Neuronal activity within the ventrolateral periaqueductal gray during simulated hemorrhage in conscious rabbits

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Schadt, James C., Heidi L. Shafford, and Michael D. McKown. Neuronal activity within the ventrolateral periaqueductal gray during simulated hemorrhage in conscious rabbits. Am J Physiol Regul Integr Comp Physiol 290: R715–R725, 2006. First published September 29, 2005; doi:10.1152/ajpregu.00374.2004.—The ventrolateral (vl) periaqueductal gray (PAG) has been proposed as a site responsible for the active process triggering the onset of hypotension (i.e., phase 2) during blood loss in conscious animals (Cavun S and Millington WR, Am J Physiol Regul Integr Comp Physiol 281: R747–R752, 2001). We recorded the extracellular activity of PAG neurons in conscious rabbits to test the hypothesis that vIPAG neurons change their firing frequency before the onset of hypotension during simulated hemorrhage. Arterial and venous catheters, an intrathoracic vena caval occluder, and midbrain microelectrodes on a microdrive were implanted in 10 rabbits. During simulated hemorrhage, the occluder was inflated until arterial pressure ≤40 mmHg. We compared changes in neuronal activity during simulated hemorrhage with those during a similar length control period for 64 vIPAG and 29 dorsolateral (dl) PAG neurons. Arterial pressure pulse modulation of neuronal activity was present in 45 and 76% of vIPAG and dlPAG neurons, respectively. When we evaluated the absolute change in activity, thus accounting for both increases and decreases, simulated hemorrhage had a significant effect on activity of vIPAG but not dlPAG neurons. The majority (56%) of vIPAG neurons did not appear to respond to simulated hemorrhage. Of the 28 responsive vIPAG neurons, 11 showed an abrupt change in firing frequency during the time interval preceding the onset of hypotension; 13 responded after the onset of hypotension; and 4 showed a consistent direction of change across the entire simulated hemorrhage. Thus 24 (38%) of the vIPAG neurons recorded responded at a time consistent with a contribution to the hypotension associated with simulated hemorrhage.

The cardiovascular response to blood loss in conscious animals is biphasic (38). Phase 1 is characterized by maintenance of arterial pressure primarily through sympathetic activation. Although renin release increases during phase 1, there is no increase in release of vasopressin or adrenal catecholamines (38). At a critical point during continued blood loss, arterial pressure drops precipitously, initiating phase 2. This phase 2 hypotension is accompanied by a profound sympathoinhibition. An important question from a clinical and physiological point of view is what triggers the rapid fall in blood pressure during hemorrhage. The transition to phase 2 appears to be an active process, rather than simply a result of threshold volume depletion or exhaustion of compensatory mechanisms. For example, exposure of conscious animals to stressful sensory stimuli during hemorrhage can prolong phase 1, thus extending maintenance of arterial pressure in the face of a larger volume loss (36, 37). In addition, phase 2 hypotension is reversed or blocked by a variety of compounds that act on endogenous systems (38; e.g., endogenous opioids (10)), indicating the animal still has compensatory reserve at the time arterial pressure falls (13).

Active modulation of sympathetic activity, and ultimately blood flow and arterial pressure during hypotensive hemorrhage, occurs within the central nervous system. Thus it has been proposed that the ventrolateral (vl) periaqueductal gray (PAG) triggers the onset of hypotension (4) and sympathoinhibition (6) characteristic of phase 2. Any central nervous system area involved in triggering the onset of hypotension during blood loss might manifest certain characteristics. For example, activation of the area might result in hypotension and sympathoinhibition. In anesthetized rats, chemical activation of the vIPAG with excitatory amino acids results in hypotension, bradycardia (3, 14, 15, 18), and sympathoinhibition (1). Similarly, blockade of this area should alter the response to blood loss. Here, it is known that chemical inactivation of the vIPAG in conscious and anesthetized rats during hemorrhage delays or prevents phase 2 hypotension and sympathoinhibition (4, 6). Some central nervous system transmitter systems have been implicated in the biphasic response to blood loss [e.g., endogenous opioids and serotonin (38)]. These transmitters should be anatomically and functionally represented in the vIPAG or somewhere along the pathway that ultimately produces hemorrhagic hypotension. For the vIPAG to be involved in cardiovascular control during blood loss, vIPAG neurons should receive, directly or indirectly, sensory information related to cardiovascular function. Two potential indicators of such sensory input might be some evidence of modulation of vIPAG neuronal activity coincident with the cardiac cycle or a response to increases or decreases in arterial pressure. Finally, there should be changes in activity of neurons in this area during blood loss.

The goal of this study was to evaluate the response of vIPAG neurons to simulated hypotensive hemorrhage (21) in conscious, chronically prepared rabbits. If the vIPAG is important in the initiation of hypotension during phase 2, then vIPAG neurons should modify their discharge rate immediately before the onset of hemorrhagic hypotension. Thus we hypothesized that individual vIPAG neurons would change their firing frequency before the onset of hypotension during simulated hemorrhage. We recorded responses of dorsolateral PAG (dlPAG) neurons to simulated hemorrhage as an anatomic control.

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METHODS

Animals and Instrumentation

General. All procedures were approved by the University of Missouri Animal Care and Use Committee and conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals [DHHS (DHHS) Publication No. (NIH) 85–23, revised 1985, Office of Science Health Reports, DRR/NIH, Bethesda, MD 20205]. Thirteen New Zealand White rabbits (4 female) weighing 2.75 ± 0.09 (SE) kg were chronically prepared.

