c-Fos expression in the midbrain periaqueductal gray after chemoreceptor and baroreceptor activation

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Hayward, Linda F., and Marcela von Reitzenstein. c-Fos expression in the midbrain periaqueductal gray after chemoreceptor and baroreceptor activation. Am J Physiol Heart Circ Physiol 283: H1975–H1984, 2002. First published July 18, 2002; 10.1152/ajpheart.00300.2002.—The pattern of Fos-like immunoreactivity (FLI) in the periaqueductal gray (PAG) associated with activation of arterial chemoreceptors versus baroreceptor afferents was examined in urethane-anesthetized rats. Chemoreflex responses elicited by repeat intravenous injections of potassium cyanide ( KCN; 90 μg/kg) significantly increased FLI in all columns of the PAG relative to saline-injected animals. Pressor responses elicited by intravenous phenylephrine (PE) produced a similar pattern of increased FLI throughout the PAG except in the dorsomedial and lateral columns of the caudal PAG, where FLI was minimal. Chemoreflex responses were unaltered by blockade of excitatory amino acid receptors in the dorsomedial PAG, and <10% of the neurons of the caudal PAG that expressed FLI after KCN stimulation were retrogradely labeled from the A5 region of the caudal ventrolateral pons. These results indicate that integration of chemoreceptor inputs occurs primarily in the dorsal and lateral columns of the caudal PAG, but these neurons have little direct descending influence over lower brain stem regions integral to the central arterial chemoreflex arc.

midbrain central gray; A5; potassium cyanide; baroreceptors

IN MANY ANIMALS, brief stimulation of arterial chemoreceptors with cyanide evokes a stereotypic pattern of hyperventilation coupled with vasodilation in muscle vascular beds and vasoconstriction in the renal and mesenteric beds (19, 30). In conscious animals, cardiorespiratory adjustments are coupled with behavioral responses that range from mild arousal to running or escape-like behavior (13). Both cardiorespiratory and arousal responses are eliminated by selective denervation of the carotid sinus nerve (12). This suggests these responses are mediated through selective activation of arterial chemoreceptors in the carotid body. On the basis of similarities between the response to peripheral chemoreceptor activation and the alerting response evoked by threatening stimuli, it has been hypothesized that activation of “defense areas” in the brain, including the midbrain periaqueductal gray (PAG) (3, 4, 6, 10, 27), must be an integral component of the central arterial chemoreflex arc (19, 29–31). This hypothesis has been substantiated by numerous studies demonstrating that c-Fos protein expression increases within different columns of the PAG after prolonged stimulation of arterial chemoreceptors (7, 11, 21, 22, 41).

Yet, our understanding of the role of the PAG in the central arterial chemoreflex pathway remains relatively rudimentary. It remains to be determined whether lesion of the PAG has any influence on cardiorespiratory or behavioral responses to chemoreceptor activation. Furthermore, none of the previous studies examining the effects of chemoreceptor stimulation on c-Fos expression in the PAG has controlled for the influence of baroreceptor afferents. Exposure to systemic hypoxia or direct chemoreceptor afferent nerve stimulation can produce profound changes in blood pressure and heart rate (HR) (13, 18, 20). Associated fluctuations in baroreceptor inputs may independently trigger increased c-Fos expression in the PAG (25, 33, 36, 38). The importance of taking into account the influence of baroreceptor-mediated changes was recently highlighted in a study examining the c-Fos expression in the PAG after muscle afferent stimulation (26). In that study, sustained muscle contractions were shown to significantly increase c-Fos expression throughout the middle and caudal regions of the PAG. Yet when the same experimental conditions were applied in baroreceptor-denervated animals, c-Fos expression declined by 50% or more. This suggested that changes in baroreceptor input during experimental manipulation of cardiorespiratory reflexes could play a significant role in c-Fos expression patterns in the PAG.

The present study was undertaken to examine the contribution of baroreceptor inputs to c-Fos expression in the PAG after prolonged arterial chemoreceptor activation. On the basis of previous studies, it was hypothesized that approximately one-half of the c-Fos expression in the PAG observed after chemoreceptor afferent stimulation was likely to be associated with reflex-mediated changes in baroreceptor afferent input. The second objective of the present study was to "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact."
identify whether PAG neurons activated by chemoreceptor input play a significant role in modulating arterial chemoreflex responses in the rat. It was hypothesized that chemoreceptor activation of PAG neurons plays a significant role in the arterial chemoreflex response of the rat.

**METHODS**

All experiments were carried out on adult male Sprague-Dawley rats (weight, 350–420 g) housed in the university animal care facility and exposed to a normal 12-h light (6 AM to 6 PM) to 12-h dark (6 PM to 6 AM) cycle. All experimental procedures were preapproved by the University of Florida Institutional Animal Care and Use Committee.

