PACAP is expressed in sympathoexcitatory bulbospinal C1 neurons of the brain stem and increases sympathetic nerve activity in vivo

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The rostral ventrolateral medulla (RVLM) is a major nucleus involved in the tonic and reflex regulation of the cardiovascular system (26). Within the RVLM there are many neurons that have a spinal axon and are inhibited by baroreceptor activation; it is generally accepted that these neurons are presypathetic sympathoexcitatory (2, 14, 15). Approximately 60–80% of presypathetic neurons can be defined phenotypically as C1 adrenaline-synthesizing neurons (15, 25, 33, 34). Of the remaining non-C1 bulbospinal neurons, many, but not all, express preproenkephalin (32). At the present time there is no adequate marker for all populations of presypathetic neurons in the RVLM.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38 amino acid peptide originally identified in the ovine hypothalamus (18). The distribution of PACAP within the medulla oblongata and spinal cord has been identified using immunohistochemistry (IHC) and in situ hybridization (ISH; 6–9, 12). PACAP-containing cells exist in several brain stem areas that influence cardiovascular function such as the dorsal motor nucleus of the vagus, the ventral medulla, and the raphe (6, 8, 9, 12). Although some physiological studies report changes in blood pressure following the central administration of PACAP (10, 21, 28, 37) the results are highly variable.

PACAP is associated with many catecholaminergic neurons and also adrenal chromaffin cells in the adrenal gland (6, 12, 29). Specifically, PACAP is found in C1 and A1 neurons that project to the hypothalamus (6). PACAP as a neurotransmitter alters catecholamine biosynthetic enzyme [including tyrosine hydroxylase (TH), dopamine-β-hydroxylase, and phenylethanolamine N-methyltransferase (PNMT)] gene expression and activity (5, 39) in adrenal chromaffin cells, including phaeochromocytoma cells, and in dopaminergic cell groups within the brain (1, 5, 20, 23). The association of PACAP with other catecholaminergic neurons in the brain stem, many of which influence cardiovascular function, is undetermined.

Thus the aims of this study are 1) to confirm the localization of PACAP mRNA containing (PACAP+) neurons in areas of the brain stem involved in cardiovascular regulation, 2) to determine whether PACAP mRNA is colocalized with bulbospinal catecholamine cell groups, and 3) to determine whether activation of PACAP receptors in the spinal cord leads to sympathoexcitation.

METHODS

All procedures and protocols were approved by the Animal Care and Ethics Committee of the Royal North Shore Hospital and the University of Technology, Sydney, Australia. Experiments were conducted on adult male Sprague-Dawley rats (350–500 g; Gilead Research Laboratories, Sydney, Australia) in accordance with the Australian code of practice for the care and use of animals for scientific purposes.

Surgical preparation. Rats (n = 10) used in the intrathecal protocol were anesthetized with urethane (1.0–1.5 g/kg ip, with additional doses of 30–40 mg as required to suppress nociceptive reflexes). Rats (n = 3) used in the retrograde tracing protocol were anesthetized with pentobarbital sodium (60 mg/kg ip; Nembutal, Merial), and carprofen (0.1 ml/kg, Norocarp; Norbrook Laboratories) was administered to reduce postoperative pain. In both protocols atropine sulfate (0.2 ml/kg ip; Astra Pharmaceuticals) was administered to reduce bronchial secretions. All rats (n = 13) were secured in a stereotaxic frame, and temperature was maintained between 36.5°C and 37.5°C by using a rectal probe connected to a homeothermic heating blanket (Harvard Apparatus). The depth of anesthesia was monitored by observing reflex responses to noicceptive and tactile stimuli (periodic tail/paw pinches), pupillary responses to light stimuli, and the corneal touch reflex. Additional anesthetic was then administered as required.