Anesthesia, analgesia, and recovery. Ten rabbits underwent the following three surgical procedures: a laparotomy, a thoracotomy, and a craniotomy. At least 2 wk were allowed between procedures. Three additional rabbits underwent the laparotomy and thoracotomy but no craniotomy. Antibiotics (22.7 mg enrofloxacin sc; Baytril; Bayer) were administered the day before surgery. Food but not water was withheld 15–20 h before surgery. For the laparotomy, anesthesia was induced and maintained with halothane in oxygen via face mask (5% for induction, 0.5–3.5% for maintenance). Anesthesia for the thoracotomy or craniotomy was induced with pentobarbital sodium (total dose 42 ± 3 and 49 ± 5 mg/kg iv Nembutal, respectively; Abbott). After induction with pentobarbital sodium, the rabbit was orally intubated and mechanically ventilated with room air. Adequacy of anesthesia was assessed by the absence of withdrawal or blood pressure changes in response to noxious stimuli. During thoracotomy and craniotomy, supplemental pentobarbital sodium for maintenance of anesthesia was given as needed (12 ± 2 and 40 ± 6 mg/kg iv, respectively). The analgesic buprenorphine hydrochloride (0.06 mg sc Buprenex; Reckitt Benckiser) was administered to all rabbits after each surgery. Rabbits were allowed to recover a minimum of 2 wk after the last surgery before the start of experiments.

Laparotomy. A midline laparotomy was performed for implantation of venous (vena cava) and arterial (aorta) catheters (12). Catheters were tunneled under the skin and exteriorized at the base of the neck. We used the venous and arterial catheters for injection of drugs and recording of arterial pressure, respectively.

Thoracotomy. A right lateral thoracotomy was performed at the third intercostal space for placement of an intrathoracic vena cava occluder. The cuff of the pneumatic occluder was placed around the caudal vena cava. Tubing enabling inflation of the occlusive balloon was tunneled under the skin and exteriorized at the base of the neck. Inflation of the occluder simulated hemorrhage by reducing venous return (21). Rapid, manual inflation of the occluder was performed three times a week during recovery to limit connective tissue attachments.

Craniotomy. Rabbits were mounted in a rabbit stereotaxic frame (David Kopf). The head was leveled and fixed with lambda 1.5 mm below bregma. Four stainless steel machine screws (1–72 × 1/8 in.; Small Parts) were implanted in the skull and covered with dental acrylic (Stoelting). A midline craniotomy was performed, and the dura was opened to expose the cortex. A bundle of electrodes attached to a microdrive was implanted in the midbrain. The electrode bundle consisted of 10 nichrome microwires (5 each of 32 and 64 μm diameter; California Fine Wire; see Ref. 11). The blunt, cut end of the wire served as the recording surface of the electrode. A single ground electrode was implanted in the subcutaneous layers of the neck. Each microwire, in addition to the ground electrode, was connected to a multipin plug (Viking TKR24–101-V). The hole in the skull was filled with rehydrated absorbable gelatin powder (Gelfoam; Pharmacia & Upjohn) and then covered with dental acrylic. The dental acrylic head plate was shaped to secure the microdrive and electrode plug and to incorporate the four screws. The stereotaxic location of the electrode bundle at the time of implantation was 1 mm lateral to midline (right = 5 animals; left = 5 animals), 10.5–11 mm caudal to bregma, and 10.5–12 mm below bregma (skull zero). One turn of the microdrive advanced the electrode bundle 0.3 mm.

Experimental Procedures

General. All experiments were performed on conscious rabbits accustomed to sitting quietly in a box that restricted their movement (33 × 15 × 18 cm). Animals were fasted for 15–20 h before each experiment. We performed experiments as often as every day in individual rabbits. On the day of the experiment, rabbits were heparinized (sodium heparin, 2,000 U iv; Elkins-Sinn), the arterial catheter was connected to a pressure transducer, and the electrode plug was connected to a flexible cable. Arterial blood pressure and extracellular neuronal activity were monitored on an oscilloscope and recorded using commercially available software (Spike2; CED). An audio speaker was used to monitor neuron activity. Heart rate was triggered on-line by pulsatile arterial pressure. Heart rate and blood pressure were allowed to stabilize before each experiment. The experimental protocol we used involved a control period, the simulated hemorrhage, and, finally, responses to intravenous pressor and depressor agents.

Multiple neuron extracellular recording. We recorded the voltage difference across a pair of microwire electrodes. A custom-built amplifier (10×; incorporated into head plug) and switch box allowed us to select electrode combinations displaying neuron activity. The presence of one or more spikes with >2:1 signal-to-noise ratio was required for recording from a particular electrode combination. Neuron activity was amplified (gain 1,000–10,000), filtered (0.5–10 kHz bandpass), and digitized (1401 system; CED). We acquired one or two channels of neuron activity at 10–20 kHz.

Software (Spike2; CED) was used to generate templates of spike waveforms off-line. The threshold for spike detection was set at two times the noise level. Principal component analysis was applied to the templates and used to aid in spike discrimination. Templates were considered to represent single unit activity if principal component analysis yielded a discrete cluster and the minimum interspike interval was >1 ms (27). For cases in which the interspike interval was <1 ms and spikes could not reliably describe the activity of a single unit, the templates were considered to represent a population of neurons with similar waveforms. Fifteen of 64 vlPAG and 2 of 29 dlPAG neurons were classified as multiple units. In this study, we describe the activity of single and multiple unit populations and refer to both as “neuron” activity.

