Vasopressin Produces Inhibition on Phrenic Nerve Activity and Apnea through V_{1A} Receptors in the Area Postrema in Rats

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Abstract

The area postrema (AP) is the most caudal circumventricular organ in the central nervous system and contains arginine vasopressin (AVP) receptors. To investigate that AVP receptors in the AP might participate in the modulation of respiration, the adult rat was anesthetized with urethane (1.2 g/kg, i.p.), paralyzed, ventilated artificially, and maintained at normocapnia in hyperoxia. The phrenic nerve was separated at C_4 level. Phrenic burst was amplified, filtered, integrated, and then stored in the hard disc *via* the PowerLab system. Three doses of AVP and an AVP V_{1A} receptor antagonist, [β-mercapto-β,β-cyclopentamethylenepropionyl^1,-O-Me-Tyr^2,Arg^8]-vasopressin, were microinjected into the AP through a pair of microelectrodes. The moderate and high doses of AVP reduced the PNA to 72 % and 45% of the control ($P < 0.05$), extended the mean T_E from 1.4 s before AVP to 4.0 s and 7.6 s, ($P < 0.05$), and decrease in BP by 26 and 37 mmHg ($P < 0.05$), respectively. These significant reductions in PNA and BP and elongation of T_E were totally abolished by the pre-treatment of the AVP V_{1A} receptor antagonist and by application of lidocaine or $CoCl_2$ at the nucleus tractus solitarius (NTS). Moreover, pulmonary inhibition caused by AVP was significantly attenuated by hypercapnia. These results strongly suggest that AVP V_{1A} receptors in the AP may participate in the modulation of cardiopulmonary functions through the activation of V_{1A} receptors and the pathway connected to the NTS. They may also indicate that a putative vasopressinergic pathway has a projection to the AP to alter the excitability of neurons having AVP V_{1A} receptors and results in an inhibition of cardiopulmonary functions *via* the connection between the AP and NTS.

Key Words: area postrema, arginine vasopressin, V_{1A} receptor, phrenic nerve, the nucleus of the tractus solitarius, rat

Introduction

The area postrema (AP) is a small area located on the dorsal surface of the medulla at the level of the obex and represents the most caudal circumventricular organ in the central nervous system (CNS), and has been reported to be a chemosensitive trigger zone for initiating emetic reflex (8). It is involved in a number of physiological functions, such as fluid intake (16), gastric motility (4, 5, 30), and cardiovascular regulation (18, 34). Available evidence indicates that the AP can attenuate exercise pressor effect (7, 49). Blockade of AVP V_1 receptor could attenuate the postexercise hypotensive effect (13). Receptors for various neuropeptides are found on neurons within the AP including those for angiotensin, arginine vasopressin (AVP), cholecystokinin, endothelin, nerve growth factor, and calcitonin gene-related peptide (14). Activation of these receptors may change the excitability of AP neurons and modulate its physiological functions (14, 19). In this regard, circulating AVP has been reported to enhance baroreflex-induced decreases in heart rate (HR) and blood pressure (BP) (15, 41). It appears that AVP and its receptors in the AP may play a role in the modulation of cardiovascular functions.

Central loci that modulate cardiovascular

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functions such as the ventrolateral medulla (VLM) may also participate in the regulation of respiration (10-12). Whether specific receptor on the neurons of the AP participated in the modulation of respiration is unclear. In this regards, microinjection of DL-homocysteic acid (DLH) and glutamate (Glu) into the AP has been demonstrated to initiate an increase in respiratory frequency (37, 47) and tidal volume (5) in rabbits. However, DLH is a non-specific excitatory amino acid (EAA) and may indiscriminately excite neurons having all kinds of receptors in the AP. Glu is also an EAA and activates NMDA and AMPA receptors (5). Thus, application of specific agonist, such as AVP, to the AP may produce specific effect on cardiopulmonary functions. We have recently reported that AVP-induced activation of V_{1A} receptors in the VLM produces inhibition on phrenic burst in rats (10-12). We therefore proposed that AVP-induced activation of AVP receptors in the AP might produce inhibition on respiration rather than excitation.

Neural pathway connected between the AP and the nucleus of the tractus solitarius (NTS) has been documented to enhance cardiovascular response to baroreflex (6, 9, 23, 36). Specifically, the AP is lack of blood-brain-barrier and can detect the circulating AVP to improve baroreflex-induced decreases in HR and BP (15, 41). Whether this neural connection participates in the modulation of respiration during AVP-induced activation of AVP receptors in the AP is unknown. If true, blockade of synaptic transmission in the NTS might eliminate the modulation of pulmonary functions caused by AVP microinjected into the AP.

Based on the mention above, aims of the present study include: [1] to examine if AVP could produce inhibition on phrenic burst amplitude in a dose-dependent manner; [2] to determine whether the inhibition of AVP on phrenic burst, if any, was through the activation of V_{1A} receptors in the AP neurons; [3] to check if AVP-induced inhibition on phernic burst was through the NTS; and [4] to study if increase in CO_2 concentration might attenuate AVP-induced inhibition on phrneic burst. The results obtained supported our hypothesis that AVP-induced activation of V_{1A} receptors in the AP neurons could produce inhibition on respiration reflecting on a decrease of phrenic amplitude and an immediately transient elongation of expiratory period. This inhibition could be attenuated by hypercapnia.

Materials and Methods

Animal Preparation

Male Wistar rats (379 ± 5 g) were purchased from the Animal Center of the Medical School of National Taiwan University and housed in a room with temperature maintained at 25°C. Water and food were provided *ad libitum*. The experimental protocols described in the present study were approved by the Animal Care and Use Committee of National Taiwan Normal University.

There were fifty-three rats used in this study: three for the pilot study, nine for examining the dose-response of AVP, fourteen for experiments involving the use of AVP receptor antagonist and V_2 receptor agonist, six for examining the effect of AVP response of hypercapnia, and the remaining twenty-one was for the investigation of the transmission of the NTS. During the experiment, rats were pre-treated with atropine (0.5 mg/kg, i.m., Sigma, St. Louis, MO, USA) and then anesthetized with urethane (1.2 g/kg i.p.). The depth of anesthesia was assessed by a stable recording of blood pressure (BP) and phrenic nerve activity (PNA). Additional dose of urethane (0.12 g/kg, i.p.) was administered when changes in BP and PNA were observed by applying a nociceptive stimulus to the paw. Catheterization was performed on the femoral artery and vein for monitoring of BP and administration of drugs, respectively. Blood pressure was recorded *via* a pressure transducer (Statham P23D, Grass Instrument, Quincy, MA, USA) that was connected to an amplifier (AC amplifier 7P1, Grass Instrument, Quincy, MA, USA). After calibration, signal of BP was digitized and stored in hard disc *via* the PowerLab system (ADInstrument Pty Ltd. NSW, Australia). Tracheostomy and bilateral cervical vagotomy were performed. The rat was then mounted on a prone position in a stereotaxic instrument (Stoelting, Wood Dale, IL, USA), paralyzed with gallamine triethiodide (5 mg/kg, i.v., Sigma), and ventilated artificially. A portion of the occipital bone and dura mater was removed; the obex was exposed to access the AP. End-tidal fractional concentration of CO_2 ($F_{ET}CO_2$) was continuously monitored with a CO_2 analyzer (Gemini End-tidal O_2 and CO_2 analyzer, CWE, PA, USA). The animal was maintained at normocapnia in hyperoxia (pure oxygen). Body temperature was maintained at 37-38°C with a heating pad or lamp.

Recording of Phrenic Nerve Activity

The phrenic nerve (PN) was isolated *via* a dorso-lateral approach at the level of spinal C_4~C_5 and cut peripherally. The central cut end of the PN was placed on a stainless bipolar electrode, which was connected to the input of a preamplifier (P511, Grass Instrument, Quincy, MA, USA), filtered (0.3~3 kHz), and then integrated (R-C circuits, time constant = 0.5 sec) (10-12). Integrated PNA was digitized and stored in hard disc *via* the PowerLab system

(ADInstrument Pty Ltd, NSW, Australia).

Microinjection Technique

Glass capillary tube (A-M system, #No 6250) with an inner diameter of 0.4 mm was heated and pulled with a puller (Model P-97, Sutter Instrument Co., Novato, CA, USA) into microelectrode. The tip of the microelectrode was trimmed to approximate 20-30 μm in diameter under the microscope. AVP was carefully infused into the microelectrode by a 32-gauge syringe and was microinjected into the brain tissue by a pressure injector (Picosprizer IID, General Valve Corporation, Fairfield, NJ, USA). The microelectrode filled with AVP or its receptor antagonist was fixed on a microelectrode holder (Stoelting, Wood Dale, IL, USA), which was mounted on the stereotaxic instrument. The microelectrode was then positioned at midline, 0.2 mm rostral to the calamus sriptorius (CS), and 0.2 mm below the surface. The AP at this coordinate is approximately 0.4-0.5 mm in its dorsal-ventral dimension according to the atlas of Paxinous and Watson (40). The injected volume was between 15 and 45 nl, which was controlled by the repeated injections of a 10-ms interval. The exact injected volume was estimated by the displacement of fluid meniscus in the microelectrode under the binocular microscope (Wild, Heerbrugg, Switzerland) with an ocular meter in one eyepiece.