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Retrograde cholera toxin B subunit labeling of bulbospinal neurons. The spinal cord between the dorsal processes of the T1 and T2 vertebrae was exposed by dorsal laminectomy. Cholera toxin B subunit (CTB; 1%, 200 nl; List Biologicals) was microinjected bilaterally into the intermediolateral cell column (IML) (0.6 mm lateral, 1.0 mm ventral to the dorsal surface). Two injections were made on each side of the spinal cord. The wound was then closed, and iodine (Betadine, Faulding Pharmaceuticals, Australia) was applied to prevent infection.

After 2 days, rats were reanesthetized with pentobarbital sodium (80 mg/kg ip) and were perfused through the left ventricle ascending aorta with 300 ml of ice-cold heparinized 0.9% saline followed by 300 ml of ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and thoracic spinal cord were removed and postfixed overnight in the same fixative. Brain stems were sectioned coronally (40 μm) by using a vibrating microtome (model VT 1000S; Leica) and collected sequentially into four pots containing phosphate buffer with 0.1% Tween-20.

ISH combined with fluorescence IHC. Methods were conducted as described previously (13, 22). Sense and antisense probes were synthesized by first amplifying a DNA fragment of the PACAP gene from rat brain cDNA (purified DNA showed a single band when run on a 1.2% agarose gel) using forward and reverse primers with Sp6 and T7 promoters attached at the 5’ end, respectively (PACAP-ISH).

Fig. 1. Pituitary adenylate cyclase-activating polypeptide (PACAP) association with tyrosine hydroxylase (TH) in the medulla oblongata. A: PACAP+ neurons (black), TH-immunoreactive (ir) neurons (red), and cholera toxin B-ir (CTB-ir) neurons (green) can be seen in panels i to vii. A1-PACAP mRNA is colocalized with TH-ir but not CTB-ir (i); nucleus tractus solitarius (NTS)-lightly stained PACAP+ neurons are colocalized with TH-ir in the NTS, but intensely stained PACAP+ neurons are found in the dorsal motor nucleus of the vagus (DMNX), which is not TH-ir (ii); A5-PACAP+ neurons are not colocalized with CTB-ir neurons (iii); locus coeruleus (LC)-PACAP+ is not colocalized with TH-ir (iv). The CTB-ir is most likely from labeling of the dorsal horn, not the IML; C2-PACAP+ is colocalized with TH-ir (v); rostral ventrolateral medulla (RVLM)-PACAP+ is colocalized with both TH-ir and CTB-ir (vi); and C3-PACAP+ is colocalized with TH-ir (vii). B: A high-power image of RVLM neurons showing colocalization of PACAP+, TH-ir, and CTB-ir. The RVLM is the only nucleus to have a high proportion of triple-stained neurons: TH-ir (i), CTB-ir (ii), and PACAP+ (iii). The 3 images are merged in panel iv showing colocalization of PACAP+ with TH-ir and CTB-ir. Arrowheads depict triple-stained neurons. Scale bars = 100 μm (A); 10 μm (B).
alkaline buffer NTMT (0.1 M NaCl, 0.1 M Tris with 2 mM levamisole. Following 2 h at 58°C, sections were then washed 3 times in TPBS buffer (10 mM HCl, pH 9.5, 0.1 M phosphate, 0.1% Tween-20) without the PACAP riboprobe and then at 58°C for 1 h. The PACAP probe was then added to the buffer to a final concentration of 100 ng/ml, and sections were incubated by shaking at 58°C overnight. Subsequently, the sections were washed 2× 30 min in 2× SSC, 0.1% Tween-20 at 58°C, followed by 2× 30 min in 0.1× SSC, 0.1% Tween-20 at 58°C. To reveal DIG-labeled (mRNA) neurons simultaneously with TH and CTB, sections were rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-20) 2× 15 min at room temperature and incubated for 2 h in maleic acid buffer containing 2% BSA blocking reagent (Roche) and 10% normal horse serum. Primary antibodies: alkaline phosphatase-conjugated sheep anti-DIG (1:1,000; Dako), mouse anti-TH (1:2,000; Sigma-Aldrich), and rabbit anti-CTB (1:2,000; List Biological Laboratories) were added to the buffer and incubated for 48 h at 4°C. Sections were then washed 3× 30 min in TPBS buffer (10 mM Tris-HCl, 10 mM sodium phosphate buffer, 0.9% NaCl, pH 7.4). TH was revealed by incubation overnight with Cy3-conjugated donkey anti-mouse IgG (1:500, Jackson), and CTB with a FITC-conjugated donkey anti-rabbit IgG (1:1,500, Jackson). To reveal DIG-labeled neurons, the sections were then washed 5× 1 h in maleic acid buffer with 2 mM levamisole. Following 2× 15 min equilibration in the alkaline buffer NTMT (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 0.1 M MgCl2, 0.1% Tween-20, 2 mM levamisole), a colorimetric reaction using nitroblue tetrazolium (Roche), and 5-bromo-4-chloro-3-indolyl phosphate salts (Roche) in NTMT revealed DIG-labeled neurons as those containing dark purple precipitants. Sections were mounted sequentially on glass slides, coverslipped with Vectashield (Vector Laboratories), and sealed with nail polish.

