Brain Natriuretic Peptide–like Immunoreactive Innervation of the Cardiovascular and Cerebrovascular Systems in the Rat

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Atrial natriuretic peptide is a potent dilator of aorta and renal and cerebral arteries and inhibits sympathetic tone in the heart in several mammalian species. We examined the possibility that a molecule related to porcine brain natriuretic peptide (pBNP), which acts at the same receptor sites as atrial natriuretic peptide, might provide an alternative source of natriuretic peptide to the cardiovascular system in the rat. An antiserum against pBNP demonstrated profuse immunoreactive innervation of the heart, cerebrovascular tree, and renal arteries. pBNP-like immunoreactive fibers ran in bundles along the surface of the heart, innervating the atria most heavily and penetrating the ventricular myocardium along the coronary arteries. There was greater density of innervation of the right side of the heart compared with the left, particularly in the ventricles, suggesting a parasympathetic origin. The entire cerebrovascular tree was innervated by immunoreactive pBNP fibers, with the densest concentration of immunoreactive fibers along the surface of the internal carotid, middle cerebral, posterior communicating, and anterior cerebral arteries. The proximal renal arteries were not innervated, but as they approached the kidney, they were invested by bundles of immunoreactive pBNP fibers. These axons followed the major branches of the renal artery into the kidney parenchyma, running along the surface of the arteries up to their entrance into the renal glomeruli. No immunoreactive innervation of the aorta or proximal brachiocephalic, subclavian, or carotid arteries was seen. A substance related to pBNP may serve as a neuromodulator regulating cardiac output as well as blood flow in certain vascular beds. (Circulation Research 1990;67:1345–1354)

Although atrial natriuretic peptide (ANP) is both a vasodilator and a hypotensive agent, the major mechanism for its hypotensive action is a decrease in cardiac output.\(^1\)\(^-\)\(^4\) ANP has complex effects on different vascular beds, causing dilatation of the aorta, coronary and cerebral arteries, and renal afferent arterioles but constriction of renal efferent arterioles and certain veins, and no net effect in many vascular beds.\(^1\)\(^-\)\(^4\)\(^-\)\(^10\)

The fall in cardiac output during ANP administration results from a complex combination of a decrease in venous return\(^7\) and absence of a baroreceptor reflex response,\(^9\) which ordinarily would increase cardiac sympathetic drive to compensate for the fall in arterial blood pressure. The baroreceptor reflex, in turn, is thought to be suppressed both by ANP effects on baroreceptor afferent transmission and by inhibitory effects of ANP on sympathetic drive to the heart.\(^11\)\(^-\)\(^15\)

Recently, Sudoh and colleagues\(^6\) isolated a new peptide from pig brain, porcine brain natriuretic pep-

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tide (pBNP). pBNP causes natriuresis, diuresis, hypotension, and smooth muscle relaxation at doses similar to those of ANP. It is virtually equipotent with ANP at the classical ANP-A receptor and is up to seven times more potent at the newly discovered ANP-B receptor. In porcine heart, the ANP-B receptor predominates, suggesting that pBNP rather than ANP may play a physiological role in this tissue. Recently, we used an antiserum against pBNP to examine the distribution of pBNP-like immunoreactive (pBNPir) neurons in the rat nervous system.16 During these studies, we found pBNPir cells in both sympathetic and parasympathetic preganglionic and postganglionic populations, as well as many pBNPir small dorsal root ganglion cells. pBNPir fibers were also seen investing the blood vessels of the circle of Willis.

These observations raise the possibility that the cardiovascular system may respond physiologically to a BNP provided as an autonomic or sensory neuromodulator. We now have used this pBNP antiserum to examine systematically the pBNPir innervation of the cardiovascular and cerebrovascular systems.

