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PACAP causes PAC_1/$VPAC_2$ receptor mediated hypertension and sympathoexcitation in normal and hypertensive rats

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Farnham MM, Lung MS, Tallapragada VJ, Pilowsky PM. PACAP causes PAC_1/$VPAC_2$ receptor mediated hypertension and sympathoexcitation in normal and hypertensive rats. *Am J Physiol Heart Circ Physiol* 303: H910–H917, 2012. First published August 10, 2012; doi:10.1152/ajpheart.00464.2012.—Pituitary adenylate cyclase-activating polypeptide (PACAP) is an excitatory neuropeptide that plays an important role in hypertension and stress responses. PACAP acts at three G protein-coupled receptors [PACAP type 1 receptor (PAC_1) and vasoactive intestinal peptide receptor types 1 and 2 ($VPAC_1$ and $VPAC_2$)] and is localized to sites involved in cardiovascular control, most significantly the rostral ventrolateral medulla (RVLM). The RVLM is crucial for the tonic and reflex control of efferent sympathetic activity. Increases in sympathetic activity are observed in most types of hypertension and heart failure. PACAP delivered intrathecally also causes massive sympathoexcitation. We aimed to determine the presence and abundance of the three PACAP receptors in the RVLM, the role, in vivo, of PACAP in the RVLM on tonic and reflex cardiovascular control, and the contribution of PACAP to hypertension in the spontaneously hypertensive rat (SHR). Data were obtained using quantitative PCR and microinjection of PACAP and its antagonist, PACAP(6–38), into the RVLM of anesthetized artificially ventilated normotensive rats or SHRs. All three receptors were present in the RVLM. PACAP microinjection into the RVLM caused sustained sympathoexcitation and tachycardia with a transient hypertension but did not affect homeostatic reflexes. The responses were partially mediated through PAC_1/$VPAC_2$ receptors since the effect of PACAP was attenuated (~50%) by PACAP(6–38). PACAP was not tonically active in the RVLM in this preparation because PACAP(6–38) on its own had no inhibitory effect. PACAP has long-lasting cardiovascular effects, but altered PACAP signaling within the RVLM is not a cause of hypertension in the SHR.

blood pressure; rostral ventrolateral medulla; sympathetic; neuropeptides; pituitary adenylate cyclase-activating polypeptide; vasoactive intestinal peptide receptor

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) exists in discrete regions of the brain stem and spinal cord involved in cardiovascular control, one of which is the rostral ventrolateral medulla (RVLM) (7, 12, 17, 22, 35, 59). The RVLM is crucial for cardiovascular control (20, 40, 48, 49) as it contains the neurons that determine resting mean arterial pressure (MAP) and responds to the activation of adaptive reflexes (11, 21, 37, 38, 51). Changes in autonomic function resulting from activation of the baroreceptor, somatosympathetic, and chemoreceptor reflexes are all principally mediated by increasing or decreasing the activity of RVLM neurons, which, in turn, project to and excite sympathetic preganglionic neurons in the thoracolumbar spinal cord (28, 36, 39, 43, 44, 49, 62).

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Peripheral administration of PACAP-38 (PACAP) dilates blood vessels, causing hypotension (42, 53). Centrally acting PACAP is sympathoexcitatory and, in most studies, pressor (13, 16–18, 26, 30, 56). In the central nervous system, the PACAP type 1 receptor (PAC_1) is the predominant PACAP receptor (3, 7, 8, 32, 63), but the effect of activating PACAP receptors in the RVLM of normotensive or hypertensive rat models is unknown. Around 80% of spinally projecting presympathetic neurons in the RVLM contain PACAP mRNA (17). Intrathecal infusion of PACAP causes sustained tachycardia and widespread sympathoexcitation in both normotensive and hypertensive rat strains (16, 17, 26, 33). PACAP acting at receptors in the spinal cord has effects on MAP (33) that vary in different strains but that do not contribute to the hypertension of the spontaneously hypertensive rat (SHR) (16). These variable effects on MAP may possibly be due to differences in PAC_1 versus vasoactive intestinal peptide receptor (VPAC) expression in the spinal cord (27). Here, we investigated a role for PACAP in the RVLM in normotension and hypertension.

Increased sympathetic nerve activity (SNA) is common in most forms of hypertension, including essential hypertension (54, 55). The SHR is a model of essential hypertension (29, 46) that exhibits elevated sympathetic tone well before hypertension is manifest (60). Similarly, intrathecal PACAP causes prolonged sympathoexcitation with variable effects on MAP (16, 17, 26, 33).

We propose that PACAP acting in the RVLM is a potent sympathoexcitatory agent in normotensive and hypertensive rats. To test this idea, we first determined PACAP receptor mRNA content within the RVLM. Second, we determined the functional role of PACAP in the RVLM on tonic and reflex sympathetic regulation, and, finally, we investigated a role for PACAP within the RVLM in the sympathoexcitation and hypertension seen in the SHR. The results revealed that PACAP in the RVLM increases SNA and blood pressure differentially in Sprague-Dawley (SD) rats, Wistar-Kyoto (WKY) rats, and SHRs without impairing adaptive reflex function.