Analysis and imaging. Sections of the brain stem extending from the locus coeruleus (Bregma −9.0 mm) to the first cervical spinal segment were examined using an epifluorescence microscope (AxioImager Z1; Zeiss, Germany) under both brightfield and fluorescence conditions. In situ hybridization labeling was visualized using brightfield, Cy3-stained TH neurons were visualized using a Cy3-4040B filter set (Semrock, Rochester, NY), and FITC-stained CTB neurons were visualized using a FITC-3540B filter set (Semrock). Cell counts within the RVLM of each replicate were performed bilaterally on eight sections spaced 160 μm apart, extending from Bregma −11.6 mm caudally to Bregma −12.7 mm. The RVLM was defined as a triangular area ventral to the nucleus ambiguus, medial to the spinal trigeminal tract, and lateral to the inferior olive or the pyramidal tracts. Results were plotted as the means ± SE at the 160-μm interval. Counts were made for PACAP+, TH-immunoreactive (TH-ir) and CTB-ir neurons, as well as all double- and triple-labeling combinations.

Images were captured in grey scale with an Axioskop MR3 digital camera. Pseudocoloring was applied to the fluorescence images for better visualization of distribution and colocalization. The images were adjusted individually for brightness and contrast with Axiovision 4.5 software to best reflect the appearance of the original images.

Intrathecal injection of PACAP-38. In five animals, the right jugular vein and carotid artery were cannulated for the administration of drugs and fluids and recording of blood pressure, respectively. An ECG was recorded, and a tracheal cannula was inserted to permit artificial ventilation. The left splanchnic sympathetic nerve was isolated via a retroperitoneal incision, dissected, and prepared for recording (2 kHz, 1–100 k × gain, 0.1–2 kHz filtering). The rats were bilaterally vagotomized, connected to the ventilator, and then paralyzed with pancuronium bromide (2 mg iv, Astra Pharmaceuticals).

A catheter (polyvinylchloride tubing; ID, 0.2 mm; OD, 0.5 mm, Critchley Electrical Products) with a dead space of ~6 μl was inserted into the intrathecal space through a slit in the dura at the atlantoaxial joint and advanced caudally to the level of T5/T6. A control injection of 10 μl of 10 mM PBS (0.9%) was washed in with 6 μl PBS. Next, 10 μl of 1 mM PACAP-38 (Auspep) was administered and flushed in with a further 6 μl of PBS. Injections were done over a 10- to 15-s period. Responses were recorded for 3 h. At the conclusion of the experiments, the rats were killed by using 0.5 ml of 3 M potassium chloride (KCl) iv. Postmortem verification of the location of the catheter tip was achieved by exposing the spinal cord and then flushing 10 μl of India ink followed by 6 μl of 1× PBS through the intrathecal catheter. The spinal segment level of the catheter was recorded as the level where the blue spot appeared on the spinal cord.

