Activation of NADPH-diaphorase-positive projections to the rostral ventrolateral medulla following cardiac mechanoreceptor stimulation in the conscious rat

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Submitted 21 July 2005; accepted in final form 7 January 2006

Kantzides, A., and E. Badoer. Activation of NADPH-diaphorase-positive projections to the rostral ventrolateral medulla following cardiac mechanoreceptor stimulation in the conscious rat. Am J Physiol Regul Integr Comp Physiol 290: R1626–R1638, 2006; doi:10.1152/ajpregu.00532.2005.—Stimulation of cardiac mechanoreceptors during volume expansion elicits reflex compensatory changes in sympathetic nerve activity (SNA). The hypothalamic paraventricular nucleus (PVN) and nucleus of the tractus solitarius (NTS) are autonomic regions known to contribute to this reflex. Both of these nuclei project to the rostral ventrolateral medulla (RVLM), critical in the tonic generation of SNA. Recent reports from our laboratory show that these pathways 1) are activated following cardiac mechanoreceptor stimulation, and 2) produce nitric oxide, known to influence SNA. The aims of the present study were to determine whether 1) the activated neurons within the PVN and NTS were nitrergic and 2) these neurons projected to the RVLM. Animals were prepared, under general anesthesia, by microinjection of a retrogradely transported tracer into the pressor region of the RVLM and the placement of a balloon at the right venoatrial junction. In conscious rats, the balloon was inflated to stimulate the cardiac mechanoreceptors or was left uninflated. Balloon inflation elicited a significant increase in Fos-positive neurons in the paravascular PVN (sevenfold) and NTS (fivefold). In the PVN, 51% of nitrergic neurons and 61% of RVLM-projecting nitrergic neurons were activated. In the NTS, these proportions were 8% and 18%, respectively. The data suggest that nitrergic neurons within the PVN and, to a lesser extent, in the NTS, some of which project to the RVLM, may contribute to the central pathways influencing SNA elicited by cardiac mechanoreceptor stimulation.

hypothalamic paraventricular nucleus; nucleus tractus solitarius; cardiac mechanoreceptors; Fos; nitric oxide synthase

ACUTE ELEVATIONS IN PLASMA volume result in increased atrial pressure and distension, which activates the cardiopulmonary receptors located on or near the heart (2, 4, 25). Volume expansion evokes compensatory reflex responses, including a decrease in renal sympathetic nerve activity (RSNA) and vasopressin secretion, and an increase in renal blood flow, urinary flow rate, sodium excretion, and the release of atrial natriuretic peptide (2, 8, 27). The impairment of this reflex is believed to contribute to the autonomic disturbances observed in some disease states, including heart failure where, for example, there is inappropriate elevation in sympathetic nerve activity (SNA) to the kidneys.

The afferent limb of the cardiac mechanoreceptor reflex relays information about blood volume from the heart to the central nervous system via the vagus nerve and terminates in the nucleus of the solitary tract (NTS) within the dorsomedial medulla oblongata. The central pathways subsequently involved in the reflex arc have not been elucidated; however, there is strong evidence that the autonomic responses depend on neurons within the paraventricular nucleus (PVN) of the hypothalamus. Volume expansion produces an increase in Fos expression in the PVN (15, 33). This effect is significantly reduced when intrapericardial procaine is administered to block the cardiac mechanoreceptors (5). Furthermore, destruction of neurons within the paravascular division of the PVN, or acute inhibition of these neurons by microinjection of muscimol, markedly attenuates the reflex reduction in RSNA and the reflex increase in renal blood flow normally observed in response to acute volume expansion (9, 24, 28). In addition, chemical activation of the PVN using discrete microinjections of α,β-homocysteic acid evokes a pattern of SNA, including a decrease in RSNA and an increase in splanchnic, adrenal, and cardiac SNA, that closely mimics that seen following acute volume expansion (7).

The hypothalamic PVN is composed of functionally different subgroups of neurons, including parvocellular neurons that project to important autonomic sites, i.e., preautonomic neurons. Subpopulations of parvocellular preautonomic neurons project to autonomic targets in the brain stem and spinal cord that are critical to cardiovascular regulation, such as the intermediolateral cell column and rostral ventrolateral medulla (RVLM) (32, 34, 35). Furthermore, there are PVN neurons that send collaterals to both the spinal cord and RVLM (32, 35).