METHODS

Animals

Procedures and protocols were approved by the Animal Care and Ethics Committee of Macquarie University. Experiments were conducted on adult male SD rats, WKY rats, and SHRs (350–500 g, Gore Hill Research Laboratories, Sydney, NSW, Australia, and the Animal Resource Centre, Perth, WA, Australia) in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

Tail-Cuff Phenotyping of WKY Rats and SHRs

The blood pressure of hypertensive and normotensive rats was measured at >18 wk of age by tail-cuff sphygmomanometry. Hypertension was defined as tail-cuff systolic pressure ≥ 150 mmHg and normotension as ≤140 mmHg. All SHRs in this study were hypertensive (mean: 183 mmHg, range: 164–202 mmHg), and all WKY rats were normotensive (mean: 124 mmHg, range: 115–133 mmHg).

Anesthesia

For the real-time quantitative PCR (qPCR) experiments, rats were anesthetized with urethane ($n = 3$ SD rats, 3 WKY rats, and 3 SHRs, 1.5 g/kg) or pentobarbital sodium ($n = 3$ SD rats, 3 WKY rats, and 3 SHRs, 80 mg/kg) and perfused with ice-cold sterile saline (0.9% NaCl).

For the in vivo physiological experiments, rats ($n = 22$ SD rats, 10 WKY rats, and 10 SHRs) were anesthetized with 10% urethane (1.0–1.5 g/kg ip). Atropine sulfate (100 μg/kg ip) was administered in the same injection to reduce bronchial secretions before vagotomy. The surgical level of anesthesia was defined as the absence of any withdrawal reflex to any nociceptive or tactile stimuli, such as a tail pinch or corneal touch. While indexes of respiration and corneal and flexor withdrawal reflexes can no longer be used to assess the depth of anesthesia under neuromuscular blockade, our continuous monitoring of heart rate (HR) and blood pressure and response of the above to sensory stimuli, such as the paw pinch, allowed us to determine the depth of anesthesia and respond to potentially painful stimuli. A steady resting level of these variables, in conjunction with a <20% change in response to sensory stimuli, indicated an adequate depth of anesthesia. This is the standard of care recommended by Hildebrand in *Anesthesia and Analgesia in Laboratory Animals* (24).

To assess the degree of paralysis, the animal was monitored for voluntary respiratory efforts and a withdrawal response to mild sensory stimuli.

Additional anaesthetic (30–40 mg iv) was administered as required.

Real-Time qPCR for PACAP Receptors in the RVLM of SD Rats, WKY Rats, and SHRs

The RVLM ($n = 6$ SD rats, 6 WKY rats, and 6 SHRs) was excised bilaterally (Fig. 1*A*) and combined, and total RNA was extracted for each animal. Only RNA samples with an optical densiometry 260-to-280-nm absorption ratio higher than 1.95 were used. Total RNA (150 ng) was reverse transcribed into cDNA in a 40-μl reaction. The resulting reverse transcription products were used in subsequent real-time qPCR experiments for the quantification of mRNA expression of PACAP receptors.

Optimization of real-time qPCR. The reference gene was hydroxymethylbilane synthase (*Hmbs*), which has very stable expression in rat brain tissue (10). Primers for *Hmbs*, PAC_1, and vasoactive intestinal peptide receptor types 1 and 2 ($VPAC_1$ and $VPAC_2$, respectively) were designed using Primer 3 software based on published sequences. The sequences and properties of each primer pair are shown in Table 1.

Real-time qPCR. Each reaction for real-time qPCR experiments contained 2 μl of reverse transcription product, 200 nM of each primer, and 10 μl of the 2× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), made up to 20 μl with sterile Milli-Q water. After a 50°C hold for 2 min (UDG incubation) and a 95°C denaturation for 2 min, the reactions were cycled 45 times with a 95°C denaturation step for 15 s and 61°C combined annealing and extension step for 30 s with a single fluorescence measurement. Melting curve analysis was conducted after the final cycle to verify that only the specific product was amplified.

Analysis. All qPCR experiments were set up using the relative standard curve method. The slopes of all standard curves and efficiencies of different primer pairs were calculated to ensure they were comparable. The values of quantification cycles were determined using the auto-find threshold function of Rotor-Gene software, and the relative amount of gene expression of each sample was determined from the standard curve. Values obtained from each sample were first normalized to a calibrator sample to allow accurate comparison of samples between different runs and then normalized to their corresponding expression level of the reference gene. Samples from three animals from each strain were run in duplicate for each of the two anesthetics used.