Intrathecal injection of PACAP-38 with barodenervation. In a separate group of five rats (instrumented in the same way as the 5 rats above), acute barodenervation was accomplished by cutting the aortic and glossopharyngeal nerves bilaterally. To ensure a complete denervation, polyethylene cannulae (OD, 0.96 mm; ID, 0.58 mm) were placed outside the carotid artery with the tip at the bifurcation on both the right and left sides and secured to the muscle wall. Before the administration of intrathecal PBS, a baroreflex test was conducted. Phenylephrine (PE; 100 μl, 10 μg/kg iv) was administered, and responses were measured until readings returned to baseline. The long-acting local anesthetic, bupivacaine (500 μl Marcaine 0.5%; AstraZeneca), was administered to each carotid bifurcation and given 5 min to act. PE was then administered intravenously again, and responses were recorded until readings returned to baseline. PBS was then administered intrathecally, and the full protocol was repeated. After recording the response to PACAP-38 for 3 h, the baroreflex was again tested with PE. When all responses returned to baseline, the animal was killed and the location of the catheter tip verified as described above.

Data acquisition and analysis. Data was acquired using a Cambridge Electronic Design ADC system (model 1401; Cambridge Electronic Design, Cambridge, UK) and Spike 2 acquisition and analysis software (Cambridge Electronic Design, UK). Responses were sampled at 10 kHz and filtered with a 1 kHz low-pass filter (Blackman) and a 1 kHz high-pass filter (FIR). Each response was averaged, and the area under the curve was computed. The area under the curve was then normalized to the baseline area under the curve.

Fig. 2. Rostrocaudal distribution of TH-ir neurons and PACAP + and TH-ir neurons in the RVLM. TH-ir neurons and neurons positive for both TH-ir and PACAP + (n = 3) are plotted on the left axis against the Bregma level (mm) and rostrocaudal distribution [VII (facial nucleus), RVLM, and caudal ventrolateral medulla (CVLVM)]. The mean percentage of TH-ir, PACAP + over the whole RVLM, was 84.4 ± 4.2% (average of 58 ± 8.5 neurons out of 67 ± 8.7 across the entire region counted from −11.6 to −12.6).

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analysis software (version 6.03). Blood pressure, splanchnic sympathetic nerve activity, and heart rate were analyzed from 5 min blocs taken 5 min prior to and 5, 10, 20, 30, 40, 50, 60, 90, 120, 150, and 180 min after intrathecal injections of PBS (up to 60 min) or PACAP (up to 180 min). Analysis was conducted with GraphPad Prism (version 4.0). A one-way repeated-measures ANOVA with Dunnett’s multiple comparison tests was used to compare postvalues with the prevalue for both the nonbarodenervated and barodenervated groups. The effect of barodenervation compared with nonbarodenervation on the PACAP response was analyzed with a t-test on area under the curve.

RESULTS

PACAP mRNA distribution in the brain stem. PACAP mRNA was found in several compact cell clusters in the brain stem with few neurons found outside these areas, in agreement with earlier studies (9). Colocalization of TH-ir and PACAP mRNA was seen throughout the ventral medulla from the caudal pole of facial nucleus to the cervical cord as well as in the C2 and C3 cells. Lightly stained neurons were found colocalized with A2 neurons in the NTS (Fig. 1A, ii). A compact cluster of PACAP mRNA-containing neurons was also found close to the A5 cell group (Fig. 1A, iii) and in a nucleus ventromedial to the locus coeruleus (Fig. 1A, iv), but neither were colocalized with TH-ir neurons. PACAP mRNA was found densely in the dorsal motor nucleus of the vagus (Fig. 1A, ii) and in the supragenual nucleus. A few faintly labeled PACAP+ neurons were found in the caudal raphé nuclei.

