PACAP release from the canine adrenal gland in vivo: its functional role in severe hypotension

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PACAP release from the canine adrenal gland in vivo: its functional role in severe hypotension. Am J Physiol Regul Integr Comp Physiol 284: R588–R597, 2003. First published October 31, 2002; 10.1152/ajpregu.00466.2002.—This study was to investigate if endogenous pituitary adenylate cyclase-activating polypeptide (PACAP) can be released during direct splanchnic nerve stimulation in vivo and to determine whether PACAP in the adrenal gland can modulate the medullary response to sympathoadrenal reflex. The output of adrenal catecholamine and PACAP-38-like immunoreactivity (PACAP-38-ir) increased in a frequency-dependent manner after direct splanchnic nerve stimulation (0.2–20 Hz). Both responses were highly reproducible, and PACAP-38-ir output closely correlated with catecholamine output. Sodium nitroprusside (SNP; 0.1 mg/kg iv bolus) caused a severe hypotension resulting in marked increases in catecholamine secretion. In the presence of local PACAP-27 (125 ng), the maximum catecholamine response to SNP was significantly potentiated in a synergistic manner compared with that obtained in the group receiving SNP or PACAP-27 alone. The study indicates that endogenous PACAP-38 can be released particularly when the sympathoadrenal system is highly activated and that the local exogenous PACAP-27 enhanced the reflex-induced catecholamine release, suggesting collectively a facilitating role of PACAP as neuromodulator in the sympathoadrenal function in vivo.

catecholamine; pituitary adenylate cyclase-activating polypeptide; splanchnic nerve; anesthetized dogs

PACAP is a neuropeptide having two isoforms composed of 27 and 38 amino acids (PACAP-27 and PACAP-38), which are both widely distributed in the central and peripheral nervous systems. The rat adrenal gland contains high concentrations of PACAP as well as PAC1 receptors (29). PACAP immunoreactive nerve fibers ending on chromaffin cells have also been identified in the rat adrenal gland (6, 12, 27). A few studies in vitro have shown that endogenous PACAP was released in response to direct splanchnic nerve stimulation in isolated, perfused porcine and rat adrenal glands (27, 30) and that the direct nerve stimulation-induced catecholamine secretion was diminished by a PACAP antagonist (8). These previous observations in vitro have suggested that PACAP may play a role of controlling adrenomedullary function during an actual stimulation of the sympathoadrenal system in vivo. However, such a functional implication of PACAP in the sympathoadrenal system in physiological and/or pathophysiological situations still remains mostly conjectural in the limited number of experimental demonstrations under in vivo conditions. Therefore, the present study was carried out 1) to determine whether endogenous PACAP can actually be released into the adrenal venous effluent during a direct splanchnic nerve stimulation in vivo and 2) to investigate if locally administered PACAP can effectively modulate the adrenal catecholamine secretion in response to a reflex activation of the sympathoadrenal system in anesthetized dogs.

METHODS

Surgical preparations of the animals. Adult mongrel dogs, fasted overnight but allowed free access to water, were anesthetized with pentobarbital sodium (30 mg/kg iv followed by 4 mg/kg as needed). Artificial respiration was maintained through an endotracheal tube with a Harvard pump (model 607). The rectal temperature was monitored and kept constant at 37.5 ± 0.5°C by means of a thermoregulator (model 74, Yellow Springs Instruments, Yellow Springs, OH) connected to a heating pad. Physiological saline was slowly administered intravenously during the whole period of the experiment to prevent dehydration. The pH of the physiological saline was adjusted at 7.38 immediately before use. Both

THE ADRENAL MEDULLAE RELEASE catecholamines in response to various stresses that activate the sympathetic nervous system. It is well established that ACh, the classical cholinergic neurotransmitter released from splanchnic nerve terminals, evokes catecholamine secretion (7). In addition to ACh, it has been acknowledged that various endogenous neuropeptides are coreleased along with ACh to mediate and/or modulate catecholamine secretion in response to varying stress. Among several neuropeptides playing a potential role as a noncholinergic neurotransmitter and/or neuromodulator in the adrenal medulla, pituitary adenylate cyclase-activating polypeptide (PACAP) is likely to play a major role.
femoral arteries were cannulated: the right femoral artery was used to measure aortic pressure and the left femoral artery to obtain aortic blood samples.