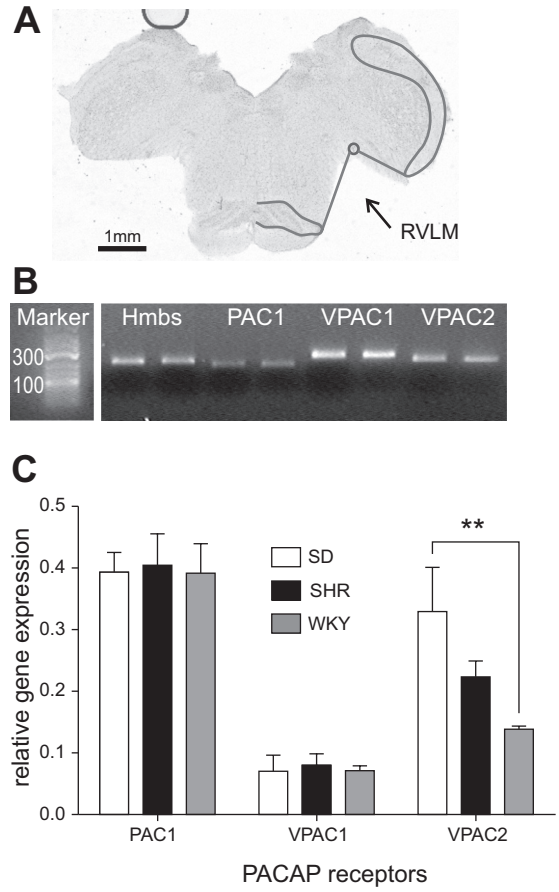

Fig. 1. Relative gene expression of pituitary adenylate cyclase-activating polypeptide (PACAP) receptors [PACAP type 1 receptor (PAC_1) and vasoactive intestinal peptide receptor types 1 and 2 ($VPAC_1$ and $VPAC_2$)] and in the rostral ventrolateral medulla (RVLM) of Sprague-Dawley (SD) rats, Wistar-Kyoto (WKY) rats, and spontaneously hypertensive rats (SHRs). *A*: cresyl violet stained coronal section of the brain stem with the RVLM cut out for use in quantitative PCR (qPCR; bregma: −12.36 mm). *B*: a 2% Tris-borate-EDTA-agarose gel showing the correct sizes of PCR products produced by all four primer pairs [hydroxymethylbilane synthase (*Hmbs*), 176 bp; PAC_1, 150 bp; $VPAC_1$, 216 bp; and $VPAC_2$, 183 bp]. *C*: PAC_1 was the most highly expressed receptor type ($P < 0.05$). $VPAC_2$ was significantly less expressed in the WKY rat compared with the SD rat. $^{**}P < 0.01$. Digital images in *A* and *B* were adjusted for brightness and contrast only. The marker lane is demarcated by a white space.

Statistical analyses were performed using Microsoft Excel and GraphPad Prism software. To determine if the different anesthetics affected PACAP receptor gene expression, a nonparametric Mann-Whitney *U*-test was performed. To determine if the levels of gene expression of the three PACAP receptors were significantly different in the three strains of rats tested, one-way ANOVA was performed for each receptor.

Table 1. *Sequences and properties of gene-specific real-time quantitative PCR primers*

Gene	GenBank Accession Number	Sequence		Amplicon Size, bp
		Forward	Reverse	
Hmbs	NM_013168	5′-TCCTGGCTTTACCATTGGAG-3′	5′-TGAATTCCAGGTGAGGGAAC-3′	176
PAC_1	NM_133511	5′-TCTTGAATGGGGAGGTACAGG-3′	5′-TCTTGCTCAGGATGGACAGC-3′	150
$VPAC_1$	NM_012685	5′-CAGCAAGATGTGGGACAACC-3′	5′-TGCTGCTCATCCAGACTCG-3′	216
$VPAC_2$	NM_017238	5′-CCGAGGATGAGAGTAAGATCACG-3′	5′-AGATGGCTCTCAGCATGAAGG-3′	183

Hmbs, hydroxymethylbilane synthase; PAC1, pituitary adenylate cyclase-activating polypeptide type 1 receptor; $VPAC_1$ and $VPAC_2$, vasoactive intestinal peptide receptor types 1 and 2, respectively. Exon-intron boundaries are underlined.

Surgical Preparation

Rats (n = 22 SD rats, 10 WKY rats, and 10 SHRs) were anesthetized as described above, and surgical preparation and data-acquisition methods were as described elsewhere (16, 17, 26, 50, 57).

Briefly, rats were secured in a stereotaxic frame, and temperature was maintained at 37 ± 0.5°C. The right carotid artery and jugular vein were cannulated for the measurement of MAP and administration of drugs and fluids, respectively. The trachea was cannulated to permit artificial ventilation. Leads were attached to the forepaws to obtain ECG and derive HR. The left greater splanchnic sympathetic nerve was isolated, and activity was recorded (sampling rate: 2 kHz, gain: 20,000, filtering: 100–2,000 Hz). The left sciatic nerve (n = 8 SD rats, 9 WKY rats, and 8 SHRs) and aortic depressor nerves (n = 10 SD rats) were isolated and prepared for stimulation. The dorsal surface of the medulla was exposed by occipital craniotomy, and the dura was removed for the microinjection of drugs into the RVLM. All rats were bilaterally vagotomized, ventilated with oxygen-enriched room air, and paralyzed with pancuronium bromide (Astra Zeneca, 0.8 mg/kg iv, followed by an infusion of 0.8 $mg \cdot kg^{-1} \cdot h^{-1}$ pancuronium in 0.9% saline at a rate of 2 ml/h).