Experimental Protocol

The AP in the rat is very small area located at the dorsal part of the medulla (Fig. 1A). In a pilot study performed on three animals, AVP with a dose of 3.0 $\times 10^{-5}$ IU, which was reported in our recent studies to produce inhibition on respiration (10-12), was microinjected into the AP to find a region that may elicit changes in cardiopulmonary functions. In this pilot study, several penetrations were systematically schemed according to Fig. 1, B and C. With a vertical position of the microelectrode holder, the first penetration was placed in midline at 0.1 mm rostral to the CS according to the atlas for rats (40). The second penetration was set at 0.2 mm rostral to the CS and the third penetration was at 0.3 mm ahead of the CS according to the scheme in Fig. 1A. For each penetration point, AVP was injected at 0.2, and 0.4 mm below the surface or sometimes at 0.1, 0.3, and 0. 5 mm below the surface (Fig. 1B). Following this systematic search, a small region producing decreases in PNA and BP was localized at 0.2-0.3 mm rostral to the CS and 0.2-0.3 mm below the surface as shown in the small black point in Fig. 1B. In addition, another area having a coordinate of 0.4-0.5 mm rostral to the CS and 0.2-0.3 mm below the surface at the midline could initiate increase in BP but no changes in PNA. Usually, six to eight injection points were completed in one animal.

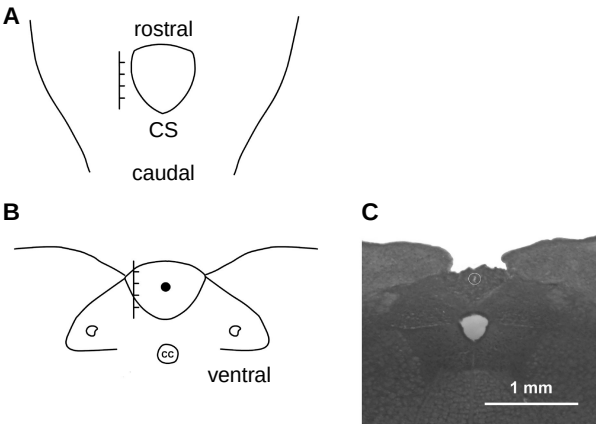

Fig. 1. Graphic presentation showing the dorsal view of the medulla and its cross section. By using the calamus scriptorius (CS) as reference, the area postrema (AP) was schemed for 4 penetrations, each separated by 0.1 mm, for microinjection of AVP (panel A). Panels B is the cross section representing 0.2 mm from the CS. Ruler with a length of 0.5 mm was set on each panel. Panel C represents the cross section at a level of 0.2 mm rostral to the CS taken from one animal to show the exact injection site of the AVP at the AP. CC is the central canal.

Four experimental protocols were performed. First, three doses of AVP, 1.5×10^{-5}, 3.0×10^{-5}, and 4.5×10^{-5} IU, were randomly microinjected into the effective region of the AP, which was determined in the pilot study, at normocapnia in hyperoxia. AVP injections were administered with a 30-min interval to ensure complete recovery from the response and also to avoid anaphylaxis, if any. The aim of this protocol was to examine whether AVP could produce a dose-dependent inhibition on respiration and also to evaluate BP responses. From the result obtained, the moderate dose of AVP (3.0×10^{-5} IU) was chosen as a threshold dosage for the subsequent studies.

Second, the inhibitory action of AVP on respiration was evaluated before and after pre-treatment of AVP V_{1A} antagonist. The purpose of the second protocol was to determine whether AVP V_{1A} receptor subtypes mediate this inhibitory effect of AVP on cardiopulmonary functions. The threshold dose of AVP (3.0×10^{-5} IU) determined in the first protocol was chosen. To achieve this specific goal, AVP, AVP V_{1A}-receptor antagonist, or DDAVP, a V_2 receptor agonist, were administered into the same point by using pairs of glass microelectrodes that were made and trimmed the same way as our recent studies (11, 12). One of the microelectrodes was used

for microinjection of AVP and the other was used for delivery of its antagonist or the DDAVP. The AVP V_{1A}-receptor antagonist was microinjected 30 min after the first dose of AVP. Then, a second injection of the same dose of AVP was immediately followed. Thirty min later, another similar dose of AVP was microinjected to examine if the effects of the receptor antagonist have dissipated and to observe if the modulating effects of AVP were reproducible.

In the third protocol, microinjection of AVP into the AP was performed before and after synaptic blockade at the level of the NTS. After microinjection of lidocaine or cobalt chloride into the NTS to block the synaptic transmission, AVP was microinjected into the AP to evaluate if the decrease in PNA, apnea, and hypotension caused by AVP were abolished. Another threshold dose of AVP was microinjected into the AP within 30 or 60 min after synaptic blockade to determine if the blockade of synaptic transmission was dissipated. Aim of this protocol was to identify whether respiratory inhibition by AVP-injected into the AP was mediated through a neural pathway connected between the AP and the NTS.

The fourth protocol was to examine if the inhibition of respiration by AVP injection could be attenuated by hypercapnia. This study was aimed to observe whether a possible interaction between the action of AVP and of chemoreceptor might be accessible.

Drug Preparation

Arginine vasopressin (Sigma, St. Louis, MO, USA) was dissolved in saline (pH = 7.4) to make a stock solution with a concentration 10 IU/ml and stored at -20°C. This stock was then diluted with saline (pH = 7.4) containing 1% pontamine sky blue during experiment. AVP V_{1A}-receptor antagonist, [β-mercapto-β,β-cyclopentamethylenepropionyl[1],-O-Me-Tyr[2],Arg[8]]-vasopressin (Sigma) was dissolved in saline to make a solution of 100 μg/ml and stored at -20°C as stock. This stock was diluted 10 times with saline at the day of experiment.

Lidocaine or $CoCl_2$ was dissolved in saline. The dose of either 15 nM (lidocaine) or 5 mM ($CoCl_2$) was microinjected into the NTS by adjusting the injection volume on each side.

Statistical Analysis

Data in the hard disc were retrieved and then analyzed with software written by Visual C^{++}. In general, ten consecutive respiratory cycles before AVP treatment were taken and averaged as the control. Neural activities following AVP treatment was chosen as the experimental data and were further transformed into percent (%) of the control. Mean BP before and after AVP treatment were retrieved and analyzed by Data pad module of the PowerLab system. All data were expressed as mean ± S.E.M. (standard error of the mean).

T_I (period for phrenic inspiration), T_E (period between phrenic inspiration) and T_{TOT} (sum of T_I + T_E) were also analyzed and computed before and after AVP treatment. T_{EE} represents the extension of T_E during the period of cessation of PNA or apnea caused by AVP treatment.

Multiple comparisons were performed (56), of which one-way or two-way ANOVA was first executed and then a Dunnett's modified *t*-test (17) was followed as a post hoc test. A "*P*" value of less than 0.05 denotes statistical significance.

Histological Verification

The rat was sacrificed by a high dose of anesthetics and a saturation solution of potassium chloride after completion of the experiment. The brain stem was quickly removed and stored in formalin solution (10%) for at least one week. Serial sections of the brainstem were transversely cut at 50 micron using a frozen microtome. The thin brain tissue slice was stained with Cresyl violet. Microinjection site of AVP was verified by comparison with the rat brain atlas (40). Data were excluded if microinjections were administered outside the AP or the NTS.

Results

Phrenic Nerve Response to Microinjection of AVP into the AP

Microinjection of AVP into the AP with a coordinate of 0.2 mm rostral to the CS and 0.2 mm below the surface at the midline (Fig. 1, A, B, and C) produced inhibition of PNA showing decrease in amplitude (Fig. 2, Aa, Ba, and Ca). This inhibition was dose-dependent. The low or sub-threshold dose of AVP evoked no effect on PNA (Fig. 3A) while moderate or threshold dose of AVP (3.0×10^{-5} IU) induced significant decreases in PNA (Fig. 3A). The high dose of AVP (4.5×10^{-5} IU) produced greater decreases in PNA (Fig. 3A). Microinjection of AVP into other areas such as the medullary raphe nucleus (Fig. 2Ac) or at the edge of the AP (Fig. 2Ad) produced no cardiopulmonary inhibition. Microinjection of AVP into an area with a coordinate of 0.4 mm rostral to the CS at the midline evoked hypertension without changes in PNA (Fig. 2Ab). The same volume of vehicle (normal saline) or of vehicle containing Pontamine sky blue into the same area as did by the AVP in the AP evoked no changes in PNA (Fig. 6Ab).

