Blockade of Delta Opioid Receptors in the Ventrolateral Periaqueductal Gray Region Inhibits the Fall in Arterial Pressure Evoked by Hemorrhage

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ABBREVIATIONS: viPAG, ventrolateral periaqueductal gray; PAG, periaqueductal gray; diPAG, dorsolateral periaqueductal gray; DLH, δ-homocysteic acid; CTP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide; nor-BNI, nor-binaltorphimine; bpm, beats per minute; DAMGO, [D-Ala², N-Me-Phe³, Gly⁵-ol]enkephalin.

ABSTRACT

Severe hemorrhage lowers arterial pressure by suppressing sympathetic activity. The central mechanism that initially triggers the fall in arterial pressure evoked by hemorrhage is not well understood, although opioid neurons are thought to play a role. This study tested the hypothesis that hemorrhagic hypotension is mediated by delta opioid receptors in the ventrolateral periaqueductal gray (viPAG), a region importantly involved in opioid analgesia. Depressor sites were first identified by microinjecting δ-homocysteic acid (20 nmol/0.1 μl) or β-endorphin (0.5 nmol/0.1 μl) into the viPAG of halothane-anesthetized rats. Consistent with earlier reports, δ-homocysteic acid injection into the caudal viPAG lowered arterial pressure and heart rate; β-endorphin evoked a comparable depressor response, but did not affect heart rate. Naloxone or selective opioid receptor antagonists were subsequently injected into the viPAG 5 min before hemorrhage (1.9 or 2.5 ml/100 g of body weight over 20 min) was initiated using the same stereotaxic coordinates. Naloxone injection into the caudal viPAG completely prevented the fall in arterial pressure evoked by hemorrhage. The response was dose-dependent and evident with both fixed volume and fixed pressure hemorrhage. The delta opioid receptor antagonist naltrindole inhibited hemorrhagic hypotension significantly in both conscious and anesthetized rats but mu and kappa receptor antagonists were ineffective. β-Endorphin³⁴, an endogenous opioid receptor antagonist, was also significantly inhibitory. Naltrindole was ineffective when injected into the dorsolateral periaqueductal gray and did not influence cardiovascular function in nonhemorrhaged animals. These data support the hypothesis that hemorrhagic hypotension is mediated by delta opioid receptors in the viPAG.

In 1979, Faden and Holiday reported that intravenous injection of naloxone, an opioid receptor antagonist, inhibited the fall in arterial pressure caused by hemorrhage in conscious rats (Faden and Holaday, 1979). Their data have since been reproduced in animal models of both hemorrhagic (Sandor et al., 1987; Schadt and Ludbrook, 1991; Ludbrook and Ventura, 1994) and endotoxic (D’Amato and Holaday, 1984) shock and have been shown to result from a central mechanism (Sandor et al., 1987; Schadt and Ludbrook, 1991; Owen et al., 1997) that involves delta, rather than mu or kappa, opioid receptors (D’Amato and Holaday, 1984; Ludbrook and Ventura, 1994). The finding that opioid receptor blockade prevents hemorrhagic hypotension was initially controversial, however, because opioid receptor antagonists produce little or no change in cardiovascular function in normotensive animals (Gordon, 1986; Morilak et al., 1990) and because delta opioid receptor agonists produce hypertension when administered centrally to conscious animals (May et al., 1989), not the hypotension predicted by the effects of delta receptor antagonists in hemorrhage. It thus appears that naloxone prevents hemorrhagic hypotension through a central mechanism that is different from the baroreceptor reflex that normally regulates cardiovascular homeostasis.

This conclusion is consistent with extensive evidence that severe hemorrhage initially lowers arterial pressure through a central mechanism that overrides the baroreceptor reflex (Schadt and Ludbrook, 1991). Although mild hemorrhage activates the baroreflex, which thus maintains arterial pressure within normal limits, severe hemorrhage produces the opposite response; it abruptly inhibits sympathetic activity and thereby initiates the precipitous fall in arterial pressure that ultimately leads to hemorrhagic shock (Schadt and Ludbrook, 1991). The ability of delta receptor antagonists to prevent the sympathoinhibition caused by hemorrhage means that opioid peptide neurons play a pivotal role in the response. But little is known about the anatomical pathway that initially triggers the sympathoinhibitory phase of hem-
orrage and neither the identity nor the location of the opioid
neurons that mediate the response is known.

