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Abstract

Our objective was to investigate in conscious Sprague-Dawley (6-8 weeks, 250-300 g) female rats (N = 7 in each group) the effects of intracerebroventricularly (*icv*) injected adrenomedullin (ADM) on blood pressure and heart rate (HR), and to determine if ADM and calcitonin gene-related peptide (CGRP) receptors, peripheral V_1 receptors or the central cholinergic system play roles in these cardiovascular effects. Blood pressure and HR were observed before and for 30 min following drug injections. The following results were obtained: 1) *icv* ADM (750 ng/10 µL) caused an increase in both blood pressure and HR (∆MAP = 11.8 ± 2.3 mmHg and ∆HR = 39.7 ± 4.8 bpm). 2) Pretreatment with a CGRP receptor antagonist ($CGRP_{8-37}$) and ADM receptor antagonist (ADM_{22-52}) blocked the effect of central ADM on blood pressure and HR. 3) The nicotinic receptor antagonist mecamylamine (25 µg/10 µL, *icv*) and the muscarinic receptor antagonist atropine (5 µg/10 µL, *icv*) prevented the stimulating effect of ADM on blood pressure. The effect of ADM on HR was blocked only by atropine (5 µg/10 µL, *icv*). 4) The V_1 receptor antagonist [β-mercapto-β-β-cyclopentamethylenepropionyl[1], O-me-Tyr[2],Arg[8]]-vasopressin (V2255; 10 µg/kg), that was applied intravenously, prevented the effect of ADM on blood pressure and HR. This is the first study reporting the role of specific ADM and CGRP receptors, especially the role of nicotinic and muscarinic central cholinergic receptors and the role of peripheral V_1 receptors in the increasing effects of *icv* ADM on blood pressure and HR.

Key words: Adrenomedullin; Blood pressure; Vasopressin; Atropine; Mecamylamine

Introduction

Adrenomedullin (ADM), a calcitonin/calcitonin gene-related peptide (CGRP) with vasodilatory properties, has multiple functions in regulating cardiovascular homeostasis, and is of particular interest in the pathophysiology of hypertension (1-3). ADM shows structural homology to CGRP, and promotes its effects via activation of specific ADM and CGRP1 receptors (3,4). Some of the central effects of this peptide are thought to be mediated by specific receptors and CGRP1 receptors, similar to the peripheral mechanism (5,6). ADM was originally isolated from pheochromocytoma tissue and subsequently found to be produced in many peripheral tissues as well as in the central nervous system (CNS) (7-10). Intravenous (*iv*) administration of ADM causes vasodilatation and a reduction of arterial pressure (11), but intracerebroventricular (*icv*) administration of ADM elevates sympathetic output and increases arterial pressure (12-15). Additionally, *icv* ADM stimulates the hypothalamo-neurohypophyseal axis

of rats and the production of hypothalamic nitric oxide (16). Many studies have demonstrated that *icv* ADM increased blood pressure and heart rate (HR) via central sympathetic stimulation. The action of central ADM might involve other central mechanisms such as central cholinergic stimulation. The central sympathetic stimulation and central cholinergic neurotransmission relationship has not been demonstrated in the mechanisms of central ADM. Enhancement of central cholinergic neurotransmission is very effective in increasing blood pressure. Previous studies have shown that central cholinergic stimulation or central cholinomimetic injection causes increases in blood pressure via the activation of sympathetic discharge (17-20). The action of centrally administered ADM might involve other mechanisms with peripheral V_1 receptors since there are several reports that *icv* administration of ADM increased c-fos mRNA and FOS protein in many areas of the hypothalamus, including the paraventricular nucleus (PVN) and supraoptic nucleus

(SON) (7,8). Both the PVN and SON contain neurons that produce arginine vasopressin (AVP) and/or oxytocin. Taylor at al. (21) have previously demonstrated that brain-derived ADM is a physiologic regulator of AVP secretion. *Icv* administration of ADM stimulated AVP release, while ribozyme impairment of brain-derived ADM prevented an appropriate AVP response to dehydration challenges. A previous study by our group demonstrated that peripheral V_1 receptors act as mediators of the central effects of other vasoactive peptides such as *icv* glucagon-like peptide-1 (22) and therefore peripheral V_1 receptors may act as mediators for the central cardiovascular effects of ADM. In this study, we present data that suggest roles of ADM and CGRP receptors, peripheral vasopressin and central cholinergic system in the cardiovascular responses to ADM.