Simulated hemorrhage/occlusion. A typical simulated hemorrhage experiment with midbrain recording is illustrated in Fig. 1. Before each experiment, we rapidly inflated the vena cava occluder to free the Silastic balloon of connective tissue. During the experiment, the balloon was slowly inflated using a syringe pump (Sage Instruments model 351). We attempted to inflate the occluder at a rate that would mimic changes in arterial pressure and heart rate observed during blood loss in conscious rabbits (36, 37, 39). When mean arterial pressure fell to 40 mmHg, the rate of inflation of the vena cava occluder was reduced to maintain mean arterial pressure ≥40 mmHg for 1 min. Pressure in the occluder was then released, allowing arterial pressure to return rapidly to normal. The time required to produce hypotension varied both within a rabbit and among rabbits. Therefore, for analysis, we normalized the time for the simulated hemorrhage from 0 through 120% of total occlusion. The beginning of the simulated hemorrhage (0 in Fig. 1) was defined as the point when
heart rate first began to increase. The time point when mean arterial pressure reached 40 mmHg was defined as 100%. We evaluated neuronal activity, mean arterial pressure, and heart rate at 0, 20, 40, 60, 80, 100, and 120% of total occlusion (see Fig. 1, for example). The 120% point was after the precipitous decrease in arterial pressure and, thus, during the hypotensive period. The value taken at each point was the average from 10% before to 10% after the indicated point (e.g., the 20% values were the average between 10 and 30% of occlusion). The average elapsed times between 0 and 100% of total occlusion in 39 vlPAG and 18 dlPAG recording sessions were 117 ± 110 and 113 ± 18 s, respectively. Control measurements in each experiment were taken over a similar time period immediately before the period of simulated hemorrhage.

Response of neurons to simulated hemorrhage/occlusion. We first calculated a baseline firing frequency for each neuron as the average firing frequency over the entire control period (see above). We then subtracted the baseline value from the actual value at each point during the occlusion and control periods. We used this “change from baseline” value to analyze the change in firing frequency during the control and occlusion periods for an individual neuron.

Pulse modulation of neuronal activity. Using the arterial pressure pulse as a trigger, we constructed cardiac cycle-triggered histograms of neuron activity, as well as cardiac cycle autocorrelation histograms. We overlaid and compared these histograms and visually assessed the existence of pulse modulation during the control period. Pulse modulation was defined as the presence of visible peaks in the cycle-triggered histogram of unit activity coincident with or at a constant distance from peaks in the cardiac cycle autocorrelation histogram (33).

Neuron barosensitivity. A subset of vlPAG (n = 41) and dlPAG (n = 17) neurons was tested for sensitivity to pharmacological changes in blood pressure. Cardiovascular variables and neuronal activity were recorded for 1 min before and 2 min after intravenous injection of a pressor (10 μg/kg phenylephrine; Sigma) or depressor (0.1 mg/kg sodium nitroprusside; Fisher Scientific; or 80 μg/kg nitroglycerin, Nitrostat; Parke-Davis) agent. All injection volumes equaled 0.2 ml/kg and were followed by 2 ml heparinized (10 U/ml) 0.9% sodium chloride flush.

Histology. The end of the electrode track was marked using the Prussian blue reaction. Iron ions were deposited at the electrode tips (50 μA DC for 30 s; brain electrode positive). Rabbits were killed with an overdose of pentobarbital sodium (780 mg iv Sleepaway; Fort Dodge Animal Health) and perfused transcardially with 250 ml of 0.9% sodium chloride followed by 1 liter of 15% potassium ferrocyanide in 10% formaldehyde. The result was a blue reaction product at the site of the electrode tips. The brain was removed and stored in 10% formaldehyde followed by 15% sucrose in 10% formaldehyde. Frozen sections (40 – 60 μm) were stained with neutral red and evaluated every 100 μm. The blue dot at the bottom of the electrode track was used as the reference to back-calculate the location of recording sites. A horizontal line bisecting the cerebral aqueduct was used to define the border between the dlPAG and vlPAG. The shape of the PAG and anatomical landmarks (e.g., red nucleus, rostral and caudal colliculi) were used to determine the relative rostrocaudal location of each recording site (43).

Statistical analysis. We used two-way, repeated-measures ANOVA to compare results between control and simulated hemorrhage. We analyzed changes from baseline in neuronal firing frequency as raw
changes (i.e., positive and negative values) and as the absolute value of the changes (i.e., all changes treated as positive values). For the ANOVA, change in firing frequency (raw and absolute values), mean arterial pressure, or heart rate was the dependent variable. The independent variables were time (i.e., percent of total occlusion from 0 to 120%) and treatment (i.e., control or simulated hemorrhage). We used a Chi-square analysis to test for proportional differences between the vlPAG and dlPAG in terms of pulse modulation. Significance was set at $P < 0.05$. Data are presented as means ± SE. The SE was calculated from the pooled estimate of the sample variance calculated in the ANOVA.

RESULTS

Simulated Hemorrhage/Occlusion

We compared the hemodynamic effects of simulated hemorrhage (3/rabbit) with actual blood loss (2/rabbit) in three rabbits (Fig. 2). Simulated hemorrhage or actual blood loss produced similar biphasic changes in mean arterial pressure ($P = 0.99$), heart rate ($P = 0.99$), and hindquarters ($P = 0.62$) or cranial mesenteric vascular conductance ($P = 0.85$).