Preparation for direct splanchnic nerve stimulation. The direct splanchnic nerve stimulation was applied in the group of animals in which endogenous PACAP-38 was determined in the left adrenal venous effluent. After a median laparotomy and a left flank incision, the left splanchnic nerve was dissected free from surrounding tissues, double ligated ~2 cm from the adrenal gland, and protected from dryness by applying mineral oil. Direct electrical stimulation was applied to the distal end of this nerve. All the other nerves to the left adrenal gland from the lumbar paravertebral sympathetic ganglia and from the celiac-superior mesenteric plexus were double ligated and cut within ~2 cm from the gland to prevent undesired retrograde nerve conduction during direct splanchnic nerve stimulation (9, 35).

Preparation of local intra-arterial drug infusion to the left adrenal gland. After a median laparotomy and a left flank incision, the left adrenolumbar artery was dissected free from surrounding tissue for installation of a cannula in the adrenal gland. The volume of this catheter was fixed at 0.5 ml, and the catheter was connected to an infusion pump (model 1140–001, Harvard). PACAP-27 was locally infused to the left adrenal gland through this catheter at a fixed rate of 0.5 ml/min.

Preparation of the extracorporeal adrenal venous circuit. Adrenal venous blood was sampled through a polyethylene catheter (PE-240) specially shaped at one end for easy introduction and inserted into the left adrenolumbar vein via the left femoral vein. The catheter was tied at the adrenoadominal-vena cava junction to prevent adrenal venous blood contamination with abdominal vena cava blood. The left adrenolumbar vein, distal to the gland, was ligated to obtain actual adrenal venous blood. Venous blood from the gland was drained into a small blood reservoir filled with saline. The volume of this catheter was fixed at 1.5 ml. Blood volume in the reservoir was kept as small as possible and the venous blood was returned to the dog using an automatic blood level controller connected to an infusion pump (Masterflex model 7016–52, Cole-Parmer Instrument, Chicago, IL) through a catheter inserted into the right femoral vein at a perfusion rate adjusted to a stabilized initial venous flow (34).

Preparation of acute surgical denervation of the left adrenal gland. In a separate group, a relative contribution of the local splanchnic input to the adrenal medulla was tested during a reflex activation of the sympathoadrenal system induced by a severe hypotension with an intravenous injection of sodium nitroprusside (SNP; Sigma Chemical, St. Louis, MO). To this end, acute surgical denervation of the left adrenal gland was performed by cutting the left splanchnic nerve according to our previously reported procedure (35). All the other nerves to the left adrenal gland were cut after the same method described for preparing the direct splanchnic nerve stimulation. After all surgical procedures were completed, sodium heparin (200 U/kg iv) was administered, followed by 100 U/kg every hour thereafter. Dogs were then allowed a stabilization period of ~60 min.

