

# Adrenomedullin in the rostral ventrolateral medulla inhibits baroreflex control of heart rate: a role for protein kinase A

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**1** The rostral ventrolateral medulla (RVLM) is an essential vasomotor center in the brainstem which participates in maintaining resting levels of arterial pressure and for regulating baroreflex activity. We have demonstrated that microinjections of adrenomedullin (ADM), a vasoactive neuropeptide, into the RVLM cause increased resting mean arterial pressure (MAP) and heart rate (HR). However, the effect of ADM on baroreflex function remains unclear.

**2** The purposes of the present study were to investigate the effect of ADM in the RVLM on the regulation of baroreflex activity and to identify the underlying mechanisms. Baroreflex curves were generated with intravenous injections of multiple doses of phenylephrine and nitroprusside. The upper and lower plateaus, reflex range, MAP at the midpoint of HR range (MAP<sub>50</sub>), and gain were evaluated before and after various microinjections were made into the RVLM of urethane-anesthetized rats.

**3** Microinjections of ADM decreased the upper plateau, reflex range, and gain, and increased MAP<sub>50</sub>, indicating that ADM in the RVLM impairs baroreflex function.

**4** ADM<sub>22–52</sub>, a putative ADM receptor antagonist, significantly increased the baroreflex gain and upper plateau, demonstrating that endogenous ADM tonically inhibits the baroreflex. Coinjections of ADM<sub>22–52</sub> with ADM blocked the ADM-induced baroreflex responses.

**5** ADM's effect was abolished with H-89, a protein kinase A (PKA) inhibitor.

**6** Our results show that ADM in the RVLM exerts an inhibitory effect on baroreflex activity *via* an ADM receptor-mediated mechanism, and that activation of PKA is involved in this event.

*British Journal of Pharmacology* (2006) **148**, 70–77. doi:10.1038/sj.bjp.0706698;

published online 27 February 2006

**Keywords:** Adrenomedullin; blood pressure; baroreflex control; neuropeptides; sympathetic nervous system

**Abbreviations:** ADM, adrenomedullin; AP, area postrema; cAMP, cyclic adenosine monophosphate; CVLM, caudal ventrolateral medulla; HR, heart rate; MAP, mean arterial pressure; NP, nitroprusside; NTS, nucleus tractus solitarius; PHEN, phenylephrine; PKA, protein kinase A; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla

## Introduction

Adrenomedullin (ADM), a vasoactive neuropeptide, has attracted a great deal of attention because of its potent peripheral vasodilatory actions (Kitamura *et al.*, 1993; Shimekake *et al.*, 1995; Terata *et al.*, 2000; Champion *et al.*, 2003). Indeed, recent studies have shown that subcutaneous administration of ADM attenuates high blood pressure in salt-sensitive hypertensive rats (Yoshihara *et al.*, 2005), and that intravenous injections or inhalation of ADM ameliorate pulmonary hypertension in humans (Nagaya & Kangawa, 2004). Besides its effects in the periphery, ADM also modulates mean arterial pressure (MAP) and heart rate (HR) through central mechanisms. Thus, ADM administered into the paraventricular nucleus (PVN) of the hypothalamus causes a transient depressor effect (Smith & Ferguson, 2001; Xu & Krukoff, 2004b), whereas elevations in MAP and/or HR are evoked by intracerebroventricular (i.c.v.) injections of ADM (Takahashi *et al.*, 1994; Saita *et al.*, 1998; Samson *et al.*, 1998; Matsumura *et al.*, 1999; Shan & Krukoff, 2001b), or when ADM is

microinjected into the area postrema (AP) (Allen *et al.*, 1997) or the rostral ventrolateral medulla (RVLM) (Xu & Krukoff, 2004a) in the brainstem. In addition, it has been shown that i.c.v. injections of ADM regulate the baroreflex (Matsumura *et al.*, 1999; Taylor *et al.*, 2003), a central reflex vital to maintaining normal MAP (Malpas, 2004; Dampney *et al.*, 2005). However, ADM's effects on the baroreflex in specific regions in the brain remain to be elucidated.

The RVLM is an essential component of the neural circuit that controls baroreflex function (Dampney *et al.*, 2003a, b; Milner & Pickel, 2003). When MAP is altered, information about changes in pressure originating from baroreceptors in the aortic arch and carotid sinus is relayed to the nucleus tractus solitarius (NTS) of the brainstem. Sensory neurons in the NTS then project to neurons in the caudal ventrolateral medulla (CVLM), which, in turn, project to presympathetic neurons in the RVLM. RVLM presympathetic neurons modulate not only HR but also sympathetic vasomotor discharge and thus peripheral resistance; as a result of these effects, normal levels of MAP can be re-established (Dampney *et al.*, 2003a, b; Milner & Pickel, 2003).

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We have shown that ADM receptors are abundant in the RVLM (Stachniak & Krukoff, 2003) and that ADM-producing neurons are found in other autonomic nuclei that project to the RVLM such as the PVN, NTS, and AP (Serrano *et al.*, 2000; Shan & Krukoff, 2001a). Furthermore, in a previous study we demonstrated that microinjections of ADM into the RVLM induce robust and long-lasting increases in both resting MAP and HR levels, suggesting that ADM exerts a sympathoexcitatory effect in the RVLM to influence cardiovascular functions (Xu & Krukoff, 2004a). However, it is not known whether ADM in the RVLM also contributes to the regulation of baroreflex function, particularly because the RVLM regulates the homeostasis of arterial pressure by both baroreflex-dependent and baroreflex-independent mechanisms (Dampney *et al.*, 2003b; Schreihofer *et al.*, 2005).