*Retrograde labeling.* A small subset of rats underwent placement of a retrograde tracer into the ventrolateral pons 1 wk before experimentation. Each animal was deeply anesthetized with a mixture of ketamine-acepromazine (100:10 mg/kg ip). With the use of sterile procedures, the animal was placed in a stereotaxic head holder (Kopf; Tujunga, CA), and a small hole was drilled in the skull. A small-diameter glass microinjection pipette (tip diameter, 15–30 μm) was filled with 2.5% Fluoro-Gold (Fluorochrome; Denver, CO) diluted in physiological saline. The microinjection pipette was attached to a microinjection pressure system (model PPS-2, Medical Systems; Greenvalle, NY) and lowered into the left side of the brain using a micropositioner (Kopf MP660). Coordinates for microinjection into the ventrolateral pons/A5 cellular region were based on Paxinos and Watson’s *Rat Brain in Stereotaxic Coordinates* (37). The caudal ventrolateral pons was chosen as a target site based on previous work suggesting that the PAG has more descending projections to the caudal versus rostral ventrolateral pons (1, 8). The retrograde tracer was then pressure injected into the left side of the brain over the course of 30–60 s. The volume injected was determined by carefully monitoring the movement of the meniscus with a calibrated 40× monocular (Titan Tools; Buffalo, NY). The pipette remained in position for 3 min after fluid ejection and was then retracted. The wound was sutured (Ethilon, 4-0), and an antibiotic ointment was applied to the skin. Supplemental doses of anesthesia were administered intraperitoneally during surgery as needed. Body temperature was continuously monitored with a rectal probe and was maintained at 38 ± 1°C with a heating pad. To help relieve postoperative pain, all animals received a subcutaneous injection of buprenorphine (0.1–0.2 mg/kg). Animals were closely monitored during recovery for any signs of discomfort or infection. All animals recovered well from surgery, maintaining steady weight gain and water consumption.

*General preparations.* At the time of the terminal experiment, all animals were anesthetized with urethane (1.3–1.5 gm/kg ip). After the induction of anesthesia, animals were placed in the supine position and instrumented with a right carotid arterial catheter (polyethylene-50 tubing) to record arterial pressure and a right jugular venous catheter for intravenous fluid administration. A tracheotomy was performed, and the animals were intubated. A small incision was made in the skin subcostally. Two small (0.003 mm diameter) Teflon-coated, stainless steel wires with bare tips were inserted through the abdominal musculature into the right side of the diaphragm for measurement of spontaneous inspiratory electromyographic (EMG) activity. Body temperature was monitored continuously with a rectal temperature probe (Harvard Apparatus; Holliston, MA) and kept within a normal range (38 ± 1°C) with a heating blanket. Supplemental anesthesia was continued (0.05–0.1 g·kg⁻¹·h⁻¹) thereafter until the experiment was terminated.

The arterial catheter was attached to a calibrated pressure transducer (Statham) connected to an amplifier (Stoelting; Wood Dale, IL) for continuous monitoring of arterial pressure. The analog output from the blood pressure amplifier was connected to a computer data sampling system [Cambridge Electronics Design (CED) 1401 computer interface; Cambridge, UK]. The diaphragm EMG (dEMG) wires were connected to a Grass preamplifier probe (H1P5, Grass Instruments; West Warwick, RI) in series with a signal amplifier (P511). The dEMG signal was amplified (5,000–50,000 times) and bandpass filtered (0.3–3.0 kHz). The signal was rectified, integrated (Paynter Filter, 50-ms time constant, BAK Electronics; Rockville, MD), and then sampled using CED software (Spike2). The baseline dEMG was arbitrarily adjusted to a value of 1.0–2.0 (arbitrary units) at the beginning of the experiment. dEMG and arterial pressure were recorded simultaneously.

**Experimental procedures for chemoreceptor or baroreceptor stimulation in c-Fos experiments.** Animals were placed in the supine position after the completion of all instrumentation. A rest period of 10–15 min was given before drug administration. Each animal then received 11 bolus intravenous injections of a single type of solution, repeated every 2 min. This was followed by a 90-min rest period before euthanization. Chemoreflex responses were elicited by intravenous injection of potassium cyanide (KCN; 0.5 mg/ml dissolved in saline). Individual KCN doses were flushed through the catheter with 150 μl of saline. KCN was chosen as a stimulus because it provides brief and potent activation of arterial chemoreceptors and elicits reproducible reflex responses upon repeated administration (18, 19, 23). Furthermore, the cardiorespiratory response to KCN in both conscious and anesthetized rats is dependent on an intact carotid sinus nerve (13, 17, 18). KCN also has little influence on baroreceptor afferents (12). This suggests that the effects of KCN administration on c-Fos expression in our protocols would primarily be a function of changes in peripheral chemoreceptor input and not the direct influence of KCN on central neurons. The dose range used in the present study was derived from previous studies that have demonstrated doses ≥60 μg/kg elicit marked cardiorespiratory changes in both conscious and anesthetized rats (12, 18). In conscious animals, intravenous injections above 60 μg/kg also elicit arousal and/or escapelike behavior (12). Because anesthesia is known to dampen the cardiorespiratory response to KCN (12, 18), 60 μg/kg was chosen as the lowest dose to be tested in the present study.