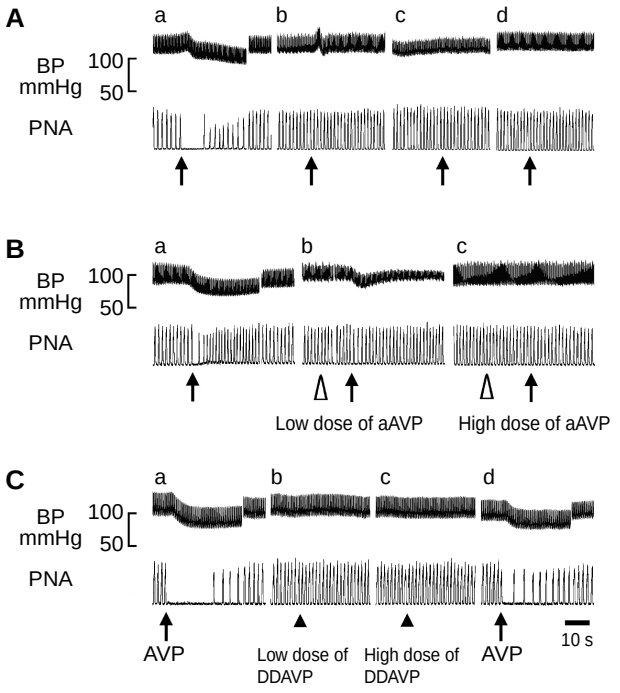

Fig. 2. Effect of AVP-induced cardiopulmonary modulation mediated through the V_{1A} receptor subtypes. Cardiopulmonary inhibition induced by AVP microinjection of 3.0×10^{-5} IU was only observed in an area with a coordinate of 0.2 mm rostral to the CS and 0.2-0.3 mm below the surface at the midline (panel Aa). An area having a coordinate of 0.4 mm rostral to the CS at the midline and 0.2-0.3 mm below the surface and receiving the same dose of AVP produced hypertensive effect without changes in PNA (panel Ab). Microinjection of AVP into the regions outside these two areas, such as medullary raphe (panel Ac) or at the edge of the AP (panel Ad), produced no cardiopulmonary modulations. Microinjection of low-dose AVP V_{1A} receptor antagonist alone (aAVP, 150 pg) into the area identified by AVP to induce cardiopulmonary inhibition (panel Ba) did not evoke any changes in cardiopulmonary functions but reversed the inhibition of PNA and part of the hypotension caused by AVP injection (panel Bb). Microinjection of high-dose aAVP (300 pg) into the same area did not provoke any cardiopulmonary modulation (panel Bc) whereas completely abolished the cardiopulmonary inhibition caused by AVP. Microinjection of low- and high-dose (panels Cb and Cc, respectively) DDAVP, an AVP V_2 receptor agonist, into the area identified by AVP-induced cardiopulmonary inhibition (Ca) neither initiated any cardiopulmonary effect nor impeded the cardiopulmonary response to AVP microinjection (panel Cd). Panels A, B, and C are taken from different animals. Blank and black upward arrowheads represent microinjection of aAVP and DDAVP, respectively, while upward arrow represents injection of AVP.

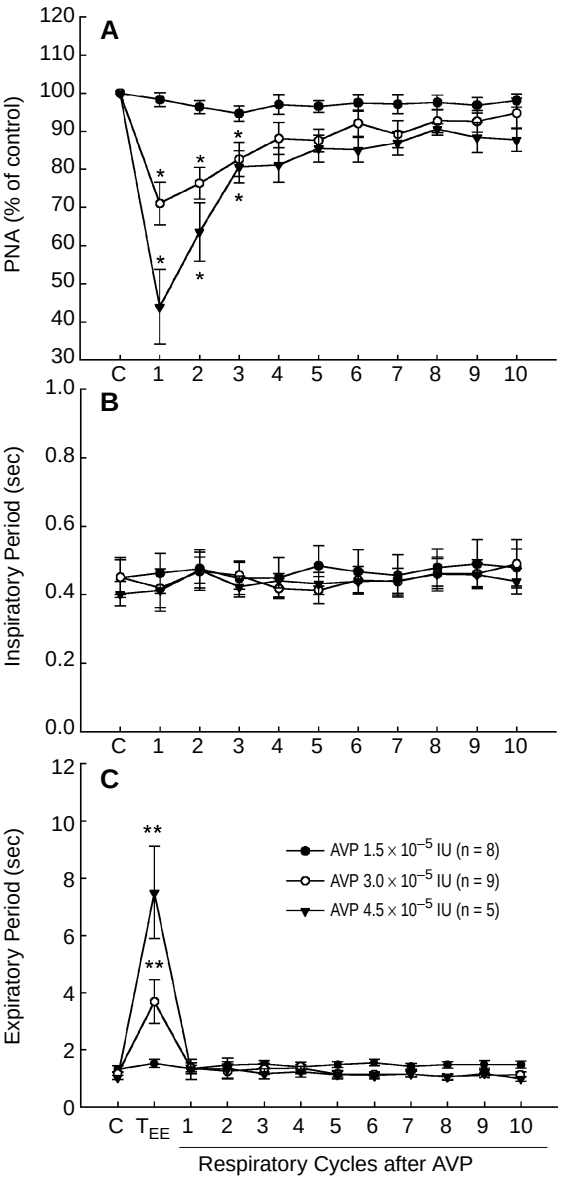

Fig. 3. Respiratory inhibition induced by AVP injection into the area postrema (AP). Decreases in mean PNA caused by AVP-induced activation of neurons in the AP was dose-dependent. The moderate and high doses of AVP evoked significant decreases in PNA for three respiratory cycles and then gradually recovered while low dose of AVP produced no significant changes in PNA (panel A). Microinjection of AVP produced no change in T_I (panel B) but an immediate elongation of the expiratory period (T_{EE}) in a dose-dependent manner (panel C). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control by one-way ANOVA and then by Dunnet's modified *t*-test.

In the group data, the average decrease of PNA in response to the threshold and high dose of AVP was 72 % of the control ($P < 0.05$, Fig. 3A, n = 9) and 45% of the control ($P < 0.05$, Fig. 3A), respectively. These reductions of PNA remained for several breaths following AVP administration and then returned to the baseline (Figs. 2 and 3A). Low dose of AVP

produced no changes in PNA ($P > 0.05$, Fig. 3A).

Alternation of Respiratory Pattern with Microinjection of AVP into the AP

AVP-activated neurons in the AP resulted in apnea, which was the extension of the T_E (Fig. 2, Aa, Ba, and Ca). This extension of T_E was dose-dependent. Thus, mean T_E was 1.4 s before AVP treatment and was extended to 4.0 s and 7.6 s after the administration of the moderate and high dose of AVP, respectively ($P < 0.01$, Fig. 3B). T_I and T_E after recovery from apnea tended to prolong, but insignificantly ($P > 0.05$, Fig. 3B).

Changes in Blood Pressure with AVP Microinjection into the AP

AVP-induced activation of neurons in the AP evoked two different types of responses of the BP, hypertension and hypotension (Figs. 2, Aa and Ab). Hypotensive response to AVP-injection was seen in an area with a coordinate of 0.2 mm rostral to the CS and 0.2-0.3 mm below surface at the midline (Fig. 2Aa, n = 5) while hypertensive response was observed in an area having 0.4 mm rostral to the CS, 0.2-0.3 mm below surface at the midline (Fig. 2Ab, n = 5). These two areas were only separated with a distance of 0.2 mm apart but displayed opposite effects on BP. Hypotensive response to AVP microinjection was accompanied by a decrease in phrenic burst and apnea while hypertensive response was not. Since we were attempted to explore how the AP participates in the modulation respiration, most of our works were hence concentrated in the hypotensive area. The low dose of AP produced only mild hypotensive effect ($P > 0.05$, Fig. 4A), while the moderate dose of AVP produced a significant decrease in BP of 26 mmHg ($P < 0.05$, Fig. 4A). The high dose of AVP resulted in a more extended decrease in BP of 37 mmHg ($P < 0.05$, Fig. 4A). There was a non-significant decrease in heart rate following microinjection of AVP ($P > 0.05$).