Vena caval occluders failed in 2 of the 10 rabbits implanted with midbrain recording electrodes. Thus we evaluated the response of 93 PAG neurons (64 vlPAG and 29 dlPAG) to simulated hemorrhage during 57 experiments (39 vlPAG and 18 dlPAG) in 8 rabbits (3–25 neurons/rabbit). The anatomical distribution of vlPAG and dlPAG recording sites is shown in Fig. 3. Mean values for heart rate and mean arterial pressure during the 57 simulated hemorrhage and corresponding control periods are shown in Table 1. Simulated hemorrhage resulted in a typical biphasic response (e.g., Fig. 1). Mean arterial pressure was relatively well maintained through ~80% of the occlusion (phase 1). This period of relative pressure maintenance was followed by an abrupt decline (phase 2) starting at ~80% and reaching a nadir near 120% of the simulated hemorrhage. Heart rate increased gradually during simulated hemorrhage and reached a maximum ~80% before decreasing back toward preocclusion levels. Mean arterial pressure and heart rate did not change during the control (i.e., no occlusion) period preceding the simulated hemorrhage.

vlPAG Recordings

The control firing frequency for vlPAG neurons ranged from 0.1 to 111 Hz (mean ± SE = 18 ± 3 Hz; $n = 64$). To evaluate changes in firing frequency during simulated hemorrhage, we subtracted the baseline firing rate (i.e., the average firing rate during the control period) from the observed value at each point (i.e., from 0 through 120%) during the control and simulated hemorrhage periods. This produced control and simulated hemorrhage values reflecting the change from baseline in firing frequency (Fig. 4A). We then statistically compared the control period with the simulated hemorrhage period using ANOVA. Neither the main effects of time (i.e., percent of total occlusion, $P = 0.8$) and treatment (i.e., control or occlusion) nor the interaction of time with treatment ($P = 0.98$) was significant. Thus, as a group, the firing rate for the recorded vlPAG neurons did not change during simulated hemorrhage.

One possible reason for the apparent lack of response of vlPAG neurons during simulated hemorrhage could be the variable direction of the response in individual neurons. For

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example, a decrease in firing in one neuron might cancel out an increase in another. To address this possibility, we considered the absolute value of changes from baseline (Fig. 5A). The interaction of time and treatment had a significant effect (P = 0.007) on the absolute change in firing frequency in vlPAG neurons. Here, the significant interaction effect indicates a nonparallel relationship between data from the control and that from the simulated hemorrhage period. Thus there was a significant effect of simulated hemorrhage on neuronal firing. We chose to test this single global hypothesis rather than make multiple comparisons of individual points (19, 20).

Our initial examination of the data suggested that the baseline discharge frequency might have some predictive value in terms of a neuron’s response to simulated hemorrhage (42). Therefore, we divided the vlPAG neurons into the following three frequency groups: low, ≤3 Hz, n = 25; middle, >3 and ≤15 Hz, n = 18; and high, >15 Hz, n = 21. The average baseline discharge frequencies for the low-, mid-, and high-frequency groups were 1.3 ± 0.2, 6.9 ± 0.6, and 45 ± 7 Hz.

### Table 1. Hemodynamic values during simulated hemorrhage

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Occlusion, %</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
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<tbody>
<tr>
<td>vIPAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>occlusion</td>
<td>MAP, mm Hg</td>
<td>66±1</td>
<td>64</td>
<td>64</td>
<td>62</td>
<td>56</td>
<td>41</td>
<td>32</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>142±3</td>
<td>183</td>
<td>225</td>
<td>261</td>
<td>272</td>
<td>257</td>
<td>242</td>
<td></td>
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<tr>
<td>vIPAG control</td>
<td>MAP, mm Hg</td>
<td>66±1</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>139±4</td>
<td>138</td>
<td>135</td>
<td>137</td>
<td>136</td>
<td>137</td>
<td>134</td>
<td></td>
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<tr>
<td>dIPAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>occlusion</td>
<td>MAP, mm Hg</td>
<td>62±1</td>
<td>59</td>
<td>60</td>
<td>59</td>
<td>55</td>
<td>40</td>
<td>32</td>
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<tr>
<td>HR, beats/min</td>
<td>152±5</td>
<td>194</td>
<td>218</td>
<td>241</td>
<td>263</td>
<td>250</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td>dIPAG control</td>
<td>MAP, mm Hg</td>
<td>65±1</td>
<td>65</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>142±5</td>
<td>144</td>
<td>141</td>
<td>141</td>
<td>142</td>
<td>142</td>
<td>143</td>
<td>140</td>
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</table>

Values are means for the simulated hemorrhages and respective control periods during 57 recording sessions [39 ventrolateral (vl) periaqueductal gray (PAG) and 18 dorsolateral (dl) PAG]. Mean ± SE values (from pooled estimate of sample variance in ANOVA) for each row are shown in the “0” column. The ANOVA demonstrated a significant interaction effect between treatment (occlusion or control) and %total occlusion for mean arterial pressure [MAP; F(6, 494); P < 0.00009] and heart rate [HR; F(6, 494); P < 0.00009] for the vIPAG and for MAP [F(6, 221); P < 0.00009] and HR [F(6, 221); P < 0.00009] for the dIPAG. Analyses were done separately for vIPAG and dIPAG.