PACAP mRNA colocalization with TH in the RVLM. PACAP mRNA and TH-ir were frequently colocalized in neurons in the RVLM (Fig. 1B). PACAP mRNA was found in 84.4 ± 4.2% (average of 58 ± 9 neurons out of 67 ± 9 across the entire region counted from −11.6 to −12.6; n = 3) of TH-ir neurons throughout the RVLM (Fig. 2). PACAP+ and

![Fig. 3. Rostrocaudal distribution of bulbospinal presympathetic neurons in the RVLM. Three different bulbospinal (CTB-ir) neuron groups (n = 3) plotted on the left axis against the Bregma level (mm) and rostrocaudal distribution (VII, RVLM, and CVLM). Note: The percentage of bulbospinal TH-ir (CTB-ir, TH-ir) neurons also positive for PACAP mRNA was 82.3 ± 5.0% over the whole RVLM (average of 20 ± 4.7 neurons out of 22 ± 5.3 across the entire region counted from −11.6 to −12.6).](image)

![Fig. 4. Rostrocaudal distribution of the proportion of PACAP+ neurons negative for both TH-ir and CTB-ir in the RVLM. The number of solely PACAP+ neurons (n = 3), calculated as a percentage of the total PACAP+ population, plotted against the Bregma level (mm) and rostrocaudal distribution (VII, RVLM, and CVLM). The total percentage of PACAP+ neurons negative for both TH-ir and CTB-ir was 38.5 ± 2.7% over the entire RVLM (average of 45 ± 6.5 neurons out of 114 ± 14 across the entire region counted from −11.6 to −12.6).](image)

![Fig. 5. In vivo effects of intrathecal administration of vehicle or PACAP-38. Splanchnic sympathetic nerve activity (sSNA) percentage change (A), change in heart rate (HR; B) and change in mean arterial pressure (MAP; C), before and following administration of PACAP-38. Arrow indicates time of PACAP-38 injection. Pre, period before injection of any solution; PBS, period after intrathecal injection of PBS; PACAP-38, period after intrathecal injection of PACAP-38.](image)
TH-ir had a similar distribution in the RVLM (Fig. 2) with the greatest concentration found rostrally, as previously described for TH-ir (25). Within the RVLM, however, not all neurons containing PACAP mRNA were found to be TH-ir. There were ~40% more PACAP+ neurons than there were TH-ir neurons in the RVLM.

PACAP mRNA colocalization within bulbospinal (CTB) neurons in the RVLM. As described previously, the greatest number of bulbospinal neurons in the RVLM was clustered in the most rostral 500 μm close to the caudal border of the facial nucleus: level I of Stornetta et al., (32) near the center of this region. There was a sharp decline in the number of bulbospinal neurons beyond this point (13, 31, 32). Thus we also found that the distribution of PACAP+ bulbospinal neurons, bulbospinal TH-ir neurons, and PACAP+ bulbospinal TH-ir neurons, were also clustered within the rostral half of the RVLM (Fig. 3).

An average of 30 ± 6% (31 ± 6 neurons out of 114 ± 14 across the entire region counted from −11.6 to −12.6; n = 3) of the PACAP+ neurons in the RVLM were bulbospinal (Fig. 3). Some of the remaining 50% of PACAP+, non-TH-ir neurons can be accounted for with CTB labeling. PACAP+ non-TH-ir bulbospinal (CTB+) neurons account for an additional 11.4 ± 3.1% (11 ± 2 neurons out of 114 ± 14 across the entire region counted from −11.6 to −12.6; n = 3) of the gross PACAP+ number in the RVLM. Although the numbers changed rostrocaudally, the distribution of TH-ir bulbospinal neurons closely paralleled the distribution of PACAP+ bulbospinal neurons. Hence the total proportion of PACAP+ neurons that were not colocalized with either TH-ir or CTB-ir was 38.5 ± 2.7% (45 ± 7 neurons out of 114 ± 14 across the entire region counted from −11.6 to −12.6; n = 3) in the RVLM (Fig. 4).