The RVLM is an important autonomic region that projects directly to the intermediolateral column and is believed to be responsible for the tonic generation of SNA (6, 39). The direct connection between the PVN and the RVLM is likely to be involved in the sympathetic nerve responses elicited by activating the PVN. This pathway may also contribute to the reflex sympathetic nerve responses elicited by disturbances in blood volume. In a recent report from our laboratory, we provided evidence supporting the involvement of a subpopulation of the RVLM-projecting neurons within the PVN in mediating the reflex responses elicited by cardiac mechanoreceptor stimulation (15).

Nitric oxide (NO) is a ubiquitous modulatory molecule, which acts as a nonconventional neurotransmitter in the central nervous system, particularly within the central autonomic regions, where it generally exerts an inhibitory influence on SNA.

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whether a subpopulation of those project to the RVLM. The NTS contains a dense concentration of neurons that express NO synthase (NOS), the enzyme responsible for the production of NO. In the anesthetized rat, NO within the PVN appears to tonically inhibit RSNA as well as contribute to the reflex reduction in RSNA elicited by volume expansion (15, 31). Moreover, the NTS is known to contain neurons that are activated by stimulation of the cardiac mechanoreceptors (15), particularly in the caudal NTS. Neurons containing NOS are present in this region of the NTS, and NO within the NTS can influence cardiovascular regulation (14, 26, 40). Thus an additional aim of the present study was to examine whether 1) NO is present in neurons in the PVN that are activated by stimulation of the cardiac mechanoreceptors; and 2) whether a subpopulation of those neurons project to the RVLM.

The NTS is an important brain region critical in cardiovascular regulation and is the site of termination for primary cardiovascular afferents. Neurons within the NTS are activated by volume expansion and by cardiac mechanoreceptor stimulation (15, 31). Moreover, the NTS is known to contain neurons that provide a dense innervation of the RVLM, and we have previously found that some of those neurons are activated following stimulation of the cardiac mechanoreceptors (15), particularly in the caudal NTS. Neurons containing NOS are present in this region of the NTS, and NO within the NTS can influence cardiovascular regulation (14, 26, 40). Thus an additional aim of the present study was to examine whether stimulation of cardiac mechanoreceptors resulted in the activation of nitrergic neurons in the NTS and to determine whether a subpopulation of those project to the RVLM.

**METHODS**

Male Sprague-Dawley rats (200–250 g) obtained from Monash University Animal Services were used in the present study. The experimental protocols were approved by RMIT University Animal Ethics committee, and all protocols conform to the guidelines set out by the American Physiological Society (1) and the National Health and Medical Research Council of Australia, as well as the Australian government regulations. Every attempt was made to minimize animal suffering and to reduce the number of animals needed. Surgical procedures were performed under general anesthesia [intraperitoneal pentobarbitone sodium 60 mg/kg (Nembutal 60 mg/ml, Boehringer Ingelheim, NSW, Australia), with supplemental doses of 20 mg/kg ip every 50 min if required] and subcutaneous Buscopan Compositum (N-butyrobromide, 12.5 mg/kg, and dipyrone, 0.1 mg/kg; Boehringer Ingelheim) was administered to prevent excessive salivary secretions. The antibiotic oxytetracycline (200 mg/kg sc Terramycin, Provet, Victoria, Australia), and the analgesic buprenorphine HCl (15 μg ip, Temgesic, Reckitt and Colman Pharmaceuticals, NSW, Australia) were routinely administered at the conclusion of each procedure.

**Injections of Retrogradely Transported Tracer into the RVLM**

Under general anesthesia, the right femoral artery was cannulated to enable blood pressure monitoring. The left RVLM was functionally located by microinjection of L-glutamate (50 nl, 0.1 M in saline) using a glass micropipette (tip diameter 50–70 μm). After the RVLM was located (an increase of 20–50 mmHg in the arterial blood pressure was observed when glutamate was microinjected in the RVLM), the pipette was withdrawn, emptied of the glutamate solution, and filled with retrogradely transported tracer (rhodamine-tagged microspheres, LumaFluor, NY, diluted 1:1 in 0.9% sterile normal saline). Typically, the coordinates of the RVLM were 2.5–3.5 mm caudal to the lambdoid suture, 1.8–2.2 mm lateral of the midline (sagittal suture), and 8.9 mm from the dural surface. The micropipette was reinserted into the RVLM pressor site, and 250 nl of the tracer were unilaterally injected over 5–10 min. The pipette was left in place for 10 min after completion of the injection before being gradually removed over 5–10 min. Subsequently, the arterial cannula was removed from the femoral artery, and the head and groin wounds were closed with sutures. The animals were left to recover in a warmed enclosure before being returned to their home cages. Two weeks were allowed to elapse before implantation of the balloon-tipped cannula. At the end of the experiment, the site of the microinjection was confirmed histologically to encompass the RVLM.
Implantation of Balloon-Tipped Cannula at the Right Atrial-Vena Caval Junction