![Fig. 3. Rostrocaudal distribution of the vIPAG (○) and dorsolateral (dl) PAG (□) recording sites drawn on representative transverse sections of the rabbit midbrain (43). The PAG was subdivided into the dl-PAG and vIPAG using a horizontal line that bisected the cerebral aqueduct (AQ). RC, rostral colliculus; RN, red nucleus; ST, supratrochlear nucleus; III, oculomotor nucleus; IV, trochlear nucleus.](image)

![Fig. 4. Average change in firing rate (Firing) for vIPAG (A) and dIPAG (B) neurons during simulated hemorrhage (Occlusion) and control plotted against the %total occlusion. The change in firing was calculated by subtracting the baseline value (average for control) from the measured value at each point during occlusion or control. MAP changes are shown for reference. Mean ± SE values (from pooled estimate by ANOVA) are shown on the first symbol of each curve. See METHODS for details. The repeated-measures ANOVA failed to show a significant interaction between treatment (occlusion or control) and %total occlusion for vIPAG [F(6, 819) = 0.2, P > 0.98] or dIPAG [F(6, 364) = 0.5, P > 0.8] neurons. In addition, neither of the main effects was significant for either group.](image)
The interaction of time and treatment significantly affected the absolute change in firing frequency in mid ($P = 0.01$) and high ($P = 0.04$)-frequency neurons, consistent with an effect of simulated hemorrhage on neuronal firing. Although it did not reach statistical significance, there appeared to be a trend for an interaction effect in low-frequency neurons as well ($P = 0.09$).

Although the response of individual PAG neurons to simulated hemorrhage was quite variable, we identified five general patterns of response as follows: late increase, late decrease, steady increase, steady decrease, and no change in firing frequency (Figs. 1 and 6). Late-increase or -decrease neurons were characterized by changes in firing rate that began rather abruptly sometime after 40% of the occlusion period (Fig. 6, A and B). The firing rate for the steady-increase or -decrease neurons began changing near the beginning of the occlusion (Fig. 6, C and D). The fifth response type included those neurons that appeared to be unresponsive to occlusion (e.g., either neuron in Fig. 1). The number of vlPAG neurons in each of these groups is given in Table 2. The majority (56%) of the vlPAG neurons tested did not show a systematic change in discharge during simulated hemorrhage. The remaining 44% showed one of the four patterns of systematic change during simulated hemorrhage. In this group of responsive neurons, 11 also showed changes consistent with some role in triggering the fall in arterial pressure (e.g., Fig. 6, A and C). That is, they altered their discharge in some recognizable way immediately before or coincident with the point during simulated hemorrhage at which arterial pressure began its precipitous decline (i.e., ~80% of the total occlusion). Some neurons changed well after the transition to phase 2 (e.g., Fig. 6B) or exhibited a steady change across hemorrhage (e.g., Fig. 6D). These neurons were regarded as responding to the simulated hemorrhage or hypotension but not as potentially responsible for the precipitous fall in arterial pressure.

**dlPAG Recordings**

The control firing frequency for dlPAG neurons ranged from 1.4 to 69 Hz (mean ± SE = 12 ± 3 Hz, $n = 29$). In considering changes in firing frequency of dlPAG neurons during simulated hemorrhage, the main effects of time ($P = 0.75$) and the interaction effect of time and treatment ($P = 0.82$) were not significant. Therefore, the discharge of dlPAG neurons as a group was not affected by simulated hemorrhage (Fig. 4B).

To address the possibility of different directional changes limiting our ability to detect responsive dlPAG neurons, we considered the absolute value of changes during simulated hemorrhage (Fig. 7A). The main effects of time ($P = 0.04$) and treatment ($P < 0.0001$) were significant, but their interaction ($P = 0.34$) was not. This statistical result is not consistent with an effect of simulated hemorrhage on dlPAG neuronal activity.

Considering the relatively broad range of resting discharge rates observed in the dlPAG (like the vlPAG), we divided the dlPAG neurons into the following two frequency groups: low, ≤10 Hz, $n = 19$; and high, >10 Hz, $n = 10$. The average, baseline discharge frequencies for the low- and high-frequency dlPAG neurons were $4.4 ± 0.5$ and $27 ± 7$ Hz, respectively. The results of this analysis are shown in Fig. 7, B and C. The
main effect of time was not significant for the low (P = 0.15)- or high (P = 0.09)-frequency groups. The main effect of treatment was significant for both low (P = 0.0001)- and high (P = 0.001)-frequency dlPAG neurons. The interaction of time and treatment did not significantly affect the change in firing in low (P = 0.99)- or high (P = 0.20)-frequency dlPAG neurons. These results suggest that any effect of simulated hemorrhage was constant over the course of the vena caval occlusion. Therefore, as a population, it is unlikely that dlPAG neurons contributed to the transition from phase 1 to phase 2 (i.e., neuron that showed a prominent change in firing around the beginning of the drop in arterial pressure at 60–80% of the occlusion).

Pulse Modulation of Neuronal Activity

An example of arterial pulse-modulated activity is shown in Fig. 8. Pulse modulation of neuronal activity was present in 29 of 64 vlPAG units and 22 of 29 dlPAG units. Thus a smaller proportion of vlPAG units (45%) than dlPAG units (76%) demonstrated pulse-modulated activity (P = 0.01).