Baroreflex responses were elicited by intravenous injection of phenylephrine (PE). PE doses (3–5 μg/kg) were sufficient to raise mean arterial pressure (MAP) ~10 mmHg. Individual doses of PE were also flushed through the catheter with 150 μl of saline. In several animals, the effects of repeated bolus injections of 150 μl of saline alone were tested. During the rest period, all animals were put on supplemental oxygen.

**Experimental procedures for chemical blockade of the PAG.** After the induction of urethane anesthesia, a subset of naive animals were instrumented and placed in a stereotaxic head holder. A small hole was drilled in the region of the skull overlying the PAG, and a single-barrel glass microinjection pipette was lowered into the PAG. Arterial chemoreflex responses evoked by 90 μg/kg iv KCN were tested before and 5 and 45 min after microinjection of kynurenic acid into the dorsal PAG. Kynurenic acid is a broad-spectrum excitatory amino acid receptor antagonist (10 mM, Sigma; St. Louis,
MO. Each animal received two central injections (150–200 nl each) into the left dorsal PAG, including one at −7.5 mm and one at −7.9 mm caudal to the bregma.

In a separate group of animals, the efficacy of 10 mM kynurenic acid to block cardiorespiratory responses evoked by excitatory amino acid receptors was tested. First, 100 nl kynurenic acid (10 mM) was microinjected into the dorsal PAG (−7.8 mm caudal to the bregma, 0.2–0.3 mm lateral from midline, and 4.0 mm ventral from the surface of the brain). The single-barrel pipette was then withdrawn. A second pipette containing the GABA-A receptor antagonist bicuculline methobromide (0.3 mM, Sigma) was immediately repositioned in the dorsal PAG. Two separate microinjections of 40–60 nl bicuculline were made into the dorsal PAG, separated in time from the preceding kynurenic acid injection by 5 and 45 min. Bicuculline, a GABA-A receptor antagonist, was used to disinhibit PAG neurons and uncover tonic endogenous excitation (5). All microinjection drugs were diluted in artificial cerebrospinal fluid (aCSF), which contained (in mM) 122 NaCl, 3 KCl, 25.7 NaHCO3, and 1 CaCl2, with the pH adjusted to 7.4, mixed with a small quantity of fluid (−0.01 N HCl). Bicuculline or bicuculline were directly mounted on slides, coverslipped (Vectamount).

Tissue processing. At the end of the experiment, animals were given supplemental urethane (0.2–4 g/kg) and transcardially perfused. Animals were first perfused with 100–200 ml of ice-cold saline containing 0.9% NaCl, 0.2 g/ml sodium nitroprusside (Sigma), and 100 IU/ml of heparin. This was followed by 100–200 ml of ice-cold 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.2). The brains were removed, soaked overnight in 4% paraformaldehyde, and then placed in a 30% sucrose (in water) solution for 24–48 h. A small cut was made on the right, ventral surface of the brain. The single-barrel pipette was then withdrawn. A second pipette containing the GABA-A receptor antagonist bicuculline methobromide (0.3 mM, Sigma) or a blue-gray reaction product (Fluoro-Gold antibodies). After exposure to the secondary antibody, no FLI or Fluoro-Gold staining was found.

Microscope analysis. All tissue sections were imaged with a light microscope (Zeiss). Fluorescence images were captured with a video camera connected to a personal computer with image-capturing software (AIS). For each animal, a total of six brain sections from the PAG were imaged, including both representative sections each from the caudal, middle, and rostral PAG. The criteria for choosing specific sections were based on anatomic characteristics of the PAG, including 1) the shape of the central aqueduct; 2) the shape and width of the dorsal and ventrolateral columns; and 3) the presence of the oculomotor nucleus. The PAG image files were transferred to a second computer program (CorelDraw). Standardized outlines or “masks” of the PAG were superimposed over the images. These standardized masks outlined the boundaries of the different columns of the PAG as described by Paxinos and Watson (37) and Beitz (6). Images were magnified, and the numbers of FLI neurons in each PAG column, per tissue section, were counted. The criterion for the presence of FLI included the presence of dark black or brown label in a round structure between 7 and 10 μm in diameter, corresponding to the cell nucleus. Cells containing only lightly shaded labeling were not considered activated by the stimulus. Tissue slices that were double labeled for both c-Fos and Fluoro-Gold were imaged and analyzed in a similar manner. The presence of double labeling, however, was confirmed by viewing the tissue section directly under the microscope at high magnification (×40–60).