Material and Methods

We used Sprague-Dawley rats weighing 250-300 g, from the Uludag University Experimental Animal Raising, Application and Research Center. The rats were taken from the experimental animal center and kept under a 12-h light/dark cycle, with a temperature of 18-24°C, and 4-6 rats to a cage with food and water provided *ad libidum*. Experiments began after permission was obtained from the Uludag University Animal Care and Use Committee.

For blood pressure recordings, a catheter (PE 50) filled with heparinated saline (100 U/mL) was placed into the right femoral artery of rats under ether anesthesia. Another catheter (PE 10) for *iv* injections was placed into the femoral vein. The arterial and venous cannulas were then passed through the skin of the back of the neck and fixed there. For *icv* cannulation, a hole was created 1.5 mm to the right of the midline and 1-1.5 mm behind the bregma in the skull and a cannula (prepared by cutting a 20-G hypodermic stainless steel needle) 10 mm in length was placed through this hole below the skull surface, with the exposed part fixed to the skull with dental acrylic.

After the surgical procedures, the rats were placed in individual boxes and allowed to recover for 3-4 h. No signs of pain were observed in the rats during the recovery period. After recovery, the arterial catheter was connected to the volumetric pressure transducer (BPT300). Arterial blood pressure and HR were monitored continuously using a BIOPAC Data Acquisition Unit (MP30) to which this transducer was connected. Mean arterial blood pressure (MAP, mmHg) and HR (bpm) were monitored. The rats were placed in a box where they could move freely, with the arterial catheter positioned at the same level as the volumetric pressure transducer. They were then stabilized 30 min later. Baseline arterial pressure and HR values were recorded for 10-15 min before drug administration.

The study was planned as four series of experiments. The first series investigated the effect of ADM injected *icv* on blood pressure and HR. Rats received *icv* ADM (250,

500, and 750 ng/10 μL) or *icv* physiological saline (10 μL; N = 21).

In the second series, the rats were injected with the ADM receptor antagonist $ADM_{22\text{-}52}$ (1 μg/10 μL, *icv*) or $CGRP_{8\text{-}37}$ (5 μg/10 μL, *icv*) to determine whether the effects of *icv* ADM on blood pressure and HR were mediated by central receptors. Physiological saline (10 μL, *icv*) or ADM (750 ng/10 μL, *icv*) was administered 10 min after the $ADM_{22\text{-}52}$ injection and 20 min after the $CGRP_{8\text{-}37}$ injection (N = 28).

The third series investigated the role of the central cholinergic system in mediating the effects of *icv* ADM on blood pressure and HR. Physiological saline (10 μL, *icv*) or ADM (750 ng/10 mL, *icv*) was administered 15 min after injection of the muscarinic receptor antagonist atropine (5 μg/10 μL, *icv*) or the nicotinic receptor antagonist mecamylamine (25 μg/10 μL, *icv*; N = 28).

In the fourth series of experiments, the vasopressin V_1 antagonist [β-mercapto-β-β-cyclopentamethylenepropionyl[1], O-me-Tyr[2],Arg[8]]-vasopressin (V2255; 10 μg/kg, *iv*), was injected 5 min before the injection of ADM (750 ng/10 μL, *icv*) or physiological saline (10 μL, *icv*) to investigate the role of the peripheral V_1 receptor on the cardiovascular effects of ADM (N = 14).