The aim of this study was to determine whether ADM in the RVLM modulates baroreflex control of HR, and, if so, to identify the mechanisms for this modulation. Thus, baroreflex curves were generated using a pharmacological pressor agent (phenylephrine, PHEN) and a depressor agent (nitroprusside, NP) before and after ADM and/or its antagonist were microinjected into the RVLM of anesthetized rats. Furthermore, since the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway has been shown to mediate ADM's actions *in vitro* (Drake *et al.*, 1999; Spampinato *et al.*, 1999; Xu & Krukoff, 2005), we coinjected a PKA inhibitor (H-89) with ADM to determine whether PKA is required for ADM's effects on baroreflex activity.

## Methods

### Animals

A total of 55 male Sprague-Dawley rats (250–350 g), purchased from the Biological Animal Center, University of Alberta, were used in this study. They were housed in a 12:12 h light/dark cycle at 22°C, and given free access to standard lab chow and water. All experimental protocols were approved by the local Animal Welfare Committee.

### Surgery

Rats were instrumented for MAP/HR recording, intravenous (i.v.) injections, and RVLM microinjections as described previously (Xu & Krukoff, 2004a, b). Briefly, rats were anesthetized with urethane (intraperitoneal, 1.75 g kg<sup>-1</sup>, Sigma Chemical Co, Oakville, ON, Canada). Body temperature was monitored with a rectal thermometer and maintained at 37°C with a heating pad. The left femoral artery was cannulated using PE50 tubing (Becton Dickinson & Co., Sparks, MD, U.S.A.) for measurement of MAP and HR, and the left femoral vein was cannulated using PE10 tubing (Becton Dickinson & Co.) for i.v. injections. The rats were then placed onto a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) with the toothbar set at -3.3 mm and a microinjection guide cannula (C315G; Plastics One, Wallingford, CT, U.S.A.) was inserted into the left RVLM (3.5–3.8 mm posterior, 1.9–2.1 mm lateral, and 0.7–0.9 mm dorsal to the interaural zero) (Paxinos & Watson, 1986; Xu & Krukoff, 2004a).

### Measurement of MAP and HR

The arterial tubing, filled with heparinized saline, was connected to a pressure transducer (Abbott Laboratories, North Chicago, IL, U.S.A.). Digitalized arterial pressure and HR data were acquired and analyzed using WinDaq Acquisition & Playback Software (Dataq Instruments, Akron, OH, U.S.A.). After a 30 min stabilization period, the MAP and HR were measured for 10 min to obtain basal levels of MAP and HR.

### Microinjections into the RVLM

Unilateral RVLM injections were performed as previously described (Xu & Krukoff, 2004a). A syringe filled with saline solution, containing a variety of pharmacological agents or combinations thereof, was connected *via* PE10 tubing to an internal cannula (C315I; Plastics One), which is 1 mm longer than the guide cannula. With the internal cannula inserted into the guide cannula, 100 nl solution was injected into the left RVLM with an electronic infusion pump (Harvard Apparatus, Inc. Holliston, MA, U.S.A.). All microinjections were made over 1 min. To demonstrate the effect of ADM in the RVLM on the baroreflex, ADM (American Peptide Company, Inc., Sunnyvale, CA, U.S.A.) was injected into the RVLM at subpressor doses (0.1 and 1 fmol), which were determined based on our previous study (Xu & Krukoff, 2004a) and on preliminary experiments. Animals that received injections of saline into the RVLM or of 1 fmol ADM outside the RVLM were used as controls. An ADM receptor antagonist, ADM<sub>22-52</sub> (0.1 and 1 fmol; American Peptide Company, Inc.), was injected into the RVLM in order to investigate the effects of endogenous ADM on the baroreflex. To determine whether ADM's effect is receptor-mediated, 1 fmol ADM was coinjected with 0.1 fmol ADM<sub>22-52</sub>. A PKA inhibitor, H-89 (100 pmol; Sigma) (Chan *et al.*, 2003), was coinjected with 1 fmol ADM to evaluate the importance of PKA activity in ADM's effect.

### Evaluation of baroreflex activity

The baroreflex control of HR was evaluated before and after RVLM microinjections as previously described by others (Wong *et al.*, 1993; Miki *et al.*, 2003). In baroreflex test trials, blood pressure was increased or decreased by i.v. bolus injections of PHEN (0.5, 1.5, 2, and 2.5 µg; Sigma) or NP (1, 3, 4, 5 µg; Sigma), respectively. Different doses of PHEN and NP were delivered in random order and two subsequent injections were separated by a 5-min interval to ensure that MAP and HR returned to the baselines before the next injection. In most experiments, the baroreflex test was performed before and during the first hour (from 20 to 60 min) after RVLM microinjections. In the investigation of the time course of ADM's effect on the baroreflex, additional baroreflex tests were carried out during the second hour (from 60 to 120 min) and third hour (from 120 to 180 min) after RVLM microinjections.