The procedure for implantation of a balloon-tipped cannula at the right atrial-superior vena caval junction has been previously described (15). In brief, under general anesthesia, a balloon-tipped cannula was inserted into the jugular vein. The tip was placed ~21 mm from the clavicle, a distance that corresponded with the junction of the right atrium and superior vena cava. The balloon catheter was tied in position, and the free end of the Silastic tubing was passed subcutaneously to emerge between the scapulae. The wounds were then sutured closed, and the animal was allowed to recover in a warmed cage. The manufacture of the balloon was based on a previous description (17). The procedure used to manufacture the balloons used in the present study has been described in detail previously (15).

Experimental Protocol

The animals were allowed to recover for 4 days after the balloon implantation surgery, before any experimental procedures were performed. On the experimental day, the rats were brought into the laboratory 2 h before use to habituate to their surroundings. In the treatment group, the balloon was inflated for 90 min, using previously boiled saline that was cooled to room temperature to minimize bubble formation in the balloon. After this time, the animal was deeply anesthetized with pentobarbitone (100 mg/kg ip) and perfused through the aorta with 300 ml of 0.1 M phosphate buffered saline (pH 7.4) containing heparin (50 U/ml), followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were then carefully removed, postfixed in the same fixative (for 1–3 h), and left overnight in 0.1M phosphate buffer containing 20% sucrose. The position of the inflated balloon was confirmed by postmortem examination. The control group underwent similar procedures to the treatment group, except the balloon was not inflated.

Detection of Activated Nitrergic Neurons by Combined Fos Immunohistochemistry and NADPH-Diaphorase Histochemistry

One in two serial sections of the hypothalamus encompassing the PVN and one in three serial sections of the medulla encompassing the NTS were cut (40 μm) and collected. Medullary and hypothalamic

![Diagram](image-url)
series were processed (in groups of three for each region) for the detection of activated nitricergic neurons by combining Fos immunohistochemistry with NADPH-diaphorase (NADPH-d) histochemistry. In brief, routine immunohistochemical labeling of Fos (1:20,000, Ab5, Oncogene Research Products, Cambridge, MA) was performed through to the stage of incubating the sections in ExtrAvidin (1:400, Sigma-Aldrich) for 60 min (13). At this point, the process for the detection of NADPH-d was begun. The sections were washed only once in Tris buffer (0.05 M, pH 7.6) before being incubated for ~13 min in 0.2% Triton X-100, 0.1 mg/ml nitroblue tetrazolium (Sigma), and 1.0 mg/ml β-NADPH (Sigma) made in 0.05 M Tris buffer, at 37°C. The coloration (blue) of NO-producing neurons was stopped by two washes in Tris buffer, each of 5-min duration. At this stage, the process for the detection of Fos was continued, and the sections were incubated for 10 min in 0.05% 3,3′-diaminobenzidine hydrochloride (to produce a brown precipitate) in Tris buffer before the addition of 5 μl of 30% H2O2. The reaction was terminated ~5 min later by three washes (5-min duration each) with Tris buffer, and the sections were subsequently mounted onto subbed slides.

Analysis

Retrogradely labeled neurons were detected using a fluorescent light source while NADPH-d-positive cells and Fos-positive cell nuclei were identified under normal bright-field illumination. Detection of retrogradely labeled neurons that also stained positive for NADPH-d and/or contained a Fos-positive cell nucleus was performed by rapidly switching between the two light sources. Labeled neurons and Fos-positive cell nuclei were counted on one side of the brain, ipsilateral to the injection, in a total of 15 hypothalamic sections encompassing the entire rostral-caudal extent of the PVN, and 15 medullary sections, extending 1.2 mm caudal and 0.5 mm rostral of the obex, to encompass the NTS.

The data were expressed as the average number of labeled neurons per section at each level (defined by the group of three sections processed). The overall mean values for each group were calculated, and comparisons between treated and control groups were performed using Student’s t-test. Post hoc analysis of the means of each level was performed using Student’s unpaired t-test, and a Bonferroni correction was applied for multiple comparisons.

Image Acquisition and Mapping

Images were acquired using a digital SPOT camera on an Olympus BX60 microscope. The digital images were imported into PhotoShop (version 5.5 Adobe), and only the contrast and lightness were modified for presentation purposes.