PACAP mRNA colocalization with bulbospinal, TH neurons in the RVLM. Our final anatomical aim was to determine whether PACAP mRNA was present in presynaptic neurons, which we define as neurons with both TH-ir and a spinal projection as determined by the presence of CTB in the RVLM and in what proportion. We found that PACAP mRNA was indeed present in bulbospinal TH-ir neurons (Figs. 1B and 3). PACAP mRNA was present in 82.3 ± 5.0% (20 ± 5 neurons out of 22 ± 5 across the entire region counted from −11.6 to −12.6; n = 3) of these neurons averaged over the whole RVLM (Fig. 3). These PACAP+ bulbospinal TH-ir neurons comprise 20.1 ± 4.1% (20 ± 5 neurons out of 114 ± 14 across the entire region counted from −11.6 to −12.6; n = 3) of the total PACAP mRNA expressing neurons in the RVLM.

PACAP mRNA colocalization with bulbospinal TH neurons in the C2 and C3 regions. The C2 and C3 areas of the medulla oblongata contained few neurons that were positive for PACAP mRNA, TH-ir, and CTB-ir. The C2 and C3 regions were counted caudally from Bregma level −11.60 for 320 μm where the last of the CTB-ir neurons were seen. In C2 a total of 123 TH-ir neurons were counted across n = 3 rats, and 91 of these also contained PACAP mRNA. Fifteen TH-ir neurons were positive for CTB and of these, 14 also contained PACAP mRNA (~5 neurons/rat). In the C3 region, a total of 82 TH-ir neurons were counted across n = 3 rats. Of these, 77 contained PACAP mRNA. Two neurons were positive for both CTB-ir and TH-ir and both of these also contained PACAP mRNA (approximately < 1 neuron/rat).

PACAP mRNA colocalization with TH neurons in the A5 region. In the A5 region, a total of 174 ± 15 TH-positive neurons (n = 3) were counted. These neurons were counted bilaterally in a 1-mm region rostral to the caudal pole of the A5 nucleus. Of these TH-positive neurons none were also positive for PACAP mRNA.

Effects of intrathecal 1 mM PACAP-38 on sympathetic nerve activity, heart rate, and blood pressure. Since PACAP mRNA was present in the bulbospinal presynaptic neurons in the RVLM, we wished to ascertain the functional effects of activating PACAP receptors in the spinal cord. Intrathecal administration of 10 μL of 1 mM PACAP-38 (n = 5) evoked increases (P < 0.001) in both splanchnic sympathetic nerve activity (110.2 ± 42.1% and heart rate (baseline 421.6 ± 11.82 beats/min; increased 81.0 ± 14.6 beats/min after PACAP) but no change in blood pressure (109.5 ± 5.7 mmHg before PACAP-38 to 112.1 ± 8.5 mmHg 60 min after PACAP-38) (Fig. 5). Injections of 100 μM PACAP-38 did not elicit any significant effects on blood pressure, heart rate, or splanchnic sympathetic nerve activity (data not shown).

Splanchnic sympathetic nerve activity was significantly increased between 40 and 180 min postinjection compared with the control period (P < 0.05) (Fig. 5). Heart rate was significantly increased (P < 0.05) compared with the control period at every time point (Fig. 5).

Effect of barodenervation on the effect of intrathecal 1 mM PACAP-38 on sympathetic nerve activity, heart rate, and blood pressure. Intrathecal PACAP-38 had no clear effect on blood pressure even though both heart rate and splanchnic sympathetic nerve activity were markedly increased. We therefore sought to determine whether the lack of change in blood pressure was due to action of the arterial baroreflex. However,
complete barodenervation (Fig. 6) had no effect on the responses of blood pressure (baseline, −90.24 ± 5.97 mmHg), heart rate (baseline, −381.40 ± 13.61 beats/min), or splanchnic sympathetic nerve activity to intrathecal PACAP-38 (Fig. 7).

