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Essential role of hypothalamic muscarinic and α-adrenergic receptors in atrial natriuretic peptide release induced by blood volume expansion

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ABSTRACT **Expansion of the blood volume induces natriuresis, which tends to return the blood volume to normal. This response is mediated at least in part by the release of atrial natriuretic peptide (ANP) into the circulation. Previous experiments have shown the participation of the anterior ventral third ventricular (AV3V) region of the hypothalamus in the ANP release that follows volume expansion. When injected into the AV3V region, the cholinergic drug carbachol induces natriuresis and the release of ANP. In the present experiments, microinjection of norepinephrine into the AV3V region induced natriuresis and an increase in plasma ANP. To determine whether cholinergic and α-adrenergic pathways are crucial to the volume expansion-induced release of ANP, certain receptor-blocking drugs were injected into the AV3V region of conscious rats. Thirty minutes later blood volume was expanded by intravenous injection of 2.0 ml/100 g of body weight of hypertonic saline (0.3 M NaCl). Microinjection of isotonic saline (2 μl) into AV3V region of control animals 30 min prior to volume expansion had no effect on the 3-fold increase in plasma ANP concentrations measured 5 min after volume expansion. In contrast, although the receptor-blocking drugs did not alter the initial concentrations of plasma ANP 30 min later, just prior to volume expansion, blockade of muscarinic cholinergic receptors by intraventricular injection of 5 nmol (2 μl) of atropine sulfate or methylatropine markedly reduced the response to volume expansion but did not obliterate it. Microinjection of the α receptor blocker phentolamine (5 nmol) into the AV3V 30 min prior to volume expansion also markedly suppressed the ANP response. Intraperitoneal (i.p.) injection of methylatropine (0.01 mmol/100 g of body weight), which does not cross the blood–brain barrier, also did not affect the basal levels of ANP 30 min after i.p. injection. But, in striking contrast with the blockade of the response to volume expansion induced by intraventricular injection of methylatropine, the response to volume expansion was markedly enhanced by i.p. injection of methylatropine. The results therefore indicate that hypothalamic muscarinic and α-adrenergic synapses are essential to release of ANP in response to volume expansion. These results are consistent with a hypothetical pathway for physiological control of ANP release which involves distension of baroreceptors within the right atria, carotid and aortic sinuses, and kidney which alters afferent input to brain stem noradrenergic neurons with axons projecting to the AV3V region. There they activate cholinergic interneurons by an α_1-adrenergic synapse. The cholinergic neurons in turn stimulate ANP neurons in this brain region via muscarinic receptors. The stimulation of these neurons activates efferent pathways which induce the release of ANP.**

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The participation of the central nervous system (CNS) in the control of sodium excretion has been demonstrated in several studies (1–3). Injection of carbachol into the third cerebral ventricle (3V), into the medial septal area, and into several other CNS structures induces natriuresis and kaliuresis through the activation of muscarinic receptors (4–7). The hypothalamic natriuretic system is also controlled by a stimulatory α_1-adrenergic and an inhibitory β-adrenergic pathway. Thus, the adrenergic mechanism elicits two types of response: α_1-adrenergic stimulation causes natriuresis and kaliuresis, which are antagonized by pretreatment with α_1 receptor-blocking agents. On the other hand, the β-adrenergic agonist isoproterenol inhibits natriuresis and a β-blocker, propranolol, augments it. This supports the concept that β receptors exercise a tonic inhibitory control on sodium and potassium excretion (4–7).

We have recently shown that injection of carbachol into the anterior ventral third ventricular (AV3V) region induces release of atrial natriuretic peptide (ANP) into the circulation (7). These injections also induced natriuresis. In those earlier experiments before the discovery of the brain ANP system, it was possible to block the natriuresis induced by microinjection of hypertonic saline into the 3V with atropine or phentolamine (4–7).