Microinjection of PACAP and/or PACAP(6–38) into the RVLM of SD rats, WKY rats, and SHRs. The RVLM was located by stereotaxic coordinates and confirmed if a 50-nl injection of 100 mmol/l glutamate (Sigma-Aldrich) raised blood pressure > 30 mmHg. A dose-response curve was constructed for 50-nl injections of 10 μmol/l (n = 3 SD rats), 30 μmol/l (n = 3 SD rats), 50 μmol/l (n = 3 SD rats), and 100 μmol/l of PACAP [PACAP(1–38), Auspep, Melbourne, VIC, Australia, and Selleck, Houston, TX, n = 7 SD rats]. The 100 μmol/l dose of PACAP was used for the remainder of the study.

After glutamate confirmation, PBS was injected bilaterally into the RVLM, and physiological parameters were recorded for 35 min. Five picomoles of PACAP (100 μmol/l in 50 nl, n = 7 SD rats, 5 WKY rats, and 5 SHRs) or 15 picomoles of the PACAP antagonist PACAP(6–38) (Auspep, 100 μmol/l in 150 nl, n = 15 SD rats, 5 WKY rats, and 5 SHRs) were injected. Five of the PACAP(6–38)-treated SD rats had 5 pmol PACAP (100 μmol/l in 50 nl) injected 15 min after the administration of PACAP(6–38), and five SD rats were used only for baroreflex testing. Reflexes were tested 15 min after bilateral injections. After the final drug injection, recordings continued for 125 min. At the conclusion of the experiments, rats were killed with 3 M KCl (intravenous, Sigma-Aldrich), injection sites were marked, and brains were removed and postfixed in 10% formalin overnight. Brains were sectioned coronally (100 μm) and stained with cresyl violet for histological verification of the injection sites (Fig. 2*A*).

Reflex activation. Baroreceptors were activated with phenylephrine (Sigma-Aldrich, 5 μg in 0.1 ml iv) or by stimulating the aortic depressor nerve for 10 s [100 Hz, 0.2-ms pulse width, 3× threshold (41)]. The sciatic nerve (somatosympathetic reflex) was stimulated intermittently (0.2-ms pulse width, 50 pulses, 1 Hz) at a voltage sufficient to generate two distinct peaks in the rectified, averaged splanchic SNA (sSNA) trace over the stimulus period (20–50 V).

Data acquisition and analysis. Data were acquired using a CED 1401 and Spike 2 data acquisition and analysis software (versions 6

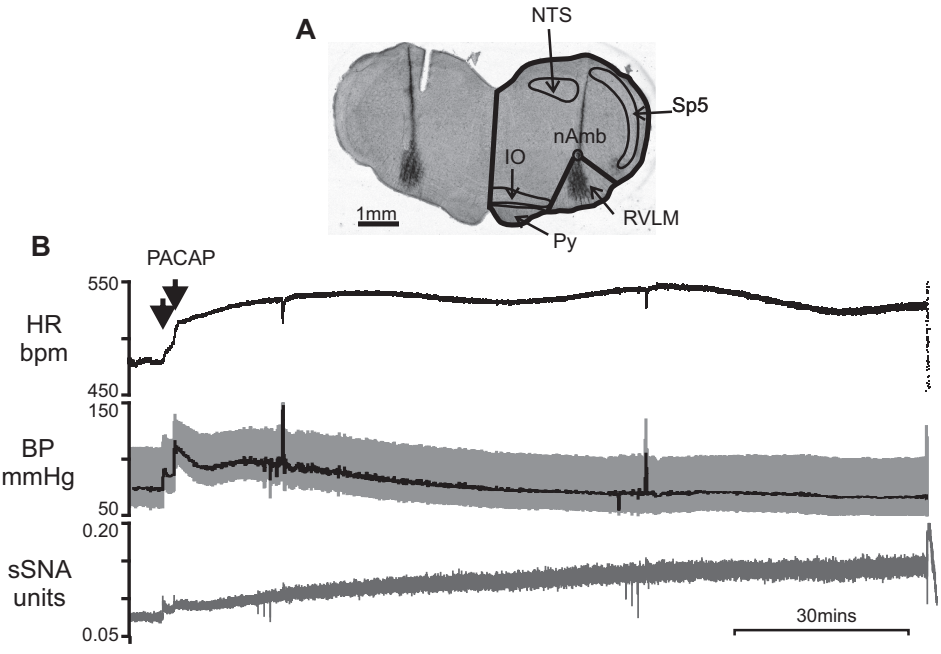

Fig. 2. In vivo effect of PACAP in the RVLM. *A*: bilateral RVLM injection sites of PACAP (bregma: −11.9 mm). The RVLM was defined as a triangular area ventral to the nucleus ambiguus (nAmb), lateral to the inferior olive (IO) or pyramidals (Py) and medial to the spinal trigeminal sensory nucleus (Sp5). NTS, nucleus tractus solitarius. *B*: heart rate [HR; in beats/min (bpm); *top*], blood pressure (BP; *middle*; red: pulsatile and black: mean), and splanchnic sympathetic nerve activity (sSNA; *bottom*) after the bilateral microinjection of PACAP into the RVLM of a SD rat. Arrows indicate points of injection. HR and sSNA remained elevated, whereas BP returned to baseline, 60 min after injection.

and 7). MAP, HR, and sSNA were analyzed from 5-min blocks taken 5 min before and 5, 10, 20, 30, 40, 50, 60, 90, and 120 min after RVLM injections of PBS (up to 30 min) or PACAP/PACAP(6–38) (up to 120 min). Statistical analysis was conducted with GraphPad Prism software (version 5).

