observations that pretreatment with an analgesic dose of PL017 (fig. 4, top) or Dyn (fig. 4, bottom) inhibits the cold-evoked SP increase in this region and that this inhibition can be reversed by the opioid receptor antagonist Nal support the hypothesis that increased SP within the PAG carries noxious input. It has been proposed that the PAG is one of several brain regions involved in the modulation of nociceptive input through descending inhibitory neuronal processes that project to the spinal cord (Basbaum and Fields, 1984). Administration of opioids into the PAG (Jensen and Yaksh, 1986; Smith et al., 1992) or electrical stimulation of the PAG (Basbaum et al., 1977) produces antinoceception and reduces the firing of nociceptive dorsal horn neurons (Bennett and Mayer, 1979; Gebhart and Jones, 1988). Both mu and kappa opioid receptors exist within the PAG (Man-sour et al., 1995). Our previous reports have shown that activation of the mu and kappa opioid receptors in the PAG by administration of PL017 and Dyn, respectively, into this region produces dose-related analgesia in the CWT (Xin et al., 1993). These findings suggest that opioid administration suppresses the neuronal activities and the neurotransmitters which transmit pain input. A reasonable explanation for the inhibition of SP increase after pretreatment with PL017 and Dyn would be that increased SP release within the PAG represents the noxious information during the cold-water stimulation. Similar results have been reported in studies by Tiseo et al. (1988, 1990) in which i.c.v. injection of Dyn induced analgesia in the CWT and prevented the increase of SP released from the spinal cord during noxious cold. Also, Yaksh et al. (1980) reported a 4.9-fold increase in SP release from the spinal cord by activating the sciatic nerve and a naloxone-reversible inhibition of the SP release by morphine at concentrations known to elicit analgesia. Additional evidence for opioid action on SP levels in the PAG was reported by Rosen and Brodin (1989). They found that morphine, given systemically in doses that are known to produce increasing degrees of analgesia (3 or 10 mg/kg s.c.), dose-dependently elevates the tissue level of SP in the PAG of rats. The authors explained that one possible mechanism for the increased tissue level of SP in the PAG after morphine is an accumulation of the peptide caused by inhibition of its release. Our present results support this suggestion in that opioids inhibited the increased SP release evoked by noxious cold.

We further investigated the direct effect of SP within the PAG on pain threshold by microdialyzing different doses of SP into this region. SP, in a dose of 27.1 fmol/μl over a 30-min perfusion (which delivers a total of 65 fmol), induced a slight but nonsignificant increase in MPA (fig. 5). However, higher doses of SP (271 fmol/μl to 271 pmol/μl) produced a significant increase of MPA in a dose-related manner. The latter result is consistent with the reports from other laboratories (Stewart et al., 1976; Malick and Goldstein, 1978; Mohrland and Gebhart, 1979; Li et al., 1991), which suggests that these doses of SP within the brain have analgesic effects. An analgesic effect was also seen in an experiment conducted in mice with i.c.v. injection of 1.25 to 5 ng SP (Frederickson et al., 1978). Malick and Goldstein (1978) reported that the analgesic effect of SP microinjected into the PAG in rats was five times as potent as morphine on a weight basis (25 times as potent on a molar basis). Our present results show that SP microdialyzed into the rat’s PAG in a dose of 271 fmol/μl (cumulative dose, 650 fmol or 0.9 ng in the PAG, on the basis of the recovery rate) can induce analgesia. The lowest dose of SP (27.1 fmol/μl or 65 fmol total) was in the range of the estimated peak extracellular SP level in the PAG evoked by noxious cold (1.3 fmol/120 μl in dialysis samples or 65 fmol in the PAG, on the basis of the recovery rate). The failure of this dose to produce a significant analgesic effect could be explained in two ways. First, SP evoked by the cold may only present the noxious signal but not affect the pain threshold, so that the analgesic effect of higher doses of SP may be a pharmacological action. Second, the real amount of SP in the synapses evoked by noxious cold may be higher than that of estimated extracellular SP, because the latter was calculated based on the amount of SP that escaped from the synapses...
and was captured by the probe. Therefore, the lowest dose of SP microdialyzed into the PAG may not be high enough to mimic the real action of endogenous SP in the synapses. If this were true, SP in a dose 10 times higher than the lowest dose may be close to the physiological range of SP acting in the synapses during noxious cold. Because this dose of SP produced analgesia, the SP evoked by noxious cold not only signals noxious information to the PAG but also appears to further trigger the antinociceptive function of the PAG.