It was therefore of interest to determine if the ANP release induced by volume expansion could be blocked by the muscarinic receptor blocker atropine. If so, this would indicate the essentiality of a muscarinic cholinergic synapse in the mechanism. The participation of α_1-adrenergic receptors in this response was also evaluated by microinjecting the α_1-adrenergic antagonist phentolamine as well. Both of these agents were injected into the AV3V of conscious rats, and their effects on basal plasma ANP and volume expansion-induced increases in ANP were determined.

MATERIALS AND METHODS

Animals. Male, albino, Wistar-derived rats weighing 250–300 g were used. The rats were housed in individual cages and maintained in a temperature-controlled room (23 ± 2°C) with controlled lighting (on 0700–1900). They had free access to standard laboratory rat chow and tap water. The animals were implanted with a stainless steel cannula (23 gauge) in the AV3V region while anesthetized with tribromoethanol (2.5%) (Aldrich, 1 ml/10 g of body weight, i.p.), according to the procedures described previously (8). The rats received a prophylactic injection of penicillin (60,000 units, i.m.) at the end of surgery. During the week following the operation, the animals were handled daily and habituated to the removal of

Abbreviations: 3V, third cerebral ventricle; AV3V, anterior ventral third ventricular; ANP, atrial natriuretic peptide.

the stylette of the intracerebral cannula. At the termination of the experiments the site of the cannula location was verified by examining frozen serial frontal sections (7 μm) of the brains.

Blood Volume Expansion. Twenty-four hours before the experiments, a catheter was inserted into the right external jugular vein and advanced to the atrium as described by Harms and Ojeda (10). Blood volume expansion was performed in conscious, freely moving rats by i.v. injection of 0.3 M NaCl in 2 ml/100 g of body weight, over 1 min. In this and subsequent experiments trunk blood samples were collected in tubes cooled in crushed ice just before 3V injection or 5 or 15 min after blood volume expansion. The tubes contained proteolytic enzyme inhibitors: 2 mg of EDTA, 20 μl of 1 mM phenylmethylsulfonyl fluoride, and 20 μl of 500 μM pepstatin A.

Effects of Central Receptor Blockers on Blood Volume Expansion-Induced ANP Release. The animals utilized in these experiments had AV3V cannulae placed 5–7 days earlier. On the day of the experiment 2 μl of isotonic saline (0.15 M NaCl) or 2 μl of isotonic saline containing 5 nmol of atropine sulfate, 5 nmol of methylatropine, or 100 nmol of phentolamine was microinjected into the AV3V cannula 30 min prior to blood volume expansion. The injections were made during a 60-sec period. These experiments were designed to investigate the influence of central blockade of muscarinic (atropine or methylatropine) or α-adrenergic (phentolamine) receptors in the mediation of the blood volume expansion-induced ANP release.

Effect of Norepinephrine on Plasma ANP. Because the effect of intraventricular norepinephrine on ANP release was unknown, norepinephrine (norepinephrine hydrochloride, 40 nmol in 2 μl) was injected into the AV3V region in a group of animals.

Effect of i.p. Injection of Methylatropine (0.01 mmol) on Blood Volume Expansion-Induced Release of ANP. To see if the observed effects of atropine on blood volume expansion-induced ANP release could be due to its diffusion from the AV3V region into the systemic circulation, we evaluated the effect of methylatropine, a quaternary ammonium derivative of atropine that lacks central nervous system effects because of its poor capacity to cross the blood–brain barrier. This substance was injected i.p. and the rats were subjected to blood volume expansion 30 min later. The blood samples were collected just before drug administration (basal) and 5 and 15 min after blood volume expansion. The effects on ANP release observed after i.p. administration of methylatropine were compared with those obtained after its microinjection into the AV3V.