Abolition of Cardiopulmonary Responses to AVP by V_{1A} Receptor Antagonist

Microinjection of V_{1A} receptor antagonist into the same area that was microinjected with AVP induced no change in cardiopulmonary responses (Figs. 2, Bb and Bc, upward blank arrowhead). However, microinjection of low- (150 pg, Fig. 2Bb) and high-dose (300 pg, Fig. 2Bc) V_{1A} receptor antagonist abolished the decreases in PNA caused by the subsequent microinjection of AVP ($P > 0.05$, Fig. 5A). The high dose of the V_{1A} receptor antagonist also prevented the elongation of T_E (T_{EE}) ($P > 0.05$,

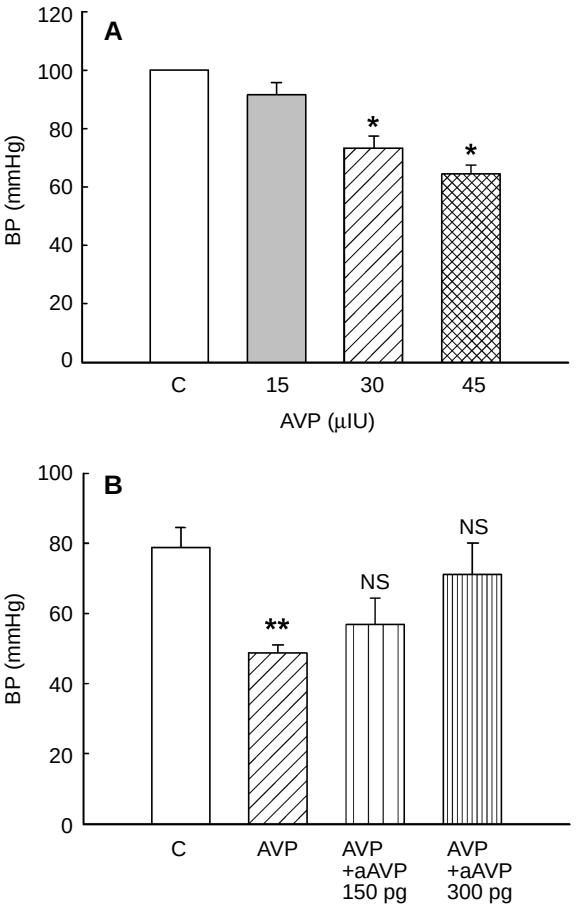

Fig. 4. Blood pressure (BP) response to microinjection of AVP into the AP before and after pre-treatment of AVP antagonist. AVP administration produced a decrease in BP in a dose-dependent manner (panel A). This hypotensive effect of AVP was partially antagonized by low-dose AVP V_{1A} receptor antagonist (aAVP) and was totally abolished by high-dose aAVP (panel B). *$P < 0.05$, **$P < 0.01$ compared with the control before AVP administration; NS represents non-significant difference compared with the control (C).

Fig. 5B) caused by AVP although the low dose of the V_{1A} receptor antagonist was not ($P < 0.05$, Fig. 5B). Hypotensive effect induced by the threshold dose of AVP was still evident following the administration of low dose V_{1A} receptor antagonist (Fig. 2Bb). However, the AVP-induced hypotensive effect was abolished by pre-treatment of high dose of V_{1A} receptor antagonist ($P > 0.05$, Fig. 4B and Fig. 2Bc). Microinjection of DDAVP into the same area that AVP injected produced neither decrease in phrenic burst nor apnea or hypotension (Figs. 2, Cb and Cc, and Fig. 5, C and D). However, the presence of DDAVP did not hinder AVP-induced decrease in PNA and apnea or hypotension (Fig. 2Cd). Thus, the decrease of PNA and apnea evoked by AVP injection

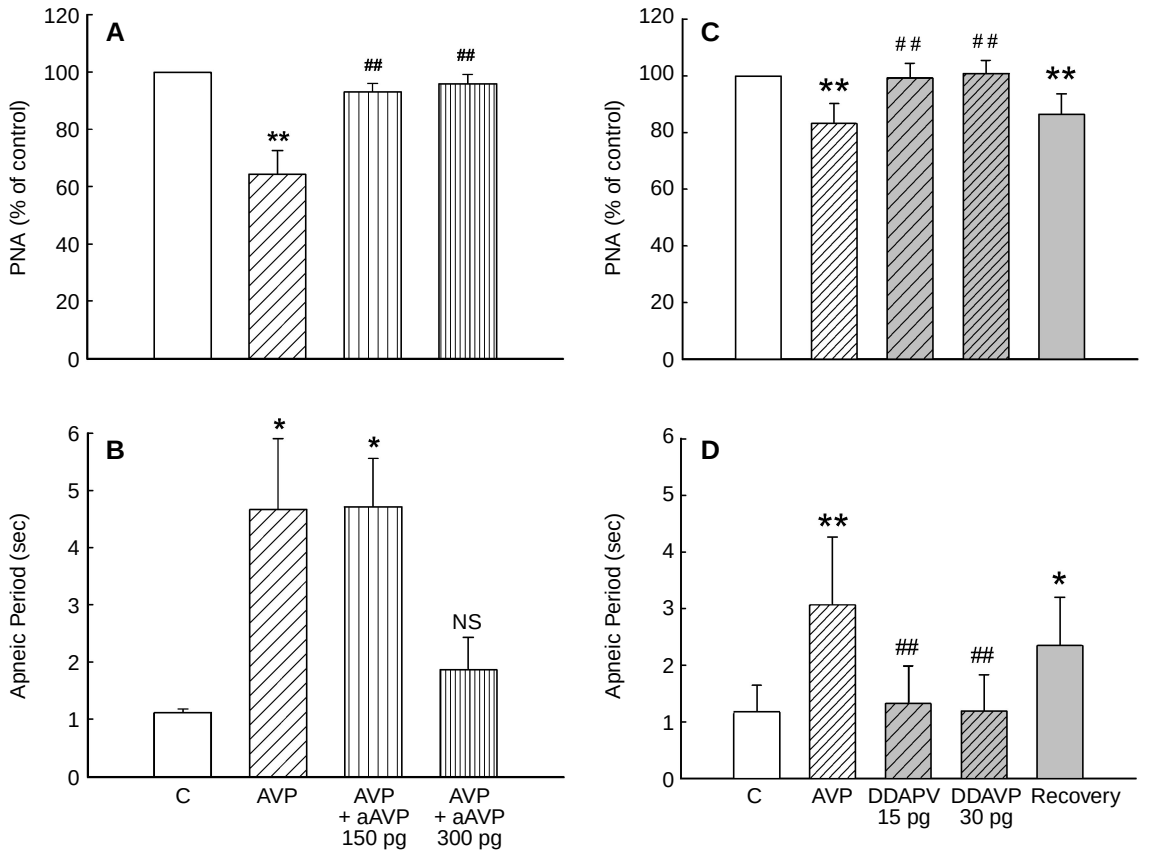

Fig. 5. Respiratory inhibition induced by AVP mediated through the activation of AVP V_{1A} receptor. Decreases in PNA (panel A) and elongation of T_E (panel B) caused by AVP administration was abolished by pre-treatment with AVP V_{1A} antagonist (aAVP). Microinjection of DDAVP into the area that was identified by AVP to produce respiratory inhibition produced effect on neither PNA (panel C) nor T_E (panel D). $*P < 0.05$, $**P < 0.01$ compared with the control before AVP injection; $\#\#P < 0.01$ compared with the value obtained from AVP administration without pre-treatment of AVP V_{1A} receptor antagonist. NS represents non-significant difference compared with the control before AVP injection.

was still significantly persistent (Fig. 5, C and D).

Involvement of the NTS in AVP-Induced Cardiopulmonary Inhibition

To determine if AVP-induced inhibition on respiration (decrease in PNA and apnea) and hypotension were mediated through the pathway connected between the AP and the NTS, we initially identified the area in the NTS that might cause a similar cardiopulmonary effects to those induced by AVP-injected into the AP (Fig. 6Aa). By way of microinjection of AVP into the NTS, area in the NTS that produced a decrease in PNA, apnea, and hypotension was localized in the region that was coordinated by 0.6-0.7 mm right to the midline, 0.5 mm rostral to the CS, and 0.4-0.5 mm below the surface (Fig. 6Ad). Other regions of the NTS rather than this small area could only produce mild or no effect on cardiopulmonary functions (Fig. 6, Ae and Af). Similar observations were consistently repeated in six animals. Thus, AVP-injected into the NTS reduced the mean PNA to 82% of the control ($P < 0.05$ *vs*. the control, n = 6) and prolonged the mean T_E from 1.0 ±0.1 s to 2.9 ±0.6 s ($P < 0.05$ *vs*. the control, n = 6). Mean BP was significantly decreased by 36 mmHg compared with the control ($P < 0.05$).