This study tested the hypothesis that naloxone inhibits
hemorrhagic hypotension by blocking delta opioid receptors
in the ventrolateral column of the midbrain periaqueductal
gray (vIPAG) region. The vIPAG is densely innervated by
opioid peptide neurons (Khachaturian et al., 1985; Martin-
Schild et al., 1999) and has long been known to play an
important role in the antinociception caused by severe stress
and injury (Lovick, 1993). More recent investigations indi-
cate that antinociception is but one component of a coor-
dinated autonomic and behavioral repertoire that is generated
by the PAG. Activation of neurons in the vIPAG with excitato-
yary amino acids produces hypotension, bradycardia, and
sympatho-inhibition accompanied by behavioral quiescence,
hyporeactivity, and opioid-dependent antinociception (Lov-
wick, 1993; Bandler et al., 2000). Similarly, visceral and deep
somatic pain and injury lower arterial pressure and heart
rate, produce behavioral quiescence, and activate vIPAG neu-
rons, as evidenced by expression of the immediate/early gene,
c-fos (Clement et al., 1996). In marked contrast to the vIPAG,
activation of the lateral and dorsolateral PAG (dIPAG) col-
umns with excitatory amino acids produces hypertension,
tachycardia, and behavioral activation, and dIPAG neurons
express c-fos in response to somatic, rather than visceral,
pain (Lovick, 1993; Bandler et al., 2000). These findings are
consistent with the hypothesis that hemorrhagic hypotension
is mediated by the vIPAG, although not the dIPAG.

We tested this hypothesis, in an earlier study, by deter-
mining whether inhibition of neuronal activity in the vIPAG
with lidocaine would prevent the fall in arterial pressure
evoked by hemorrhage. Microinjection of lidocaine into the
caudal vIPAG delayed the onset and reduced the degree of
hypotension produced by hemorrhage (Cavun and Milling-
ton, 2000). Hemorrhagic hypotension was also attenuated by
coalt chloride which, unlike lidocaine, does not inhibit ax-
onal conductance and thus demonstrates that synaptic trans-
mission within the vIPAG is necessary for hemorrhage to
lower arterial pressure. Neither lidocaine nor cobalt chloride
influenced cardiovascular function in normotensive animals.

These data support the concept that the vIPAG is a compo-
ext of a descending anatomical pathway that is activated by
hemorrhage but does not participate in the tonic regulation of
cardiovascular homeostasis.

Here, we report that bilateral injection of naloxone into the
caudal vIPAG essentially abolished the fall in arterial pres-
sure evoked by hemorrhage. Naltrendole, a selective delta
opioid receptor antagonist, was also effective, but mu and
kappa receptor antagonists were inactive. These data provide
additional evidence that the vIPAG is a critical component of
a descending pathway that initiates hemorrhagic hypoten-
sion and show that delta opioid receptors in the vIPAG play
an important role in the response.

Materials and Methods

Animals and Surgery. Male Sprague-Dawley rats (250–300 g;
Sasco, Inc., Omaha, NE, or Taconic Farms, Germantown, NY) were
housed under a 12-h light/dark cycle with free access to food and
water. Rats were anesthetized with halothane (1.5–4% in 100% O₂),
and the right carotid artery was cannulated with PE-50 tubing filled
with heparinized saline (100 U/ml). The cannula was exteriorized at
the nape of the neck and sealed until use. At the beginning of each
experiment, the cannula was attached to a volumetric pressure
transducer and arterial pressure and heart rate were recorded at
1-min intervals using a MicroMed BPA-200 blood pressure analyzer
(Micro-Med, Louisville, KY). Body temperature was not monitored or
controlled. Experiments with conscious animals were conducted 4 to
6 h after rats recovered from anesthesia. The animal protocols were
conducted in accordance with the National Institutes of Health
Guide for the Care and Use of Laboratory Animals.

DL-Homocysteic Acid (DLH) and β-Endorphin Injections. Depressor sites were identified by injecting DLH, β-endorphin, or
saline (pH 7.3) unilaterally into the vIPAG. Halothane-anesthetized
rats were positioned in a stereotoxic apparatus, and a 32-gauge
stainless steel cannula was lowered vertically through a small cra-
niotomy 0.8-mm lateral and 8.3-mm posterior to bregma to a depth of
6.2 mm below the skull surface according to the atlas of Paxinos
and Watson (1998). DLH (20 nmol/0.1 μl of saline; Sigma Chemical Co.,
St. Louis, MO) was injected over a 1-min period, and arterial pres-
sure and heart rate were recorded for 15 min. If no change in arterial
pressure occurred, the injection cannula was lowered 0.2 mm and the
injection was repeated; up to three DLH injections were made in the
same animal. Criteria included a greater than 5% change in, and
restoration to, baseline arterial pressure. When a depressor site was
identified, β-endorphin (0.5 nmol/0.1 μl of saline; Peninsula Labora-
tories, Belmont, CA) was injected into the same site.