For illustration of the different levels of the PVN and NTS, maps were drawn representing each of the rostral-caudal levels. The position of the neurons that were 1) Fos positive, 2) RVLM projecting, 3) NADPH-d positive, or contained a combination of these labels, were captured using the software package MD Plot (version 4.0) and a MD3 microscope digitizer stage (Minnesota Datametric) attached to a Leica DMLB microscope and then overlaid onto representative sections containing subnuclei within the PVN and NTS (as defined in Refs. 11 and 38, respectively).

RESULTS

PVN

Distribution of NADPH-d-positive neurons. NADPH-d-positive neurons were distributed throughout the rostral-caudal extent of the PVN, including the magnocellular and all subnuclei of the parvocellular PVN (Figs. 1 and 2). In the present study, only the parvocellular PVN has been analyzed in detail. The maximum distribution of NADPH-d-positive neurons was found in the midcaudal levels of the parvocellular PVN (Figs. 2 and 3). On average, a total of 463 ± 89 neurons were counted unilaterally, which stained positively for NADPH-d in the treated group (n = 6), and a similar number was found in the control group of animals (492 ± 48; n = 6).

Distribution of Fos-positive nuclei following balloon inflation. Fos production in the parvocellular PVN was markedly increased by balloon inflation (Figs. 1–3). Overall, a significant

![Fig. 3. Average numbers of NADPH-d-positive neurons (top), Fos-positive neurons (middle), and NADPH-d-positive neurons that also contain a Fos-positive nucleus (bottom) in five different rostral-caudal levels of the PVN (see text for details). Solid bars show data from rats in which the atriovenous balloon was inflated. Hatched bars represent data from controls. *P < 0.05 and **P < 0.01 compared with respective control level.](http://ajpregu.physiology.org/)
six- to sevenfold increase in the total number of Fos-positive neurons was observed in the treated group (761 ± 137) compared with control (115 ± 8; P < 0.001; unilateral counts). The number of Fos-positive neurons was maximal in the mid-to caudal levels of the PVN, and these neurons were located predominantly in the medial and lateral parvocellular PVN and to a lesser extent in the dorsal parvocellular PVN (Figs. 2 and 3).

Distribution of NADPH-d-positive neurons containing a Fos-positive nucleus. The number of neurons within the parvocellular PVN that stained positive for NADPH-d and also contained a Fos-positive nucleus increased dramatically by 14-fold following balloon inflation compared with control (P < 0.001). On average, the total number of these double-labeled neurons unilaterally in the PVN of the treated group was 235 ± 41, compared with 17 ± 3 in the control group. The maximum number of these double-labeled neurons was found in the midlevel of the PVN (Figs. 1 and 3). The distribution of the NADPH-d-positive neurons containing Fos in the PVN is exemplified in Fig. 2 and shows that these double-labeled neurons were observed in all subnuclei. The population of double-labeled neurons represented, on average, over one-half (51%) of the NADPH-d-positive neurons within the parvocellular PVN.

Distribution of RVLM-projecting neurons. RVLM-projecting neurons were found throughout the PVN and were concentrated in the mid- to caudal levels of the PVN (Figs. 1, 4, and 5). The distribution pattern of these neurons was similar in the balloon-inflated and control groups of animals and is shown schematically in Fig. 5. On average, a total of 273 ± 82 neurons, counted unilaterally, in the PVN were RVLM projecting in the stimulated group. In the control group, 457 ± 42 RVLM-projecting neurons were found. Although this number was slightly greater, there was no statistically significant difference between the groups.

![Diagrammatic illustration of the distribution of rostral ventrolateral medulla (RVLM)-projecting neurons (first row), NADPH-d-positive neurons that are also RVLM-projecting (second row), RVLM-projecting neurons containing a Fos-positive nucleus (third row), and triple-labeled neurons (fourth row) in five different rostral (A) to caudal (E) levels of the PVN. Approximate anterior-posterior levels caudal to bregma are shown in millimeters on the right. For simplicity, not all cells could be represented by dots in regions of high density of the RVLM-projecting cells.](image-url)
Distribution of NADPH-d-positive neurons projecting to the RVLM. NADPH-d-positive neurons in the PVN that were also retrogradely labeled were present at all levels of the PVN, but the maximum number were found in the mid- to caudal levels (Figs. 1, 4, and 5). Overall, in the treated group, 38 ± 6 of the RVLM-projecting neurons within the PVN (unilaterally) were NADPH-d positive. In the control group of animals, 47 ± 5 of the RVLM-projecting neurons were double labeled.