DISCUSSION

The key findings of this study are first, that PACAP mRNA was found in specific cell groups in the brain stem, some of which were catecholaminergic, confirming previous studies (6, 12, 30). Specifically PACAP was found in TH-ir neurons in the C1-C3 cell groups and in the A1 and A2 cell groups but not in A5 or the locus coeruleus. In expansion upon previous findings, the data here are quantified and PACAP was found at least 84% of TH-ir neurons in the RVLM. The novel finding of this study was that PACAP was found in at least 82% of the bulbospinal TH-ir neurons in the RVLM. These results indicate that pontomedullary neurons containing TH and PACAP that project to the IML must originate from C1-C3 and cannot project from the A5 nucleus, which is the only other catecholaminergic cell group that projects to the IML of the spinal cord. The observation that A5 can be distinguished from C1 by its lack of PACAP mRNA is interesting and remains to be fully investigated and understood.

All PACAP-containing areas identified here have been reported previously (8, 9, 12). The key advantages of this study over previous ones lie in the nature and sensitivity of the techniques and the combination with IHC used here. Earlier studies used colchicine to improve the detectability of PACAP by IHC, since it could not be detected in the C1-C3 regions in the resting state (8, 12). Even using ISH, only small numbers of PACAP neurons were detected (9). With our approach, extensive labeling for PACAP mRNA was found in these areas under normal conditions, highlighting the sensitivity of our technique. Evidence for PACAP-ir in the raphe is controversial. Our study supports previous findings (9, 12) that PACAPergic neurons are uncommon in the raphe nuclei in contrast to previous work (8).

PACAP mRNA, as opposed to protein, is present in many, but not all, catecholaminergic nuclei. PACAP mRNA was found in many A1 neurons confirming an earlier study (30). Our data show that PACAP mRNA is present within many neurons in the C1 cell group in both the rostrally projecting (caudal C1) and spinally projecting (rostral C1) parts (4, 32). Our finding that caudal C1 neurons (15, 36) contain PACAP mRNA confirms the findings of Das et al., (6) who demonstrated PACAP-ir, PNMT-ir neurons at the level of the RVLM that sends projections to the paraventricular nucleus. C2 and C3 cell groups also project to the spinal cord (17) and were found to contain PACAP mRNA, but the role of PACAP in these neurons is unknown. It was found that the amount of spinally projecting PNMT-ir neurons varies with the level at which the retrograde tracer was injected (16). Our own study, while only injecting at the level of T1-T2 is consistent with these results, with C1 containing the largest proportion of spinally projecting TH-ir neurons and C2 and C3 containing a much smaller proportion.

Our major finding is that PACAP is found in four populations of neurons in the RVLM: in TH-ir nonbulbospinal neurons, in TH-ir bulbospinal neurons, in non-TH-ir bulbospinal neurons, and in neurons that are neither TH-ir nor bulbospinal. The first population represents the previously reported (6) rostral extension of the hypothalamically projecting caudal C1 neurons (36, 38). The bulbospinal PACAP mRNA-containing populations include a significant population of the presympathetic neurons. Our data show that more than 80% of bulbospinal TH-ir neurons, which are known to be barosensitive and therefore presympathetic (27), contain PACAP+. A population of TH-ir bulbospinal neurons that control sympathetic preganglionic neurons innervating adrenaline-containing chromaffin cells are not barosensitive (19) but could also contain PACAP+. Since PACAP is found in a large proportion of spinally projecting non-TH-ir neurons, this would represent the presympathetic enkephalin neurons (32). The question is, are the other ~38% of PACAP neurons presympathetic neurons? This is certainly a possibility due to inadequate labeling of all bulbospinal neurons. Some neurons may project only to spinal levels that we did not inject tracer into and thus did not receive coverage from fibers of passage labeling. Functional identifi-
cation of presympathetic neurons has largely been based upon barosensitivity, and it is well established that not all sympathetic outflows have this characteristic. A further technical consideration concerns the combined ISH with IHC staining. The PACAP+ and ir neurons were observed to range in staining intensity (Fig. 1B). Thus the percentage of PACAP+ neurons in the RVLM that are neither TH-ir nor CTB-ir may be underestimated. It remains to be established to what extent PACAP+ is specific for presympathetic cell groups rather than for other spinally projecting cell populations. Although CART labels presympathetic neurons (3) it is present throughout the medulla oblongata and does not therefore make a single useful indicator. It does however, remain a possibility that a functionally independent, nonpresympathetic population of PACAP mRNA-containing neurons is present in the RVLM.