At the end of the experiments, 5 μL methylene blue solution was injected into the cerebral ventricle through the cannula and the location of the inner end of the cannula was confirmed for each rat. The brains were removed following decapitation and the location of the *icv* cannula was checked again.

Adrenomedullin, $CGRP_{8\text{-}37}$, $ADM_{22\text{-}52}$, atropine sulfate, mecamylamine chloride, and V2255 were purchased from Sigma (USA). All drugs were prepared in saline (0.9% NaCl). The doses of the drugs provided in the text were prepared in 10 μL for *icv* injections, and the injections were performed with a Hamilton microinjector. The doses of *iv* administered drugs were calculated to final volumes of 1 mL/kg.

Blood pressure and HR values were recorded over a period of 30 min. The data recorded for each animal before the injections are the baseline values. ΔMAP and ΔHR differences were calculated by substracting the baseline values from the recorded values at each 5 min. The groups were then compared to the control group. Data are reported as means ± SEM and analysis of variance (ANOVA) was used to determine statistical significance. Differences were considered to be significant at $P < 0.05$.

Results

Cardiovascular effects of *icv* adrenomedullin

Prior to drug injection, the resting blood pressure and HR of the conscious, freely moving rats were 120 ± 2 mmHg and 303 ± 6 bpm, respectively. These values were compared to the values obtained every fifth minute for a total period of 30 min. Injection of *icv* ADM (250, 500 ng/10 μL) produced increases in both blood pressure and HR but these changes

did not differ statistically from basal values.

Administration of 750 ng/10 µL *icv* ADM produced statistically significant increases in both MAP and HR. MAP was highest (ΔMAP = 11.8 ± 2.3 mmHg) at 5-15 min, then decreased gradually, but was still significantly higher at 30 min compared to saline-treated rats (Figure 1A). ADM (750 ng/10 µL, *icv*) also induced an increase in HR, which was maximal (ΔHR = 39.7 ± 4.8 bpm) at 30 min (Figure 1B). These increases were statistically significant compared to the saline-treated rats. Therefore, we used this dose in the other series of our study.

Effects of pretreatment with adrenomedullin antagonists on the pressor effects of *icv* adrenomedullin

ADM$_{22-52}$ and CGRP$_{8-37}$ are receptor antagonists of CGRP and ADM. Pretreatment with both ADM$_{22-52}$ (1 µg/10 µL, *icv*) and CGRP$_{8-37}$ (5 µg/10 µL, *icv*) significantly attenuated ADM (750 ng/10 µL, *icv*)-induced changes in

ΔMAP and ΔHR (Figure 2).

Effects of pretreatment with central cholinergic system antagonists on the pressor effects of *icv* adrenomedullin