Drugs. Atropine sulfate (Sigma, A 0257), phentolamine (Sigma, P 7547), methylatropine (Sigma A 6883), norepinephrine hydrochloride (Sigma A 7381), pepstatin A (Sigma P 7626), phenylmethylsulfonyl fluoride (Sigma P 7626), and EDTA (Sigma E 9884) were used. The receptor-blocking drugs and norepinephrine were dissolved in 0.15 M NaCl solution before 3V injection. A volume of 2 μl of drug or control solution was injected during 1 min by means of a hand-driven microsyringe (Hamilton), connected to a 30-gauge injection needle by polyethylene tubing (20 cm).

Determination of Plasma ANP Concentrations. The immunoreactive plasma ANP levels were measured by radioimmunoassay as described by Gutkowska *et al.* (11).

Statistical Analysis. Plasma ANP values from each experiment were analyzed by two-way analysis of variance with repeated measures, and the significance of the differences between group means was determined by the Newman–Keuls test.

RESULTS

Effect of Intraventricular Norepinephrine on Plasma ANP. Although intraventricular injection of norepinephrine was previously shown to evoke natriuresis (4), this had not been shown to involve ANP release. Consequently, in the first experiment we evaluated the effect of injection of 40 nmol of norepinephrine into the 3V of conscious rats. A blood sample was removed just prior to injection and at 5, 20, and 40 min after the injection of the catecholamine. There was no significant increase in plasma ANP at 5 min after norepinephrine injection; however, by 20 min the concentrations had increased more than 3-fold. By 40 min the values had declined and were not distinguishable from the initial values (Fig. 1).

Effect of Intraventricular Isotonic Saline on Blood Volume Expansion-Induced ANP Release. Microinjection of 2 μl of saline into the AV3V of control animals 30 min before blood volume expansion had no effect on the over 3-fold increase in plasma ANP concentrations at 5 min after the onset of expansion (Fig. 2). These values were similar to those obtained in other control experiments (data not shown). Plasma ANP concentrations had returned toward control by 15 min after the onset of volume expansion. The initial plasma ANP concentrations in the experimental groups 30 min after the injection of the various drugs into the AV3V were almost identical to those obtained in the rats injected with saline.

Effect of Intraventricular Atropine Sulfate on Blood Volume Expansion-Induced ANP Release. The microinjection of 5 nmol of atropine sulfate into the AV3V caused a significant decrease in the ANP release normally induced by blood volume expansion ($P < 0.001$). The residual response at 5 min was still significant in comparison with the starting values; however, by 15 min there was no significant response.

Effect of Methylatropine Injected into the 3V or i.p. on Blood Volume Expansion-Induced ANP Release. Microinjection of 5 nmol of methylatropine into the AV3V 30 min before blood volume expansion similarly produced no alteration in the plasma ANP concentrations just before expansion, but it dramatically reduced the response to blood volume expansion at 5 min. In this case because of greater variability than in the prior experiment with atropine sulfate, the residual response was not significant (Fig. 2).

When methylatropine was injected i.p. 30 min prior to the experiment, there was no effect on resting levels as in the other experiments. In striking contrast to the results with

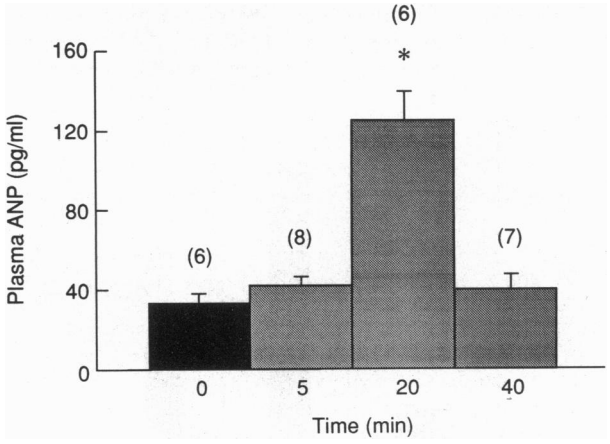

FIG. 1. Effect of the microinjection of norepinephrine [40 nmol in 2 μl of 0.15 M NaCl (saline)] into the AV3V region on plasma ANP concentrations of conscious male rats. In this and subsequent figures the height of the bar represents the mean value; vertical lines indicate the SEM. Numbers in parentheses are number of animals per group. *, $P < 0.001$ versus 0 time value.