Brain slices from those animals that only underwent central microinjections were examined separately with a microscope equipped with epifluorescence (Zeiss). Microinjection sites were recovered and imaged by identifying the location of the fluorophore.

Data analysis of cardiorespiratory responses. All data were analyzed off-line using Spike2 software (CED). Peak changes in MAP, HR, and respiratory rate (RR) during chemoreceptor or baroreceptor stimulation were calculated from the difference between the preceding baseline (a 10-s average measured just before each bolus injection) and the peak deviation from baseline during chemoreceptor or baroreceptor stimulation (a 3-s average). HR was derived from the average interval between heartbeats, and RR was derived from the average interval between arterial blood pressure pulses. Changes in baseline activity after central microinjection of bicuculline were calculated from the difference between a 10-s average taken just before central microinjection and a 10-s average taken between 70 and 80 s after onset of central injection.
ANOVA with repeated measures demonstrated no significant difference in the number of FLI-positive cells within specific columns of the PAG as a function of side (right or left). Because retrograde labeling from the ventrolateral pons was primarily unilateral, a decision was made to count FLI-positive neurons on only the left side of the PAG.

All statistical comparisons were made between tissue sections in the same rostral-caudal position of the PAG. The effect of stimulation condition (KCN, PE, or saline) and PAG column (dorsal (dorsal medial and dorsal lateral), lateral, and ventrolateral) on FLI was tested using a two-way ANOVA (effect of stimulation condition and PAG column, StatView software). Significant differences in cardiorespiratory responses to KCN, PE, and saline were compared using one-way ANOVA. Main effects and interactions were examined with Scheffe’s post hoc tests. Significant differences in cardiorespiratory responses to KCN or bicuculline before and after central microinjections of kynurenic acid were combined with Scheffe one-way ANOVA. Main effects and interactions were examined with Scheffe’s post hoc tests. Significant differences in cardiorespiratory responses to KCN or bicuculline before and after central microinjections of kynurenic acid were compared using a paired t-test. Changes were considered significant when $P < 0.05$. All data are reported as means ± SE.

RESULTS

Cardiorespiratory responses and FLI labeling after exposure to increasing doses of KCN. To identify whether a dose-response relationship between FLI labeling and KCN-evoked chemoreflex responses could be demonstrated in the PAG, three groups of animals were subjected to repeat injections of KCN (60, 90, or 120 $\mu$g/kg KCN). Table 1 shows the average resting parameters from all three groups. Figure 1A shows a representative cardiorespiratory response to intravenous injection of KCN from a single animal (90 $\mu$g/kg). The chemoreflex response typically included an increase in RR that began ~5 s after the onset of the intravenous injection. This was followed by a brief change in both MAP and HR. Changes in RR and HR were sustained for 15–30 s. All cardiorespiratory changes associated with KCN stimulation returned to preinjection levels within 40 s.

Figure 1B shows the average peak response of all animals exposed to 60 ($n = 7$), 90 ($n = 7$), or 120 $\mu$g/kg KCN. Between animals, RR increased significantly as KCN administration increased. In contrast, the peak change in MAP and HR induced by 60 $\mu$g/kg KCN was significantly different from that recorded in response to 90 $\mu$g/kg KCN but was not significantly different from responses associated with 120 $\mu$g/kg KCN. This lack of a dose-response-like relationship in the cardiovascular component of the reflex reflects a more variable response to 120 $\mu$g/kg KCN between animals. For instance, 120 $\mu$g/kg KCN produced a decrease in MAP in three of seven animals and a bradycardia in two of seven animals.

As illustrated in Fig. 2, repeat exposure to 90 or 120 $\mu$g/kg KCN elicited a significant ($P < 0.001$) increase in FLI in all columns of the PAG relative to 60 $\mu$g/kg KCN, with two exceptions. First, there was no significant difference in the average number of FLI cells between groups in the dorsal column of the caudal PAG (both dorsomedial and dorsolateral columns were combined during initial analysis). Second, there was no significant difference between the number of FLI cells in the lateral column of animals exposed to 60 versus 120 $\mu$g/kg KCN in the rostral and caudal PAG. Further comparisons demonstrated that 90 $\mu$g/kg KCN consis-

**Table 1. Resting cardiorespiratory values from animals receiving KCN, PE, or saline injections**

<table>
<thead>
<tr>
<th></th>
<th>Mean Arterial Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Respiratory Rate, breaths/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 $\mu$g/kg KCN</td>
<td>7</td>
<td>$101 \pm 6$</td>
<td>$365 \pm 29$</td>
</tr>
<tr>
<td>90 $\mu$g/kg KCN</td>
<td>14*</td>
<td>$98 \pm 6^*$</td>
<td>$355 \pm 23^*$</td>
</tr>
<tr>
<td>120 $\mu$g/kg KCN</td>
<td>7</td>
<td>$94 \pm 5$</td>
<td>$363 \pm 22$</td>
</tr>
<tr>
<td>PE</td>
<td>5</td>
<td>$97 \pm 2$</td>
<td>$364 \pm 29$</td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>$94 \pm 6$</td>
<td>$363 \pm 22$</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n =$ no. of rats. PE, phenylephrine.