Neuron Barosensitivity

We tested 41 vlPAG and 17 dlPAG units for barosensitivity. Injection of phenylephrine increased mean arterial blood pres-

Table 2. PAG neuronal response to simulated hemorrhage

<table>
<thead>
<tr>
<th>Frequency Group</th>
<th>Late Increase</th>
<th>Late Decrease</th>
<th>Steady Increase</th>
<th>Steady Decrease</th>
<th>No Change</th>
<th>Total Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>vlPAG ≤3 Hz</td>
<td>1 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>17</td>
<td>18 (0)</td>
</tr>
<tr>
<td>&gt;3 and ≤15 Hz</td>
<td>7 (3)</td>
<td>5 (3)</td>
<td>1 (0)</td>
<td>1 (0)</td>
<td>11</td>
<td>25 (6)</td>
</tr>
<tr>
<td>&gt;15 Hz</td>
<td>6 (2)</td>
<td>2 (0)</td>
<td>3 (2)</td>
<td>2 (1)</td>
<td>8</td>
<td>21 (5)</td>
</tr>
<tr>
<td>Total</td>
<td>14 (5)</td>
<td>7 (3)</td>
<td>4 (2)</td>
<td>3 (1)</td>
<td>36</td>
<td>64 (11)</td>
</tr>
<tr>
<td>dlPAG ≤10 Hz</td>
<td>4 (0)</td>
<td>1 (1)</td>
<td>1 (0)</td>
<td>1 (0)</td>
<td>12</td>
<td>19 (1)</td>
</tr>
<tr>
<td>&gt;10 Hz</td>
<td>1 (1)</td>
<td>2 (0)</td>
<td>3 (2)</td>
<td>2 (1)</td>
<td>8</td>
<td>10 (1)</td>
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<tr>
<td>Total</td>
<td>5 (1)</td>
<td>3 (1)</td>
<td>2 (0)</td>
<td>2 (0)</td>
<td>17</td>
<td>29 (2)</td>
</tr>
</tbody>
</table>

Values are no. of PAG neurons in various categories. Late increase, increase in firing after 40% of occlusion (Fig. 6A); late decrease, decrease in firing after 40% of occlusion (Fig. 6B); steady increase, gradual increase in firing starting before 40% of occlusion (Fig. 6C); steady decrease, gradual decrease in firing starting before 40% of occlusion (Fig. 6D). Nos. in parentheses indicate no. of neurons that showed discharge characteristics consistent with triggering phase 2 (i.e., neuron that showed a prominent change in firing around the beginning of the drop in arterial pressure at 60–80% of the occlusion).
sure 41 ± 3 mmHg and decreased heart rate 59 ± 7 beats/min. Administration of nitroprusside or nitroglycerin decreased mean arterial blood pressure 20 ± 3 mmHg and increased heart rate 166 ± 9 beats/min. Only two vlPAG and no dlPAG neurons appeared to be sensitive to pharmacological changes in arterial pressure (data not shown). These two neurons responded to decreases in arterial pressure by increasing their discharge rate.

DISCUSSION

We recorded extracellular unit activity in the vlPAG and dIPAG in conscious, chronically prepared rabbits during simulated hemorrhage. This approach yielded new information, uncomplicated by anesthesia, related to the potential role of the midbrain in integrative cardiovascular control during simulated hemorrhage. First, during simulated hemorrhage, the activity pattern of some populations of vlPAG neurons is altered in a way consistent with a role in initiating the active transition to phase 2 (i.e., to hypotension). Second, other populations of vlPAG neurons change their activity later in simulated hemorrhage, suggesting a responsive rather than a causal role during hypotension associated with simulated blood loss. Importantly, both of these populations could contribute to the phase 2 hypotension. Finally, we were able to identify individual vlPAG neurons in which activity was modulated by the cardiac cycle.

During blood loss in conscious animals, arterial pressure is initially maintained (phase 1) by sympathetic vasoconstriction due in large part to unloading of arterial baroreceptors (38). After a critical blood loss, there is a rapid decline in arterial blood pressure accompanied by a simultaneous decrease in sympathetic nerve activity (phase 2). Although it is generally assumed that the sympathoinhibition contributes to the decrease in arterial pressure, the central nervous system origin of the hypotension is not clear. Recent studies in anesthetized (6) and conscious rats (4, 5) have suggested that the site of the trigger for the decrease in arterial pressure and the sympathoinhibition during phase 2 may be the vlPAG.

If the vlPAG is indeed the site of the trigger that produces hypotension during hemorrhage, certain characteristic features should be present. First, vlPAG activation should result in arterial pressure and heart rate changes characteristic of phase 2. In anesthetized rats, this is the case where vlPAG activation with excitatory amino acids results in hypotension, bradycardia (3, 14, 15, 18), and sympathoinhibition (1). In conscious rats, chemical activation of the vlPAG produces bradycardia with (5) and without (28) simultaneous hypotension. A second characteristic might be that blockade of neurotransmission in the vlPAG should alter the response to blood loss. In conscious rats, microinjection of the local anesthetic, lidocaine, or cobalt.
chloride to block synaptic transmission delays or prevents phase 2 hypotension during hemorrhage (4). Similarly, vIPAG microinjection of muscimol, a GABA$_A$ agonist, before hemorrhage in anesthetized rats abolishes phase 2 sympathoinhibition and attenuates the hypotension (6).

If the vIPAG is involved in phase 2 of the response to blood loss, the neurotransmitter systems previously implicated in phase 2 should be represented in the vIPAG or somewhere along the pathway producing hypotension. Two examples of such systems are endogenous opioids and serotonin. For example, it is known that the hypertensive response to hemorrhage can be delayed or reversed by opiate antagonists administered centrally or peripherally (38). Importantly, microinjection of the opiate antagonist naloxone or the specific delta opiate receptor antagonist naltrindole in the vIPAG reduces and/or delays the hypotension associated with hemorrhage in conscious cats (5). In addition, vIPAG microinjection of selective opiate receptor agonists increases (mu agonist) or decreases (delta and kappa agonists) arterial pressure and heart rate, depending on the receptor targeted (15). The presence of opioids and their receptors in the vIPAG has been demonstrated in cats (30) and rats (24). Serotonergic mechanisms also appear to be involved in production of hemorrhagic hypotension. Peripheral or central administration of methysergide, presumably acting as a serotonin 5-HT$_{1A}$ agonist, reverses phase 2 hypotension (9, 41) through an action in the hindbrain (40). In addition, the sympathoinhibition associated either with activation of the vIPAG (1) or hemorrhagic hypotension (7) in anesthetized rats is abolished by microinjection of a specific 5-HT$_{1A}$ receptor antagonist in the rostroventrolateral medulla. The vIPAG also appears to have a variety of afferent and efferent anatomic connections appropriate for an important integrative role in cardiovascular control during simulated hemorrhage (25, 26). Importantly, the vIPAG projects to the caudal midline medulla and caudal ventrolateral medulla, two areas capable of producing profound sympathoinhibition (14).