Distribution of RVLM-projecting neurons containing a Fos-positive nucleus. The number of retrogradely labeled neurons that also contained a Fos-positive nucleus was significantly increased following balloon inflation. Overall, Fos-positive RVLM-projecting neurons within the PVN in the treated group increased sevenfold compared with the control group (P < 0.05; Fig. 5). These double-labeled neurons were concentrated in the midcaudal levels of the PVN, which coincided with levels at which the maximum number of RVLM-projecting neurons were observed (Figs. 1, 4, and 5). In the balloon-inflated animals, the average number of Fos-positive RVLM-projecting neurons counted unilaterally was 30 ± 6 and represented 11% of the total number of RVLM-projecting neurons in the PVN. In the control group, there were very few Fos-positive RVLM-projecting neurons (4 ± 1), and these represented only 1% of the total number of RVLM-projecting neurons in the PVN.

Distribution of retrogradely labeled, NADPH-d-positive neurons containing a Fos-positive nucleus. Following balloon inflation, there was a marked increase in the number of Fos-positive NADPH-d-positive neurons projecting to the RVLM (i.e., triple labeled), compared with control (P < 0.05; Figs. 1, 4, and 5). These neurons were scattered primarily in the midcaudal levels of the PVN. Overall, the number of triple-labeled neurons counted unilaterally averaged 23 ± 9, which represented 61% of the RVLM-projecting, NADPH-d-positive neurons in the treated group compared with only 3 ± 1 neurons (i.e., 6%) in controls.

Distribution of NADPH-d-positive neurons. NADPH-d-positive neurons were detected throughout the rostral-caudal extent of the NTS (Figs. 6 and 7). The majority of NADPH-d-positive neurons, however, were found in the rostral levels of the NTS (Figs. 6, 7, and 8). On average, 474 ± 86 neurons were stained positively for NADPH-d in the treated group (n = 8), and a similar number was found in the control group of animals: 611 ± 114 (unilaterally) (n = 6).

Distribution of Fos-positive nuclei following balloon inflation. Fos production in the NTS was markedly increased by balloon inflation (Fig. 8). Overall, a significant threefold increase in the number of Fos-positive neurons was observed in the treated group (average total = 592 ± 54) compared with control (217 ± 68; P < 0.001; unilateral counts). In the treated group, Fos-positive neurons were present throughout the NTS, including a prominent band in the commissural subnucleus in the caudal NTS (Figs. 6 and 7). In the control group, neurons expressing Fos were scattered in all rostral-caudal levels (Fig. 8).

Distribution of NADPH-d-positive neurons containing a Fos-positive nucleus. The total number of neurons counted unilaterally within the NTS that stained positive for NADPH-d and also contained a Fos-positive nucleus increased significantly by fourfold following balloon inflation (39 ± 10) compared with control (10 ± 4; P < 0.05). However, when the individual levels of the NTS were examined, the differences between the groups at each level did not attain statistical significance (P > 0.05, Bonferroni modified t-statistic; Fig. 8). The double-labeled neurons were found predominantly in the rostral levels of the NTS, where NADPH-d-positive neurons
were most abundant (Figs. 7 and 8). Double-labeled neurons were also found in the commissural subnucleus of the caudal NTS (Fig. 7).

**Distribution of RVLM-projecting neurons.** RVLM-projecting neurons were found throughout the NTS and at all rostral-caudal levels (Figs. 9 and 10). The distribution of these neurons was similar in the balloon and control groups of animals (Fig. 10). The total number of RVLM-projecting neurons unilaterally in the NTS was 831 \( \pm \) 74 in the balloon-inflated group compared with 913 \( \pm \) 364 in the control group.

**Distribution of NADPH-d-positive neurons projecting to the RVLM.** The distribution of the RVLM-projecting neurons overlapped with that of the NADPH-d-positive neurons; however, there were few double-labeled neurons (Figs. 9 and 10). Overall, 38 \( \pm \) 6 of the retrogradely labeled neurons were also NADPH-d positive in the treated group. This represented \( \sim 4\% \) of the retrogradely labeled neurons. A similar number of such double-labeled neurons were found in the NTS of the control group of animals (47 \( \pm \) 5, or 4\% of retrogradely labeled neurons; \( P > 0.05 \) between groups; unilateral counts).