Measured parameters. Mean aortic pressure and heart rate were measured and recorded with a polygraph system (model RM-6000, Nihon-Kohden, Tokyo, Japan). Aortic and left adrenal venous blood was simultaneously sampled into graded, chilled tubes for catecholamine analyses. In experiments with direct splanchnic nerve stimulation, plasma concentrations of endogenous PACAP-38 were also determined in the same blood samples that served for catecholamine measurements. Adrenal venous blood flow was determined by a gravimetric method at each sampling time point (34). Hematocrit was measured in all adrenal venous blood samples. For the catecholamine measurements, an aliquot of 1.5 ml of blood was transferred to a centrifuge tube containing 30 μl of preservative solution (pH 6.5) consisting of ethylene glycol-bis (β-amino-ethyl ether)-N,N,N’,N’-tetraacetic acid (95 mg/ml) and glutathione (60 mg/ml). These blood samples were immediately centrifuged at 4°C for 5 min at 14,000 revolutions/min with a refrigerated centrifuge (model 5402, Eppendorf, Hamburg, Germany). Plasma was stored at ~80°C until assayed. Plasma concentrations of epinephrine and norepinephrine were quantified by means of an isocratic high-performance liquid chromatographic system (Gilson, Villiers-Le-Bel, France) coupled with an electrochemical detector “Coulomem II” (model 5200; ESA, Bedford, MA) (34). At the end of each experiment, the left adrenal gland was removed and weighed. Adrenal catecholamine data were obtained in net catecholamine output calculated as follows: net output of adrenal catecholamine (ng·min⁻¹·g⁻¹ of gland⁻¹) = [CA]_{AV} − [CA]_{AO})/(BF_{AV} × (1-Hct_{AV})/wet wt of gland, where [CA]_{AV} is plasma catecholamine concentration in adrenal venous blood, [CA]_{AO} is plasma catecholamine concentration in aortic blood, BF_{AV} is adrenal venous blood flow, and Hct_{AV} is adrenal venous blood hematocrit. For aortic and adrenal venous PACAP-38 measurements, an aliquot of 4 ml of blood was transferred to a chilled polypropylene test tube containing disodium EDTA (1 mg/ml of blood). Plasma PACAP-38 concentration was determined by radioimmunoassay with a commercial kit (R1K-8920, Peninsula Laboratories, San Carlos, CA). The protease inhibitor aprotinin was not used in this study, because we previously showed that peptide concentrations in plasma were similar either in the presence or absence of aprotinin at the concentration of 500 KIU/ml of blood (10).

Experimental protocol. The present study consisted of three separate series of experiments. The first series contained one experimental group (29/1 ± 1.2 kg; n = 6) that was used to determine if endogenous PACAP-38 is actually released into the adrenal venous effluent during a direct splanchnic nerve stimulation under in vivo conditions. In this group, the distal end of tightly double-ligated left splanchnic nerve was stimulated with bipolar platinum electrodes by rectangular pulses of supramaximal voltage (12 V) by means of an electronic stimulator (model SEN-3301; Nihon Kohden). The stimulation period consisted of three successive stimuli with three different frequencies, 0.2, 2, and 20 Hz, each lasting 2 min, without interruption between each stimulus. The pulse duration was also modified in such a manner that the splanchnic nerves received the identical total pulse duration during each stimulus, which corresponds to a single pulse duration of 20, 2, and 0.2 ms for each frequency of 0.2, 2, and 20 Hz, respectively (10). Adrenal venous blood was separately collected into a chilled, graded tube during each stimulus (2 min), for a total period of 6 min. The dead volume of the adrenal venous catheter (1.5 ml) was taken into account for blood collection during each stimulus. Aortic blood samples (4 ml) were simultaneously obtained during adrenal venous sample collections at every sampling time point. After a 30-min stabilization period, another control sample of aortic and adrenal venous blood was taken, and a second stim-
lation period was applied under exactly the same conditions to ensure the reproducibility of the peptide response.

The second series involved four groups of experiments. This series served to evaluate whether the presence of locally administered PACAP-27 (Sigma) could modulate the adrenal catecholamine secretion in response to a reflex activation of the sympathoadrenal system during the SNP-induced severe hypotension. The experiments of this series were designed so that a possible interaction between SNP (depicting the reflex-mediated splanchnic neural drive to the adrenal medulla) and PACAP-27 can be evaluated by means of a two-way analysis of variance.

The first group (21.5 ± 1.2 kg, n = 8) received a local infusion of the vehicle (saline 0.9%, pH 7.38) combined with a simultaneous intravenous bolus injection of the vehicle and served as the control. One minute after the initial control sample was taken, the vehicle was infused into the left adrenolumbar artery at a rate of 0.5 ml/min for 5 min. The dead volume of the adrenal arterial catheter (0.5 ml) was taken into account in relation to the infusion rate. Similarly, the dead volume of the adrenal venous catheter (1.5 ml) was taken into consideration in relation to adrenal venous blood flow while adrenal venous blood samples were collected. At the onset of vehicle infusion (designated min 0), a bolus injection of the vehicle was given intravenously. An adrenal venous blood sample was obtained during the first minute of vehicle infusion (0–1 min) with a delay depending on the venous blood flow, followed by sample collections at 1–2, 2–3, 3–4, and 4–5 min during the whole period of vehicle infusion lasting for 5 min. Samples were also collected at 5–6, 6–7, 10–11, 15–16, 30–31, and 45–46 min after cessation of the vehicle infusion. The duration of each adrenal venous sample collection was thus fixed to 1 min. Aortic blood samples (1.5 ml each) were simultaneously obtained during adrenal venous sample collection at these sampling points.

