Volume expansion does not activate neuronal projections from the NTS or depressor VLM to the RVLM

ANTHONY D. SHAFTON, ANDREW RYAN, BARRY McGRATH, AND EMILIO BADOER
Department of Medicine, Monash Medical Centre, Monash University, Clayton 3168, Melbourne, Victoria, Australia

Shafton, Anthony D., Andrew Ryan, Barry McGrath, and Emilio Badoer. Volume expansion does not activate neuronal projections from the NTS or depressor VLM to the RVLM. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R39–R46, 1999.—We investigated whether a monosynaptic connection from the nucleus tractus solitarius (NTS) or the depressor ventrolateral medulla (VLM) to the pressor region of the rostral VLM (RVLM) constituted part of the reflex pathway activated by cardiopulmonary baroreceptors. Volume expansion in the conscious rabbit, which elicits renal nerve inhibition predominantly via cardiac mechanoreceptors, was used as the stimulus. The protein Fos was used as a marker of neuronal activation. The retrogradely transported tracer rhodamine-tagged microspheres, previously injected into the pressor region of the RVLM, identified medullary neurons that projected to that region. Volume expansion significantly increased the number of Fos-positive cell nuclei in the NTS and in the depressor VLM. Neurons that projected to the RVLM were found throughout the depressor region of the VLM and in the NTS but were not activated by volume expansion. Thus, although the central reflex pathways activated by volume expansion include the NTS and the depressor region of the VLM, we could not find evidence for a monosynaptic connection between those regions and the RVLM.

Cardiopulmonary mechanoreceptors; medulla oblongata; Fos immunohistochemistry; central nervous system pathways; nucleus tractus solitarius; rostral ventrolateral medulla

ARTERIAL BARORECEPTOR INPUT has a dominant influence on normal nervous control of blood pressure. Activation of the cardiopulmonary baroreceptors also has marked effects on sympathetic nerve activity and plays an important role in the reflex inhibitory responses elicited when blood volume is increased. In conditions of chronic fluid overload like congestive heart failure (CHF), there is an attenuation of the normal sympathetic inhibition elicited by the activation of the arterial and cardiac baroreceptors (14, 15, 42, 43). This may contribute to the abnormal elevation of sympathetic nerve activity that occurs early in the course of this condition and that is a key factor in the pathophysiology of CHF (14, 15, 35, 42, 43).

Cardiopulmonary mechanoreceptors are activated by volume expansion and result in a reduction of sympathetic nerve activity (3, 7, 10, 13, 29, 39). In the conscious rabbit, these mechanoreceptors are located on the heart (7). The sympathoinhibition induced by cardiac mechanoreceptors is similar to the response elicited after stimulation of the arterial baroreceptors.

The central nervous system pathways utilized by the arterial baroreceptors have been extensively studied, and there is considerable evidence indicating that the essential pathways mediating the arterial baroreceptor reflex reside within the medulla oblongata (5, 9, 12, 32, 37). In brief, the arterial baroreceptor afferents terminate in the nucleus tractus solitarius (NTS). Excitatory neurons project from there to the caudal and intermediate ventrolateral medulla (VLM). In this region, depressor neurons project to the rostral ventrolateral medulla (RVLM) to inhibit the pressor neurons that project to the sympathetic preganglionic motoneurons in the spinal cord.

In contrast, very little is known about the central pathways utilized by the cardiac afferents. In a recent report, in which we examined the regions in the brain that were activated in response to volume expansion, we found that neurons in the NTS and intermediate VLM were activated, and there was no marked activation of neurons in the RVLM (3). This distribution of activated neurons in the medulla oblongata was similar to that observed after stimulation of arterial baroreceptors induced by an elevation of arterial pressure (5, 24). The findings suggested that similar central pathways may be utilized by the cardiac and arterial baroreceptor-sympathetic nerve reflexes. Thus the aim of the present study was to determine whether neurons in the VLM that projected to the RVLM were activated by volume expansion in the conscious rabbit. We also determined whether NTS neurons that project directly to the RVLM were activated after volume expansion.

This study used the protein product Fos as a marker of neuronal activation combined with neuroanatomic tract tracing techniques (4, 5). Fos is elevated markedly in neurons after their activation, and this technique has gained widespread popular support as a functional marker of neuronal pathways (16). Using these combined techniques, we were able to identify the population of neurons in the VLM and the NTS that projected to the RVLM and to determine whether they were activated after volume expansion.