For each baroreflex test, PHEN- and NP-induced responses were recorded and HR and MAP data were averaged over each 1 mmHg bin of MAP. Using SigmaPlot (Systat Software

Inc., Point Richmond, CA, U.S.A.), HR and MAP data were fitted to a logistic sigmoid function (Kent *et al.*, 1972):

$$HR = (A - D) / \{1 + \exp[B(MAP - C)]\} + D$$

where  $A$  and  $D$  are the upper and lower plateaus of the baroreflex curve and represent the maximal and minimal levels of HR, respectively.  $C$  is MAP at the midpoint of the HR range ( $MAP_{50}$ ), which represents the set-point of the baroreflex.  $B$  is the gain coefficient. The reflex range, threshold blood pressure ( $P_{thr}$ ), saturation blood pressure ( $P_{sat}$ ), the first derivative of the curve, and maximal gain were then calculated according to the following equations (Miki *et al.*, 2003):

$$\text{Reflex range} = A - D$$

$$P_{thr} = -2.0/B + C$$

$$P_{sat} = 2.0/B + C$$

$$Y' = \Delta HR / \Delta MAP = -B(A - D) \times \exp(MAP - C) / \{1 + \exp[B(MAP - C)]\}^2$$

$$\text{Maximal gain} = -(A - D)B/4.$$

The reflex range is the difference between the upper plateau and the lower plateau.  $P_{thr}$  and  $P_{sat}$  are the MAP values at which HR was within 5% of maximum or minimum response, respectively. The first derivative shows the baroreflex gain at different MAP levels, while the maximal gain is defined as the gain value when MAP is equal to  $MAP_{50}$ .

### Brain histology

As previously described (Xu & Krukoff, 2004a), 1% Evans blue (Sigma) was microinjected into the RVLM *via* the same guide cannula at the end of each experiment. The rats were decapitated, and brains were removed and fixed in 4% paraformaldehyde for 48–72 h. The brains were then frozen and coronal brainstem sections ( $40 \mu\text{m}$ ) were cut using a cryostat ( $-20^\circ\text{C}$ ). The sections were thaw-mounted onto slides, stained with neutral red (Allied Chemical Co., New York, NY, U.S.A.), and observed under a microscope. Accurate RVLM microinjections were confirmed when Evans blue was present within the RVLM.

### Statistical analyses

For each treatment group ( $N \geq 4$ ), averaged parameters from the baroreflex curves before and after RVLM microinjections were expressed as mean  $\pm$  s.e.m. Effects of various RVLM microinjections on the baroreflex parameters and differences among groups were assessed using one-way or two-way ANOVA analysis followed by the Student–Newman–Keuls test for *post hoc* comparisons of individual means.  $P < 0.05$  indicated statistical significance.

## Results

### Microinjection accuracy

Microinjections were accurately placed within the RVLM in about 70% of rats. Figure 1a shows a representative photomicrograph indicating the termination site of an injection

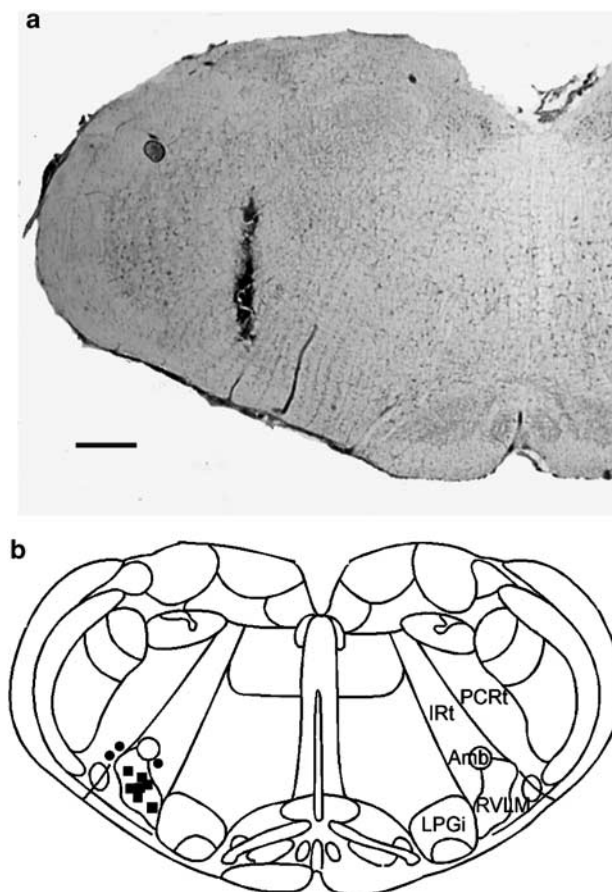
that is considered to be within the RVLM, and Figure 1b summarizes the termination sites of microinjections in 10 rats.

### Effects of microinjections on resting MAP and HR

The baseline values of MAP and HR were  $92 \pm 8$  mmHg and  $355 \pm 16$  b.p.m., respectively. Microinjections of saline or ADM alone, and ADM<sub>22–52</sub> alone or in combination with ADM, had no significant effects on resting MAP or HR. Microinjections of H-89, alone or in combination with ADM, induced significant increases in resting levels of MAP ( $23 \pm 7$  or  $28 \pm 10$  mmHg) and HR ( $54 \pm 15$  or  $55 \pm 16$  b.p.m.), respectively. However, these effects lasted fewer than 20 min and MAP was allowed to return to baseline values before the baroreflex tests were initiated.