Reports showed that some types of noxious stimuli can inhibit neuronal and behavioral responses to noxious stimulation by activation of an opioid-mediated mechanism which involves the PAG network (Watkins and Mayer, 1986; Bodnar, 1986). These results led to a suggestion that the endogenous pain-suppression system, which includes the descending modulatory control pathways, can be activated by stimuli such as stress and pain (Fields and Basbaum, 1994). In the present experimental procedure, we attempted to minimize the influence of stress, such as allowing the rat to acclimate to the experimental environment, to move freely during the experiment and by administering SP by microdialysis without disturbing the animal and injecting opioids by microinjection at a slow speed and without animal restraint. Although handling of the animal during the test might be stressful, we compared the post-treatment data with the animal's own pretreatment base-line data. In this way, any significant difference between the pre- and post-treatment data is more likely caused by the effects of treatments, i.e., noxious cold stimulation and the drugs. In addition, the result that no significant change in SP release before and after the 25°C water test (in which the animal handling procedure was the same as that used in the −10°C water test) indicates that the significant increase of SP release during the −10°C water test is evoked by noxious cold itself rather than handling stress. Our present findings that noxious cold evokes SP release in the PAG and that SP given into the PAG induces analgesia provide further evidence for the hypothesis (Basbaum and Fields, 1984) that “pain inhibits pain,” i.e., pain itself is a critical factor that activates these pathways. SP-induced analgesia is related to the endogenous opioid system in the brain because it can be blocked by naloxone (Stewart et al., 1976; Malick and Goldstein, 1978; Stewart et al., 1982). Our preliminary data also showed that the selective mu opioid receptor antagonist CTAP (cyclic d-phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂) and the delta opioid receptor antagonist naltrindole can block or attenuate the analgesic effect of SP administered into the PAG (Xin et al., 1996). Because SP itself does not bind to the opioid receptor (Terenius, 1975), it is generally believed that it may be affecting opioid receptors indirectly through mediation of the release of endogenous opioid peptides. Malick and Goldstein (1978) suggested that the most likely candidates among the opioid peptides would be β-endorphin (because it has been shown to exhibit a potent long-lasting analgesic response and a rapid onset) and enkephalins (because antibodies to met-enkephalin also antagonize SP analgesia). Cell bodies and terminals containing these opioid peptides exist within the PAG (Watson and Barchas, 1979; Fallon and Leslie, 1986; Mansour et al., 1995). Indeed, we observed an increase of β-endorphin release from the PAG after both SP administration into this region and noxious cold stimulation, and this increase was attenuated by pretreatment with a SP receptor antagonist, which indicates involvement of this opioid peptide in SP analgesia (Xin et al., 1994). A possible mechanism for SP within the PAG interacting with opioid peptides and inducing analgesia has been proposed by Basbaum and Fields (1984). Their hypothesis is that SP, released either from local PAG neurons activated by ascending nociceptive pathways or directly from the terminals of those pathways, acts upon local opioid peptide neurons to cause release of opioid peptides. They, in turn, activate PAG output neurons by inhibiting an inhibitory interneuron. It should be mentioned that although SP evoked by noxious cold could trigger the antinociceptive function of the PAG, this function was still not strong enough to totally suppress the tail-flick response of the rat to cold water. Hence, the weak analgesic action of endogenous SP seems to be one of several feedback procedures involved in the response to noxious cold.