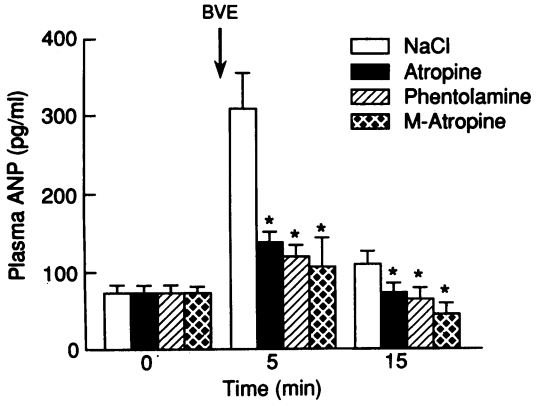

FIG. 2. Effect of blood volume expansion (2 ml/100 g of body weight) of hypertonic saline (0.3 M NaCl) solution injected into the jugular catheter over a period of 1 min on plasma ANP concentrations. Thirty minutes prior to blood volume expansion, the animals were microinjected in the AV3V region with 2 μl of saline or an equal volume of atropine, methylatropine (M-Atropine), or phentolamine (5 nmol each). The 3-fold increase in plasma ANP after blood volume expansion (BVE) in the animals preinjected with saline in the AV3V was highly significant. Each of the receptor-blocking drugs significantly inhibited ($P < 0.01$) the response as indicated by the asterisks.

intraventricular injection of atropine sulfate, not only was the response to volume expansion present after i.p. injection of methylatropine, but it was significantly increased compared with that in the control animals injected i.p. with a similar volume of saline (Fig. 3).

Effect of Phentolamine on Blood Volume Expansion-Induced ANP Release. As with atropine, 100 nmol of phentolamine microinjected into the AV3V 30 min before blood volume expansion did not alter the initial values of plasma ANP but markedly reduced the release of ANP ($P < 0.001$) that occurred in the control animals at 5 min after volume expansion (Fig. 2). Again, as with atropine, there was a small but significant response at 5 min (Fig. 2).

DISCUSSION

We have previously shown the importance of the AV3V region in the control of volume expansion-induced increases in plasma ANP and the resultant natriuresis. Lesions of the AV3V region which include the cell bodies of the brain ANP-containing neurons ("ANPergic neurons"), or of the median eminence or posterior lobe of the pituitary which contain the caudal projections of the brain ANPergic neurons, blocked the increase in plasma ANP concentrations

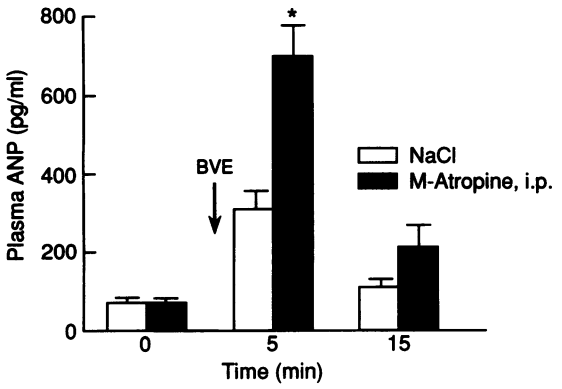

FIG. 3. The effect of i.p. injection of methylatropine (0.01 mmol/100 g of body weight) 30 min prior to the experiment on the response to blood volume expansion. The response was highly significantly augmented ($P < 0.01$) at 5 min.

induced by volume expansion (12). That this effect is related to blockade of the activity of the brain ANPergic neurons is supported by findings in sheep (13) and in rats (14) that the injection of antiserum directed against ANP into the 3V at least partially blocked volume expansion-induced release of ANP.