Materials and Methods

Heart

Nine Sprague-Dawley rats were deeply anesthetized with chloral hydrate and were then perfused with 50 ml saline followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at pH 6.5 followed by 250 ml of the same fixative at pH 8.5. Fixative was introduced either retrogradely through the descending aorta or via a cannula inserted through the left ventricle into the ascending aorta. Tissue was allowed to fix in situ for 1 hour. Then, the animals were perfused with 250 ml of 20% sucrose in phosphate buffer. The hearts were then dissected free, and sections were prepared by one of two methods. In some cases, 50-μm frozen sections were cut through the heart and every 10th section was stained immunohistochemically with antiserum against pBNP. The adjacent sections were mounted immediately in the proper anatomical orientation for later reference. The free-floating sections were incubated first in 0.1 M phosphate buffer containing 0.9% saline and 3% goat serum (PBS-G) and 0.1% Triton X-100 at room temperature for 1 hour and then overnight at 4°C in PBS-G containing rabbit antiserum (BNP33) against pBNP at a dilution of 1:500. The next day, sections were washed in PBS and incubated at room temperature for 1 hour in PBS-G containing a 1:50 dilution of goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. Sections were then washed again in PBS and mounted onto gelatin-coated glass slides by using the series of adjacent sections to maintain proper anatomical orientation. After thorough drying, sections were cleared in xylene and coverslipped with Histoclad (Clay-Adams, N.J.) containing 2% β-mercaptoethanol to retard fluorescent fading.

In other cases, immunohistochemical staining of 10-μm sections was done on the slide. These hearts were cut on a cryostat, and every 50th section was mounted and stained with antiserum against pBNP by using the same sequence of incubations as with the free-floating sections. Adjacent sections were mounted and stained with 0.1% thionin for visualization of the tissue landmarks.

Sections were examined under a Leitz fluorescence microscope (E. Leitz, Wetzlar, FRG) with an I2 filter. Relative density of pBNPir fibers in different parts of the heart was estimated by counting the number of immunoreactive fibers crossing the lines in a 10×10 grid in an eyepiece reticle. The tissue was viewed at ×500 magnification.

Blood Vessels

After perfusion and removal of the heart as above, the great vessels and cerebrovascular tree were dissected. The aorta and great vessels were cut into segments about 5–8 mm in length and stained free floating, as above. After the vessel segments were stained, they were cut open longitudinally and flat-mounted onto glass slides. Then, they were dried and coverslipped as above. The cerebral vascular tree was dissected intact, stained free floating, flat-mounted, and coverslipped.

In some cases, the blood vessels were stained by the immunoperoxidase method rather than fluorescence. In these experiments, tissue was preincubated with 1% hydrogen peroxide in PBS for 1 hour to suppress tissue peroxidase. The second antiserum was a sheep anti-rabbit IgG conjugated to horseradish peroxidase. After the tissues were washed in PBS, they were incubated for 8–10 minutes at room temperature in 0.01% hydrogen peroxide and 0.05% diaminobenzidine in 0.1 M phosphate buffer, mounted, and coverslipped with Histoclad.

Kidneys

Kidneys were dissected from seven perfused rats and cut at 50 μm on a freezing microtome. Sections were stained free floating either with the immunofluorescence or the immunoperoxidase methods described above.

Characterization of Antiserum

We have previously reported studies on the specificity of this antiserum (BNP33).18 Staining of brain and heart sections with this antiserum is abolished by preadsorption of 1 ml diluted antiserum with as little as 100 ng pBNP. Adsorption with 10 μg/ml ANP or with 50 μg/ml rat cardiac natriuretic peptide (see “Discussion”) or bovine thyroglobulin only slightly reduced staining. Sensitive antiserum against ANP (e.g., AP11)19 did not stain fibers innervating the heart or cerebral blood vessels in these preparations.

Results

Heart

Myocardium. In atrial myocytes, clusters of immunoreactive granules were seen in a perinuclear pattern
FIGURE 1. Fluorescence photomicrographs of porcine brain natriuretic peptide-like immunoreactive staining in the rat heart. Panel A: At high magnification, atrial myocytes can be seen to contain clumps of bright granular material (arrows). Bar, 10 μm. Panel B: At lower magnification, large bundles of immunoreactive axons and individual fibers can be seen along the surface of the atrial myocardium. Small bright spots (small arrows) represent clusters of immunoreactive granules in atrial myocytes, as shown at higher magnification in panel A. Bar, 50 μm. Panel C: Bundles of immunoreactive fibers (arrowheads) accompany a large coronary artery as it penetrates the ventricular myocardium. Individual immunoreactive axons can also be traced (arrows) forming a plexus along the surface of the vessel. Bar, 50 μm. Panel D: Bundles of immunoreactive axons running along the epicardial surface of the left ventricle. Bar, 50 μm.