Once an effective area in the NTS was identified by AVP microinjection to induce cardiopulmonary inhibition as was observed during AP activation (Fig. 6, Ba, Bb, Ca, and Cb), bilateral injections of lidocaine (Fig. 6, Bc and Bd) and/or of $CoCl_2$ (Fig. 6, Cc and Cd) into this effective area were then completed. In this protocol, microinjections of AVP into the AP and into the NTS were independently completed by two sets of manipulators. Following bilateral injections of lidocaine (Fig. Bc and Bd, upward black arrowhead) and/or of $CoCl_2$ (Fig. 6, Cc and Cd, upward black arrowhead), a second microinjection of AVP into the AP was then performed to observe any attenuation of decreases in phrenic burst, apnea, and hypotension caused by AVP-induced activation of the AP (Fig. 6,

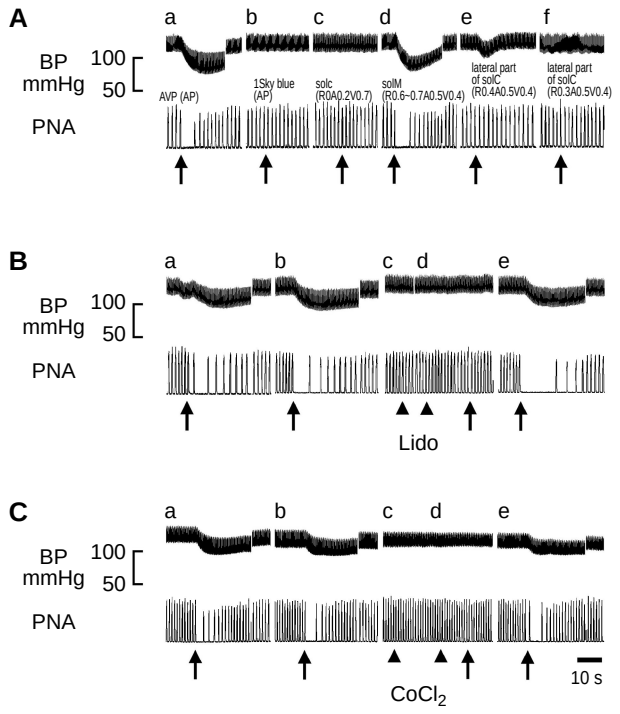

Fig. 6. Examples of AVP-induced respiratory inhibition mediating through the NTS. To identify an area in the NTS producing similar cardiopulmonary response to AVP injection into the AP (panel Aa), microinjection of AVP into to the NTS was localized in an region having a coordinate of 0.6-0.7 mm right to the midline, 0.5 mm rostral to the CS, and 0.4-0.5 mm below the surface could produce decrease in PNA, apnea, and hypotension (panel Ad). Other regions, such as 0.4 mm or 0.3 mm lateral to the midline at a level of 0.5 mm rostral to the CS and 0.4 mm below the surface, produced mild (panel Ae) or no effect (panel Af) on cardiopulmonary functions. Once a region in the NTS was confirmed to induce cardiopulmonary inhibition (panels Bb and Cb) similar to those caused by the AP (panels Ba and Ca), bilateral applications of lidocaine, a local anesthesia, and/or $CoCl_2$, a synaptic blockade, were then completed (panels Bc, Bd, Cc, and Cd) and AVP microinjection into the AP was subsequently accomplished to verify if a complete abolition of respiratory inhibition caused by AVP-induced activation neurons in the AP was obtained. These complete abolitions were reproducible after 30-60 minutes so that AVP microinjection into the AP could evoke again decreases in PNA, apnea, and hypotension (panels Be and Ce).

Bc and Cc, upward arrow) when compared with the responses before lidocaine and $CoCl_2$ (Fig. 6, Ba and Ca, upward arrow). After 30~60 min, a third microinjection of AVP into the AP was completed to verify if the synaptic blockade was dissipated. As shown in figure 6, Be and Ce, AVP-induced decreases in PNA, apnea, and hypotension were reappeared, suggesting that synaptic blockade by lidocaine or $CoCl_2$ was reversible. It appears that bilateral blockade of synaptic transmission significantly abolished phrenic inhibition (Fig. 7, A and C) and apnea (Fig. 7, B and D) caused by AVP microinjection into the AP. However, unilateral injections of lidocaine and $CoCl_2$ did not produce significant blockade (data not shown).

Attenuation of AVP-Induced Cardiopulmonary Inhibition by Hypercapnia

Increase in CO_2 concentration ($F_{ET}CO_2 = 0.08$) produced reflex enhancement of PNA ($P < 0.01$, Fig. 8A) but no effect on apneic period ($P > 0.04$, Fig. 8B). Under this hypercanic condition, threshold-dose AVP produced no longer significant inhibition on PNA when compared with the response before hypercapnia (Fig. 8A). Hypercapnia also attenuated the prolongation of T_E caused by AVP microinjection into the AP (Fig. 8B).

Discussion

We report for the first time that activation of AVP receptors in the AP evokes a substantial inhibition on respiration, reflecting on the decrease of phrenic amplitude and the elongation of T_E. This inhibition was mediated through a neural pathway between the AP and the NTS and also through the activation of V_{1A} receptor but not the V_2 receptor subtypes. This inhibitory effect caused by AVP was considerably attenuated by hypercapnia. These results suggest that an endogenous vasopressinergic pathway may project to the AP to participate in the modulation of respiration by way of a pathway through the NTS and the activation of V_{1A} receptors in the AP.

Critique of Method

Microinjection technique. The AP is a small area that is approximately 0.5-0.6 mm in length, 0.6 mm in width, and about 0.5 mm in depth (40). The tip of the microelectrode was positioned at the center of the AP. The largest volume of injection used in the present study was 45 nl, which slightly exceeded that used in our recent studies (10-12) and was also slightly greater than that recommended by Lipski *et al.* (32) who discussed the limitations of the technique of microinjection of excitatory amino acids in stimulating neurons within the CNS. However, this volume is less than that used by others, -- 50 nl by Bhatnagar, *et al.*, (1) and 60 nl by Lin *et al.* (31). The reason we injected over 30 nl in the present study was that low dose AVP did not produce any substantial cardiopulmonary responses and we wanted to examine whether a dose-dependent cardiopulmonary response could be obtained. It is possible that the larger volume of AVP used in the present study may diffuse out of

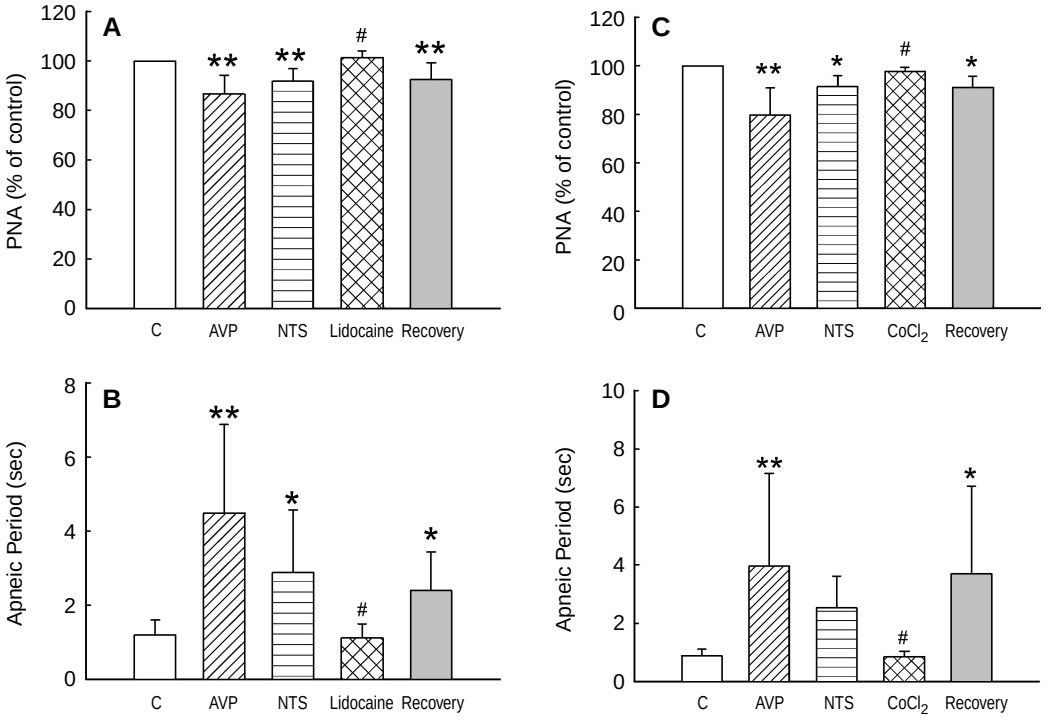

Fig. 7. Mean values (± SEM) of PNA and apneic period with AVP microinjection were attenuated by synaptic blockade at the NTS. Mean values of decreases in PNA (tripped bar in panel A) and of the elongation of apnea (tripped bar in panel B) with AVP-induced activation of AP neurons were abolished by a bilateral treatment of lidocaine (crossed bars in panels A and B) into the area at the NTS that produced similar responses (horizontal bar in panels A and B) to those caused by AP activation. Bilateral microinjection of $CoCl_2$ into the NTS also evoked a similar abolition of phrenic reduction and apneic period as was to lidocaine (crossed bars in panels C and D). *$P < 0.01$, **$P < 0.01$, #$P > 0.05$, compared with the control by one-way ANOVA and then followed by Dunnet's modified *t*-test.