EXPERIMENTAL PROCEDURES

New Zealand White male rabbits weighing between 2.5 and 3 kg, obtained from Monash Animal Services (Monash University, Clayton, Victoria, Australia), were used in the study. All experimental protocols were approved by the Monash Medical Centre and Monash University Animal Ethics committees and conform to the guidelines set by the National Health and Medical Research Council and all govern-

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ment regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Injection of rhodamine-tagged microspheres into the RVLM. The rabbits were initially sedated with diazepam (10 mg im) and 15 min later anesthetized with a mixture of ketamine hydrochloride (50 mg/kg im) and xylazine hydrochloride (7 mg/kg im). Supplementary doses of ketamine (50 mg/kg im) were given as required, usually at 50-min intervals. The central ear artery and the marginal vein were cannulated for recording blood pressure or administering intravenous infusions, respectively. The head of the rabbit was placed in a David Kopf stereotaxic frame with the dorsal surface uppermost and the head ventrificleaxed 90° (2). A mid sagittal incision of ~7 cm was made from the occipital protuberance to the level of the second cervical vertebra. The underlying muscles were retracted to expose the atlanto-occipital membrane that was cut and retracted to expose the dorsal surface of the medulla oblongata.

The pressor region of the RVLM was located by stereotaxic microinjection of 50 nl of the excitatory amino acid L-glutamate (0.1 M) using a graduated glass micropipette. The coordinates used were 3.5 mm lateral from the midline, 0.2 mm rostral to the tip of the area postrema, defined as the obex, and 5.5 mm ventral to the brain surface. The increase in blood pressure produced by glutamate ranged between 15 and 40 mmHg. After the pressor region in the medulla oblongata was identified, the glass micropipette was removed from the brain, flushed with saline, and refilled with a solution of rhodamine-tagged microspheres diluted 1:1 with normal saline and reinserted into the pressor region. One hundred thirty to 250 nl of the tracer were injected over 10 min, and the pipette was left in place for a further 15 min after the injection before being slowly removed from the brain. The atlanto-occipital membrane was then sutured closed as were the overlying muscles and the skin. At the end of the surgical procedures, 10 ml sodium lactate compound (Hartman’s solution) was infused into the rabbit intravenously over 10 min, and the arterial and venous cannulas were removed. Immediately postoperatively, all rabbits were given the analgesic buprenorphine hydrochloride (90 µg im) and an antibiotic (chloramphenicol, 150 mg sc).

Experimental protocol. The experiment was performed in conscious rabbits at least 10 days after the tracer injection. On the experimental day, the marginal ear vein was cannulated with a sterile nonpyrogenic catheter. At least 1 h elapsed before the plasma volume expander Haemaccel (Hoechst) was infused intravenously into five rabbits at a rate of 2 ml/min for 60 min. Control rabbits (n = 5) underwent the same procedures except they were not infused with Haemaccel. Ninety minutes after the start of the infusion, the animals were deeply anesthetized with pentobarbitone sodium (60 mg/kg), administered 25,000 units heparin sodium (5 ml), and perfused via the ascending aorta with 1 liter of 0.1 M sodium (60 mg/kg), administered 25,000 units heparin sodium (5 ml), and perfused via the ascending aorta with 1 liter of ice-cold PBS (0.1 M, pH 7.4) followed by 1 liter of ice-cold 4% paraformaldehyde and 250 ml of 30% sucrose in PBS. All solutions were infused at pressures of 90–120 mmHg.

The brain and first two segments of the spinal cord were removed and postfixed in a mixture of 30% sucrose and 4% paraformaldehyde in PBS for 3 h at room temperature, and then in 30% sucrose-PBS solution overnight at 4°C.

Histological processing. On the following day, the brain was frozen, and 40-µm-thick sections of the medulla oblongata were cut. One in every five sections was collected in PBS and processed free floating in groups of five representing 1-mm blocks ranging from 3 mm caudal to the obex and 1 mm rostral to the obex. Fos-positive cell nuclei were detected using standard immunohistochemical procedures. The sections were washed in PBS, and after a 60-min incubation in 10% normal horse serum (NHS), the sections were incubated for 24 h with a primary antibody raised in sheep against a conserved region of the human and mouse Fos (1:4,000 in 2% NHS; OA-11–824, Genosys, UK). After washes in PBS, sections were incubated for 60 min with a biotinylated anti-goat secondary antibody that was raised in the horse (1:200, Vector). After further washes, the sections were incubated for 45 min using an avidin-biotin peroxidase complex (Vector). After washes in Tris buffer (0.05 M, pH 7.6), the sections were incubated for 10 min with 0.05% 3,3'-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate in Tris buffer. Finally, the reaction was begun by the addition of 5 µl of 30% H2O2 and was terminated 6–12 min later by washes with Tris buffer. Sections were subsequently mounted onto subbed slides and allowed to dry. The next day the sections were washed in distilled water and allowed to dry before coverslipping with DePex mounting medium.