Rats were injected with either the nicotinic receptor antagonist, mecamylamine (25 µg/10 µL; *icv*), or the muscarinic receptor, atropine (5 µg/10 µL; *icv*), followed 15 min later by 750 ng/10 µL *icv* ADM or saline. Both mecamylamine and atropine prevented the rise in blood pressure induced by ADM (Figure 3A). The ADM-induced increase in HR was
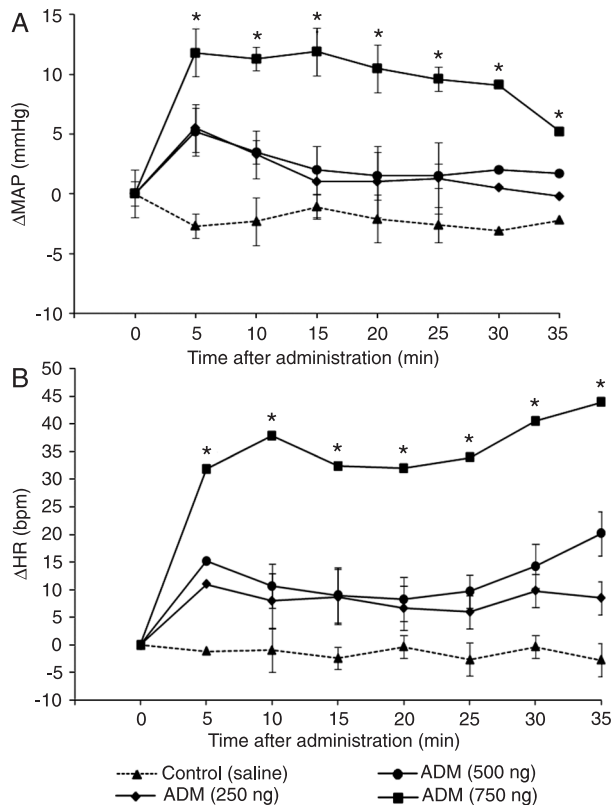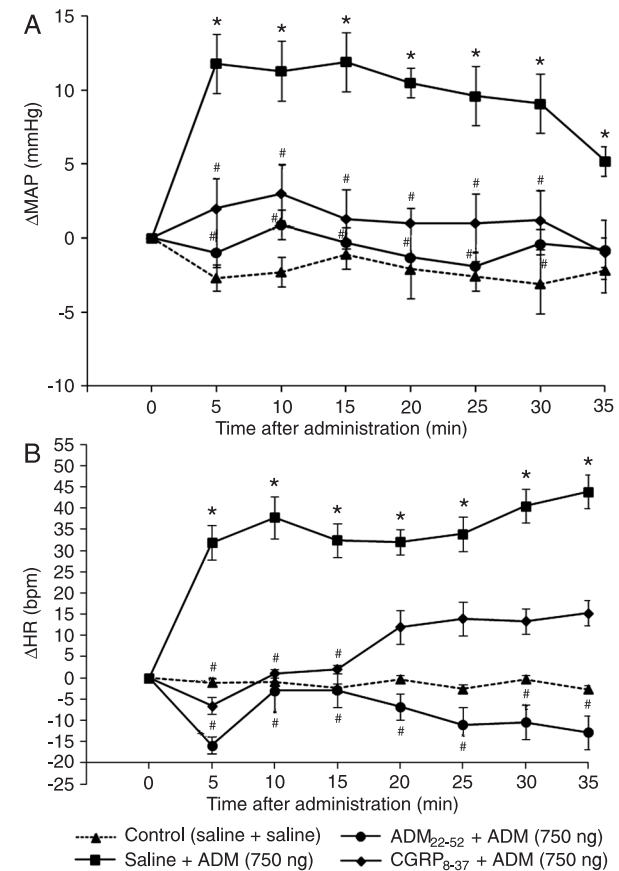
Figure 1. Time course of changes in mean arterial pressure (*A*; ΔMAP) and heart rate (*B*; ΔHR) after *icv* injection of adrenomedullin (ADM); 250, 500, and 750 ng/10 µL) and saline (10 µL). ΔMAP and ΔHR values were calculated by substracting the baseline values from the recorded values at each 5 min. Data are reported as means ± SEM for 7 rats in each group. *P < 0.05 compared to the respective values for saline treatment (Tukey-Kramer multiple comparisons test).