Peak responses of drug treatments between strains were compared using two-way ANOVA with post hoc *t*-tests and Bonferroni's correction. Time courses of drug responses were analyzed with one-way repeated-measures ANOVA. The effect of drug on the slope of the baroreflex was analyzed using Student's *t*-test.

Slopes of the baroreflex were generated by plotting MAP against rectified sSNA during phenylephrine trials. Data were fitted to a straight line, and the slope was calculated.

RESULTS

Real-Time qPCR for PACAP Receptors in the RVLM of SD Rats, WKY Rats, and SHRs

Levels of gene expression of PAC_1, $VPAC_1$, and $VPAC_2$ were measured in SD rats ($n = 6$), WKY rats ($n = 6$), and SHRs ($n = 6$; Fig. 1*C*). Comparison of the relative expression of each of the three PACAP receptor mRNAs across the strains revealed no differences in PAC_1 or $VPAC_1$ expression. $VPAC_2$ gene expression was significantly less in WKY rats (0.14 ± 0.01) compared with SD rats (0.33 ± 0.07, $P < 0.01$; Fig. 1*C*).

Effect of PACAP Microinjection Into the RVLM on sSNA, MAP, and HR

Four doses of PACAP were tested by microinjection into the RVLM of separate rats to determine a suitable dose for subsequent use: 10 μmol/l ($n = 3$ SD rats), 30 μmol/l ($n = 3$ SD rats), 50 μmol/l ($n = 3$ SD rats), and 100 μmol/l PACAP. The 50 μmol/l concentration caused a significant increase in HR (51 ± 13 beats/min, $P < 0.05$). Only the 100 μmol/l concentration was sufficient to cause significant elevations in MAP ($P < 0.05$), HR ($P < 0.05$), and SNA ($P < 0.05$) compared with PBS and was therefore used in all subsequent experiments. These effects are described in detail below.

Microinjection of PACAP in the RVLM was sympathoexcitatory, pressor, and caused tachycardia in normotensive and hypertensive rats.

MAP. Baseline MAP was 103 ± 6 mmHg in SD rats ($n = 7$), 83 ± 7 mmHg in WKY rats ($n = 5$), and 123 ± 8 mmHg in SHRs ($n = 5$, $P < 0.05$ between strains). Bilateral microinjection of PACAP into the RVLM significantly increased MAP in all strains (ΔMAP: 37 ± 5 mmHg in SD rats, 37 ± 3 mmHg in WKY rats, and 54 ± 7 mmHg in SHRs, $P < 0.001$; Figs. 3*B* and 4, *A* and *C*). MAP returned to baseline in all strains by 1 h postinjection.