We have shown here that microinjection of norepinephrine into the AV3V region stimulates ANP release. We previously demonstrated that muscarinic agonists also increase ANP release (7). Herein, we have demonstrated that the injection into the AV3V region of the muscarinic receptor blockers atropine or methylatropine or the α-adrenergic receptor blocker phentolamine can dramatically inhibit the increase in plasma ANP following blood volume expansion. Since i.p. injection of methylatropine, a muscarinic receptor blocker that does not cross the blood–brain barrier, was ineffective, whereas intraventricular injection of methylatropine inhibited the response to blood volume expansion, it is clear that the blocking action is on receptors within the brain, presumably near the AV3V region. Thus, these results indicate an essential role of muscarinic and α-adrenergic receptors in the vicinity of the AV3V region in the mediation of the release of ANP induced by blood volume expansion.

Surprisingly, the response to volume expansion was significantly augmented after the i.p. injection of methylatropine. This dose should have blocked the cholinergic vasodilatory control of the vascular system and also would have increased the heart rate by blocking the negative chronotropic effects of the vagus on the heart. The result would be an elevation of blood pressure which might render the baroreceptors more sensitive to further expansion of the blood volume and thereby provoke an excessive release of ANP.

We (15) and others (16) have previously shown that denervation of baroreceptors inhibits ANP release caused by blood volume expansion. Presumably, expansion of the blood volume alters afferent input into the brain by stretching baroreceptors in the carotid-aortic sinus regions (15, 16) and in the kidney (16), resulting in increases in the release of ANP.

Resting release of ANP was actually depressed by transection of the right vagus nerve, but not the left vagus; however, severing both vagi failed to block the ANP release caused by volume expansion. This leads us to conclude that expansion of blood volume alters the afferent input to the brain that originates from baroreceptors in the carotid-aortic sinuses, the kidney, and possibly in the right atrium. This sensory input reaches the brain stem and activates ascending noradrenergic pathways to the AV3V region (Fig. 4). Indeed, lesions of the locus ceruleus, a major site of noradrenergic neurons that project to the hypothalamus, lowered plasma ANP concentrations (J.A.-R., unpublished data). The axons of these noradrenergic neurons arriving in the vicinity of the AV3V region are visualized to synapse with local cholinergic interneurons, which in turn activate the ANPergic neurons in the AV3V region. This speculation is based on the findings of the present research that both the muscarinic cholinergic receptor blockers atropine and methylatropine and the α_1 receptor blocker phentolamine inhibit the response after microinjection into the AV3V. We postulate that these neurons are arranged in series rather than in parallel. However, studies with receptor blockade will be required to test this idea. If our theory is correct, then atropine should block the response to norepinephrine, but phentolamine should not block the response to a muscarinic agonist.

We postulate on the basis of the antiserum studies mentioned above (14) that activation of the brain ANPergic neurons is required to induce the release of ANP that follows volume expansion. What are the efferent pathways involved? Some of these neurons terminate in the median eminence and