Opioid Receptor Antagonist Injections and Hemorrhage. Rats were anesthetized with halothane and two 26-gauge guide
cannulae were implanted bilaterally in the caudal or rostral vIPAG or the caudal dIPAG at a 27° rostro-caudal angle. The tips of the
guide cannulae were positioned 0.8-mm lateral and 8.3-mm posterior
to bregma and 6.2-mm below the skull surface for caudal vIPAG
injections, 0.8-mm lateral and 7.3-mm posterior to bregma and 6.2-
mm below the skull surface for rostral vIPAG injections, and 0.8-
millimeter lateral and 8.3-mm posterior to bregma and 4.6-mm below
the skull surface for caudal dIPAG injections (Paxinos and Watson,
1998). After obtaining stable baseline arterial pressure and heart rate
measurements, naloxone HCl (1, 3, 10, or 100 nmol), naltrindole HCl
(0.2, 2, or 20 nmol), d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr amide
(CTOP; 1 or 10 nmol), nor-binaltorphimine dihydrochloride (nor-
BNI; 1 or 6 nmol) (all from Sigma Chemical Co., St. Louis, MO), or
saline was injected bilaterally (one-half the dose in each cannula) in
a volume of 0.5 μl of 0.9% saline (pH 7.3) delivered at a constant rate
over a 1-min period. The injection volume was monitored by observ-
ing the movement of an air bubble placed in the tubing. Five minutes
later, hemorrhage was initiated by withdrawing blood (1.9 ml or 2.5
ml/100 g of body weight) through the carotid cannula over a 20-min
period. At the end of each experiment, the injection site was marked
with 0.1 μl of India Ink for DLH and β-endorphin injections or 0.5
μl of India Ink for opioid receptor antagonist experiments. The brain
was removed, immersed in 10% paraformaldehyde, imbedded with
paraffin, sectioned (50 μm) with a sliding microtome, and stained
with eosin.

Statistical Analyses. Data were analyzed by analysis of variance
followed by Dunnett’s multiple comparisons test or two-tailed Stu-
dent’s t test. The criteria for statistical significance was P < 0.05.

Results

Microinjection of DLH or β-Endorphin into the
VIPAG Lowers Arterial Pressure. The hypothesis that
microinjection of delta opioid receptor antagonists into the
VIPAG will inhibit hemorrhagic hypotension is based on the
assumption that hemorrhage stimulates opioid peptide re-
lease from vIPAG neurons. This hypothesis is supported by
evidence that vIPAG administration of a delta receptor selec-
tive agonist lowers arterial pressure, whereas mu receptor
agonists generate a pressor response and kappa agonists
produce little or no consistent effect (Keay et al., 1997). The β-endorphin- and enkephalin-related peptides that serve as endogenous ligands for delta opioid receptors also display relatively high affinity for mu receptors, however (Paterson et al., 1983), which raises the possibility that β-endorphin- and enkephalin-related peptides could produce little or no net effect on arterial pressure in the vIPAG. We therefore tested whether β-endorphin affects arterial pressure or heart rate when microinjected into DLH-responsive depressor sites in the caudal vIPAG.

DLH (20 nmol/0.1 μl) injection produced a small but significant reduction in mean arterial pressure (−9.5 ± 1.6 mm Hg; range = −5.4 to −25.6 mm Hg) and heart rate (−20 ± 6.9 beats per minute [bpm]; range = +9 to −58 bpm) (Table 1) in 41% of sites tested, consistent with previous reports (Carrive and Bandler, 1991; Keay et al., 1997). The maximum fall in arterial pressure occurred 2.2 ± 0.2 min following DLH injection. The duration of the response was 6.0 ± 0.6 min.

β-Endorphin (0.5 nmol/0.1 μl) injection into DLH-responsive vIPAG sites evoked a depressor response comparable in magnitude to that following DLH (Table 1) in 58% of sites tested. The maximal fall in arterial pressure (−10.6 ± 1.9 mm Hg; range = −5.9 to −17.3 mm Hg) was slower to develop (4.6 ± 0.9 min) and longer in duration (11.6 ± 2.6 min) than following DLH. Analysis of variance revealed a significant effect of DLH and β-endorphin treatment on arterial pressure [F(2, 28) = 19.87, P < 0.01] and heart rate [F(2, 28) = 6.90, P < 0.01]; post hoc analysis showed that β-endorphin did not change heart rate significantly (Table 1). Intra-arterial naloxone (1 mg/kg) injection prevented the hypotension evoked by β-endorphin (+4.0 ± 1.7 mm Hg), but not DLH (−16.7 ± 3.4 mm Hg). β-Endorphin thus produced naloxone-reversible hypotension, but not bradycardia, in the caudal vIPAG.

**Naloxone Prevents Hemorrhagic Hypotension in the vIPAG.** Subsequently, we tested, in a separate group of animals, whether naloxone injection at the same vIPAG coordinates would prevent the fall in arterial pressure evoked by hemorrhage. During fixed volume hemorrhage (1.9 ml/100 g of body weight), arterial pressure was sustained at baseline levels initially, but it began to fall after 10 min and reached −37.8 ± 1.7 mm Hg below initial baseline measurements by the end of the 20-min hemorrhage period (Fig. 1). Heart rate was not affected by hemorrhage and did not differ significantly from baseline values (340 ± 17 bpm) at the end of the 20-min hemorrhage period (328 ± 44 bpm).