(Figure 1A) that was essentially identical to the pattern of staining typically seen with ANP antiserum. This similarity may be due to our antiserum cross-reacting with the very high concentrations of ANP in the atrial myocytes or may represent pBNP-like material. In the ventricle, ANP antiserum demonstrated immunoreactive granules in occasional endocardial cells, but the pBNP antiserum did not show any myocardial staining.

Innervation. Large bundles of pBNPir fibers were seen along the posterior wall of the heart (Figure 2G). These ran along the surface of the atria and the ventricles (Figures 1B and 1D), with considerably larger numbers of immunoreactive fibers coursing over the surface of the right compared with the left side of the heart.

In the atria, these bundles broke up into smaller fascicles and individual smooth fibers that innervated the entirety of both atria (Figure 1B). Occasional very fine fibers with varicosities could also be seen. Fiber densities were slightly higher in the right atrium compared with the left (see fiber counts, Figure 2). Small numbers of immunoreactive fibers could be followed along the proximal portions of the cardiac valves.

In the ventricles, bundles of fibers were seen along the adventitial surface of major coronary arteries and
piercing the epicardial surface along with the arteries. These broke up into smaller fascicles and individual smooth fibers and occasional very fine varicose fibers, which again often ran along the adventitial surface of penetrating coronary arteries (Figure 1C). The pBNPir fibers also were found scattered throughout the ventricular myocardium and innervating the endocardial surface of the ventricles. The density of pBNPir fibers was greater at the epicardial and the endocardial surfaces than in the interior of the ventricular wall. The greatest density of fibers in the entire heart was found in the atrioventricular node and running toward the sinoatrial node (fiber count of 298 in Figure 2F).

The overall density of innervation was about three times greater in the right ventricle than the left (see fiber counts, Figure 2). This difference was particularly apparent in the ventricular septum, where the right ventricular face was about three times as heavily innervated as the left ventricular face. There was extraordinarily intense innervation of the ventricular septal wall just below the atrioventricular junction in the region containing the atrioventricular node.
Aortic Arch and Great Vessels

The aortic arch and great vessels, including the brachiocephalic artery, the proximal subclavian arteries, and the common and internal carotid arteries, up to their entrance into the skull, were examined. No evidence of pBNPir innervation was seen. Similarly, the descending aorta and proximal portions of the renal and radicular arteries showed no evidence of immunoreactivity.

Cerebrovascular System

The entire cerebrovascular tree demonstrated rich pBNPir innervation (Figure 3). Fascicles and individual smooth fibers were found running both longitudinally...
and circumferentially along the adventitial surface of the large cerebral arteries. In favorable specimens, fine varicose fibers were also observed, running mainly in a circumferential pattern. The greatest density of fibers of both types was observed along the intracranial internal carotid, posterior communicating, and proximal middle and anterior cerebral arteries. Moderately dense pBNPir innervation extended distally along the middle, anterior, and posterior cerebral arteries and the ethmoidal arteries. There was only moderately dense innervation of the basilar artery at its apex, and the density of innervation gradually decreased more caudally and along the vertebral arteries. Only a few pBNPir fibers were seen along the cerebellar, pontine, or anterior spinal arteries.

Kidney

Bundles of immunoreactive fibers could be traced along the renal arteries as they entered the kidney. These broke up into fascicles and individual smooth and varicose fibers that ran along the adventitial surface of the penetrating renal arteries (Figure 4A). Fibers could be traced along individual renal arterioles up to their entry into renal glomeruli (Figure 4B). These fibers were in close proximity to the juxtaglomerular apparatus and efferent arterioles, but it was not possible to discern whether these structures were innervated as well. However, most vascular structures in the kidney were accompanied by pBNPir fibers, and there was no evidence for pBNPir innervation of structures in the renal parenchyma other than the arteriolar system.