To determine the extent of the site of the injection of the rhodamine beads, the rostral medulla oblongata was cut into 40-µm-thick sections. These sections were mounted onto
subbed slides, washed in distilled water, and allowed to dry before coverslipping with DePex mounting medium.

Analysis. Under ×200 magnification, Fos-immunoreactive cell nuclei were detected using normal bright-field illumination, and retrogradely labeled neurons were visualized using fluorescence illumination. By switching between the two light sources, it was possible to determine the neurons that contained both the retrogradely transported tracer (rhodamine) and a Fos-positive nucleus (6). The sections were processed in groups of five representing 1-mm "blocks" ranging from 3 mm caudal to the obex to 1 mm rostral to the obex, which encompassed the caudal and intermediate rostrocaudal levels of the NTS and the VLM. In each region, Fos-positive cell nuclei, retrogradely labeled neurons, and neurons that contained both markers (double labeled) were counted unilaterally in three of the five sections contained in each block and were expressed as the average number per section in each animal. The mean values for each group of animals were calculated, and comparisons between the control and volume-expanded groups were made using the Mann-Whitney U-test.

The rostrocaudal spread of the injection site in the RVLM was determined in each animal and compared between the groups using Student's unpaired t-test.

RESULTS

NTS. Fos-positive cell nuclei in the NTS of control animals were few in number and distributed at all the rostrocaudal levels examined (Fig. 1). After volume
expansion, there was a significant increase in the number of Fos-positive cell nuclei in this region of the brain. These were distributed throughout the rostrocaudal extent of the NTS examined, and the number of Fos-positive cell nuclei counted gradually increased the more rostral the level, with the maximum number attained near the obex level (Fig. 1). The Fos-positive cell nuclei were distributed throughout the NTS as depicted in the schema shown in Fig. 2, which shows the distribution of Fos-positive cell nuclei observed in a rabbit after volume expansion.

Neurons in the NTS that projected to the RVLM (i.e., contained the retrogradely transported tracer) were found at all levels of the NTS and gradually increased from an average of ∼11 neurons/section at the most caudal level examined to ∼54 neurons/section at the most rostral level examined (Fig. 1). There was no significant difference in the number of RVLM-projecting neurons in NTS found in the two groups of rabbits (Fig. 1). The distribution of the RVLM-projecting neurons found in the NTS is depicted in the schema shown in Fig. 2 and illustrates that those neurons were also distributed throughout the NTS.

NTS neurons projecting to the RVLM that also contained a Fos-positive cell nucleus were rare in control rabbits (<1 neuron/section). Volume expansion did not affect this value at any level of the NTS examined (Fig. 1). An example of the distribution of double-labeled neurons at different rostrocaudal levels of the NTS from a rabbit that was volume expanded is shown in Fig. 2. There was no consistent level at which double-labeled neurons were found.

VLM. In control animals, there were very few Fos-positive cell nuclei present in the caudal and intermediate levels of the VLM (Fig. 3). In contrast, after volume expansion, there was a significant increase in the number of Fos-positive cell nuclei observed in the VLM, with the maximum number found near the obex level (Fig. 3). An example of the distribution of Fos-positive cell nuclei in the VLM in a rabbit that was volume expanded is shown in Fig. 4.

Neurons that projected to the pressor region of the RVLM were found at all rostrocaudal levels of the VLM, and there was no marked difference in the numbers counted in treated compared with control rabbits (Fig. 3). In the most caudal part of the VLM, ∼6 neurons/section were detected, and this value increased progressively at each rostrocaudal level examined and reached a maximum in the intermediate VLM where ∼25 neurons/section were counted (Fig. 3). These RVLM-projecting neurons were distributed throughout the VLM as shown in the schema depicted in Fig. 4 and were often intermingled with cells that contained a Fos-positive cell nucleus.

In control animals, neurons in the VLM that projected to the pressor region of the RVLM and that also contained a Fos-positive cell nucleus were not commonly encountered (i.e., <1/section) at any level of the VLM examined (Fig. 3). After volume expansion, there was no marked change in the number of the double-labeled neurons (Fig. 3). The distribution of the double-labeled neurons in the VLM of a rabbit that was volume expanded is depicted in Fig. 4.

Injection site. The injection site was examined histologically and covered the full extent of the RVLM in each animal. The rostral-caudal extent of the injection site in control animals was 1.5 ± 0.1 mm, and in the rabbits that underwent the volume expansion, the rostral-caudal spread of the injection site was 1.3 ± 0.2 mm. There was no statistically significant difference in the size of the injection between the two groups of rabbits. Figure 5 shows a photomicrograph of the injection site in the animal that was used to produce the maps shown in Figs. 2 and 4.