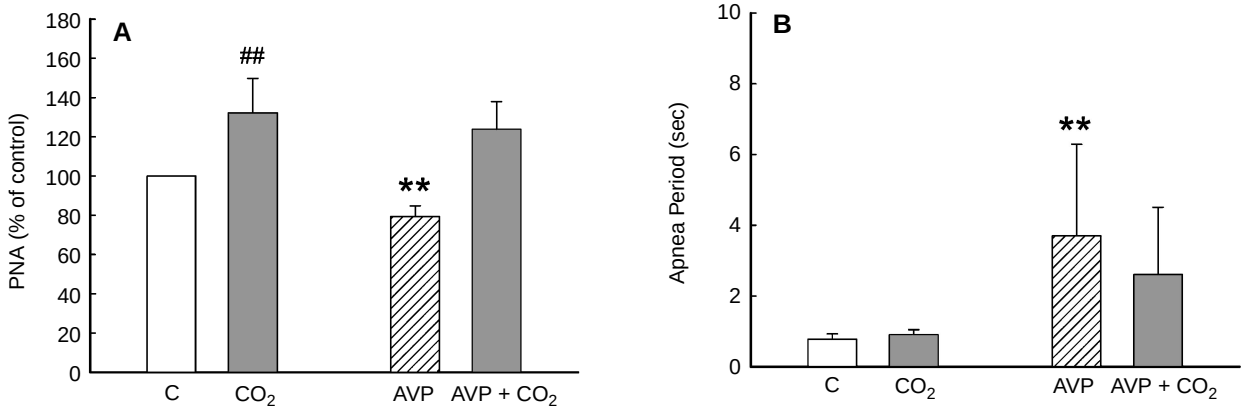

Fig. 8. Hypercapnia evoked attenuation of the respiratory inhibition caused by AVP. Increase in CO_2 concentration initiated a significant enhancement of PNA (panel A) without effect on apneic peiod (panel B) whereas abolished the phrenic reduction (panel A) and apneic period (panel B) in response to AVP microinjection. **$P < 0.01$, ##$P < 0.01$ compared with the control by Two-way ANOVA and then followed by Dunnet's modified *t*-test.

the AP. However, this did not appear to be the case here. First, there was no diffusion of the dye out of the AP. Assuming that the dye is evenly distributed from the tip of the microelectrode, it shouldn't diffuse out of the lower boarder of the AP since the tip was positioned at the center of the AP. Second, microinjection of AVP into an area 0.7 or 0.8 mm below the surface, which was the commisure subarea of the NTS having AVP receptors (43), produced no changes in PNA and BP in six animals (Fig. 6Ac). We, therefore, believe that the injected 45 nl of test substance remained on the AP such that a bulk-diffusion effect was unlikely.

Furthermore, an equivalent volume of vehicle injected into the same site did not produce any changes in cardiopulmonary responses. Moreover, microinjection of AVP into the midline raphe nucleus evoked no changes in cardiopulmonary functions (Fig. 2Ac). Thus, decreases in PNA, apnea, and hypotension evoked by AVP must be due to activation of neurons in the AP rather than a mechanical effect of volume expansion. Histological verification revealed that an injection volume of 45 nl diffused to an area that is less than 400 μm in diameter (Fig. 1C) supported our results that could mostly be caused by the activation of the AP neurons. Moreover, our physiological observations that microinjection of AVP into two areas in the AP with a distance of 0.2 mm apart produced opposite cardiopulmonary effects, of which one area evoked hypotension concomitant with an inhibition of respiration and the another induced hypertension without changes in PNA (Fig. 2, Aa and Ab). These physiological data may give extra evidence to support a notion that our results is specifically due to the activation of the AP neurons.

Another possible problem was whether AVP microinjection might cause depolarization inhibition, similar to that caused by microinjection of Glu. However, it is unlikely that depolarization inhibition occurred in the present setting. First, the effects of AVP on cardiopulmonary responses were reproducible. Moreover, these AVP-induced cardiopulmonary responses were dose-dependent. Second, the mechanism through which neurons are activated by AVP might differ from that by glutamate (45). Another concern is that desensitization of AVP may have influenced the present results. This probably did not occur because of the reproducibility of the findings. Based on these results, we believe that the AP contains neurons that possess AVP receptors and that these neurons are involved in the inhibition of cardiopulmonary functions.

Inpretation of $CoCl_2$ and lidocaine microinjections. Cobalt chloride has been widely used as a blockade in the CNS to block the synaptic transmission. Under the treatment of cobalt chloride, reflex pathway would hence be blocked and the response would certainly be abolished (22, 42). Another way to impede the synaptic transmission is the application of local anesthesia to inactivate neurons so that the synaptic transmission can be blocked. Lidocaine is frequently used as an effective agent to anesthetize the neurons (50) and results in the blockade of the synaptic transmission (51). Our data showing abolition of decreases in PNA, apnea, and hypotension caused by AVP-microinjected into the AP after pretreatment of lidocaine and $CoCl_2$, which strongly suggested that the AVP-induced cardiopulmonary inhibition is mediated through the synaptic transmission at the NTS. This blockade was reversible so that microinjection of AVP could again produce cardiopulmonary inhibition after the dissipation of drugs.

Interpretation of pontamine sky blue dye. Pontamine sky blue is frequently used as a marker to localize the tip of the stimulation microelectrode and as a tool to mark the injection site as well as the diffusion area of the drug injected in the brain (10-12). However, it may change the excitability of neurons and affect the results observed (38). In this consideration, we microinjected pontamine sky blue alone into the same area in the AP as did by AVP injection and resulted in no changes in cardiopulmonary functions as shown in one of three animals observed (Fig. 6Ab). This may be due to the low concentration of dye injected and may also be due to the tissue differences. Moreover, cardiopulmonary inhibition caused by AVP microinjection was reproducible (Fig. 6). Thus the effect of pontamine sky blue may be minor and negligible in our experimental conditions.

Respiratory Inhibition by AVP Mediated via V_{1A} Receptors

Our data showed that AVP-induced activation of neurons in the AP produced decreases in PNA and apnea, and quite differed from previous reports that excitation of AP neurons by DLH, TRH, or Glu produced increases in respiratory frequency and tonic phrenic discharges in the rabbit (5, 37, 47). This difference could be ascribed to the activation of different receptors and might also be due to different species of animals used in the studies (rats versus rabbits). AVP has been reported recently to play a role in the modulation of respiration, showing either decreased PNA (10-12) or increased respiration (29) by acting on different brain areas. Our current observations are consistent with our recent reports that AVP inhibits respiration by activating neurons in the rostral ventrolateral medulla (rVLM) (10-12). The AP has been demonstrated to connect bilaterally with the rVLM, an area in the brain stem that plays a critical role in the regulation of cardiopulmonary functions (3, 54). Electrical stimulation of the AP has been shown to produce a biphasic response -- an early excitation followed by a delayed inhibition -- on neurons that exhibit both sympathetic- and cardiac-related activities in the rVLM (56). However, activation of the AP did not produce any modulating effects on respiratory-related neurons in the rVLM (56). Thus, decreases in PNA and apnea following AVP microinjection in the present study probably were not mediated through direct activation of neurons in the rVLM.

Based on our data, respiratory inhibition by AVP-induced activation of AP neurons may be mediated through the neural connection between the AP and NTS. The NTS contains AVP receptor (43) and has been demonstrated to modulate the baroreflex control of BP and renal sympathetic nerve activity induced by

stimulation of the aortic depressor nerve (25). In the present study, microinjection of AVP into the NTS evoked a similar response of respiratory inhibition to those induced by AVP applied to the AP. Bilateral applications of lidocaine or $CoCl_2$ resulted in a total blockade of decrease in PNA and of elongation of T_E evoked by AVP microinjection into the AP. These results strongly supported our notion, regarding that respiratory inhibition induced by AVP microinjected into the AP might be mediated through the NTS.

The abolition of respiratory inhibition by the application of lidocaine or $CoCl_2$ to the NTS on both sides raises a possibility that signals from peripheral chemoreceptors and from the activation of AP neurons possibly interact at the level of the NTS, the first-order termination site of peripheral chemoreceptors. This possibility was confirmed by our current data, which show that respiratory inhibition induced by AVP microinjection into the AP was largely accentuated by hypercapnia. Nevertheless, hypercapnia alone would induce reflex excitation to PNA and certainly attenuate the respiratory inhibition caused by AVP.

There is no direct evidence to suggest a direct descending pathway from the AP to synapse with phrenic motorneurons at the spinal level. The most putative pathway mediating the respiratory inhibition during AVP-induced activation of AP neurons is probably mediated through the NTS passing the signal to the ventral respiratory group located at the ventrolateral medulla (2). Nevertheless, activation of the AP neurons with AVP receptors infers that the AP may be involved in the modulation of respiration.

Our data showed that decreases in PNA and apnea caused by AVP microinjection were totally abolished by pre-treatment of AVP V_{1A} receptor antagonist. Specifically, DDAVP, an AVP V_2 receptor, did not produce any effects on respiration. These results strongly suggest that an endogenous vasopressinergic pathway may project to the AP and produce inhibition on respiration through the activation of V_{1A} receptor subtype in AP neurons. However, the origin of this putative projection is unknown. It is conceivable that these projections may originate from the paraventricular nucleus (PVN) as suggested by physiological data (29, 44) and histological observations (20, 29).