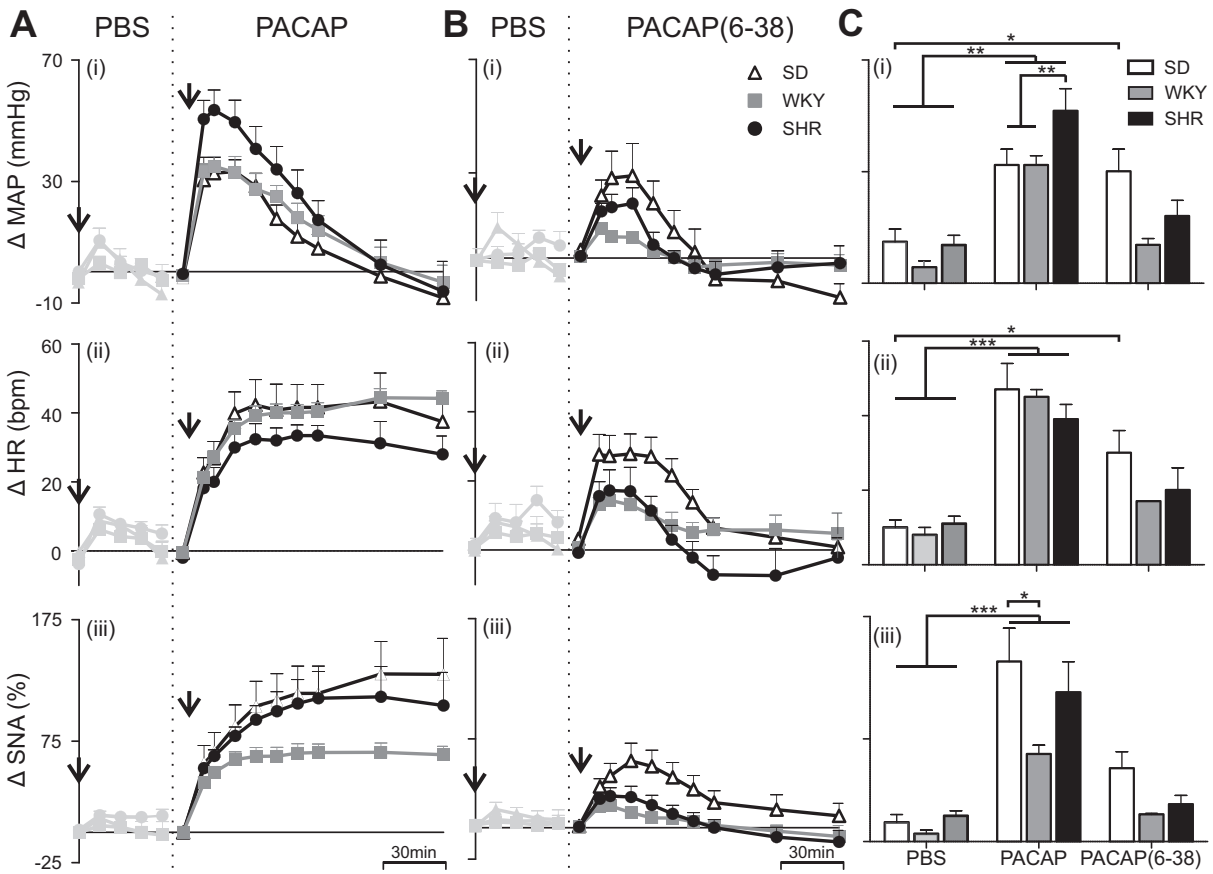

Fig. 3. Cardiovascular effects of PACAP and PACAP(6–38) in the RVLM of normotensive and hypertensive rats. *A* and *B*: changes in mean arterial pressure (MAP; *i*), HR (*ii*), and percentage of sSNA (*iii*) before and after the administration of PACAP (*A*) or PACAP(6–38) (*B*). Arrows indicate times of drug infusion. "PBS" indicates the period after the bilateral RVLM microinjection of PBS; "PACAP" and "PACAP(6–38)" indicate the periods after the bilateral RVLM microinjection of PACAP or PACAP(6–38), respectively. *C*: comparison of maximum MAP (*i*), HR (*ii*), and percentage of sSNA responses (*iii*) after PACAP or PACAP(6–38). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

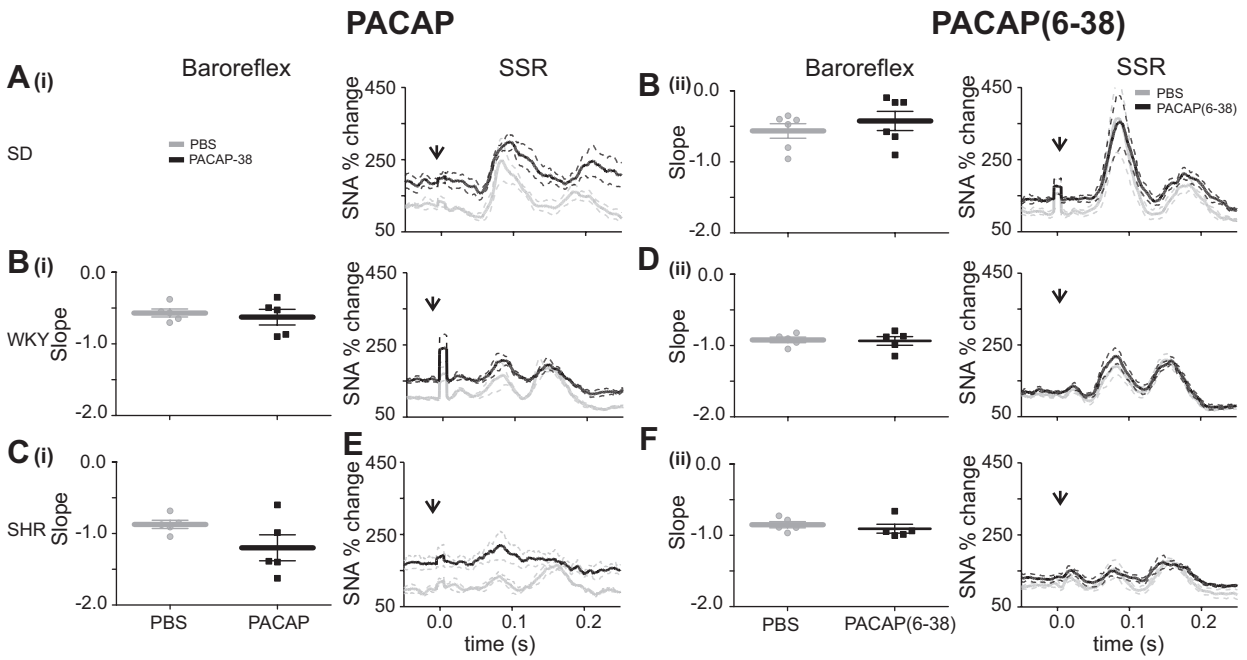

Fig. 4. PACAP and PACAP(6–38) in the RVLM does not affect baroreceptor or somatosympathetic reflexes. *A–F*: effects of PACAP (*A–C*) or PACAP(6–38) (*D–F*) on the slope of the baroreflex curve and somatosympathetic reflex (SSR) in SD rats (*A* and *B*), WKY rats (*C* and *D*), or SHRs (*E* and *F*). The slopes of the baroreflex curves were not significantly different in any strain after either drug treatment (compared with PBS). The SSR ensemble waveform averages were generated from the rectified, smoothed sSNA trace. Arrows indicate the points of stimulation of the ipsilateral sciatic nerve. The sSNA channel was calibrated so that 0% activity was the level after death and 100% was the activity level 5 min before the microinjection of PBS, PACAP, or PACAP(6–38). PACAP caused an increase in the basal sSNA, but neither drug treatment affected the responsiveness of the SSR.