DISCUSSION

This study was designed to determine whether neurons in the NTS and in the caudal and intermediate VLM that were activated following volume expansion also projected to the pressor region of the RVLM. Volume expansion elicits sympathoinhibition, particu-
larly in the nerves to the kidney, which is predomin-
nantly mediated by cardiac mechanoreceptors in the
conscious rabbit and dog (7, 29). The central pathways
involved have not been examined in any great detail,
but we have found previously that neurons in the NTS
and in the caudal and intermediate VLM are activated
following volume expansion in the conscious rabbit (3).
This has been confirmed in the present study and
recently in the conscious rat (31). The midline raphe
have also been investigated, but there is no evidence to
indicate a role for these nuclei in the reflex (3, 27, 31).

The NTS receives afferents from the cardiac mechanoreceptors that travel via the vagus (22, 23). Thus it is
not surprising to observe neurons in the NTS activated
after volume expansion. There are neurons in the NTS
that project to the pressor region of the RVLM, and
their distribution, which we have mapped in the pres-
ent study, was similar to earlier reports (8, 34). Our
findings suggest that these RVLM-projecting neurons
were not activated by volume expansion and provide
evidence that a direct inhibitory pathway from the NTS
to the pressor region of the RVLM does not mediate the
sympathoinhibition elicited by volume expansion. This
conclusion is similar to that regarding the central
pathways mediating the arterial baroreceptor reflex
sympathetic nerve responses (30). In contrast, ~50% of

Fig. 4. Typical distribution of retrogradely
labeled neurons, Fos-positive cell nuclei,
and neurons containing both markers in
depressor region of VLM in a rabbit that
was volume expanded. Seven rostrocaudal
levels are depicted with A most caudal and
G most rostral level. Distance between
each level is 600 µm for A to B, C to D, and
E to F, and 400 µm between remaining
levels. Obex is located between levels de-
picted in E and F. Neurons projecting to
pressor region of RVLM are shown on left,
Fos-positive cell nuclei are shown in
middle, and neurons with projections to
RVLM and also containing a Fos-positive
cell nucleus are shown on right. Because of
high concentration of neurons found in
some parts of VLM, for simplicity, not all
neurons in left and middle could be de-
picted by a single dot. Ion, inferior olivary
nucleus; Irn, lateral reticular nucleus; nspV,
spinal trigeminal nucleus; pyr, pyramidal
tract. Bar = 1.2 mm.
the neurons in the NTS that project to the RVLM were activated after hypoxia, suggesting that the NTS-RVLM projection is important in mediating the reflex sympathetic changes induced by arterial chemoreceptor activation (21).

The caudal and intermediate VLM contains depressor neurons and a high concentration of neurons that project to the pressor region of the RVLM. The depressor neurons that project to the RVLM are essential in mediating the sympathoinhibition elicited by activation of the arterial baroreceptors. In the conscious rabbit and rat, ~30–50% of the VLM neurons with projections to the pressor region of the RVLM were activated by stimulation of the arterial baroreceptors after an increase in blood pressure (5, 30). The majority of those neurons are found in the intermediate VLM near the obex (1, 5, 11, 30, 38).

Volume expansion activates neurons in the caudal and intermediate VLM (3, 31), and we confirmed this in the present study. We hypothesized that those activated neurons may project to the pressor region of the RVLM and mediate the sympathoinhibition elicited by volume expansion. However, we found that the neurons in the VLM that projected to the RVLM were a separate population to, but intermingled with, those that were activated after volume expansion. This finding suggests that the central pathways activated by stimulation of the cardiac mechanoreceptors differ from that activated by stimulation of the arterial baroreceptors.

A prediction of this finding would be that the caudal VLM neurons that are antidromically activated from the RVLM would be activated by arterial baroreceptor stimulation but not by cardiac mechanoreceptors. One would also predict that NTS neurons antidromically activated from the RVLM would be excited by hypoxia but not cardiac mechanoreceptors. This speculation awaits further investigation.

An alternative hypothesis consistent with the present data is that the volume expansion-related sympathoinhibition takes place at the level of the sympathetic preganglionic motoneurons in the spinal cord. This possibility could be investigated using a similar approach as we have used in the present study except that the neuroanatomic tracer would need to be injected into the spinal cord.