Central nervous system areas involved in control of arterial pressure during hemorrhage should receive sensory input from relevant receptors. Knowing the importance of arterial baroreceptors in the integrated response to hemorrhage (38), we anticipated we would find baroreflex modulation of activity in vIPAG neurons. However, most PAG units tested were unresponsive to pharmacological increases and decreases in arterial pressure. Another potential indicator of cardiac sensory input would be the presence of a cardiac rhythm in PAG unit activity. In our study, ~45% of vIPAG and 76% of dIPAG neurons demonstrated pulse-modulated activity. Thus we were able to demonstrate some cardiac cycle input to both the vIPAG and dIPAG. Our results also appear to indicate a greater degree of cardiovascular modulation of neuronal activity in the dIPAG than in the vIPAG. In addition, our results are in close agreement with a previous report that 74% of primarily dIPAG neurons showed pulse-modulated activity in conscious cats (33).

An important heretofore unreported characteristic of any neurons involved in triggering phase 2 during blood loss is that their activity should change before the decrease in arterial pressure, that is, the cause should precede the effect (23). One earlier study addresses neuronal activity in the vIPAG after 60 min of nitropresside-induced hypotension using Fos expression (31). This study demonstrated increases in Fos in the vIPAG after 60 min of hypotension. However, the results do not address a trigger for hemorrhagic hypotension in conscious animals. First, the active drop in pressure during hemorrhage is a short-lived, transient event, probably not long enough to increase Fos expression. Second, inhibitory (or disinhibitory) events are not obvious from studies of Fos expression (31). Finally, the rats in this study were anesthetized. The response to hemorrhage is significantly altered by anesthesia. Indeed, it is often difficult to even identify the transition to phase 2 in anesthetized animals (38). Thus the purpose of this study was to assess the activity of vIPAG and dIPAG single neurons during simulated hemorrhage in the conscious rabbit. Our hypothesis was that individual vIPAG neurons change their firing frequency before the onset of hypotension during simulated hemorrhage.

If neurons in the vIPAG trigger or contribute to the hemodynamic and neurohumoral changes associated with phase 2, this role should be reflected in their activity patterns during simulated hemorrhage. For example, in conscious cats, dorsal raphe neurons decrease their discharge before the onset of rapid eye movement sleep and increase their discharge with the onset of wakefulness (22). This pattern is consistent with the proposed role for these neurons in the regulation of sleep cycles (23). We were able to identify neurons that responded to the simulated hemorrhage as potential trigger neurons if their discharge increased or decreased immediately before the transition (60–80%) to phase 2. Of the 28 (out of 64) vIPAG neurons that did change their discharge during simulated hemorrhage, only 11 changed in a manner suggestive of a role in triggering the onset of hypotension. However, 13 additional vIPAG neurons responded after the onset of hypotension. Thus, although the majority of vIPAG neurons (56%) appeared to be unresponsive during simulated hemorrhage, 38% responded at a time consistent with some contribution to the hypotension. Collectively, these findings suggest that the population response, and not the response of individual neurons, might be most important in determining the cardiovascular outcome. For example, in primates, recordings from areas of the hypothalamus responsible for luteinizing hormone secretion show a clear increase in multiunit activity before the increase in plasma luteinizing hormone levels (16). With this result in mind, we focused our subsequent analyses on the population response.

When we considered all vIPAG neurons as one group, there was not a significant change in activity at a specific point during simulated hemorrhage. This result, however, does not preclude vIPAG involvement. One explanation might be that, unlike the hypothalamic example above (16), there is not a predominant increase (or decrease) in activity at the transition to phase 2. Rather, there might be simultaneous increases and decreases in discharge among vIPAG neurons. The sum of this variability in response might result in no detectable change in activity during simulated hemorrhage. In an attempt to address this possibility, we considered the absolute value of changes in activity over the course of the simulated hemorrhage. With the use of this approach, there was a gradual but significant change in vIPAG activity across the entire simulated hemorrhage (see Fig. 5A).

Our initial observations (42) had suggested that baseline firing frequency might have some predictive value in terms of the neuronal response to simulated hemorrhage. Therefore, we subdivided the vIPAG neurons into three groups based on baseline firing rate (low, mid, and high). The absolute change
in activity was significant over the simulated hemorrhage for the mid- and high-frequency groups (Fig. 5, B and C). Although the change in the low-frequency neurons (Fig. 5D) was not significant, the results were suggestive of a trend \((P = 0.09)\). An interesting difference between the three groups was the pattern of change. The high-frequency neurons showed a steady increase from the beginning through phase 2 (Fig. 5B). The mid-frequency group showed the pattern most consistent with a role in triggering phase 2 in that the change in activity began later \((i.e., \sim 60\% - 80\% \text{ of total occlusion})\) near the transition to hypotension (Fig. 5C). Although the change was not significant, the low-frequency neurons changed the latest, with the change starting after the fall in arterial pressure (Fig. 5D). The pattern shown by the low-frequency group seems most consistent with the neurons responding to the decrease in arterial pressure. We were also able to identify individual vlPAG neuronal firing patterns \((e.g., \text{Fig. 6})\) that would have contributed to the observed subpopulation responses \((\text{Fig. 5})\). For example, the absolute value of the change in firing in the neuron shown in Fig. 6D resembles the average pattern shown by the mid-frequency vlPAG neurons \((\text{Fig. 5B})\).