Naloxone (10 nmol/0.5 μl; 5 nmol/cannula) injection into the caudal vIPAG prevented the fall in arterial pressure evoked by hemorrhage completely (Fig. 1). Analysis of variance revealed a significant effect of naloxone treatment [F(1, 11) = 11.58, P < 0.01], time [F(18, 198) = 24.0, P < 0.001], and a significant treatment-time interaction [F(18, 198) = 10.0, P < 0.001] on mean arterial pressure (Fig. 1). The response was dose-related between 1 and 100 nmol (0.5–50 nmol/cannula) [F(4, 26) = 26.1, P < 0.001]; the minimal dose necessary to inhibit hemorrhagic hypotension significantly was 1 nmol (Fig. 2). Unilateral injection of 10 nmol of naloxone in a smaller injection volume (0.1 μl) was ineffective (data not shown). Heart rate was not affected significantly by any naloxone dose during hemorrhage (data not shown). Naloxone (100 nmol) did not change arterial press-

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Mean Arterial Pressure (mm Hg)</th>
<th>Change in Heart Rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (12)</td>
<td>0 ± 0.7</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>DLH (12)</td>
<td>−9.5 ± 1.6*</td>
<td>−20 ± 7*</td>
</tr>
<tr>
<td>β-Endorphin (7)</td>
<td>−10.6 ± 1.9*</td>
<td>3 ± 2</td>
</tr>
</tbody>
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* P < 0.01, differs from control.
sure or heart rate in nonhemorrhaged control animals (data not shown). Bilateral naloxone administration into the vPAG thus produces a dose-related inhibition of hemorrhage-induced hypotension without affecting cardiovascular function in normotensive animals.

Naloxone was also evaluated in fixed-pressure hemorrhage (Sandor et al., 1987). Rats were pretreated with naloxone (10 nmol; 5 nmol/cannula) and, 5 min later, sufficient blood was withdrawn to lower arterial pressure to approximately 50 mm Hg (n = 6). Following naloxone pretreatment, a significantly larger blood volume was required (2.3 ± 0.1 ml; P < 0.01) to lower arterial pressure to a comparable extent (from 86.6 ± 3.4 to 54.9 ± 0.5 mm Hg; n = 6). This shows that naloxone attenuates hemorrhagic hypotension in both fixed volume and fixed pressure models of controlled hemorrhage.

**Hemorrhagic Hypotension is Inhibited by Delta, but Not Mu or Kappa, Opioid Receptor Antagonists.** The finding that vPAG naloxone administration inhibits hemorrhagic hypotension supports the hypothesis that hemorrhage activates vPAG opioid receptors but does not establish whether delta, mu, or kappa receptors mediate the response because naloxone is not receptor selective. Previous studies have shown that intracerebroventricular (i.c.v.) administration of delta, but not mu or kappa, opioid receptor antagonists inhibit hypovolemic (Ludbrook and Ventura, 1994) and endotoxic (D’Amato and Holaday, 1984) hypotension. Receptor selectivity is thus a necessary criterion for establishing that opioid receptors in the vPAG mediate hemorrhagic hypotension. To establish this, we tested whether hemorrhagic hypotension is inhibited by naltrindole, a delta opioid receptor antagonist, CTOP, a mu receptor antagonist, or nor-BNI, a kappa receptor antagonist.

For these and subsequent experiments, hemorrhage was induced by withdrawing 2.5 ml/100 g of body weight, a somewhat larger volume than used for naloxone experiments (1.9 ml/100 g of body weight). The fall in arterial pressure induced by this hemorrhage protocol was greater in magnitude and longer in duration (Fig. 3) than the more transient hypotension produced by mild hemorrhage (Fig. 1). This, more severe hemorrhage protocol, also generated a significant reduction in heart rate [F(13,70) = 3.37, P < 0.001] (Fig. 4) in halothane-anesthetized rats, in contrast to mild hemorrhage, which did not affect heart rate (data not shown).