Discussion

Our results indicate that pBNPir nerve fibers innervate the parenchyma of the heart as well as the coronary arteries, the cerebrovascular tree, and the renal arteries and arterioles. These results suggest that a substance antigenically similar to pBNP may be a cardiovascular and cerebrovascular neuromodulator.

Technical Considerations

Our antiserum was raised against the 26 carboxy-terminal amino acid form of pBNP conjugated to bovine thyroglobulin. This antiserum has a very small amount of cross-reactivity with rat ANP-(5–28) but recognizes pBNP with at least 500 times greater affinity. In the brain, BNP33 antiserum did not recognize structures containing some of the most intense immunoreactive ANP staining, but it did demonstrate a distinct pattern of neural innervation that is consistent with reports of ANP and pBNP receptor distribution.
Nevertheless, it is possible that our pBNP antiserum may stain structures containing much higher concentrations of ANP than those found in the brain. For example, the pattern of pBNPir staining seen in atrial myocytes is identical with the pattern of most intense immunoreactive ANP staining in this tissue. Hence, it is possible that some, if not all, of the staining of atrial myocytes with the pBNP antiserum may represent ANP cross-reactivity. On the other hand, very sensitive ANP antiserum does not stain any nerve fibers in the heart or along the cerebral arterial tree (C.B. Saper, unpublished observations), so the neural staining pattern is not likely to represent ANP cross-reactivity.

The structure of the antigen recognized by the pBNP antiserum in these nerves is not yet known. Although a structure for rat BNP has been proposed (on the basis of cDNA homology), the peptide predicted by that cDNA has been demonstrated only in rat heart and apparently is not found in rat brain. More importantly, our antiserum does not recognize this peptide, which has more properly been labeled a rat cardiac natriuretic peptide.

Staining with our pBNP antiserum can be blocked by adsorption with synthetic peptides from the conserved region of the natriuretic peptide ring sequence (K.M. Hurley et al, unpublished observations). Hence, it is likely that the critical epitope for staining is in this region and that our antiserum is recognizing a natriuretic peptide in rat nervous tissue. On the other hand, the more intense staining of large, smooth fibers than the smaller varicose ones may indicate that our antiserum can also bind to an epitope present on a structural component present in certain neurons. We are currently attempting to identify chemically the molecular species that our antiserum recognizes. For the present, however, our staining is most appropriately referred to as “pBNP-like immunoreactivity.”

**pBNPir Innervation of the Heart**

Although the heart is the major source of circulating ANP (and perhaps cardiac natriuretic peptide), the ANP-B receptor, which responds best to pBNP, is the form that is present in cardiac tissue. Our results indicate that there is extensive innervation of the atrial and ventricular myocardium with pBNPir fibers. The pattern of innervation, much denser in the atria than the ventricles and in the right ventricle than the left, is distinct from other peptidergic fibers that innervate the heart, such as substance P (found mainly in sensory afferents) or neuropeptide Y (found in sympathetic efferents). The greatest similarity is with vasoactive intestinal peptide, which is mainly found in nerves innervating the atria and heart valves. Vasoactive intestinal peptide is thought to be colocalized with acetylcholine in some, but not all, parasympathetic postganglionic fibers.

Although vagal innervation of the ventricles was once thought to be inconsequential, more recent studies have indicated that it does exist in a variety of species. Cholinergic innervation and muscarinic receptor densities tend to be most intense in the atria but are also present in the ventricles at about one third the atrial levels and are more prominent on the right side. We find that pBNPir fibers in the rat heart are similarly distributed, although it is difficult to compare the absolute densities of innervation. Hence, we would predict that a substantial portion of the pBNPir innervation of the heart may represent parasympathetic postganglionic fibers. Direct exper-

**Figure 4.** A pair of fluorescence photomicrographs to illustrate porcine brain natriuretic peptide–like immunoreactive innervation of the kidney. Panel A: Large bundles of immunoreactive fibers were seen accompanying the renal arteries as they penetrated the parenchyma (arrowhead). Individual fibers often could be traced into fiber plexuses running along the surfaces of these arteries (arrows). Bar, 50 mm. Panel B: As arteries gave off branch arterioles (curved arrow), individual immunoreactive fibers left the larger bundles (arrowhead) and could be traced along the arteriole (arrows) up to its entry into a glomerulus. Bar, 50 mm.
imential evidence for the source of this innervation would be of considerable interest.