*Includes data averaged from both naive animals ($n = 7$) and animals with Fluoro-Gold retrograde labeling ($n = 7$).

**Statistical analysis.** Preliminary analysis ($n = 6$) using ANOVA with repeated measures demonstrated no significant difference in the number of FLI-positive cells within specific columns of the PAG as a function of side (right or left). Because retrograde labeling from the ventrolateral pons was primarily unilateral, a decision was made to count FLI-positive neurons on only the left side of the PAG.

**Fig. 1. Reflex responses evoked by intravenous potassium cyanide (KCN) injection in spontaneously breathing, urethane-anesthetized rats. A: cardiorespiratory responses, including arterial pressure (AP), diaphragm electromyograph (dEMG), heart rate (HR), and respiratory rate (RR), evoked by intravenous bolus injection of 90 $\mu$g/kg KCN from a single animal. Horizontal bar, time of KCN injection; AU, arbitrary units. B: averaged peak change in mean AP (MAP), HR, and RR evoked after 60 $\mu$g/kg (open bars, $n = 7$), 90 $\mu$g/kg (solid bars, $n = 7$), and 120 $\mu$g/kg (hatched bars, $n = 7$) KCN. bpm, Beats per minute; brpm, breaths/min. Peak changes were calculated relative to the preceding baseline activity levels. *Significantly different from the response evoked by 60 $\mu$g/kg KCN ($P < 0.05$).**
tently induced a greater increase in FLI compared with 120 μg/kg KCN. This increase in FLI was statistically significant ($P < 0.01$) in all columns except the dorsal column of the caudal PAG and the lateral column of the rostral PAG.

$c$-Fos expression after exposure to chemoreceptor versus baroreceptor stimulation. To test the possible role of blood pressure fluctuations and associated changes in baroreceptor input might play in FLI labeling after chemoreceptor stimulation, two additional groups of animals were tested. One group received only repeat bolus injections of saline (the vehicle of drug administration). The second group underwent repeat bolus injection of PE. The dose of PE given was sufficient to raise MAP 10–15 mmHg above baseline, similar to the MAP change associated with bolus injections of 90 μg/kg KCN. The change in blood pressure evoked by PE was set to match that elicited by 90 and not 120 μg/kg KCN, based on the more consistent pressor response evoked by 90 μg/kg. The average increases in MAP, HR, and RR evoked in response to PE, saline, and 90 μg/kg KCN are shown in Table 2. The PE-induced increase in MAP was not significantly different from that induced by 90 μg/kg KCN. The PE-induced increase in MAP was, however, significantly different from the change in MAP associated with saline injections. Bolus injections of PE also produced small decreases in HR and RR.

Figure 3 shows photomicrographs taken from animals that received repeat intravenous injections of saline (left), PE (middle), or KCN (right). Relative to saline, both PE and KCN increased FLI labeling throughout the PAG. Comparisons between groups confirmed a trend for PE to induce increased levels of FLI in all columns of the PAG relative to saline-injected animals except within the dorsomedial column of the PAG (see Fig. 4). Yet, PE-induced increases in FLI were only significantly different from saline-injected animals in the ventrolateral column of the caudal PAG. Relative to saline-injected animals, repeat exposure to 90 μg/kg KCN induced a significant increase in FLI labeling throughout all columns of the rostrocaudal extent of the PAG, with one exception. The increase in FLI observed in the dorsolateral column in the rostral PAG was not significantly different between saline- and KCN-injected animals. Relative to PE-injected animals, however, 90 μg/kg KCN only increased FLI in select regions of the PAG. These included the dorsomedial column and the lateral columns of the caudal PAG and in the dorsomedial columns of the middle and rostral PAG.

c-Fos expression in retrogradely labeled cells of the PAG with descending projections to the ventrolateral pons. To test the hypothesis that chemoreceptor-sensitive neurons in the PAG have direct descending projections to critical brain stem regions involved in arterial chemoreflex function (16), a third group of animals was tested. These animals underwent placement of 80–100 nl of the retrograde tracer Fluoro-Gold into the caudal ventrolateral pons. One week after central placement of the retrograde tracer, animals were reanesthetized and exposed to repeated injections of 90 μg/kg KCN. In four of seven animals, the retrograde tracer was positioned in the caudal ventrolateral pons, just lateral to the exit of the facial nerve and medial to the inferior olive, near the position of the A5 noradrenergic cell group (see Fig. 5A). In these animals, extensive retrograde labeling was observed within the ven-