Naltrindole microinjection (0.2, 2.0, or 20 nmol) into the vPAG bilaterally (0.1, 1.0, or 10 nmol/cannula) inhibited the hypotension (Fig. 3) and bradycardia (Fig. 4) evoked by hemorrhage significantly. The higher naltrindole doses (2 and 20 nmol) not only reduced the magnitude, but also delayed the onset and shortened the duration of hemorrhagic hypotension. Analysis of variance confirmed that the effects of naltrindole treatment [F(4,21) = 96.6, P < 0.001], time [F(16,357) = 17.9, P < 0.001], and treatment-time interaction [F(64,357) = 3.1, P < 0.001] were significant. Naltrindole also prevented the bradycardia induced by hemorrhage in anesthetized rats [F(4,21) = 19.2, P < 0.001] (Fig. 4), although time and treatment-time effects were not significant. The highest naltrindole dose (20 nmol) prevented hemorrhage-induced bradycardia completely, although it did not elevate heart rate significantly above baseline, prehemorrhage values (Fig. 4). Naltrindole administration had no effect on arterial pressure (Fig. 3) or heart rate (Fig. 4) in nonhemorrhaged animals. In contrast, vPAG microinjection of CTOP (1 or 10 nmol), a mu opioid receptor antagonist, or nor-BNI (1 or 6 nmol), a kappa receptor antagonist, had no effect whatsoever on the hypotension (Fig. 5) or bradycardia (data not shown) evoked by hemorrhage. Hemorrhagic hypotension is thus inhibited by microinjection of delta, but not mu or kappa, receptor antagonists into the vPAG.

**β-Endorphin1-27 Administration into the vPAG Inhibits Hemorrhagic Hypotension.** We also tested whether
hemorrhagic hypotension is inhibited by \( \beta \)-endorphin\(^{1-27} \), \( \beta \)-Endorphin\(^{1-27} \) is an endogenous \( \beta \)-endorphin-derived peptide that inhibits the antinociception produced by \( \beta \)-endorphin administration into the PAG (Nicolas and Li, 1985; Tseng and Tang, 1990; Monroe et al., 1996). The receptor mechanism responsible for the inhibitory effects of \( \beta \)-endorphin\(^{1-27} \) is controversial, however (Monroe et al., 1996). Receptor binding experiments indicate that \( \beta \)-endorphin\(^{1-27} \) displays relatively high affinity for both mu and delta opioid receptors (Nicolas and Li, 1985; Monroe et al., 1996), although some functional studies suggest that \( \beta \)-endorphin\(^{1-27} \) blocks a distinct, \( \beta \)-endorphin-specific epsilon receptor thought to mediate \( \beta \)-endorphin-elicited antinociception in the PAG (Tseng and Tang, 1990).

Microinjection of \( \beta \)-endorphin\(^{1-27} \) (3 nmol) into the vPAG 5 min before initiating hemorrhage (2.5 ml/100 g of body weight) inhibited the subsequent fall in arterial pressure significantly. Hemorrhage alone lowered arterial pressure by \(-70 \pm 5 \text{ mm Hg (n = 5)} \) by the end of the 20-min hemorrhage period in saline-treated controls. \( \beta \)-Endorphin\(^{1-27} \) pretreatment reduced the fall in arterial pressure significantly; mean arterial pressure fell \(-45 \pm 2 \text{ mm Hg (n = 5)} \) \((P < 0.01) \) by the end of the hemorrhage period. \( \beta \)-Endorphin\(^{1-27} \) also inhibited the response to mild hemorrhage (1.9 ml/100 g of body weight); control = \(-37.8 \pm 1.6 \text{ mm Hg (n = 7)} \); \( \beta \)-endorphin\(^{1-27} \) = \(-22.0 \pm 2.9 \text{ mm Hg (n = 5)} \); \((P < 0.01) \). \( \beta \)-Endorphin\(^{1-27} \) did not affect arterial pressure in nonhemorrhaged control animals (2 \pm 2 mm Hg; \( n = 3) \).

**Naltrindole Injection into the vPAG Prevents Hemorrhagic Hypotension in Conscious Rats.** Thus far, the data show that delta opioid receptor antagonists inhibit hemorrhagic hypotension in halothane-anesthetized rats, but anesthetics can influence the cardiovascular response to hypovolemia markedly (Schadt and Ludbrook, 1991; Owen et al., 1997). To determine whether halothane anesthesia is a significant complicating factor, we tested whether naltrindole inhibits hemorrhagic hypotension in conscious animals. We found that hemorrhage (2.5 ml/100 g of body weight over 20 min) produced essentially the same effect on arterial pressure in conscious rats (Fig. 6) as it did in halothane-anesthetized animals (Fig. 3). In both conscious and halothane-anesthetized rats, arterial pressure was initially sustained at baseline values, began to fall within 10 min, reached approximately the same low levels by the end of the 20-min hemorrhage period (conscious rats = \(-63.4 \pm 2.8 \text{ mm Hg (n = 7)} \); anesthetized rats = \(-66.3 \pm 3.6 \text{ mm Hg (n = 7)} \), and then increased spontaneously, although it did not achieve baseline values by the end of the experiment. The effect of hemorrhage on heart rate was different in conscious than in halothane-anesthetized rats, however. Severe blood loss had no significant effect on heart rate in conscious rats (Fig. 6) but produced a sustained bradycardia in halothane-anesthetized animals (Fig. 4).