In conscious rats \((41)\) and rabbits \((2, 13, 29)\), the decrease in sympathetic nerve activity during hemorrhage may slightly lag the fall in blood pressure. However, in anesthetized rats, there appears to be a substantial delay between the fall in blood pressure and the decrease in sympathetic activity \((1, 6, 44, 46)\). In some cases, the length of this delay appears to be \(>60\) s \((1, 6, 44)\), with the onset of hypotension actually accompanied by an increase in renal sympathetic nerve activity \((44, 46)\). Thus it seems possible that anesthesia may act to dissociate the onset of hypotension from the sympathoinhibition. Even if this is the case, a delayed decrease in sympathetic activity would still contribute to the phase 2 decrease in arterial pressure. In a similar fashion, vlPAG neurons increasing or decreasing their discharge after the start of the fall in pressure could still contribute to the overall decrease in sympathetic nerve activity and, ultimately, to the hypotension. Our results are consistent with a role for the vlPAG in acute hemorrhagic hypotension, with the contribution coming both during and after the initiation of the fall in pressure.

Although the number of dlPAG neurons recorded was relatively small, the comparison between vlPAG and dlPAG is interesting. The dlPAG neurons as a group did not show any clear pattern of response during hemorrhage \((\text{Fig. 4B})\). Unlike the vlPAG neurons, when we considered the absolute value of the activity changes, the change in activity in the dlPAG neurons during simulated hemorrhage was not statistically significant \((\text{Fig. 7A})\). After subdividing the dlPAG neurons based on baseline firing frequency, the activity of dlPAG neurons was still not significantly altered by simulated hemorrhage. However, in the case of the high-frequency group, the sample size \((n = 10)\) limits our ability to make a conclusion. Thus, although a similar percentage of dlPAG \((41\%)\) and vlPAG \((44\%)\) neurons appeared to respond in a systematic way to simulated hemorrhage \((\text{see Table 2 and Fig. 6})\), the group response does not reflect involvement of the dlPAG. Moreover, there is no suggestion in the literature that the dlPAG might be involved in the onset of phase 2. Chemical or electrical activation of the dlPAG consistently produces cardiovascular changes suggestive of a defense response \((i.e., \text{hypertension and tachycardia})\; \text{see Ref. 17})\). In addition, whereas lidocaine \((4)\) and the delta opiate antagonist naltrindole \((5)\) delayed or abolished phase 2 hypotension when injected in the vlPAG, they were without effect in the dlPAG. Thus the intimate involvement of dlPAG neurons in the cardiovascular adjustments associated with simulated hemorrhage seems unlikely.

A necessary limitation of the present study was the use of simulated hemorrhage rather than actual blood removal. These two hypotensive stressors are not identical. In contrast to progressive blood removal, intra-abdominal venous volume increases as the intrathoracic balloon is inflated during simulated hemorrhage. In addition, in our hands, the time course for the development of hypotension differs between simulated hemorrhage \((\sim 2\) min) and progressive blood loss \((\sim 6-7\) min). Finally, to the best of our knowledge, it has never been shown that a biphasic sympathetic response accompanies simulated hemorrhage as it does hypotensive blood loss. All of these factors may contribute to differences in central cardiovascular control between simulated and real hemorrhage. However, Ludbrook and coworkers \((21)\) compared simulated hemorrhage with actual blood loss and reported similar changes in blood pressure, heart rate, vascular conductance, and plasma levels of neurohumoral agents. In the present study, we recorded comparable hemodynamic effects with simulated and real hemorrhage in conscious rabbits \((\text{Fig. 2})\). In addition, the arterial pressure and heart rate responses to more rapid vena caval occlusions \((\text{Table 1})\) were very similar to occlusions done over a longer time period \((\text{Fig. 2})\). Finally, lower body negative pressure \((\text{LBNP})\) is a model used to study blood loss in humans. Like thoracic vena caval occlusion, LBNP decreases central blood volume by sequestering blood in the lower body. The hemodynamic and sympathetic response to hypotensive LBNP is biphasic and similar to that during blood removal \((8, 32, 34, 35)\). Thus it seems likely that the rapid fall in blood pressure seen during simulated hemorrhage is driven by central processes similar to those that trigger hypotension during blood loss.

In summary, when we considered the absolute change in activity from control, thus accounting for increases and decreases in neuronal activity, simulated hemorrhage had a significant effect on neuronal activity in the vlPAG but not in the dlPAG. In some vlPAG neurons, the change in activity is consistent with a role for these neurons in triggering phase 2. In other neurons, the activity changes occur too late to act as a trigger. However, the changes could still be mediating neurohumoral changes \((e.g., \text{sympathoinhibition})\) that contribute to the low levels of arterial pressure reached in phase 2. Alternatively, considering the integrative role of the midbrain, these late-changing neurons might simply be responding to the low arterial pressure. Thus, as with the earlier studies \((4-6, 45)\), our study in conscious rabbits supports a role for the midbrain in modulating the sympathetic and cardiovascular responses to blood loss.

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