There are reports demonstrating that various SP metabolites can influence nociceptive responses (Larson and Sun, 1994; Stewart et al., 1982). For example, the major metabolite of SP, amino-terminal SP 1–7, is able to inhibit nociceptive behavior (Hall and Stewart, 1983; Larson and Sun, 1994) and this effect can be blocked by either Nal (Stewart et al., 1982) or mu and delta opioid receptor antagonists (Larson and Sun, 1993). On the other hand, the carboxyl terminal of SP, SP 7–11, exerts an opposite effect (Hall and Stewart, 1983). An in vitro study also shows that N-terminal SP 1–7 plays a role in modifying guanine nucleotide-modulated mu opioid receptor binding, whereas the C-terminal SP 5–11 is without effect (Krumins et al., 1993). These results suggest that, unlike peripheral effects of SP, which are mediated by receptors that recognize the C-terminal part of the SP molecule, certain central actions of SP are mediated by the receptors which recognize the N-terminal part of the SP molecule. It is possible that SP may be metabolized to this active fragment before its action at these receptors, which then produce the analgesic effect observed in our present results. However, on the basis of our experimental procedure, we cannot determine which fragment of SP is involved. The result that pretreatment with Nal blocks the analgesic effect of a high dose of SP (fig. 7) is in agreement with earlier findings from other laboratories (Stewart et al., 1976; Malick and Goldstein, 1978) and provides evidence that exogenous SP induces analgesia through the action of the endogenous opioid system. Because opioids generally produce an inhibitory effect on neurons whereas SP generally produces an excitatory effect, we further investigated whether endogenous SP itself could act differently to induce hyperalgesia when opioid receptors were blocked by Nal. The result showed that, at least in the PAG, endogenous SP evoked by noxious cold or SP microdialyzed into the PAG in a low dose close to the evoked-SP level did not cause hyperalgesia in Nal-pretreated rats (fig. 7). Indeed, SP given intrathecally produces hyperalgesia (Lembeck et al., 1981; Akerman et al., 1982; Vashpal et al., 1982; Dirig and Yaksh, 1996) and SP excites those neurons in the spinal cord responsive to noxious stimuli (Henry, 1976). Thus it appears that SP may play a role in sensory transmission within the spinal cord and that this role may be related specifically to pain. SP also produces excitation of neurons in the PAG (Ogawa et al., 1992) when given into this region. However, the functional consequence of the excitation produced by SP in the PAG and in the spinal cord seems to be different because reports show that excita-
tion of the PAG by SP or by electrical stimulation (Basbaum et al., 1977) induces analgesia, whereas excitation of the spinal cord by SP causes hyperalgesia (Lembeck et al., 1981; Akerman et al., 1982; Yaish et al., 1982; Dirig and Yaksh, 1996). One explanation for this difference may be the distinct neuronal networks in the PAG and the spinal cord, because the descending pain control system originates in the PAG and excitation of the PAG output neuron results in the initiation of the descending control. In addition, as mentioned above, a different distribution of SP receptors, which recognize the N or C terminals of SP distinctively in the PAG and the spinal cord may contribute to the different results of SP in the two regions. Therefore, it appears that SP at the PAG level does not cause pain but activates the descending control system through endogenous opioids to regulate pain.

SP release in the POAH did not change significantly during noxious cold stimulation (fig. 3), although SP and its receptors have been demonstrated in the POAH of rats (Panula et al., 1984; Quirion et al., 1983). Because microdialysis of SP into the POAH does not induce analgesia (fig. 6) and there is no change of SP level after noxious cold, it indicates that this region is less important than the PAG in the integrity of pain input and antinociception. The POAH is generally believed to be a primary site for central control of Tb and a region which contains the highest population of thermosensitive neurons (Boulant et al., 1989). Opioids can affect these neurons by either increasing or decreasing their firing rate to alter the set point and integrate corresponding afferent nociceptive input. The POAH may not only carry noxious signals to the PAG but also trigger the antinociceptive function of the descending pain suppression system. Finally, our results also demonstrate that SP does not affect Tb when it is microdialyzed into the POAH or PAG.

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References


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