HR. Baseline HR was 465 ± 8 beats/min in SD rats, 455 ± 9 beats/min in WKY rats, and 431 ± 9 beats/min in SHRs ($P < 0.05$). HR was significantly increased in all strains (ΔHR: 47 ± 7 beats/min in SD rats, 45 ± 2 beats/min in WKY rats, and 39 ± 4 beats/min in SHRs) after the bilateral microinjection of PACAP into the RVLM ($P < 0.0001$; Figs. 2*B* and 3, *A* and *C*). HR remained elevated in all strains at 2 h postinjection.

sSNA. sSNA was significantly increased in SD rats by 140 ± 26% ($P < 0.001$), WKY rats by 68 ± 7% ($P < 0.05$), and SHRs by 116 ± 24% ($P < 0.001$) after the bilateral microinjection of PACAP into the RVLM (Figs. 2*B* and 3, *A* and *C*). All sSNA responses to PACAP remained elevated throughout the experiment (2 h).

PACAP(6–38) Antagonizes the Cardiovascular Effects of PACAP

Bilateral microinjection of the antagonist PACAP(6–38) before the bilateral microinjection of PACAP in SD rats significantly blunted MAP (Δ20 ± 4 mmHg, $P < 0.05$), HR (Δ15 ± 5 beats/min, $P < 0.05$), and sSNA (Δ45.5 ± 6.9%, $P < 0.05$) responses compared with PACAP alone.

PACAP(6–38) Alone Does Not Reduce MAP, sSNA, or HR in Normotensive or Hypertensive Rats

Microinjection of the antagonist PACAP(6–38) increased MAP (Δ37 ± 5 mmHg, $P < 0.01$) and HR (Δ30 ± 6 beats/min, $P < 0.01$) in SD rats (Figs. 3*B* and 4*C*). The PACAP(6–38) sSNA response (57 ± 13%, $P > 0.05$) was not different from that of PBS (Fig. 3*C*). The effects of microinjection of PACAP(6–38) into the RVLM of WKY rats and SHRs were similar to microinjection of PBS (Fig. 3*C*).

PACAP(6–38) did not reduce basal MAP, HR, or sSNA in any of the three strains (Fig. 3*B*).

Neither PACAP Nor PACAP(6–38) Altered the Function of Homeostatic Reflexes in Normotensive or Hypertensive Rats

Baroreceptor reflex. Neither PACAP nor PACAP (6–38) altered the slope of the sympathetic baroreflex in any of the three strains (Fig. 4).

Somatosympathetic reflex. Neither PACAP nor PACAP(6–38) treatment altered the somatosympathetic reflex in any of the three strains ($n = 2$–3 rats/group for each condition; Fig. 4). PACAP increased basal sSNA, but the reflex remained unchanged in all three strains. The antagonist evoked a small increase in sSNA in SD rats but not in SHRs or WKY rats.

DISCUSSION

The main findings of this study were, first, that microinjection of PACAP in the RVLM increases sSNA, HR, and MAP in SHR, WKY and SD rats. This effect was significantly attenuated by pretreatment with the $PAC_1/VPAC_2$ receptor antagonist PACAP(6–38). The antagonist itself did not reduce any measured parameter, suggesting that PACAP receptors are not tonically active. Second, unlike many other peptides (1, 2, 19, 50, 57), neither exogenous PACAP nor PACAP(6–38) affected the homeostatic reflexes tested. Finally, PACAP(6–38) did not reduce sSNA or blood pressure in the SHR, suggesting that PACAP neurotransmission in the RVLM does not maintain established hypertension in this model.

The effect of PACAP in the RVLM on sSNA and HR was similar in both magnitude and duration to the responses seen after the intrathecal administration of PACAP (17), even with

a 10 times lesser concentration. This difference is likely due to pharmacokinetic factors, since microinjection allows peptides to gain direct access to receptors. However, RVLM microinjection evoked a large pressor response that was absent after intrathecal administration. The difference in PACAP-induced MAP responses between this study and the previous two studies (16, 17) is likely due to the route of administration. Microinjection of PACAP directly into the RVLM affects bulbospinal sympathoexcitatory neurons. Intrathecal administration, on the other hand, may activate PACAP receptors on sympathetic preganglionic neurons as well as inhibitory interneurons (47) or neurons in the dorsal and ventral horns (47) within the spinal cord. Finally, the receptor complement present on RVLM neurons compared with spinal cord neurons, and the effect of activating them, may be different in the two regions (27). While this is the first study to microinject PACAP into the RVLM, the pressor response observed is in agreement with other studies that administered PACAP centrally using other approaches (13, 31, 33, 56).