**Distribution of RVLM-projecting neurons containing Fos-positive nuclei.** Following balloon inflation, the number of retrogradely labeled neurons counted unilaterally that also contained a Fos-positive nucleus was increased significantly following balloon inflation compared with controls (\( P < 0.05 \); Figs. 9 and 10). Overall, 44 \( \pm \) 12 neurons, or 5\% of RVLM-projecting neurons in the NTS, were Fos positive following balloon inflation. The distribution of these double-labeled neurons was largely confined to the commissural nucleus in the caudal levels of the NTS (Figs. 9 and 10). In control animals, RVLM-projecting neurons that also contained Fos were few (7 \( \pm \) 3) and represented only 1\% of the total number of retrogradely labeled cells in the NTS.

**Distribution of retrogradely labeled NADPH-d-positive neurons containing a Fos-positive nucleus.** RVLM-projecting neurons that were Fos-positive and contained NADPH-d (i.e., triple-labeled) were few in number in both groups of animals (Figs. 9 and 10). In the treated group, the average total number of triple-labeled neurons counted unilaterally was 6 \( \pm \) 2, which was statistically significantly greater than in the control group (1 \( \pm \); \( P < 0.05 \)). Comparisons of the different NTS levels, however, did not show any statistically significant difference between the treated and control group (\( P > 0.05 \), Bonferroni \( t \)-statistic; Fig. 10).

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**Fig. 6.** Photomicrographs showing the distribution of Fos-positive neurons and NADPH-d-positive neurons in the rostral nucleus tractus solitarius (NTS). A: predominance of NADPH-d-positive neurons in the rostral NTS at low power. B: NADPH-d-positive neurons (examples are highlighted by arrows) in higher power, present in the area outlined in A. C: low-power photomicrograph of the caudal NTS from a balloon-inflated rat. D: high-power photomicrograph of the area outlined in C showing the predominance of Fos-positive cell nuclei in the commissural NTS. E: low-power photomicrograph of the caudal NTS (at a similar level to C) from a control rat. F: high-power micrograph of the area outlined in E showing the commissural NTS of a control rat. Scale bar = 100 \( \mu \)m in A, C, and E; 50 \( \mu \)m in B; and 40 \( \mu \)m in D and F.
Extent of Injection Site in the RVLM

To determine the extent of the spread of the injection site, the RVLM was sectioned and mounted onto gelatine-subbed slides, coverslipped, and viewed under a fluorescent light source. The injection sites were discrete (see Fig. 11 for a representative example) and encompassed the full rostral-caudal extent of the pressor region of the RVLM, defined as the area 0.6 mm caudal from the inferior pole of the facial nucleus. In control animals, the center of the injection site was located 360 ± 23 μm caudal of the inferior pole of the facial nucleus and covered an average rostral-caudal distance of 705 ± 53 μm. In the treated group of animals, the center of the injection site was located a similar distance caudal of the inferior pole of the facial nucleus (339 ± 24 μm) and covered an average rostral-caudal distance of 658 ± 34 μm.

DISCUSSION

The present study highlights several novel findings. First, in the PVN and NTS, a significant proportion of the neurons activated by cardiac mechanoreceptor stimulation were nitrergic. Moreover, within the parvocellular PVN, approximately one-half of the nitrergic neurons were activated by this stimulus. Second, in both nuclei, there was a significant increase in the number of activated nitrergic neurons that projected to the RVLM. Indeed, within the parvocellular PVN, ~61% of the activated RVLM-projecting neurons were nitrergic.

In the present work, there was a marked increase in the expression of Fos within the PVN following cardiac mechanoreceptor stimulation using a small, inflatable balloon positioned near the junction of the superior vena cava and the right...
atrium. This confirms the findings in anesthetized animals (33) and our own previous observations in conscious animals (15).

It is interesting to note that the number of Fos-positive cell nuclei in the PVN were very low following stimulation of the cardiac mechanoreceptors in the anesthetized preparation (33), compared with the conscious rat (Ref. 15 and the present observations). When blood volume is increased by intravenous fluid infusion, similar increases in Fos production in the PVN have been reported (5, 31). Thus the PVN is activated by cardiac mechanoreceptor stimulation. Taken together with findings showing that inhibition or destruction of the PVN antagonizes the reflex sympathetic renal nerve inhibition normally observed following a volume load, the evidence to date indicates that the PVN plays an essential role in blood volume regulation (9, 24, 28).