The second group (29.2 ± 3.5 kg, n = 8) received a local infusion of the vehicle combined with a simultaneous intravenous bolus injection of SNP (0.1 mg/kg; Sigma) and served as the SNP-induced hypotension group. The experimental protocol for this group followed the same procedures as those described above.

The third group (23.5 ± 1.6 kg, n = 8) received a total dose of 125 ng of PACAP-27 given locally to the left adrenal gland via the left adrenolumbar artery with the concentration of 50 ng/ml at the infusion rate of 0.5 ml/min for precisely 5 min. Instead of SNP, a bolus of saline was given intravenously at the onset of PACAP-27 infusion. This group served as the PACAP-induced catecholamine secretion group. The procedures for drug administrations and sample collections were exactly the same as those described for the vehicle control group. After taking an aliquot of 1.5 ml of adrenal venous blood (for plasma catecholamine quantification) from the first seven samples obtained during and after the drug infusion, the remaining blood was not returned to the dog to prevent potential systemic hemodynamic changes due to the released catecholamine.

Finally, the fourth group (26.8 ± 3.4 kg, n = 8) received a local infusion of PACAP-27 (125 ng as a total dose, see above) to the left adrenal gland combined with a bolus intravenous injection of SNP (0.1 mg/kg) at the onset of PACAP-27 infusion. The protocol for blood sampling followed exactly the same procedures described for the vehicle control group.

The third series consisted of one group (26.9 ± 1.6 kg, n = 8) and served to assess the relative contribution of the splanchnic neural pathway in the sympathoadrenal counter-response to SNP-induced severe hypotension. This group received both the acute surgical denervation of the left adrenal gland and intravenous bolus injection of SNP (0.1 mg/kg iv bolus). The vehicle was locally infused to the left adrenal gland. The experimental protocols for the sequence of the drug administration as well as the timing for sample collections of adrenal venous and aortic blood were exactly the same as those described for the second series of experiments.

The concentration of PACAP-27 used in this study was selected on the basis of our previous observations obtained under similar experimental conditions (36). The period of PACAP-27 infusion, however, was fixed for 5 min to ensure the presence of PACAP-27 in the adrenal gland during a period of the maximal sympathoadrenal reflex stimulation, which could be estimated to be ~5 min according to the time course of adrenal catecholamine secretion after the intravenous administration of SNP alone. The dose of SNP was chosen on the basis of several dose-finding pilot experiments in which a bolus intravenous injection of SNP caused a severe fall in arterial pressure from which the animals could recover over a given experimental period. Each of the experimental protocols described for all series was applied only once in each animal. All surgeries and experimental procedures were performed while the dogs were under full surgical anesthesia. All experiments were acute, terminal procedures. The experimental protocols have been approved by the animal research committee of the Université de Montréal. The animals used in this study were cared for and used in accordance with the “Guiding principles for research involving animals and human beings” published by the American Physiological Society.

Statistical analyses. The statistical evaluation was made using a package of statistical software (SigmaStat for Windows, version 2.03; SPSS, Chicago, IL). Differences over a given experimental period were assessed by an analysis of variance for repeated measures followed by multiple comparisons with one control using the Dunnett’s method. A two-way analysis of variance was conducted to assess a possible interaction between PACAP-27 and SNP. This test was made on the maximal net amount of catecholamines released during the first 5 min after the SNP administration and/or PACAP-27 infusion. When applicable, a preliminary logarithmic transformation was used to satisfy the condition of a normal distribution of variance (32). All results are expressed as means ± SE, and P < 0.05 was considered statistically significant.