The presence of PACAP+ in presympathetic RVLM neurons suggests that PACAP is an important peptide controlling sympathetic outflows. Our data show that intrathecal injection of PACAP-38 at T5–6 increases splanchnic sympathetic nerve activity and heart rate but has no significant effect on arterial pressure. The effects on sympathetic outflow support findings that PACAP-38 excites sympathetic preganglionic neurons in spinal cord slices from juvenile rats (11). However, our finding that blood pressure is unaffected contrasts with one previous study that found a pressor response evoked by intrathecal administration of PACAP-38. Apart from differences in spinal levels of injection and doses, the much lower baseline blood pressure of 75–80 mmHg in the Lai et al. (11) study compared with ~110 mmHg in the present study could easily account for the differences in blood pressure responses observed. Since the peak pressure generated in the Lai et al. study (11) was ~105–110 mmHg, this would certainly represent a plausible explanation. Consonant with the finding of Lai et al. (11), we did find a large increase in sympathetic nerve activity that was unaffected by acute barodenervation. Nevertheless, we need to explain why nearly all presympathetic neurons contain PACAP mRNA but intrathecal injection does not elevate blood pressure. At least three possibilities remain: 1) abundant PACAP is found in interneurons in the spinal cord (24), some of which may be inhibitory, and activation of these neurons may reverse the elevation of sympathetic activity expected in some beds; 2) it also remains a possibility that regions, such as the raphe may release PACAP evoking vasodilation of the tail, thereby masking the sympathoexcitatory effects on blood pressure; and 3) it is possible that PACAP-38 only exerts a pressor effect in vivo in the presence of another cotransmitter, such as glutamate.

Barodenervation was performed to determine whether the baroreflex was masking a pressor response to intrathecal PACAP-38. However, barodenervation had no effect on the responses to PACAP-38 and so is not involved in the response to intrathecal PACAP-38. Spinal C1 transection, adrenalec-
tomy, and regionalized sympathetic nerve recordings may all assist in understanding the regional circulatory effects of intrathecal PACAP-38.

In conclusion, we report for the first time that PACAP mRNA is present in most, if not all, C1 presympathetic neurons (defined in this study as neurons with both TH-ir and a spinal projection determined by the presence of CTB) in the RVLM. Additionally, intrathecal administration of PACAP-38 causes intense sympathoexcitation in the splanchnic bed and tachycardia in vagotomized animals without any clear overall blood pressure response, even in animals that have been baroreceptor denervated. Intrathecal PACAP-38 presumably excites sympathetic preganglionic neurons. However, PACAP is abundant in interneurons in the spinal cord and activation of this target may mask any elevation in blood pressure, so different sympathetic beds are affected differentially.

Perspectives and Significance

Our anatomical findings combined with our physiological findings suggest that endogenous PACAP-38 release from the C1-C3 cell groups plays a role in the sympathetic control of the cardiovascular system. Precisely when, in response to what stimulus, and where, PACAP-38 is released in the spinal cord to exert its effects in physiological or pathological situations remains to be determined.

GRANTS

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