### Exogenous ADM in the RVLM inhibits baroreflex control of HR

To determine the effect of exogenous ADM on the baroreflex control of HR, we microinjected ADM into the RVLM at



**Figure 1** (a) A brightfield photomicrograph showing an injection that is considered to be within the RVLM. The scale bar =  $500 \mu\text{m}$ . (b) Schematic representation of sites of microinjections in 10 rats that received microinjections. Each square represents an injection that is considered to be within the RVLM; each circle represents an injection that is considered to be outside the RVLM. Amb, ambiguous nucleus; IRt, intermediate reticular nucleus; LPGi, lateral paragigantocellular nucleus; PCRt, parvocellular reticular nucleus; RVLM, rostral ventrolateral medulla. The drawing is modified from Paxinos & Watson (1986).

a subpressor dose (1 fmol) (Xu & Krukoff, 2004a), and the baroreflex activities were compared before and during the first, second, and third hour after the microinjections. As shown in Figure 2a and Table 1, during the first hour after RVLM microinjections of ADM, the upper plateau of the baroreflex curves was significantly decreased from  $390.8 \pm 5.9$  to  $363.2 \pm 8.1$  b.p.m. ( $P < 0.05$ ), whereas the lower plateau was not affected. As a result, the reflex range was significantly reduced from  $58.0 \pm 2.4$  to  $37.2 \pm 1.4$  b.p.m. ( $P < 0.01$ ). In addition, ADM significantly decreased the maximal gain from  $-2.4 \pm 0.3$  to  $-1.4 \pm 0.1$  b.p.m. mmHg<sup>-1</sup> ( $P < 0.01$ ), increased MAP<sub>50</sub> from  $99.1 \pm 1.5$  to  $104.8 \pm 0.4$  mmHg ( $P < 0.01$ ), and increased Psat from  $111.1 \pm 0.8$  to  $118.3 \pm 1.0$  mmHg ( $P < 0.01$ ), whereas Pthr was not affected. These data indicate that exogenous ADM in the RVLM impaired the baroreflex control of HR. Furthermore, we found that the inhibitory effect of ADM on the baroreflex was time-dependent and reversible because the upper plateau, reflex range, gain, MAP<sub>50</sub>, and Psat gradually returned to baseline levels during the second and the third hours. Microinjections of ADM (1 fmol) outside the RVLM did not induce any significant changes in the baroreflex (Figure 2b and Table 1), indicating that the effects of ADM were specific to the RVLM.

ADM at a lower dose (0.1 fmol) also inhibited the baroreflex by significantly decreasing the maximal gain from  $-2.1 \pm 0.4$  to  $-1.2 \pm 0.2$  b.p.m. mmHg<sup>-1</sup> ( $P < 0.05$ ) (Figure 2c and Table 1). ADM (0.1 fmol) also tended to decrease the upper plateau and the reflex range, although these responses were not statistically

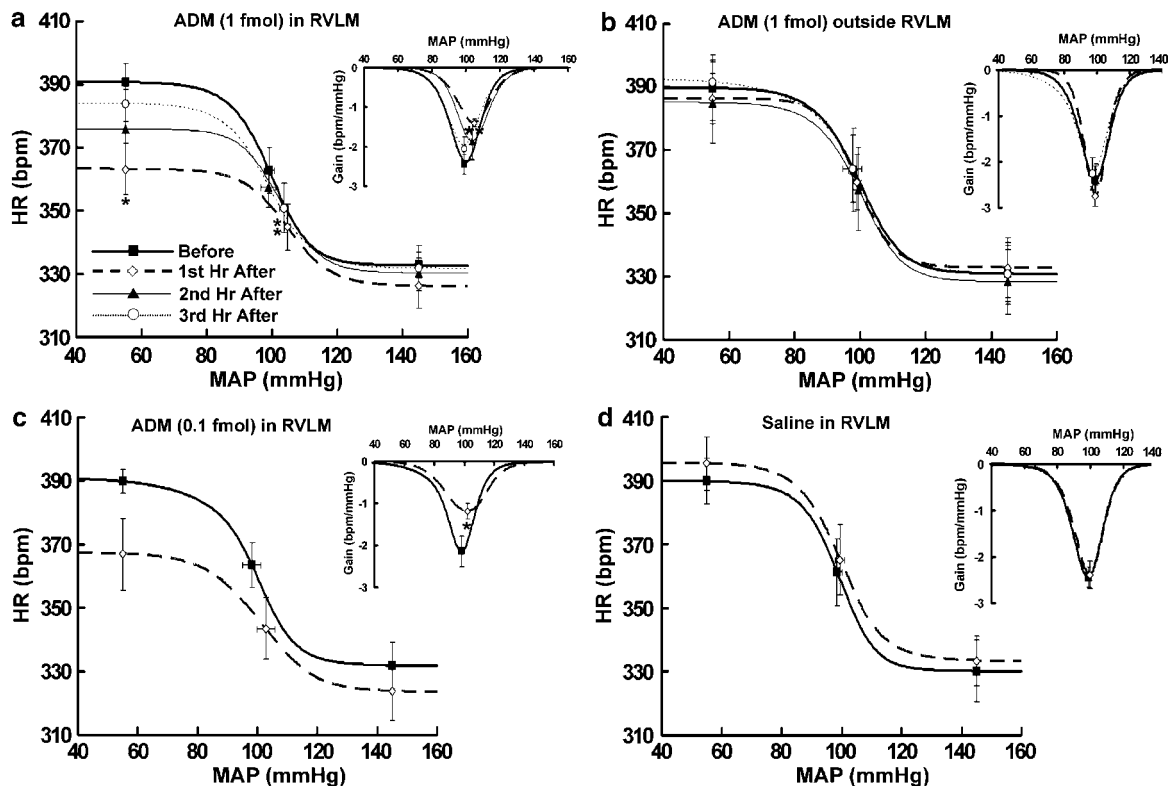
significant (Figure 2c and Table 1). Control microinjections of saline into the RVLM had no effect on the baroreflex (Figure 2d and Table 1).