Figure 2. Effect of the adrenomedullin (ADM) receptor antagonist ADM$_{22-52}$ and calcitonin gene-related peptide (CGRP$_{8-37}$) on blood pressure (*A*) and heart rate (*B*) responses to *icv* ADM. Rats received ADM$_{22-52}$ (1 µg/10 µL, *icv*) 10 min, or CGRP$_{8-37}$ (5 µg/10 µL, *icv*) 20 min before *icv* ADM (750 ng/10 µL) injection. Blood pressure and heart rate were recorded for 30 min following ADM injection. Mean arterial pressure (ΔMAP) and heart rate (ΔHR) values were calculated by substracting the baseline values from the recorded values at each 5 min. Data are reported as means ± SEM for 7 rats in each group. *P < 0.05 compared with respective values for saline treatment (Tukey-Kramer multiple comparisons test); #P < 0.05 compared with respective values for *icv* ADM-treated group (Tukey-Kramer multiple comparisons test).
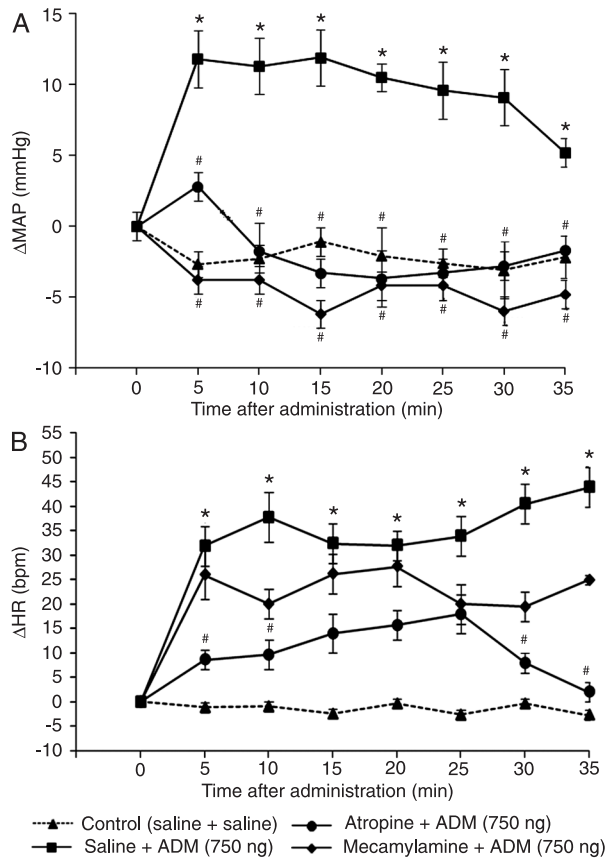
Figure 3. Effect of cholinergic muscarinic receptor blockade and cholinergic nicotinic receptor blockade on blood pressure (*A*) and heart rate (*B*) responses to *icv* adrenomedullin (ADM). Rats received atropine sulfate (5 µg/10 µL, *icv*) 15 min or mecamylamine (25 µg/10 µL, *icv*) 15 min before *icv* ADM (750 ng/10 µL) injection. Blood pressure and heart rate (HR) were recorded for 30 min following ADM injection. Mean arterial pressure (ΔMAP) and ΔHR values were calculated by substracting the baseline values from the recorded values at each 5 min. Data are reported as means ± SEM for 7 rats in each group. *P < 0.05 compared with respective values for saline treatment (Tukey-Kramer multiple comparisons test); #P < 0.05 compared with respective values for *icv* ADM-treated group (Tukey-Kramer multiple comparisons test).

blocked by atropine but not by mecamylamine (Figure 3B). The *icv* administration of each drug alone did not induce any significant changes in cardiovascular parameters (data not shown).

Effects of pretreatment with vasopressin V_1 receptor antagonists on the pressor effects of *icv* adrenomedullin