Naltrindole injection (0.2, 2, or 20 nmol) bilaterally into the vPAG of conscious rats inhibited the hypotension caused by hemorrhage significantly (Fig. 6). The response was dose-related and essentially the same as observed in halothane-anesthetized rats. Statistical analysis confirmed that the effects of naltrindole treatment \( F(4, 25) = 170.8, P < 0.001 \), time \( F(16, 366) = 42.4, P < 0.001 \) and treatment-time interaction \( F(64, 366) = 3.1, P < 0.001 \) were significant. Naltrindole had no overall effect on heart rate, although the highest naltrindole dose (20 nmol) produced a mild tachycardia. Analysis of variance indicated that the effect of naltrindole treatment on heart rate was significant \( F(4, 13) = 10.4, P < 0.001 \), but time and treatment-time interactive effects were not, and post hoc analysis did not reveal a significant treatment effect at any individual time point. These data indicate that the effects of naltrindole on arterial pressure during hemorrhage are not dependent on concurrent anesthesia.

**Effects of Naltrindole in the Rostral vPAG and Caudal dPAG.** Next, we tested whether opioid receptor antagonists inhibit hemorrhagic hypotension only in the caudal vPAG or if they are also effective in the rostral vPAG. The vPAG is viscerotopically organized to the extent that rostral and caudal vPAG regions regulate different vascular beds (Carrive and Bandler, 1991), although activation of either vPAG region with excitatory amino acids lowers arterial pressure and heart rate (Carrive and Bandler, 1991; Keay et al., 1997) (A. D. Evec and M. M. Rapaco-Baker, unpublished observations). Figure 7 shows that bilateral naltrindole in-
jection (2 nmol; 1 nmol/cannula) into the rostral vlPAG inhibited hemorrhagic hypotension significantly (P < 0.01) in conscious rats. The response was comparable in magnitude to caudal vlPAG naltrindole injection. Heart rate was not affected significantly by naltrindole in either the rostral (data not shown) or caudal (Fig. 6) vlPAG. Naltrindole thus inhibits hemorrhage-induced hypotension in either the rostral or caudal vlPAG.

In contrast to the vlPAG, excitatory amino acid injection into the lateral or dlPAG produces hypertension and tachycardia (Bandler et al., 2000). This makes it unlikely that activation of delta opioid receptors in the dlPAG triggers the hypotension that results from hemorrhage. To test this assumption, we injected naltrindole (2 nmol) bilaterally (1 nmol/cannula) into the caudal dlPAG 5 min before initiating hemorrhage. Dorsolateral PAG naltrindole injection had no effect on arterial pressure (Fig. 7) or heart rate (data not shown). Thus, delta opioid receptors in the vlPAG, but not the dlPAG, mediate hemorrhagic hypotension.

Discussion

Naloxone and delta opioid receptor antagonists inhibit hemorrhagic hypotension through a central mechanism, presumably by interrupting synaptic transmission by opioid peptide neurons in an anatomical pathway that inhibits sympathetic neuronal activity. In this study, we tested the hypothesis that the opioid receptors that initiate the fall in arterial pressure caused by hemorrhage are located in the vlPAG. We found that naloxone injection into the caudal vlPAG essentially abolished hemorrhage-induced hypotension. Naloxone was effective at doses at least an order of magnitude lower than required to inhibit hemorrhagic hypotension

**Fig. 6.** The effect of vlPAG naltrindole administration on arterial pressure and heart rate following hemorrhage in conscious rats. Naltrindole (0.2, 2 or 20 nmol) or saline (0.5 μl) was injected bilaterally (one-half the dose per cannula) into the vlPAG of conscious rats 5 min before hemorrhage (2.5 ml blood/100 g of body weight over 20 min) was initiated. Mean arterial pressure (MAP; top panel) and heart rate (bottom panel) were recorded at 5-min intervals. Numbers in parentheses indicate the number of animals in each group. Baseline mean arterial pressure values at the −25 min time point were: saline = 125.0 ± 2.5 mm Hg; naltrindole 0.2 nmol = 123.5 ± 3.2 mm Hg; naltrindole 2.0 nmol = 129.6 ± 3.6 mm Hg; naltrindole 20 nmol = 130.1 ± 4.3 mm Hg; naltrindole-treated nonhemorrhage control = 111.2 ± 11.2 mm Hg; saline-treated nonhemorrhaged control = 130.5 ± 4.3 mm Hg. Baseline heart rate values were: control = 380 ± 10 bpm; naltrindole 0.2 nmol = 305 ± 20 bpm; naltrindole 2.0 nmol = 358 ± 19 bpm; naltrindole 20 nmol = 368 ± 17 bpm; and saline-treated nonhemorrhage control = 395 ± 17 bpm. *P < 0.05, **P < 0.01 differs from control.