### Blockade of ADM receptor in the RVLM facilitates baroreflex control of HR

To determine whether endogenous ADM in the RVLM exerts a tonic regulatory effect on the baroreflex, we examined the effects of microinjections of a putative ADM receptor antagonist, ADM<sub>22-52</sub>, on baroreflex activity. While injections of 0.1 fmol ADM<sub>22-52</sub> had no effects on the baroreflex (Figure 3a and Table 1), ADM<sub>22-52</sub> at a higher dose (1 fmol) significantly increased the baroreflex gain from  $-2.3 \pm 0.2$  to  $-4.8 \pm 0.7$  b.p.m. mmHg<sup>-1</sup> ( $P < 0.05$ ). In addition, ADM<sub>22-52</sub> (1 fmol) significantly increased the upper plateau from  $387.3 \pm 4.3$  to  $403.5 \pm 3.6$  b.p.m. ( $P < 0.05$ ) and resulted in a trend towards an increased reflex range, although it was not statistically significant (Figure 3b and Table 1). ADM<sub>22-52</sub> (1 fmol) injected outside the RVLM had no effect on baroreflex activity (data not shown).

### ADM-induced inhibition of baroreflex is mediated by ADM receptor

The responses in the baroreflex parameters induced by ADM (1 fmol) were completely abolished by coinjected with 0.1 fmol ADM<sub>22-52</sub> (Figure 4 and Table 1). Similarly, coinjections of



**Figure 2** (a, b) Average baroreflex curves generated before and during the first, second, and third hour after ADM (1 fmol) was microinjected into the RVLM (a,  $n = 6$ ) or outside of RVLM (b,  $n = 4$ ). (c, d) Average baroreflex curves generated before and during the first hour after 0.1 fmol ADM (a,  $n = 6$ ) or saline (b,  $n = 4$ ) was microinjected into the RVLM. Insets, average gains of baroreflex control of HR as a function of MAP. Symbols represent the upper plateau, set-point, and lower plateau of baroreflex curves, respectively. \* $P < 0.05$  and \*\* $P < 0.01$  versus measurements before microinjections.

**Table 1** Baroreflex parameters before and after microinjections of various pharmacological agents into the RVLM

Treatment	Upper plateau (b.p.m.)	Lower plateau (b.p.m.)	Reflex range (b.p.m.)	MAP <sub>50</sub> (mmHg)	Gain <sub>Max</sub> (b.p.m.mmHg <sup>-1</sup> )	P <sub>thr</sub> (mmHg)	Psat (mmHg)
<i>Saline</i>							
Before	390.1±7.3	330.2±9.6	59.9±2.8	98.6±1.6	-2.4±0.2	86.8±2.3	110.5±1.4
After	390.6±8.8	328.1±10.7	62.5±5.0	99.5±1.6	-2.4±0.3	85.5±2.9	113.5±2.7
<i>ADM (0.1 fmol)</i>							
Before	390.8±3.7	331.3±7.6	59.4±5.9	98.1±3.0	-2.1±0.4	83.5±6.5	112.6±1.3
After	367.3±11.3	323.4±9.1	43.9±6.8	102.1±3.0	-1.2±0.2*	85.7±5.0	118.5±2.8
<i>ADM (1 fmol)</i>							
Before	390.8±5.9	332.7±4.2	58.0±2.4	99.1±1.5	-2.4±0.3	87.1±2.5	111.1±0.8
1st hour after	363.2±8.1*	326.0±7.0	37.2±1.4**	104.8±0.4**	-1.4±0.1**	91.3±1.3	118.3±1.0**
2nd hour after	375.9±12.4	330.3±4.9	45.7±8.0	103.6±2.3	-1.9±0.5	91.9±2.4	115.4±2.7
3rd hour after	384.0±5.6	329.2±7.7	52.3±4.0	98.9±1.4	-2.1±0.3	85.5±1.7	112.2±2.0
<i>ADM (1 fmol) outside of RVLM</i>							
Before	389.9±10.3	330.9±8.9	59.0±1.8	99.0±1.4	-2.4±0.3	86.0±2.6	112.1±0.7
1st hour after	386.3±7.9	332.8±9.4	53.5±3.9	99.0±1.3	-2.7±0.2	88.7±1.3	109.3±1.3
2nd hour after	385.1±12.9	328.3±10.3	56.8±3.5	99.4±1.5	-2.4±0.3	86.4±2.5	112.4±1.0
3rd hour after	392.4±6.4	330.4±10.1	62.0±4.6	97.4±2.8	-2.2±0.3	83.0±6.4	111.9±1.2
<i>ADM<sub>22-52</sub> (0.1 fmol)</i>							
Before	388.4±8.1	331.2±8.2	57.2±2.8	99.9±0.9	-2.5±0.3	88.1±1.6	111.8±1.1
After	394.1±6.8	332.7±10.6	61.3±4.4	97.9±2.0	-3.3±0.3	87.1±2.5	108.8±3.1
<i>ADM<sub>22-52</sub> (1 fmol)</i>							
Before	387.3±4.3	333.9±4.7	53.5±3.7	97.3±1.8	-2.3±0.2	85.8±1.7	108.8±1.9
After	403.5±3.6*	340.6±4.0	62.9±1.7	97.7±2.0	-4.8±0.7*	89.9±1.1	105.5±3.2
<i>ADM (1 fmol) + ADM<sub>22-52</sub> (0.1 fmol)</i>							
Before	387.1±10.6	333.5±9.8	53.5±3.8	99.7±1.2	-2.6±0.2	89.4±1.1	110.0±1.4
After	384.7±6.6	334.1±7.2	50.5±1.1	97.4±1.7	-2.5±0.3	87.0±1.5	107.8±2.4
<i>H-89 (100 pmol)</i>							
Before	388.5±9.4	332.5±8.6	55.9±2.8	99.0±1.3	-2.5±0.3	87.4±2.3	110.6±1.7
After	387.6±11.3	332.9±8.1	54.7±4.0	97.3±1.2	-2.3±0.4	84.9±2.1	109.6±2.3
<i>ADM (1 fmol) + H-89 (100 pmol)</i>							
Before	387.9±10.0	334.4±9.5	53.6±4.5	98.0±1.6	-2.4±0.3	86.8±2.5	109.3±1.7
After	379.1±12.0	330.1±8.6	49.0±5.2	100.5±2.0	-1.9±0.2	86.7±2.3	114.3±2.1