Rats were injected with the specific vasopressin V_1 receptor antagonist, V2255 (10 µg/kg, *iv*) through the cannula 5 min before *icv* ADM or *icv* saline injection. The vasopressin V_1 receptor antagonist significantly prevented
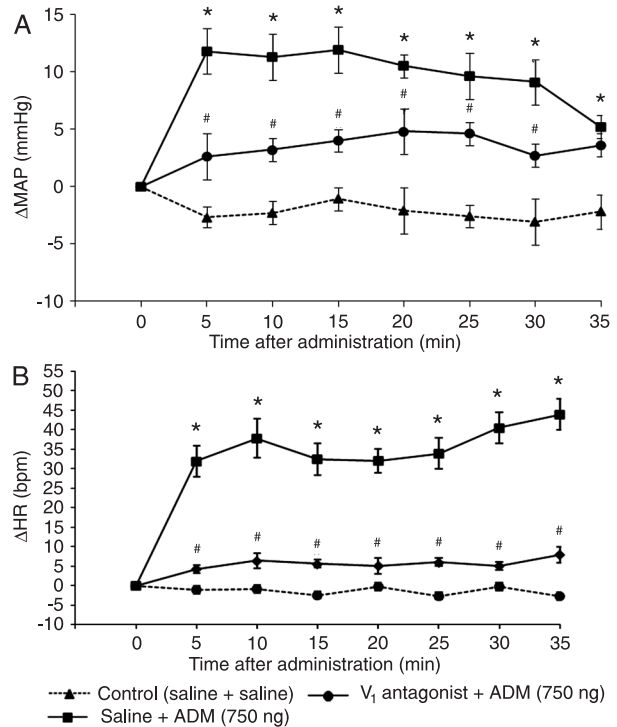

Figure 4. Role of the vasopressin V_1 receptor antagonist in the blood pressure (*A*) and heart rate (*B*) responses to *icv* adrenomedullin (ADM). Rats received the V_1 receptor antagonist (10 µg/10 µL, *icv*) 5 min before *icv* ADM (750 ng/10 µL) injection. Blood pressure and heart rate (HR) were recorded for 30 min following ADM injection. Mean arterial pressure (ΔMAP) and ΔHR values were calculated by substracting the baseline values from the recorded values at each 5 min. Data are reported as means ± SEM for 7 rats in each group. *P < 0.05 compared with respective values for saline treatment (Tukey-Kramer multiple comparisons test); #P < 0.05 compared with respective values for *icv* ADM-treated group (Tukey-Kramer multiple comparisons test).

the ADM-induced increase in blood pressure and HR (Figure 4A,B). In normal rats, pretreatment with vasopressin V_1 receptor antagonist itself did not change blood pressure (data not shown).

Discussion

Recent studies have shown that ADM is involved in the regulation of MAP although ADM has different cardiovascular effects when administered peripherally or centrally. Intravenous administration of ADM causes a long-lasting and dose-dependent decrease in arterial blood pressure (1,3,11). On the other hand, *icv* administration of ADM increases MAP, HR and sympathetic activity (12-16). However, central administration of ADM exerted multiple central effects in different areas. Microinjection of ADM into the hypothalamic PVN induced a significant decrease of blood pressure (23), whereas microinjection of ADM into the rostral

ventrolateral medulla caused a significant increase of blood pressure accompanied by an increase of renal sympathetic output (24,25). We observed that *icv* ADM administered at a dose of 750 ng/10 µL increased blood pressure and HR in rats and these increased values did not return to basal values after 30 min. ADM has structural homology with CGRP, and specific ADM and CGRP1 receptors interfere with the effects of these peptides (2-4). The CGRP1 receptor antagonist $CGRP_{8-37}$ has been observed to block some ADM effects. The central effects of this peptide are thought to be mediated by specific receptors and CGRP1 receptors, similar to the peripheral mechanism (5,6). In the present study, the effect of *icv* ADM on blood pressure and HR was blocked in rats pretreated with ADM_{22-52} and $CGRP_{8-37}$, suggesting that ADM acts through specific ADM receptors and CGRP1 receptors in agreement with other studies (5,6). Takahashi et al. (13) observed that central administration of the CGRP antagonist $CGRP_{8-37}$ blocked the effects of ADM. Saita et al. (14) showed that both $CGRP_{8-37}$ and the ADM-specific receptor antagonist ADM_{22-52} block the cardiovascular effects of ADM. On the other hand, Samson et al. (15) found that $CGRP_{8-37}$ did not prevent the hypertensive effects of central ADM. It is not known which receptor binds more strongly or is more specific in regulating the effects of ADM. We found that the specific receptor antagonist, ADM_{22-52}, blocked both ADM-induced increases in blood pressure and HR more efficiently than $CGRP_{8-37}$. This result is consistent with those reported by Saita et al. (14).