The AP is one of the circumventricular organs and lacks the blood-brain-barrier. Thus, neurons within the AP are subject to the influence of substances in the peripheral circulation (14). Peripheral AVP may activate the AP neurons having V_{1A} receptors and evokes a modulating influence on respiration. In this regard, we have previously observed that infusion of AVP causes a significant decrease in lung compliance in the rat, implicating a decrease in respiratory volume (26), and a decrease in diaphragmatic activity (53). Walker and Jennings (55) have also reported that AVP V_1-receptor blockade can enhance the excitation of angiotensin on respiration in dogs. Thus, our present data may provide a neurophysiological basis for peripheral AVP's modulating effects on respiration through the AP.

Cardiovascular Modulation by AVP-Activated Neurons in the AP

The AP has been reported to play a role in the regulation of cardiovascular functions and is essential for the maintenance of resting BP (46). Electrical stimulation of the AP in anesthetized rats produced hypotensive effect (24). The AP has been demonstrated to inhibit exercise-induced pressor reflex (7, 49). Chemical lesion of the AP can eliminate AVP-induced baroreflex enhancement of bradycardia (15). Our data are consistent with these reports. Interestingly, dynamic and static exercises have been demonstrated to stimulate AVP secretion (34, 35). In the present study, we did not observe a significant decrease in heart rate after AVP microinjection. This may be due to: [1] decrease in BP may contribute to an inhibition of sympathetic nerve discharges caused by AVP treatment (21), and [2] bradycardia was probably reversed by the use of gallamine triethiodide, an antagonist of cholinergic M_2 receptor subtypes, which have been reported to be associated with bradycardia and hypotension induced by capsaicin administration in the rat (52).

Data inconsistent with the notion mentioned above are that Glu injection into the AP cause increase in BP (1, 24). Our data showing direct application of AVP to activate AP neurons could produce either hypotension or hypertension dependent on the sites that AVP was injected. These data suggested that there might be two different areas in the AP participating in the modulation of cardiovascular functions. If true, our data provide evidence that AVP is probably an excellent agent to activate AP neurons and to distinguish them into two subgroups of which caused either enhance or diminish the BP upon activation. Since our most interest was to determine how the AP participates in the modulation of respiration, we hence focused only on the hypotensive area of the AP.

Physiological Considerations

What is the physiological significance of this AVP's modulating influence on neurons in the AP? There is insufficient information to indicate that the AP modulates respiration. To our knowledge, there are very few reports that injection of DLH, TRH, and Glu into the AP facilitates the respiratory frequency in rabbits (5, 37, 47). Unfortunately, these previous data are quite different from our current results. At this juncture, we would like to speculate on the role of

the AP, if any, on respiration. First, the PVN may project to the AP to modulate cardiopulmonary functions by releasing AVP as a neurotransmitter. This pathway is activated mainly by an increase in plasma osmolality. It is unlikely that respiration should decrease or even temporally cease during the regulation of plasma osmolality. Second, AVP has been demonstrated to be involved in modulating cardiovascular functions such as the pressor effect and the redistribution of blood flow during exercise (28, 39, 48). The release of AVP into the general circulation is well-correlated with the rise in plasma osmolality during submaximal exercise. This release of AVP is enhanced by high-intensity exercise and is thought to activate the hypothalamic-pituitary-adrenal axis to maintain ACTH release during prolonged exercise (28). Hence, AVP may play a role in the modulation of respiration during exercise. More interesting is that endogenous AVP has been demonstrated to increase in secretion during exercise (33, 35). However, this putative modulating role seems unfavorable because of its inhibition on respiration. Third, the AP can inhibit static exercise-induced pressor reflex (7). In concert with the inhibition of respiration, activation of AVP V_{1A} receptors may play a role in the exercise-intolerance. Respiratory inhibition by AVP may signal the need of oxygen, which in turn, protects the subject from oxygen deprivation during heavy exercise. Fourth, chronic stress may lead to secretion of AVP, which is necessary for the maintenance of the function of hypothalamo-hypophyseal-adrenal axis (45). Under chronic stress, secretion of AVP replaces the role of corticotrophin-releasing hormone (CRH). Highly elevated levels of circulating AVP under chronic stress may produce modulating effects on respiratory. Unfortunately, there is inadequate data showing how endogenous AVP modulates respiration under some circumstance. Further investigation is needed to ascertain the role of AVP receptors-containing neurons in the AP on respiration.

In conclusion, our present data show that AVP-induced activation of the V_{1A} receptors found on the AP neurons produces inhibitory modulation on pulmonary functions by way of the NTS. This inhibition may play a role during some physiological conditions such as heavy exercise. More studies are needed to elucidate that the circumstance in which AVP may activate the V_{1A} receptors in the AP and maintain homeostasis.

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References

1. Bhatnager, T., Chtrvanshi, V.C. and Sapru, H.N. Cardiovascular responses to microinjections of excitatory amino acids into the area postrema of the rat. *Brain Res.* 822: 192-199, 1999.
2. Bianchi, A.L., Denavit-Saubie, M. and Champagnat, J. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. *Physiol. Rev*. 75: 1-45, 1995.
3. Blessing, W.W., Hedger, S.C. Joh, T.H. and Willoghby, J.O. Neurons in the area postrema are the only catecholamine-synthesizing cells in the medulla or pons with projections to the rostral ventrolateral medulla (C1-area) in the rabbit. *Brain Res*. 419: 336-340, 1987.
4. Bongianni, F., Mutolo, D., Srinivasan, M., Staderini, G., Baccari, M.C., Calamai, F. and Pantaleo, T. Gastric relaxation in response to chemical stimulation of the area postrema in the rabbit. *Brain Res.* 646: 307-311, 1994.
5. Bongianni, F., Mutolo, D., Carfi, M. and Pantaleo, T. Area postrema glutamate receptors mediate respiratory and gastric responses in the rabbit. *NeuroReport* 9: 2051-2062, 1998.
6. Bonham, A.C. and Hasser, E.M. Area postrema and aortic or vagal afferents converge to excite cells in nucleus tractus solitarius. *Am. J. Physiol*. 264: Hl674-Hl685, 1993.
7. Bonigut, S., Bonham, A.C. and Stebbins, C.L. Area postrema-induced inhibition of the exercise pressor reflex. *Am. J. Physiol.* 272: Hl650-Hl655, 1997.
8. Borison, H.L. and Wang, S.C. Physiology and pharmacology of vomiting. *Pharmacol. Rev*. 5: 193-230, 1953.
9. Cai,Y., Hay, M. and Bishop, V.S. Synaptic connections and interactions between area postrema and nucleus tractus solitarius. *Brain Res.* 724: 121-124, 1996.
10. Chuang, C.-W., Cheng, M.-T., Lin, J.-T., Hsien, Y.-Y., Hung, H.-Y. and Hwang, J.-C. Arginine vasopressin produces inhibition upon respiration without pressor effect in the rat. *Chinese J. Physiol*. 46: 71-78, 2003.
11. Chuang, C.-W., Cheng, M.-T., Yang, S.-J. and Hwang, J.-C. Activation of ventrolateral medulla neurons by arginine vasopressin *via* V_{1A} receptors produces inhibition on respiratory-related hypoglossal nerve discharge in the rat. *Chinese J. Physiol*. 48: 1-11, 2005.
12. Cheng, M.-T., Chuang, C.-W., Lin, J.-T. and Hwang, J.-C. Cardiopulmonary response to vasopressin-induced activation on V_{1A} receptors in the lateral ventrolateral medulla in the rat. *Chinese J. Physiol*. 47: 31-34, 2004.
13. Collins, H.L., Rodenbaugh, D.W. and DiCarlo, S.E. Central blockade of vasopressin V_1 receptors attenuates postexercise hypotension. *Am. J. Physiol*. 281: R375-R380, 2001.
14. Cottrell, G.T. and Ferguson, A.V. Sensory circumventricular organs: central roles in integrated autonomic regulation. *Regul. Pept*. 117: 11-23, 2004.
15. Cox, B.F., Hay, M. and Bishop, V.S. Neurons in area postrema mediate vasopressin-induced enhancement of the baroreflex. *Am. J. Physiol*. 258: H1943-H1946, 1990.
16. Curtis, K.S., Verbalis, J.G. and Stricker, E.M. Area postrema lesions in rats appear to disrupt rapid feedback inhibition of fluid intake. *Brain Res.* 726: 31-38, 1996.
17. Dunnett, C. W. New tables for multiple comparisons. *Biometrics* 20: 482-491, 1964.
18. Ferguson, A.V. and Marcus, P. Area postrema stimulation induced cardiovascular changes in the rat. *Am. J. Physiol.* 255: R855-R860, 1988.
19. Ferguson, A.V. and Bains, J.S. Electrophysiology of the circumventricular organs. *Front. Neuroendocrin.* 17: 440-475, 1996.
20. Hardy, E. G. P. Hypothalamic projections to cardiovascular centers of the medulla. *Brain Res.* 894: 233-240, 2001.