Values are mean ± s.e.m. \**P*<0.05 and \*\**P*<0.01 versus measurements before microinjections.

1 fmol ADM<sub>22-52</sub> also eliminated the ADM-induced baroreflex inhibition (data not shown). These results indicate that ADM's effect on the baroreflex is a receptor-mediated event.

#### *ADM-induced inhibition of baroreflex is dependent on PKA*

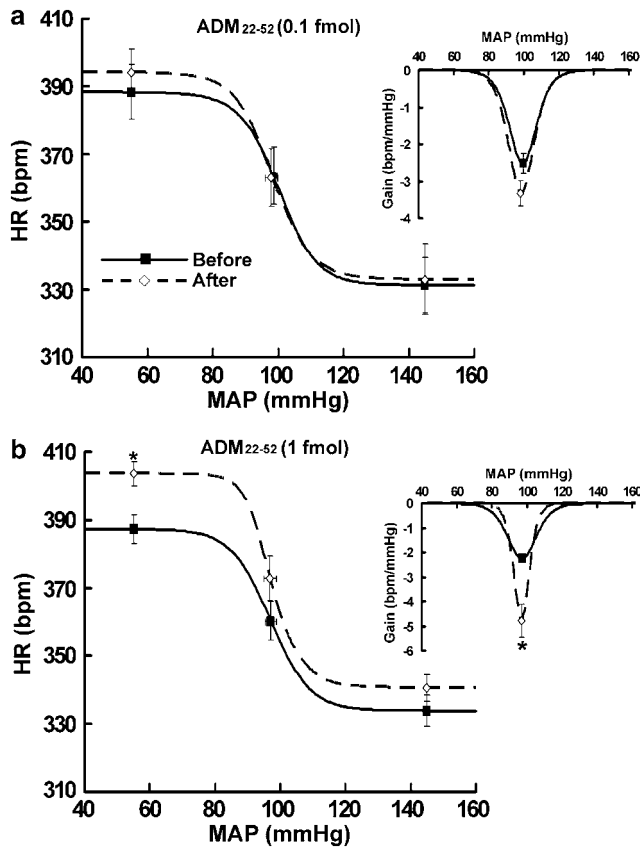
As the cAMP-PKA pathway mediates some functions of ADM *in vitro* (Drake *et al.*, 1999; Spampinato *et al.*, 1999; Xu & Krukoff, 2005), we investigated whether the PKA-related signaling is required for ADM's effect on baroreflex activity. We show that the inhibition of the baroreflex induced by 1 fmol ADM was completely eliminated by coinjections of 100 pmol H-89, a PKA inhibitor, whereas H-89 alone had no significant effect on the baroreflex (Figure 5 and Table 1). These results show that PKA is required for ADM's effect on the baroreflex control of HR.