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Table 2. Cardiorespiratory changes associated with intravenous KCN, PE, or saline injections

<table>
<thead>
<tr>
<th></th>
<th>Mean Arterial Pressure, ΔmmHg</th>
<th>Heart Rate, Δbeats/min</th>
<th>Respiratory Rate, Δbreaths/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5 (4 ± 1)</td>
<td>3 ± 5</td>
<td>-1 ± 2</td>
</tr>
<tr>
<td>PE</td>
<td>5 (13 ± 3)</td>
<td>-8 ± 3</td>
<td>-9 ± 2</td>
</tr>
<tr>
<td>90 μg/kg KCN</td>
<td>6 (12 ± 2)</td>
<td>33 ± 6*</td>
<td>41 ± 8*†</td>
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Values are means ± SE; $n$ = no. of rats. *Significantly different from saline response, $P < 0.02$; †significantly different from PE response, $P < 0.01$. 

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in the dorsomedial columns. Relatively few retrogradely labeled neurons were identified in the dorsolateral cell column. Table 3 shows the distribution of double-labeled cells in the dorsal and lateral columns of the caudal and middle PAG (those regions identified above to primarily contain selective increases in FLI related to chemoreceptor activation). Relatively few cells were double labeled with Fluoro-Gold and FLI.

In two animals, the retrograde tracer was misplaced lateral and dorsal to the exit of the facial nerve (see Fig. 6A). In these two animals, no retrograde labeling was observed in the PAG. In the third animal, the retrograde tracer was misplaced in the ventral lateral region of the dorsal pons. Only moderate retrograde tracing, restricted to the ventral quadrant of the PAG, was observed in this animal. In these three brains, further examination for double-labeled cells was not made.

Effect of unilateral excitatory amino acid receptor blockade in the PAG on chemoreflex function. The potential role of the PAG in arterial chemoreflex responses was examined further in four additional animals. These animals underwent chemoreflex testing before and after central microinjection of kynurenic acid in the PAG. Kynurenic acid is a competitive antagonist for 

![Diagram of FLI labeling in the PAG](image)

Fig. 3. FLI labeling in the PAG from urethane-anesthetized rats exposed to repeated intravenous injections of saline, phenylephrine (PE), or KCN. Photomicrographs show FLI from representative sections of the PAG from one animal that received 11 repeated intravenous injections of either saline (150 μl; left), PE (3–4 μg/kg; middle), or KCN (90 μg/kg; right). The approximate location of the representative sections relative to the bregma are −7.9 mm (top), −7.6 (middle), and −7.2 (bottom). Boundaries of different PAG columns were defined by Paxinos and Watson’s rat stereotaxic atlas (37). *, Location of the central aqueduct.
acid (10 mM) into the dorsal PAG. Each animal received two microinjections (150–200 nl each) along the rostrocaudal extent of the left side of the PAG (one at −7.9 and one at −7.5 mm caudal to the bregma; see Fig. 5B). Before central microinjection, 90 μg/kg KCN elicited an average increase in MAP, HR, and RR above baseline of 9 ± 3 mmHg, 19 ± 4 beats/min, and 64 ± 7 breaths/min, respectively. Five minutes after microinjection of kynurenic acid, the average response to 90 μg/kg KCN was unchanged [7 ± 2 mmHg, 24 ± 6 beats/min, and 67 ± 8 breaths/min (P > 0.4)]. Chemoreflex responses retested 45 min later remained unchanged (8 ± 4 mmHg, 25 ± 2 beats/min, and 62 ± 7 breaths/min).

In three additional animals, the effectiveness of kynurenic to block excitatory amino acid receptor function was tested. In these animals, the cardiorespiratory response to microinjection into the dorsal PAG of bicuculline, a GABA-A receptor antagonist, was recorded 5 and 45 min after central microinjection of kynurenic acid (100 nl, 10 mM) into the same region of the PAG (see Fig. 5B for injection sites). Microinjection of kynurenic acid alone into the dorsal PAG did not markedly change baseline MAP, HR, or RR (99 ± 2 vs. 99 ±