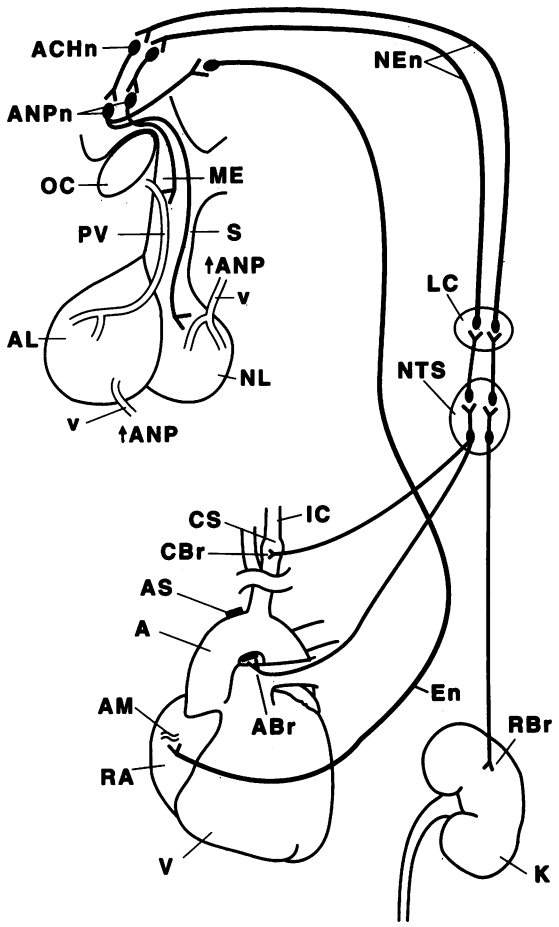

FIG. 4. Schematic diagram of the proposed mechanism of volume expansion-induced ANP release. For explanation see text. OC, optic chiasm; ME, median eminence; S, pituitary stalk; AL, anterior lobe of the pituitary gland; NL, neural lobe of the pituitary gland; PV, portal vessel; v, vein draining anterior or neural lobe; LC, locus ceruleus; NTS, nucleus tractus solitarius; IC, internal carotid artery; A, aorta; RA, right atrium; V, ventricle; K, kidney; AM, atrial myocyte; ABr, afferent from aortic baroreceptor; CBr, carotid baroreceptor afferent; RBr, renal baroreceptor afferent; ↑ANP, increased ANP concentration; ANPn, ANPergic neurons; ACHn, acetylcholinergic neurons; NEn, norepinephrinergic neurons; and En, efferent neuronal axon.

neural lobe of the hypophysis. It is probable that their activation leads to release of the peptide into the vasculature draining the median eminence or the neural lobe (7). Since the quantity of ANP in these structures is 1/1000 of that in the atria (7), we believe that this ANP plays a minor role in the response. Rather, we believe that these ANPergic neurons activate descending pathways, which then activate efferent pathways to the heart with consequent release of ANP from cardiac myocytes. The combined release from both sources then accounts for the increase in plasma ANP concentrations which mediates the ensuing natriuresis.

The efferent pathway to the heart could be completely neural; however, it cannot be cholinergic, since bilateral section of the vagi does not block the response to volume expansion (15). It is unlikely that this is a sympathetic efferent pathway, since volume expansion by elevating blood pressure should, if anything, diminish sympathetic outflow. Rather, we suspect that ANP may be released by an unknown efferent pathway reaching the atria. It could be peptidergic in nature. Alternatively, release of other brain peptides induced by ANP neurons, such as endothelin, which evokes release of ANP by direct action on the heart (17) and hypothalamus (9), and vasopressin, which has similarly been reported to be effective (12), could be involved.

Because the receptor blockers did not abolish the response to volume expansion, the residual response could be due to direct release of ANP from the atria after atrial stretch (19). Alternatively, this residual response could be due to incomplete receptor blockade.

The system is diagrammatically illustrated in Fig. 4. Volume expansion would induce stretch of baroreceptors in the right atrium (not shown), in aortic and carotid sinuses, and in the kidney. Altered afferent input from these structures to the nucleus tractus solitarius would cause activation of noradrenergic neurons located in locus ceruleus, which project axons to the AV3V region, there to synapse with cholinergic neurons, which in turn by muscarinic synapses would stimulate the ANP neurons. Activation of these neurons would activate release of ANP from the median eminence into the long portal vessels, and from the neural lobe into its venous drainage, thereby increasing ANP in the blood draining from the head. The ANP neurons would also activate the efferent nerves, which would eventually project to the right atrium to activate the release of ANP from atrial myocytes. The nature of the transmitter in the efferent nerve is not known. Also, not shown, but possible, is that the ANP neurons may activate the release of other peptides from the hypothalamus such as endothelin or vasopressin which are supposed to have direct effects on the atria to enhance ANP release.

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