## Discussion

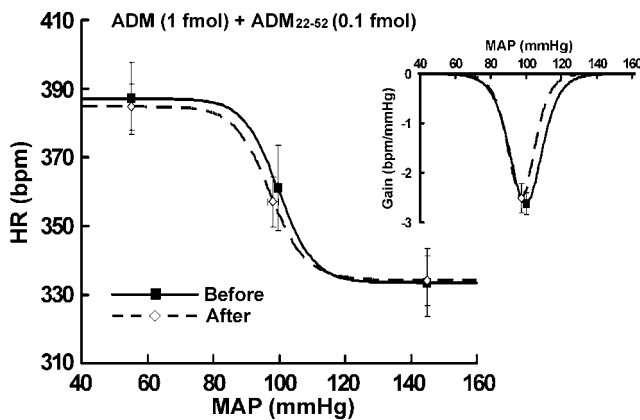
In the present study, we demonstrate that exogenous ADM applied in the RVLM inhibits the baroreflex control of HR by

decreasing the upper plateau, reflex range, and gain of baroreflex curves and by increasing MAP<sub>50</sub>; and that this inhibition is reversible and site-specific to the RVLM. In addition, we show that blockade of ADM receptors in the RVLM increases the baroreflex gain and upper plateau, suggesting that endogenous ADM in the RVLM exerts a tonic inhibitory effect on baroreflex activity. Furthermore, our data also suggest that the PKA-associated signaling pathway is recruited by ADM to regulate baroreflex function, as a PKA inhibitor abolished ADM's effect on the baroreflex.

Few studies have focused on ADM's role in the regulation of the baroreflex. I.c.v. injections of ADM were shown to facilitate the baroreflex by increasing the upper plateau and gain (Matsumura *et al.*, 1999), whereas blockade of central ADM with i.c.v. injections of anti-ADM serum was shown to alter the time course of the baroreflex (Taylor *et al.*, 2003). However, the results of these studies are difficult to interpret because of the nonspecific nature of the injections. Since central ADM is known to exert different effects on resting MAP and HR depending on where it is applied (Takahashi *et al.*, 1994; Allen *et al.*, 1997; Saita *et al.*, 1998; Samson *et al.*, 1998; Matsumura *et al.*, 1999; Shan & Krukoff, 2001b; Smith & Ferguson, 2001; Xu & Krukoff, 2004a, b), it is important to

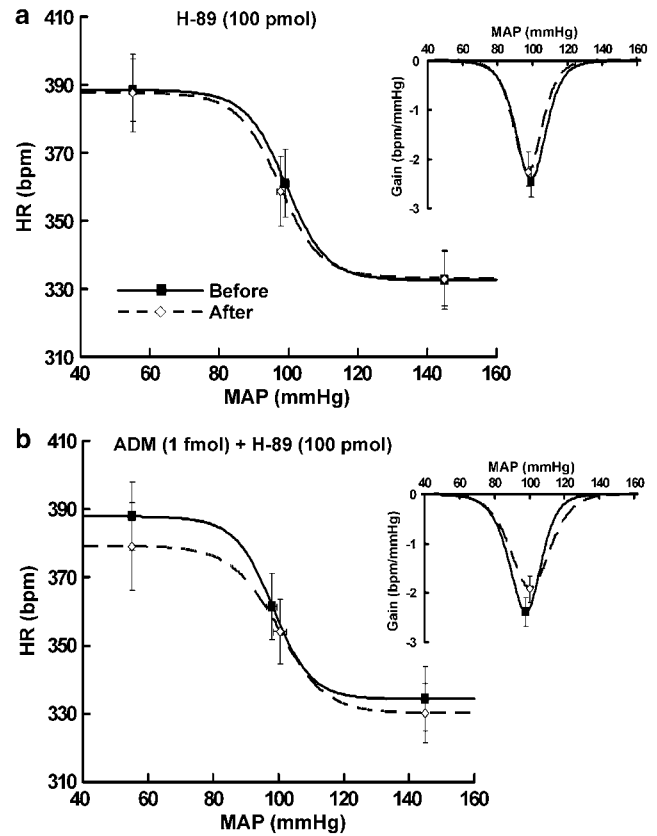


**Figure 3** Average baroreflex curves generated before and during the first hour after 0.1 fmol ADM<sub>22-52</sub> (a,  $n = 4$ ) or 1 fmol ADM<sub>22-52</sub> (b,  $n = 6$ ) was microinjected into the RVLM. Insets, average gains of baroreflex control of HR as a function of MAP. Symbols represent the upper plateau, set-point, and lower plateau of baroreflex curves, respectively. \* $P < 0.05$  versus measurements before microinjections.



**Figure 4** Average baroreflex curves generated before and during the first hour after 1 fmol ADM plus 0.1 fmol ADM<sub>22-52</sub> ( $n = 4$ ) was microinjected into the RVLM. Insets, average gains of baroreflex control of HR as a function of MAP. Symbols represent the upper plateau, set-point, and lower plateau of baroreflex curves, respectively.

study the effects of ADM in specific brain regions on the baroreflex. Interestingly, our data show that ADM in the RVLM inhibits the baroreflex and illustrate that central ADM regulates baroreflex function in a site-specific manner.



**Figure 5** Average baroreflex curves generated before and during the first hour after 100 pmol H-89 (a,  $n = 4$ ) or 1 fmol ADM plus 100 pmol H-89 (b,  $n = 4$ ) was microinjected into the RVLM. Insets, average gains of baroreflex control of HR as a function of MAP. Symbols represent the upper plateau, set-point, and lower plateau of baroreflex curves, respectively.