The PVN is among the brain nuclei that have the highest concentration of neurons that contain NOS, the enzyme responsible for the production of the atypical neurotransmitter NO (14, 19, 37, 42). Both magnocellular and parvocellular PVN neurons contain NOS. The present work shows, for the first time, that approximately one-half of the NADPH-d-positive (a marker for NOS) neurons in the parvocellular PVN were activated by cardiac mechanoreceptor stimulation. Furthermore, these activated nitrergic neurons represented approximately one-third of the activated neurons. These results suggest that the stimulation of the cardiac mechanoreceptors activates nitrergic neurons in the parvocellular PVN, which may play an important role in the central pathways mediating the reflex responses induced. This is in agreement with the findings in the rat showing that NO within the PVN inhibits RSNA (42, 43) and that stimulation of the cardiac mechanoreceptors results in decreases in RSNA (Ref. 33 and personal observations). Furthermore, the activation of nitrergic neurons suggests there is an increase in the production of NO. Since NO easily diffuses through cell membranes and is thereby capable of influencing neurons adjacent to the neurons from which it has originated, the influence of NO following its release from activated neurons could be widespread within the PVN.

The PVN contains neurons that project to the RVLM, a nucleus critically important in sympathetic nerve regulation. The present study shows that RVLM-projecting neurons within the PVN are activated by cardiac mechanoreceptor stimulation, suggesting that this connection contributes to the central pathways mediating changes in SNA mediated by disturbances in blood volume. This finding also confirms our earlier work (15). We now extend those observations by showing that some of the activated neurons in the PVN that project to the RVLM are nitrergic; indeed, of the nitrergic neurons in the parvocellular PVN that project to the RVLM, 61% were activated. Thus our work suggests that there is a population of nitrergic neurons in the parvocellular PVN that project to the RVLM and contribute to the changes in SNA elicited by disturbances in blood volume. This concurs with reports that show NO within the PVN plays an important role in the reflex reduction in RSNA induced by volume expansion (42, 43) and with recent electrophysiological studies that indicate that NO inhibits parvocellular neurons in the PVN (36, 37). These studies contrast with the conclusions of Yang and Coote (41) in the anesthetized rabbit. Interestingly, in the conscious rabbit, Nω-nitro-L-arginine methyl ester does not significantly affect the reflex reduction in RSNA in response to volume expansion (28). The presence and type of anesthesia and species differences may account for the discrepancies. It should also be noted that the activated nitrergic neurons that projected to the RVLM following balloon inflation in the present study represented ~8% of

Fig. 8. Average numbers of NADPH-d-positive neurons (top), Fos-positive neurons (middle), and NADPH-d-positive neurons that also contain a Fos-positive nucleus (bottom) in five different rostral-caudal levels of the NTS (see text for details). Solid bars show data from rats in which the atriovenous balloon was inflated. Hatched bars represent data from controls. *P < 0.05 and **P < 0.01 compared with respective control level.
the total number of RVLM-projecting neurons within the PVN (compared with <1% in controls), 5% of the nitrergic neurons in the parvocellular PVN, and 3% of the total number of activated neurons.

The role of NO in the activated RVLM-projecting neurons of the PVN is unknown. Since these neurons are activated, the effect of the inhibitory actions of NO on those neurons would counteract the activation that the neurons are experiencing. Thus the response of these neurons to cardiac mechanoreceptor stimulation is likely to be complex. One possibility is that the nitrergic neurons are activated and subsequently inhibited. However, it is unlikely that the neuron is inhibited entirely, since Fos production is increased; thus we hypothesize that NO within an activated neuron may act as a brake to reduce the degree to which the neuron increases its activity. As NO diffuses through cell membranes, adjacent neurons may be inhibited, as discussed above, and, assuming the activated neurons projecting to the RVLM are also increasing NO production at the terminal, neurons in the RVLM may also be influenced. Thus the effect of NO within the PVN is likely to be extensive.

Our findings also suggest that nitrergic neurons in the PVN that are activated by stimulation of the cardiac mechanoreceptors must include neurons projecting to brain regions other than the RVLM. The PVN projects to the dorsomedial medulla, caudal ventrolateral medulla (CVLM), and spinal cord, and NOS has been observed in those neurons in varying proportions (10, 20). It is not known whether some of those neurons may also be involved in volume regulation, since there is scant information regarding the physiological function of those nitrergic neurons. It is of interest to note, however, that hypotension and hypotensive hemorrhage have been shown to

Fig. 9. Diagrammatic illustration of the distribution of RVLM-projecting neurons (A), NADPH-d-positive neurons that also project to the RVLM (B), RVLM-projecting neurons containing a Fos-positive nucleus (C), and triple-labeled neurons (D) in five different rostral (A) to caudal (E) levels of the NTS. Approximate anterior-posterior levels rostral (+) or caudal (−) to the obex are shown in millimeters on the right. For simplicity, not all cells could be represented by dots in regions of high density of the RVLM-projecting cells.
activate a subpopulation of the nitrergic PVN neurons that project to the dorsomedial medulla and CVLM, suggesting that these neurons may be involved in reflex responses initiated by both of those stimuli (20).