Fig. 5. Schematic of reconstructed microinjection sites in the ventrolateral pons and dorsal PAG. All illustrations were adapted from Paxinos and Watson (37). A: approximate location and size of reconstructed of Fluoro-Gold microinjection sites in the ventrolateral pons. Shaded areas represent injection sites that resulted in retrogradely labeled neurons in dorsal, lateral, and ventrolateral columns of the PAG; solid area represents an injection site that only labeled neurons in the ventrolateral PAG; hatched areas illustrate reconstructed injections sites that resulted in little or no retrograde labeling in the PAG. B: centers of injection sites marked by fluorescent microspheres reconstructed from dorsal PAG microinjection studies. Shaded ovals illustrate kynurenic acid injection sites (n = 4); solid ovals represent combined kynurenic/bicuculline injection sites (n = 3). 3mn, oculomotor nucleus; 7n, facial nerve; A5, noradrenaline cell group; Dr, dorsal raphe; g7, genu of facial nerve; LC, locus coeruleus; Py, pyramidal tract; Sp5, spinal trigeminal tract; scp, superior cerebellar peduncle; Su3, supraoculomotor periaqueductal; *, location of the central aqueduct.

Fig. 6. Example of the coexistence of FLI and retrogradely labeled neurons with projections to the ventrolateral pons in the dorsal and lateral columns of the PAG after repeat exposure to KCN. Bright-field images of the middle PAG were taken at ×2.5 (A) and ×10 (B) magnification. Black dots indicate neurons with FLI labeling in the nucleus; light-stained neurons with processes indicate retrogradely labeled neurons 1 wk after microinjection of 2.5% Fluoro-Gold into the ventrolateral pons. Arrows indicate position of cells double labeled for both the retrograde tracer and FLI. The approximate location of the section shown is 8.1 mm caudal to the bregma. Boundaries of different PAG columns are indicated by dotted lines and were defined by Paxinos and Watson’s rat stereotaxic atlas (37); *, location of the central aqueduct.
4 mmHg, 376 ± 22 vs. 381 ± 15 beats/min, and 105 ± 5 vs. 105 ± 2 breaths/min, before vs. 5 min after kynurenic acid, respectively). Microinjection of bicuculline into the dorsal PAG 5 min after kynurenic acid microinjections also did not significantly alter MAP, HR, or RR (3 ± 3 mmHg, 6 ± 1 beats/min, and −2 ± 1 breaths/min). Bicuculline microinjection into the dorsal PAG at 45 min, however, induced a significant rise in MAP, HR, and RR (18 ± 6 mmHg, 39 ± 7 beats/min, and 44 ± 11 breaths/min) relative to the response evoked at 5 min (P < 0.05). This suggested that the dose of kynurenic acid used was more than sufficient to block increases in excitatory input. In two animals, the cardiorespiratory response to microinjection of bicuculline was retested 5 min after central microinjection of aCSF (the vehicle for bicuculline and kynurenic acid) into the dorsal PAG. The mean response to bicuculline in these animals was similar before and after aCSF, suggesting the process of microinjection alone did not significantly alter cardiorespiratory responses to bicuculline.

DISCUSSION

In the present study, repeat stimulation of arterial chemoreceptors evoked a significant increase in FLI throughout the PAG in the anesthetized rat. Across all three doses tested, the increase in FLI induced by KCN was greatest in the caudal PAG. This pattern of FLI is similar to that previously reported from conscious rats exposed to systemic hypoxia (7, 11, 21, 22). Yet, in our study, further comparisons between groups demonstrated that FLI in the caudal PAG was greatest in those animals exposed to a dose of KCN that consistently produced the greatest pressor response, not the highest dose of KCN. This suggested that a significant percentage of FLI observed in the PAG after chemoreceptor stimulation was induced indirectly through reflex-mediated changes in baroreceptor input.

To our knowledge, no previous studies have attempted to identify the contribution of baroreceptor inputs to c-Fos expression induced by chemoreceptor activation in the PAG. In our study, repeated exposure to small increases in blood pressure induced increased levels of FLI in the middle and rostral PAG. These increases in FLI were similar to those observed after chemoreceptor stimulation. Identification that certain regions of the PAG express increased levels of FLI after increased baroreceptor input is not a new finding. Indeed, the PAG appears to be one of the more sensitive regions of the brain to fluctuations in baroreceptor afferent input (33). Previous studies suggest that hypertension primarily induces increased FLI in the dorsolateral, lateral (35), and ventrolateral columns of the caudal and middle PAG (26). Alternatively, hypotension appears to selectively increase FLI in the ventrolateral column of the PAG (25, 35). The results of our study are in agreement with these findings. Our results also suggest that repeat exposure to increased chemoreceptor afferent input, independent of changes in baroreceptor input, only moderately increases FLI labeling in the caudal PAG (~50% above baseline). This finding is in marked contrast to previous studies that have reported FLI in the PAG increases 100–200% above baseline after exposure to systemic hypoxia (7, 22, 41). None of these previous studies, however, assessed the contribution of simultaneous changes in blood pressure to FLI. Furthermore, the increases in FLI we observed after chemoreceptor activation were primarily localized to the dorsal and lateral columns of the caudal PAG. This suggests that increases in c-Fos expression previously identified as occurring within the ventrolateral column of the caudal PAG after exposure to hypoxia (7, 22) may have been primarily related to blood pressure changes rather than chemoreceptor stimulation or the direct effects of hypoxia on neurons (7, 22).