HR responses to hypotension and hypertension are differentially regulated by the sympathetic and parasympathetic nervous systems (Stornetta *et al.*, 1987). The parasympathetic nervous system is primarily responsible for hypertension-induced bradycardia, whereas the sympathetic and parasympathetic systems both contribute to the hypotension-induced tachycardia (Stornetta *et al.*, 1987). Interestingly, our data show that exogenous ADM affected the upper plateau of the baroreflex curve, but left the lower plateau intact, suggesting that ADM in the RVLM regulates baroreflex control of HR only when the MAP is decreased. Therefore, although changes in sympathetic activities were not directly measured in the present study, our results suggest that ADM in the RVLM regulates the baroreflex mainly by modulating the sympathetic outflow from this nucleus. Indeed, we have previously shown that microinjections of ADM at higher doses (10 and 100 fmol) into the RVLM induce robust increases in HR as well as modest increases in MAP, indicating that ADM in the RVLM stimulates neurons that control cardiac sympathetic activity (Xu & Krukoff, 2004a).

In an attempt to identify the mechanisms underlying the effect of ADM on baroreflex activity, we first demonstrated that ADM's effect is receptor-mediated, because an ADM receptor antagonist, ADM<sub>22-52</sub> (0.1 fmol), eliminated the ADM-induced inhibition of baroreflex. To further determine whether PKA activity is involved in ADM's effect, we blocked

PKA activity in the RVLM with 100 pmol H-89. H-89 is a commonly used PKA inhibitor which has been shown at 100 pmol to effectively inhibit PKA in the NTS to block heat shock-induced cardiovascular responses (Chan *et al.*, 2003). Our data show that coinjections of H-89 with ADM abolished the ADM-induced inhibition of the baroreflex to implicate PKA as a mediator of ADM's effect on the baroreflex, although we cannot rule out the possibility that a small part of the effect of H-89 is due to nonspecific effects on other intracellular messengers such as protein kinase G (Satake *et al.*, 1996) and Rho kinase (Leemhuis *et al.*, 2002). Consistent with our results, it has been shown that ADM elevates intracellular cAMP levels in various types of cells (Kitamura *et al.*, 1993; Shimekake *et al.*, 1995), including neural cells (Drake *et al.*, 1999; Spampinato *et al.*, 1999; Xu & Krukoff, 2005), and that many of ADM's actions are mediated by the cAMP-PKA pathway *in vitro* (Drake *et al.*, 1999; Spampinato *et al.*, 1999; Xu & Krukoff, 2005).

We found that blockade of ADM receptors alone with 1 fmol ADM<sub>22-52</sub> significantly increased the baroreflex gain and upper plateau. A trend towards increased reflex range was also observed, although this difference was not statistically significant due to variation among animals. Thus, we propose that endogenous ADM in the RVLM exerts a tonic inhibitory effect on the baroreflex control of HR.

The sources of endogenous ADMergic inputs to the RVLM have not been definitively identified. However, we and others previously showed that either ADM-like immunoactivity or mRNA of preproadrenomedullin (ADM precursor) is present in other autonomic centers including the PVN, lateral hypothalamic area, amygdala, parabrachial nucleus, NTS, and AP (Serrano *et al.*, 2000; Shan & Krukoff, 2001a). These nuclei have also been shown to contain barosensitive neurons that project to the RVLM (Polson *et al.*, 1995; Dampney *et al.*, 2003b). Among these candidates, we speculate that the PVN, NTS, and AP are more likely to provide major ADMergic inputs to the RVLM to regulate cardiovascular activities because we found that expression of ADM in these regions is

suppressed when arterial pressure homeostasis is challenged by physiological stressors (Shan & Krukoff, 2001a). Given that ADM in the RVLM inhibits the baroreflex control of HR, we propose that the resulting decrease in ADM production may provide animals with a protective mechanism to maintain cardiovascular homeostasis.

Whereas baroreflex activity is conventionally believed to be important primarily for moment-to-moment control of arterial pressure (Cowley, 1992), recent findings show that the baroreflex also contributes to the long-term control of arterial pressure (Malpas, 2004; Dampney *et al.*, 2005) and that chronic impairment of baroreflex activity results in neurogenic hypertension (Thrasher, 2002). Therefore, the fact that ADM in the RVLM inhibits the baroreflex control of HR, especially by resetting MAP<sub>50</sub> to a higher level, together with our previous finding that ADM in the RVLM induces a robust and long-lasting vasopressor effect (Xu & Krukoff, 2004a), suggests that potentiated ADMergic neurotransmission within the RVLM (e.g. elevated ADM release or increased expression of ADM receptors) will contribute to the development of hypertension. Indeed, it has been shown that in hypertensive rats, expression of ADM receptors in the brainstem is increased compared to normotensive rats (Li *et al.*, 2004).

In conclusion, our results demonstrate that endogenous ADM in the RVLM exerts an inhibitory effect on the baroreflex control of HR through ADM receptor-mediated mechanisms. Furthermore, we suggest that activation of PKA-related signaling is involved in ADM-induced baroreflex inhibition. Together with our previous finding that ADM in the RVLM increases MAP and HR under resting conditions (Xu & Krukoff, 2004a), these data indicate that ADM in this autonomic center contributes to the control of blood pressure not only by regulating the resting MAP and HR levels but also by modulating baroreflex activity.

Y.X. is the recipient of a studentship from the Heart and Stroke Foundation of Canada. This work was supported by the Heart and Stroke Foundation of Alberta/Northwest Territories/Nunavut.

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(Received December 22, 2005

Accepted January 19, 2006

Published online 27 February 2006)