In the NTS, there was a threefold increase in the number of activated neurons following balloon inflation compared with controls, confirming our earlier observations (15). The NTS is the initial brain site in which the primary afferents of the cardiac mechanoreceptors travel in the vagus terminate. The activation of neurons in the NTS was expected, and the distribution of the Fos-positive neurons occurred in regions known to contain the terminals of vagal afferents (12). For the first time, the present study shows that Fos-positive neurons were found in regions containing NADPH-d-positive neurons, and that ~8% of NADPH-d-positive neurons were activated following cardiac mechanoreceptor stimulation, compared with 2% in controls. Some topographical distribution was suggested by the fact that the more rostral parts of the NTS and the caudal commissural NTS were the predominant sites for these activated nitrergic neurons. Thus the results suggest that a proportion of nitrergic neurons in the NTS contribute to the pathways activated by cardiac mechanoreceptor stimulation.

The NTS provides a dense innervation to the RVLM, and the present findings show that a small proportion of neurons projecting to the RVLM (~5%) are activated by cardiac mechanoreceptor stimulation, and this confirms our earlier observations (15). The present work highlights that some of these neurons contain NADPH-d. Indeed, of the nitrergic neurons that project to the RVLM, 18% were activated following cardiac mechanoreceptor stimulation. It should be noted that these triple-labeled neurons in the NTS represented <1% of the RVLM-projecting population. Thus this nitrergic pathway may contribute to blood volume regulation, but it is likely to be only a minor component of the central pathways involved.

The production of Fos highlights activated neurons, and the absence of this marker does not exclude a role for neurons in a particular response. For example, a neuron may be inhibited by a particular stimulus and will not increase Fos production. Additionally, secondary influences that result in activation of neurons may impact on the interpretation of experiments that use Fos as a marker. We have used a small balloon inserted into the junction of the superior vena cava and right atrium to...
stimulate cardiac mechanoreceptors. This stimulus simulates volume expansion and produces changes in SNA and hormonal levels that mimic the physiological response (16, 33). Inflation of the atriovenous balloon to activate cardiac mechanoreceptors is a more selective stimulus than volume expansion. In the rat, a volume load can change blood pressure and activate the arterial baroreceptor reflex. Although not measured in the present study, previous experiments show that activation of cardiac mechanoreceptors is not usually associated with significant changes in blood pressure (15, 17, 33). Indeed, following inflation of the balloon, there were few activated neurons in the CVLM that projected to the RVL (15). In stark contrast, this pathway is strongly activated following increases in blood pressure (3).

Perspectives

There is growing evidence to suggest that NO within the rat brain influences the central pathways mediating the reflex responses to volume expansion. The pathways involved have not been investigated to date. The present study suggests pathways that project to the RVL from the PVN and, to a much smaller extent, the NTS are likely to contribute. NO is an atypical neurotransmitter that passes through cell membranes easily, and its production in activated neurons suggests that it could act in an autocrine and paracrine fashion. In electrophysiological experiments, NO has been shown to inhibit PVN neurons that project to the RVL. Thus its production within an activated neuron could result in two opposing forces, an inhibitory influence on that neuron by NO, which would reduce the level of activation of that neuron. Thus we hypothesize that NO would act to brake or dampen the degree to which the neuron increases its activity. Additionally, NO could inhibit adjacent neurons. This widespread influence of NO within the PVN may provide a unique way in which the reflex responses initiated by volume expansion are integrated.

The PVN is essential for the reflex reduction in RSNA and the resultant increase in renal blood flow elicited by volume expansion, and NO contributes to this response in the rat (9, 24, 28). In heart failure, the cardiac mechanoreceptor reflex is attenuated, and this is believed to contribute to the abnormal elevation in SNA (including that to the kidneys) that is a characteristic of this debilitating condition (30, 45). Several reports indicate that downregulation of NOS within the PVN is characteristic of this debilitating condition (30, 45). Several reports indicate that downregulation of NOS within the PVN is characteristic of this debilitating condition (30, 45).

References