A molecule similar to pBNP may act as a cardiac neuromodulator to decrease sympathetic drive to the heart. Administration of ANP causes a fall in cardiac index that appears to be mediated by decreased sympathetic drive.\textsuperscript{36} In part, this response may be due to activation of vagal C fibers that act centrally to inhibit sympathetic tone.\textsuperscript{11,14} However, there is also evidence for a direct presynaptic depressive effect of ANP on sympathetic neurotransmission.\textsuperscript{12,13,15,37}

ANP has also been reported to have direct effects on the membrane potential and synthesis of neurotransmitter in sympathetic ganglion cells.\textsuperscript{38–40} We found that many sympathetic preganglionic neurons in the rat are pBNPir suggesting that a substance similar to pBNP may play a physiological role as a synaptic modulator of the activity of sympathetic ganglion cells.\textsuperscript{18} The sympathetic ganglion cells may also express natriuretic peptide receptors on their terminals, allowing presynaptic inhibition by a molecule similar to pBNP that is released locally from nerve terminals in the heart.

**pBNPir Innervation of the Cerebrovascular Tree**

Cerebral arteries respond to ANP with dilatation.\textsuperscript{5,41–43} Hence, the innervation of the cerebral arterial tree by pBNPir nerves may represent a physiological mechanism by which natriuretic peptides may cause cerebral vasodilatation.

The innervation of the cerebrovascular tree by pBNPir fibers is very similar to the innervation pattern of many other peptides, including CGRP,\textsuperscript{44–46} substance P,\textsuperscript{46–48} cholecystokinin,\textsuperscript{49} dynorphin,\textsuperscript{50} vasoactive intestinal peptide,\textsuperscript{51,52} and neuropeptide Y.\textsuperscript{53} CGRP, cholecystokinin, and substance P arise mainly from the trigeminal sensory system,\textsuperscript{45,47,49,54,55} vasoactive intestinal peptide and dynorphin from the parasympathetic system,\textsuperscript{50–52,56,57} and neuropeptide Y from the sympathetic system (although it may also be found in some nonadrenergic fibers).\textsuperscript{58} pBNPir neurons are found in sympathetic, parasympathetic, and sensory ganglia, so that all three types of neurons may contribute to cerebrovascular innervation.

The role of the innervation of the major cerebral arteries in the normal control of cerebral blood flow is controversial. Although there is considerable local control of blood flow in the brain, the large cerebral arteries account for as much as 30% of the resistance in the cerebral circulation.\textsuperscript{59} In pathological states such as migraine or subarachnoid hemorrhage, vaso-motor tone in large resistance vessels may play an important role in the regulation of cerebral blood flow.\textsuperscript{60} A great deal remains to be learned about the differential control of the cerebral arteries via their autonomic and sensory innervation.

**Innervation of the Kidney**

A major action of ANP is to cause natriuresis and diuresis. The mechanism of action is thought to be an increase in tone of renal efferent arterioles and decrease in afferent arteriolar tone, resulting in an increase in glomerular filtration rate without a net change in renal blood flow.\textsuperscript{4,6,61} In this regard, it is interesting that the renal afferent arterioles are selectively innervated by pBNPir fibers. It was not possible for us to determine if there was immunoreactive innervation of the juxtaglomerular apparatus or the efferent arterioles, as we saw no unequivocal examples of such innervation in our material. However, the immunoreactive nerve fibers did follow the afferent arterioles into the juxtaglomerular region, and most if not all of the arterioles we encountered in our preparations did appear to be innervated by pBNPir fibers. The presence of this innervation suggests that renal arteries\textsuperscript{62} and perhaps the juxtaglomerular apparatus\textsuperscript{62} may respond to natriuretic peptides that are delivered both via direct neuronal innervation as well as via the circulation.

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