**Fig. 7.** Naltrindole inhibits hemorrhagic hypotension in the caudal and rostral vlPAG, but not the dlPAG. Naltrindole (2 nmol) was injected bilaterally (1 nmol/cannula) into either the caudal vlPAG (left), rostral vlPAG (middle), or caudal dlPAG (right), and hemorrhage was induced 5 min later by withdrawing 2.5 ml/100 g of body weight blood over a 20-min period. Columns depict the change in mean arterial pressure (MAP) at the end of the 20-min hemorrhage period (n = 4–7). **P < 0.01 differs from controls.

**Fig. 8.** Schematic representation of coronal sections through the rostral (~7.3 mm from bregma) and caudal (~8.3 mm from bregma) PAG illustrating the drug and peptide injection sites. Aq = Sylvian aqueduct.
tension following i.c.v. injection (Owen et al., 1997), which makes it unlikely that naloxone acts by diffusing from the vIPAG to a distant site. Hemorrhagic hypotension was also inhibited by naltrindole, but not by CTOP or nor-BNI, which confirms that delta opioid receptors mediate the response. Naltrindole did not affect arterial pressure or heart rate in normotensive animals, consistent with evidence that opioid neurons are specifically activated by hemorrhage, but do not contribute substantially to the tonic regulation of arterial pressure (Gordon, 1986). These findings support the concept that activation of delta opioid receptors in the vIPAG plays a pivotal role in the response to hemorrhage.

Naltrindole is a relatively selective opioid receptor antagonist with an affinity for delta receptors at least 100-fold higher than for mu and kappa receptors (Portoghese et al., 1988). It is thus unlikely that naltrindole inhibits hemorrhagic hypotension through a nonselective receptor mechanism. To further rule out this possibility, we tested whether the mu and kappa receptor selective antagonists, CTOP (1 and 10 nmol) and nor-BNI (1 and 6 nmol), inhibit the response to hemorrhage following vIPAG administration at doses comparable with the naltrindole doses (0.2–20 nmol) we found to be effective. Neither drug had any discernible effect. Their lack of efficacy was not attributable to dose inequivalence because the relative affinity of CTOP ($K_i = 0.16$ nM) (Hawkins et al., 1989) and nor-BNI ($K_i = 0.06$ nM) (Emmerson et al., 1994) for mu and kappa receptors is higher than the affinity of naltrindole for delta receptors ($K_i = 1.4$ nM) (Emmerson et al., 1994). It seems safe to conclude, therefore, that naltrindole inhibits hemorrhagic hypotension by blocking delta opioid receptors in the vIPAG, and that mu and kappa receptors do not participate in the response.

This conclusion is consistent with a report by Keay et al. that microinjecting the delta receptor agonist [d-Pen]$^2$, [d-Pen]$^5$]enkephalin (DPE) into the vIPAG lowers arterial pressure and heart rate (Keay et al., 1997). [d-Pen]$^2$, [d-Pen]$^5$]Enkephalin was effective in approximately 35% of tested sites located throughout the rostro-caudal extent of the vIPAG and extending into the lateral and dIPAG. The mu receptor agonist [d-Ala]$^2$-[N-Me-Phe]$^4$-[Gly]-ol]enkephalin (DAMGO) produced the opposite response, hypertension and tachycardia, and the kappa receptor agonist, U69,593, generated a small but inconsistent depressor and bradycardic effect. The present data extend these findings by demonstrating that vIPAG delta receptors function endogenously in the regulation of arterial pressure during hemorrhage.

The finding that delta receptors in the vIPAG influence cardiovascular function is somewhat unexpected because delta receptor binding densities (Mansour et al., 1987) and mRNA levels (Kalyuzhny and Wessendorf, 1998) are quite low in the vIPAG. Furthermore, both opioid- and stress-induced antinociception are mediated primarily, if not exclusively, by mu receptors in the vIPAG, not delta receptors (Yaksh, 1997). This conclusion is derived from microinjection studies showing that selective delta receptor agonists have no effect at all on nociceptive response latencies following vIPAG administration (Smith et al., 1988; Yaksh, 1997). Subsequent studies did show, however, that delta agonists inhibit neuropathic pain (Sohn et al., 2000) and potentiate DAMGO-induced antinociception (Rossi et al., 1994) following vIPAG administration, which suggests that vIPAG delta receptors may influence nociception under specific circumstances. But despite conflicting evidence, it would appear that endogenous opioids normally influence pain perception and cardiovascular function through different receptor mechanisms, mu and delta receptors, respectively, in the vIPAG.

The opioid peptide neurons that mediate hemorrhagic hypotension remain to be identified. The vIPAG contains relatively high amounts of immunoreactive β-endorphin and met-enkephalin (Khachaturian et al., 1985), both of which serve as endogenous agonists for delta opioid receptors (Paterson et al., 1983), as well endomorphin- (Martin-Schild et al., 1999) and dynorphin- (Khachaturian et al., 1985) related peptides, which, in general, are selective for mu and kappa receptors, respectively. Although there is evidence that both β-endorphin- and met-enkephalin-releasing neurons modulate pain perception in the PAG (Yaksh, 1997), a report by Sandor et al. (1987) implicates β-endorphin-release neural nuclei as exclusive regulators of cardiovascular function during hemorrhage. They found that i.c.v. injection of a β-endorphin antiserum inhibits, and β-endorphin potentiates, the hypotension produced by hemorrhage, whereas met-enkephalin antiserum or peptide administration are ineffective (Sandor et al., 1987).