Involvement of cholinergic neurons in the regulation of blood pressure has been demonstrated (17-20). Central cholinergic stimulation or central cholinomimetic injection causes increases in blood pressure via the activation of sympathetic discharge. Sympathoadrenergic activation has been shown to be caused by *icv* ADM (13-16), indicating the importance of the role of cholinergic receptors in mediating the effects of central ADM. Previous studies have suggested that central muscarinic receptor stimulation plays an important role in cardiovascular regulation (18,26). *Icv* administration of muscarinic agonists into the posterior hypothalamic nucleus or medulla of rats evoked pressor and cardioacceleratory responses (27,28). Circulating catecholamines and sympathetic neural outflow are elevated in animals after central muscarinic stimulation (29,30).

In the present study, we have shown the relationship between the central cholinergic system and *icv* ADM. Our results suggest that the pressor effect of ADM might be mediated by the central cholinergic receptors in normotensive rats. This is the first study reporting that the ADM-induced blood pressure increase was prevented by a cholinergic receptor antagonist. Both the nicotinic receptor antagonist mecamylamine and the muscarinic receptor antagonist atropine prevented ADM-induced blood pressure increase. This result suggests that nicotinic and muscarinic receptors play

roles in the effects of ADM on blood pressure. Muscarinic receptors have been shown to be present in areas involved in regulating cardiovascular responses to cholinergic agonists and these receptors are widely expressed in the CNS, especially in autonomic centers, and they control a variety of neuronal functions (29,30). ADM-producing neurons are prominent in autonomic centers of the CNS and these centers contain muscarinic receptors (7-10,30). *Icv* ADM increases nicotinic and muscarinic neurotransmission by presynaptic mechanisms and also activates nicotinic and muscarinic receptors as a direct agonist. Buys et al. (31) have shown that ADM up-regulates the expression of the M2 muscarinic receptor in both P19 isolated atrial cells and in adult rats (31). Interestingly, the nicotinic receptor antagonist mecamylamine did not alter the increase in HR whereas the muscarinic receptor antagonist atropine blocked it. Nicotinic receptor blockage decreased blood pressure through presynaptic cholinergic activation but did not alter HR due to the possibility of a reflex tachycardiac response. The effect of ADM on HR was prevented by the central muscarinic receptor antagonist. Furthermore, central M1 receptor activation appears to have an important role in mediating the baroreflex (29) and the Bezold-Jarisch reflex (32) in rats. Thus, alterations in muscarinic receptor activity in the brain may influence the control of cardiovascular function by the autonomic nervous system.

The distribution of ADM-positive neurons in both the parvo- and magnocellular division of the PVN suggests that ADM participates in the regulation of the hypothalamus-pituitary-adrenal, neurohypophyseal functions and of autonomic responses (9-12). The PVN and SON of the hypothalamus are known to secrete vasopressin and oxytocin and to have important functions in autonomic and neuroendocrine regulation. *Icv* administered ADM localizes in the PVN and SON (9,10). A study has shown that ADM inhibits the arginine-vasopressin production induced by hypovolemia and osmotic stimulation and to stimulate oxytocin production in the rat hypothalamus (33), but Taylor et al. (21) have shown that central administration of ADM leads to a dose-related increase in plasma vasopressin levels. We observed that administration of a vasopressin V_1 receptor antagonist blocked the blood pressure and HR stimulating effects of ADM, suggesting that V_1 receptors play a role in the cardiovascular effects of central ADM.

In conclusion, *icv* ADM can effectively increase blood pressure and HR and these effects might occur by way of the specific ADM and CGRP receptors, peripheral V_1 receptors and central nicotinic and muscarinic cholinergic receptors.

Acknowledgments

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