The heart not only contains mechanosensitive receptors but also chemosensitive receptors that can be activated by chemicals such as phenyl biguanide acting on 5-HT3 receptors. Activation of cardiopulmonary chemoreceptors with phenyl biguanide elicits sympathoinhibition that has been reported, using electrophysiological recordings in the anesthetized rat, to involve activation of neurons in the NTS and in the depressor region of the VLM and inhibition of the pressor neurons in the RVLM (40). Subsequent work obtained in the conscious rabbit has found that neurons in the depressor region of the VLM that projected to the RVLM were activated (i.e., contained Fos) after intravenous phenyl biguanide (18). Unfortunately, the data were not quantified. We believe that the sympathoinhibition elicited by stimulation of the cardiac mechanoreceptors also involves activation of neurons in the NTS and in the depressor region of the VLM, but we find no evidence that a monosynaptic connection between those regions and the RVLM is activated. Therefore, our present data suggest that the central pathways mediating the cardiopulmonary chemoreceptor-induced sympathoinhibition may differ from the pathway mediating the effects of cardiac mechanoreceptor stimulation. Mechanosensitive afferents arising from the heart that were not sensitive to chemical stimulation have been described previously (36).

Methodological considerations. A major methodological consideration in the present study is the use of the protein Fos as a marker of neuronal activation. This method has been used extensively in the last decade to highlight brain regions activated by various stimuli. However, cells that are actively inhibited will not express Fos. Thus the lack of Fos, after volume expansion, in neurons that projected to the pressor region of the RVLM may not mean that those neurons do not participate in the central reflex pathways. However, if volume expansion does actively inhibit the RVLM-projecting neurons, this would indicate that those neurons provide an excitatory input into the RVLM. This would be an exciting development and requires much further investigation.

One may argue that the neurons projecting to the pressor region of the RVLM may not have been activated sufficiently to express Fos. However, this would

![Fig. 5. Photomicrograph under fluorescent illumination showing injection site in coronal section through RVLM of same rabbit used to produce maps shown in Figs. 2 and 4. Ventrolateral edge of section is shown by dashed line. Bar = 0.6 mm. Inset: low-power tracing of medullary section from which fluorescent photomicrograph was taken showing outline and relative position of injection site (inj site). Na, nucleus ambiguus; ion, inferior olivary nucleus; nspV, spinal trigeminal nucleus; pyr, pyramidal tract.](http://ajpregu.physiology.org/)
seem unlikely given that we observed many neurons in the NTS and in the VLM that contained Fos after volume expansion, and these were intermingled with neurons that projected to the RVLM.

Perspectives

A few brain regions outside the medulla oblongata have also been implicated in the central pathways utilized by cardiopulmonary mechanoreceptor afferents. Neurons in the locus ceruleus have been reported to be inhibited by volume expansion (17, 28), and in the parabrachial nucleus, some neurons are inhibited whereas others are activated by that stimulus (33, 41). However, it is the paraventricular nucleus (PVN) of the hypothalamus that has been postulated to be of particular importance in the responses to cardiopulmonary mechanoreceptor stimulation, and there is strong evidence that it has a crucial role. Ablation of the PVN has been shown to prevent the renal vasodilatation (26) and the inhibition of the renal sympathetic nerve elicited by volume expansion (19). Although the protein marker of neuronal activation, Fos, is increased in the PVN after volume expansion (3, 20), we have found that this effect is dependent on the plasma expander used, and therefore, the PVN is not likely to be activated as a result of volume expansion per se (3). Indeed, evidence indicates that neurons in the PVN are inhibited by volume expansion (4, 25). Hemorrhage activates paravascular neurons, and we have found that a proportion of spinaly projecting neurons located within the PVN were activated by a nontensive hemorrhage (4), suggesting a reduction in blood volume may activate those neurons. This is in agreement with electrophysiological recordings that show that an increase in plasma volume inhibited activated spinally projecting neurons in the PVN (25).

In conclusion, the central nervous system pathways utilized by the cardiopulmonary-sympathetic nerve reflex are not well known at present. The present study has indicated that within the medulla oblongata, neurons in the NTS and the depressor VLM are activated by volume expansion; however, in contrast to the essential pathways utilized by the arterial baroreceptors, we find no evidence for monosynaptic connections between the depressor region of the VLM and the pressor region of the RVLM. It remains for future study to determine how the different regions in the central nervous system identified to date fit into a schema of central pathways involved in the reflex sympathetic nerve responses initiated by volume expansion. Of particular interest will be the connection between the NTS and the depressor neurons in the VLM.

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Address for reprint requests: E. Badoer, Dept. of Medicine, Level 5, Block E, Monash Medical Centre, 246 Clayton Rd., Clayton 3168, Melbourne, Victoria, Australia (E-mail: emilio.badoer@med.monash.edu.au).

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