Nevertheless, the prospect that either β-endorphin or met-enkephalin lowers arterial pressure in the vIPAG seems paradoxical because these peptides display a high affinity for both mu and delta receptors (Paterson et al., 1983), which produce opposite effects on cardiovascular function in the vIPAG (Keay et al., 1997). But we found that β-endorphin generated a depressor response in the vIPAG equivalent in magnitude to, although longer in duration than, that produced by DLH. The reason for β-endorphin’s unexpected efficacy is not readily apparent, although it may be due to differences in the cellular location of mu and delta receptors in the vIPAG (Kalyuzhny and Wessendorf, 1998). Alternatively, β-endorphin may lower arterial pressure through a receptor mechanism that does not involve mu or delta receptors, as shown previously for β-endorphin-induced antinociception (Tseng and Tang, 1990; Monroe et al., 1996). The finding that β-endorphin$^{1–27}$ inhibits hemorrhagic hypotension may provide support for this contention. Tseng and Tang (1990) reported that β-endorphin$^{1–27}$ selectively inhibits the antinociception produced by β-endorphin, but not morphine, in the PAG, suggesting that it blocks a β-endorphin-specific receptor; contradictory evidence has also been reported, however (Monroe et al., 1996). In either case, the present data support the hypothesis that endogenous opioid peptides contribute to the fall in arterial pressure caused by hemorrhage, despite their lack of selectivity for delta receptors.

Anatomically, the vIPAG is strategically situated to mediate the effects of hemorrhage. Ventrolateral PAG neurons densely innervate the midline raphe nuclei, raphe obscurus, and pallidus (Cameron et al., 1995; Henderson et al., 1998). Activation of neurons in the midline raphe nuclei and adjacent regions of the caudal midline medulla lowers arterial pressure and heart rate and inhibits sympathetic neuronal activity (Coleman and Dampney, 1995; Henderson et al., 1998). The midline raphe influence cardiovascular function both directly, by innervating preganglionic sympathetic neurons in the intermediolateral cell column, and indirectly, through axonal projections to the rostral ventrolateral medulla (RVLM) pressor and caudal ventrolateral medulla (CVLM) depressor regions (Romagnano et al., 1991; Lovick, 1998).
Delta Antagonists in the PAG Inhibit Hemorrhagic Hypotension

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References

Cardioregulatory and pain control pathway and microinjection of opioids into the midline medulla that precipitates hemorrhagic hypotension. Ventrolateral PAG neurons also innervate the rostral ventrolateral medulla and the caudal ventrolateral medulla directly (Cameron et al., 1995; Chen and Aston-Jones, 1996; Henderson et al., 1998; Keay et al., 2000), which raises the possibility that multiple descending pathways may be influenced by hemorrhage. The caudal medulla is thus an important component of a descending pathway that conveys the effects of vPAG activation to pre-ganglionic sympathetic neurons, although other cardioregulatory brainstem regions may also be involved.

Opioid receptor antagonists may act at multiple locations in this descending pathway. Ang et al. (1999) recently that intrathecal injection of naloxone or a delta opioid receptor antagonist inhibits hemorrhagic hypotension without affecting resting arterial pressure or heart rate. The conclusion that opioid receptors in the spinal cord mediate the effects of hemorrhage (Ang et al., 1999) is supported by evidence that enkephalinergic neurons in the midline raphe nuclei innervate pre-ganglionic sympathetic neurons in the spinal cord (Romagnano et al., 1991). Microinjection of a delta opioid receptor antagonist into the caudal medulla depressor region also attenuates the effects of hemorrhage (Henderson et al., 1999). These findings support the hypothesis that hemorrhage stimulates opioid peptide release at multiple sites in a descending cardio-inhibitory pathway that includes the vPAG, caudal medulla, and intermediolateral cell column. In this way, the descending cardio-inhibitory pathway that precipitates hemorrhagic hypotension is similar to the descending pain control pathway that inhibits pain perception during periods of extreme stress (Yakh, 1997). Opioid receptors are situated strategically in the vPAG, midline raphe nuclei, and spinal cord in both the cardio-inhibitory and pain control pathway and microinjection of opioid receptor agonists and/or antagonists influence both nociception and cardiovascular function in each of these three regions. The spatial location of opioid receptors and receptors in the two descending pathways may, in part, explain the simultaneous hypotension and antinoceptive generation evoked by severe pain, serious injury, and hemorrhagic shock.

References


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