PACAP can act in several ways; it can function as a neurotransmitter as well as have endocrine, paracrine, or autocrine actions (see Refs. 18 and 58). It has been proposed that PACAP is released as a cotransmitter (15). Costorage of PACAP and glutamate has been demonstrated in the retinohypothalmic tract (23) and in retinal ganglion cells (14). It is plausible that glutamate, acting on PACAP-containing presympathetic neurons in the RVLM, triggers a paracrine release of PACAP within the RVLM, thereby increasing the likelihood that bulbospinal presympathetic neurons are activated. The intensity and duration of the responses may also be modulated by the quantity of PACAP released. This has been demonstrated in spinal cord slices where low concentrations of PACAP caused reversible inward currents in sympathetic preganglionic neurons, whereas higher concentrations caused sustained inward currents (64).

Given the intensity and duration of the PACAP responses, the presence of a rich receptor density within the RVLM is assumed but has not been previously demonstrated. Previous studies (32, 63, 65) have demonstrated the presence of PACAP receptors in the medulla oblongata. Our qPCR results further refine this localization to the region of the RVLM where sympathetic premotor neurons are found and confirm another report (5) of PAC_1 being the predominate receptor form in the central nervous system. Comparison of the relative expression of the receptors between the strains revealed that $VPAC_2$ receptor expression was significantly less in the WKY rat compared with the SD rat. The significance of this finding is unclear as it is not yet known which subpopulations of RVLM neurons express each of the receptors. In situ hybridization studies of PACAP receptor mRNAs and their colocalization with other RVLM markers, such as tyrosine hydroxylase, could resolve this issue.

The in vivo physiological data presented here focused on a single class of receptors. All three PACAP receptors have splice variants (4, 61, 65). The increased responsiveness of the SHR to exogenous PACAP could be due to differential expression of splice variants of PAC_1. PACAP(6–38) is a potent antagonist of PAC_1 (52) and did not reduce basal MAP, sSNA, HR, CO_2, or temperature in any of the three strains, indicating that PACAP receptors are not tonically active within the RVLM in the urethane-anesthetized, paralyzed, vagotomized, and artificially ventilated rat.

Activation or blockade of PACAP receptors did not affect homoeostatic reflexes in any strain tested. This lack of effect is particularly interesting given the robust effects of PACAP on MAP, HR, and sSNA. It indicates that PACAP release is more likely related to the long-term modulation of sympathetic tone rather than adaptive reflexes. PACAP attenuates cardiac baroreceptor reflex sensitivity in trout (34), but this reflex is controlled predominantly by the parasympathetic system. Long-term effects of PACAP have also been seen in sympathetic neurons (33, 64), adrenal medullary cells (6, 45), and tyrosine hydroxylase phosphorylation (9). We do not believe that the absence of effect on adaptive reflexes is an artifact of the preparation, since many of our earlier studies (1, 2, 19, 50, 57) with peptides have demonstrated differential effects on the reflexes studied here.

The physiological stimuli for PACAP release in RVLM are currently unknown, but it is plausible that the sympathetic cardiovascular responses observed in this study are part of a stress response (or defense reaction). In addition, given its sustained effect, it appears that PACAP may cause persistent activation of intracellular signaling pathways.

The persistent increase in SNA after PACAP administration into the RVLM is particularly interesting in light of early demonstrations that increased SNA, even in the absence of raised arterial blood pressure, may be detrimental. For example, patients with heart failure have elevated levels of SNA whether or not their arterial blood pressure is raised. Treatment of such patients with β-blockers enhances survival (25).

Perspectives

The cardiovascular responses to PACAP in the RVLM are at least partially mediated through PAC_1/$VPAC_2$ receptors since the effect of PACAP-38 was attenuated by preadministration of PACAP(6–38). PACAP is not tonically active in the RVLM as determined by the lack of effect of PACAP(6–38). The only difference in PACAP receptor expression between the strains was a lesser expression of $VPAC_2$ in the WKY rat compared with the SD rat. The functional implications of this finding are unclear. While there is still much to be learned about PACAP and the physiological mechanisms that stimulate its release, evidence from this study suggests that altered PACAP signaling within the RVLM is not an underlying cause for hypertension in the SHR.

The finding that PACAP does not affect homoeostatic reflexes but does affect tone is extremely important. Most neurotransmitters, and drugs that affect their activity, not only change SNA and blood pressure but also alter the ability of the organism to regulate its responses to stimuli from the periphery. Taken together, our findings suggest that drugs affecting PACAP receptors will be an attractive target in the treatment of disorders associated with increased SNA, including cardiac arrhythmias after myocardial infarction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.M.-J.F. and P.M.P. conception and design of research; M.M.-J.F., M.S.L., and V.J.T. performed experiments; M.M.-J.F., M.S.L., and V.J.T. analyzed data; M.M.-J.F. and P.M.P. interpreted results of experiments; M.M.-J.F., M.S.L., and V.J.T. prepared figures; M.M.-J.F. drafted manuscript; M.M.-J.F., M.S.L., V.J.T., and P.M.P. edited and revised manuscript; M.M.-J.F. and P.M.P. approved final version of manuscript.

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