To investigate further a potential role for the caudal PAG in the arterial chemoreflex, we placed a retrograde tracer in the ventrolateral pons. The ventrolateral pons was chosen as a putative target site for chemosensitive PAG neurons because this region has been identified as essential for full expression of the sympathetic response to chemoreceptor stimulation in the rat (23, 28). Similar to other reports, 5–7 days after central injection, we observed numerous retrogradely labeled neurons in the lateral and ventrolateral columns of the PAG (1, 9, 32). Despite extensive retrograde labeling in the PAG, only a small percentage of cells were double labeled after repeated exposure to KCN. This finding is in agreement with the results of another study that demonstrated relatively few double-labeled neurons in the PAG after exposure to hypoxia in conscious animals (21). In that study, PAG neurons were retrogradely labeled from the rostroventrolateral medulla, another region critical for chemoreflex modulation of sympathetic drive (16).

Next, we examined the effect of the excitatory amino acid blockade in the dorsal PAG on chemoreflex responses. On the basis of our c-Fos data, we chose to localize our microinjections sites to the dorsomedial column of the PAG. Yet, after blockade of the dorsomedial PAG, arterial chemoreflex responses were not significantly altered. This finding does not support the hypothesis that the PAG plays an important role in chemoreflex responses. Still, this finding does corrob-
rate previous observations that relatively few PAG neurons activated by chemoreceptor stimulation have direct descending projections to essential components of central arterial chemoreflex arc. Our results raise the new possibility that chemoreceptor-related activation of PAG neurons may play an important role in relaying chemoreceptor-related signals to the forebrain and in the alerting response to hypoxia. Indeed, excitotoxic lesions of the dorsal PAG have been shown to significantly alter baroreflex function (24, 40) without significantly modulating the cardiovascular response to air jet stress in conscious rats (24). This suggests that PAG neurons activated by stressful stimuli, such as hypoxia, may only be indirectly involved in the cardiorespiratory response to those stimuli.

Methodological considerations. Although the expression of FLI within neurons can be used as a marker of cellular excitation, there are limitations to the technique. First, not all neurons express c-Fos proteins when activated. Thus the lack of FLI does not necessarily indicate a lack of excitation. Second, the use of anesthetics can alter c-Fos expression patterns. In the present study, we used urethane as our anesthetic because it has been shown have the least effect on chemoreflex responses (13, 18). Interestingly, the general pattern of FLI that we observed in the PAG after repeat chemoreceptor stimulation is similar to that reported from conscious animals. For example, systemic exposure to hypoxia in conscious animals was recently reported to increase of FLI counts by 40–60 neurons above baseline per PAG section (7). We observed a similar increase in FLI above baseline in the anesthetized preparation. This suggests that chemoreceptor stimulation can activate similar populations of PAG neurons in either anesthetized or conscious animals.

Finally, it might be argued that exposure to moderate hypoxia for 1–3 h in a conscious animal is a more threatening stimuli than repeat injections in KCN because it has been shown to have the least effect on chemoreflex responses (13, 18). Interestingly, the general pattern of FLI that we observed in the PAG after repeat chemoreceptor stimulation is similar to that reported from conscious animals. For example, systemic exposure to hypoxia in conscious animals was recently reported to increase of FLI counts by 40–60 neurons above baseline per PAG section (7). We observed a similar increase in FLI above baseline in the anesthetized preparation. This suggests that chemoreceptor stimulation can activate similar populations of PAG neurons in either anesthetized or conscious animals.

In summary, the results of the present study demonstrate that repeated stimulation of arterial chemoreceptors with KCN induces significant increases in FLI throughout the PAG. These increases were greatest in the caudal PAG. These findings are in strong agreement with previous studies using systemic hypoxia to activate chemoreflex responses in conscious animals (7, 22). Yet, when the influence of reflex-induced changes in baroreceptor input was accounted for, chemoreceptor-related increases in FLI in the caudal PAG were found to be relatively modest. Results from additional retrograde labeling and chemical blockade studies support this finding and suggest for the first time that the PAG does not play a prominent role in descending modulation of cardiorespiratory components of the arterial chemoreflex. The results of the present study raise the possibility that activation of chemoreceptor-sensitive neurons in the caudal PAG may be more important in relaying information to the forebrain and/or coordinating escape behavior associated with the chemoreflex response. Further investigations are needed to identify the role of the PAG relaying chemoreceptor information to higher brain centers.

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