21. Hasser, E.M., Undesser, K.P. and Bishop, V.S. Interaction of vasopressin with area postrema during volume expansion. *Am. J. Physiol.* 253: R605-R610, 1987.
22. Haxhiu, M.A., Deal, E.C., Mathew, P.Jr., van Lunteren, N.E., Mitra, J. and Cherniack, N.S. Influence of ventrolateral surface of medulla on reflex tracheal constriction. *J. Appl. Physiol*. 61: 791-796, 1986.
23. Hay, M. and Bishop, V.S. Interactions of area postrema and solitary tract in the nucleus tractus solitarius. *Am. J. Physiol*. 260: Hl466-H1473, 1991.
24. Hegarty, A.A., Hayward, L.F. and Felder. R.B. Sympathetic responses to stimulation of area postrema in decerebrated and anesthetized rats. *Am. J. Physiol*. 268: H1086-H1095, 1995.
25. Hegarty, A.A. and Felder, R.B. Antagonism of vasopressin V_1 receptors in NTS attenuates baroreflex control of renal nerve activity. *Am. J. Physiol.* 269: H1080- H1086, 1995.
26. Hwang, J.-C. and Chen, S.M. The effect of vasopressin on respiratory mechanics in the rat. *Biol. Bull. NTNU*. 12: 15-22, 1977.
27. Hwang, J.-C., Su, C.-K., Yen, C.-T. and Chai, C.-Y. Presence of neuronal cell bodies in the sympathetic pressor areas of dorsal and ventrolateral medulla inhibiting phrenic nerve discharge in cats. *Clin. Auton. Res*. 2:189-196, 1992.
28. Inder, W. J., Hellemans, J., Swanney, M. P., Prickett, T.C.R. and Donald, R.A. Prolonged exercise increases peripheral plasma ACTH, CRH, and AVP in male athletes. *J. Appl. Physiol*. 85: 836-841, 1998.
29. Kc, P., Haxhiu, M.A., Tlentino-Silva, E.P., Wu, M.F., Tough, C.O. and Mack, S.O. Paraventricular vasopressin-containing neurons project to brain stem and spinal cord respiratory-related sties. *Respir. Physiol. Neurobi*. 133:75-88, 2002.
30. Koga, T., Kobashi, M., Mizutani, M., Tsukamoto, G. and Matsuo, R. Area postrema mediates gastric motor response induced by apomorphine in rats. *Brain Res*. 960: 122-131, 2003.
31. Lin, H.-C., Wan, F.-J., Tung, C.-S. and Tseng, C.-J. Adenosine and glutamate modulate the cardiovascular responses of angiotensins II and III in the area postrema of rats. *J. Auton. Nerv. Syst.* 51: 19-26, 1995.
32. Lipski, J., Bellingham, M. C., West, M. J. and Pilowsky, P. Limitations of the technique of excitatory amine acid for the CNS. *J. Neurosci. Meth*. 26: 169-179, 1988.
33. Liviakis, L.R. and Stebbins, C.L. Static contraction causes a reflex-induced release of arginine vasopressin in anesthetized cats. *Brain Res. Bull.* 53: 233-238, 2000.
34. Lowes, V.L., McLean, L.E., Kasting, N.W. and Ferguson, A.V. Cardiovascular consequences of microinjection of vasopressin and angiotensin II in the area postrema. *Am. J. Physiol*. 265: R625-R631, 1993.
35. Michelini, L.C. Endogenous vasopressin and the central control of heart rate during dynamic exercise. *Braz. J. Med. Biol. Res*. 31: 1185-1195, 1998.
36. Migita, K., Hori, N., Manako, J.I., Saito, R., Takano, Y. and Kamiga, H.O. Effect of arginine-vasopressin on neuronal interaction from the area postrema to nucleus tractus solitarii in rat brain slices. *Neurosci. Lett*. 256: 45-48, 1997.
37. Mutolo, D., Bongianni, F., Carfy', M. and Pantaleo, T. Respiratory responses to thyrotropin-releasing hormone microinjected into the rabbit medulla oblongata. *Am. J. Physiol*. 277: R1331-R1338, 1999.
38. Nagano, M., Ashidate, N., Yamamoto, K., Ishimizu, Y., Saitoh, S., Konishi, Y., Koga, T. and Fukuda, H. Excitatory neurotoxic properties of pontamine sky blue make it a useful tool for examining the functions of focal brain parts. *Jpn. J. Physiol.* 54: 61-70, 2004.
39. O'Leary, S., Rossi, N. F. and Churchill, F. C. Muscle metaboreflex control of vasopressin and renin release. *Am. J. Physiol*. 264: H1422-H1427; 1993.
40. Paxinous, G. and Watson, C. The Rat Brain in Stereotaxic Coordinates 2nd Ed. Academic Press Inc. 1986.
41. Peuler, J.D., Edwards, G.L., Schmid, P.G. and Johnson, A.K. Area postrema and differential reflex effects of vasopressin and phenylephrine in rats. *Am. J. Physiol*. 258: H1255-Hl259, 1990.
42. Plowey, E.D. and Waldrop, T.G. Cobalt injections into the pedunculopontine nuclei attenuate the reflex diaphragmatic responses to muscle contraction in rats. *J. Appl. Physiol.* 96: 301-307, 2004.
43. Raggenbass, M., Tribollet, E., Dubois-Dauphin, M. and Dreifuss, J.J. Vasopressin receptors of the vasopressor (V_1) type in the nucleus of the solitary tract. *J. Neurosci.* 9: 3929-3936, 1989.
44. Schlenker, E., Barnes, L., Hansen, S. and Martin, D. Cardiorespiratory and metabolic responses to injection of bicuculline into the hypothalamic paraventricular nucleus (PVN) of conscious rats. *Brain Res*. 895: 33-40, 2001.
45. Scott, L.V. and Dinan, T.G. Vasopressin and the regulation of hypothalamic- pituitary-adrenal axis function: implications for the pathophysiology of depression. *Life Sci.* 62: 1985-1998, 1998.
46. Skoog, K.M. and Mangiapane, M.L. Area postrema and cardiovascular regulation in rats. *Am. J. Physiol*. 254: H963-H969, 1988.
47. Srinivasan, M., Bongianni, F., Fontana, G.A. and Pantaleo, T. Respiratory responses to electrical and chemical stimulation of the area postrema in the rabbit. *J. Physiol*. 463: 409-420, 1993.
48. Stebbins, C.L. and Symons, J.D. Vasopressin contributes to the cardiovascular response to dynamic exercise. *Am. J. Physiol*. 264: H1701-H1707, 1993.
49. Stebbins, C.L., Bonigut, S., Liviakis, L.R. and Munch, P.A. Vasopressin acts in the area postrema to attenuate the exercise pressor reflex in anesthetized cats. *Am. J. Physiol*. 274: H2116-H2122, 1998.
50. Sullivan, J., Fuller, D. and Fregosi, R.F. Control of nasal dilator muscle activities during exercise: role of nasopharyngeal afferents. *J. Appl. Physiol*. 80: 1520-1527, 1996.
51. St John, W.M., Bledsoe, T.A. and Sokol, H.W. Identificaion of medullary loci critical for neurogenesis of gasping. *J. Appl. Physiol.* 56: 1008-1019, 1984.
52. Tsai, C.-Y., Wu, C.-C., Lee, K.-Z. and Hwang, J.-C. Muscarinic M_2 receptor mediates bradycardia and hypotension induced by capsaicin administration in the rat. *BioFormosa* 40: 25-35, 2005.
53. Tseng, Y.-L., Wu, J.-S. and Hwang, J.-C. Arginine vasopressin reduces respireatory-related activity of the alae nasi muscle in rats. *BioFormosa* 38: 37-47, 2003.
54. Yang, Z. and Coote, J.H. Influence of the hypothalamic paraventricular nucleus on cardiovascular neurons in the rostral ventrolateral medulla of the rat. *J. Physiol.* 513: 521-530, 1998.
55. Walker, J.K.L. and Jennings, D.B. During acute hypercapnia vasopressin inhibits an angiotensin drive to ventilation in conscious dogs. *J. Appl. Physiol*. 79: 786-794, 1995.
56. Wallenstein, S., Zucker, C. L. and Fleiss, J.L. Some statistical methods useful in circulation research. *Circ. Res*. 47: 1-9, 1980.
57. Wilson, C.G. and Bonham, A.C. Area postrema excites and inhibits sympathetic-related neurons in rostra1 ventrolateral medulla in rabbit. *Am. J. Physiol*. 266: H1075-H1086, 1994.