RESULTS

Responses to a direct splanchnic nerve stimulation. Values for plasma concentrations of catecholamines and PACAP-38 immunoreactivity (ir) in adrenal venous and aortic blood, mean arterial pressure, heart rate, and adrenal venous blood flow obtained before and during splanchnic nerve stimulation are summarized in Table 1. During the first stimulation period, both adrenal venous catecholamine concentrations and blood flow rose in response to the three successive stimuli following a frequency-dependent manner, reaching a statistically significant level at the frequencies of 2 and 20 Hz. PACAP-38-ir concentrations also increased in a frequency-dependent manner and reached a significant level at the highest frequency tested (20 Hz), at which the concentration of PACAP-38-ir was approximately twofold higher than the basal value (Table 1). Aortic blood catecholamine and PACAP-38-ir concentrations and hemodynamic pa-
Consequently, the output of adrenal catecholamines and PACAP-38-ir increased in a similar profile to that observed in their concentrations (Fig. 1). The catecholamine and PACAP-38-ir responses to the direct splanchnic nerve stimulation returned to their respective basal control levels by 30 min after the cessation of the first block of stimulation. These secretory responses were highly reproducible upon repetition of the subsequent stimulation given with an interval of 30 min (PAC38AV, adrenal venous PACAP-38-ir (pg/ml); PAC38AO, aortic PACAP-38-ir (pg/ml); EpiAV, adrenal venous epinephrine (ng/ml); NEAV, adrenal venous norepinephrine (ng/ml); MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min); BFAV, adrenal venous blood flow (ml/2 min); *P < 0.05 compared with corresponding control values).

Values are means ± SE obtained from 6 dogs. Samples were taken before (basal) and during splanchnic nerve stimulation at 0.2, 2, and 20 Hz. S1 and S2, 1st and 2nd stimulation given immediately after corresponding control; stimulation periods S1 and S2 were given with an interval of 30 min. PAC38AV, adrenal venous PACAP-38-ir (pg/ml); PAC38AO, aortic PACAP-38-ir (pg/ml); EpiAV, adrenal venous epinephrine (ng/ml); NEAV, adrenal venous norepinephrine (ng/ml); EpiAO, aortic epinephrine (ng/ml); NEAO, aortic norepinephrine (ng/ml); MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min); BFAV, adrenal venous blood flow (ml/2 min). *P < 0.05 compared with corresponding control levels.

### Table 1. Changes in plasma PACAP-38-ir and catecholamine concentrations and hemodynamic parameters before and during direct splanchnic nerve stimulation in dogs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Basal</th>
<th>0.2 Hz</th>
<th>2.0 Hz</th>
<th>20 Hz</th>
<th>Basal</th>
<th>0.2 Hz</th>
<th>2.0 Hz</th>
<th>20 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC38AV</td>
<td>10.2 ± 1.2</td>
<td>12.1 ± 1.6</td>
<td>15.0 ± 2.1</td>
<td>19.6 ± 2.7a</td>
<td>10.7 ± 2.3</td>
<td>11.5 ± 3.2</td>
<td>18.0 ± 4.6</td>
<td>20.4 ± 2.2a</td>
</tr>
<tr>
<td>PAC38AO</td>
<td>7.2 ± 1.1</td>
<td>8.4 ± 2.3</td>
<td>7.5 ± 0.8</td>
<td>5.9 ± 1.4</td>
<td>7.0 ± 1.0</td>
<td>7.6 ± 0.7</td>
<td>8.5 ± 1.3</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>EpiAV</td>
<td>21.2 ± 8.6</td>
<td>36.0 ± 9.0</td>
<td>312.9 ± 101.2a</td>
<td>823.6 ± 297.2a</td>
<td>18.7 ± 6.1</td>
<td>51.4 ± 11.8</td>
<td>499.6 ± 310.3a</td>
<td>1,105.5 ± 298.8a</td>
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<td>NEAV</td>
<td>3.4 ± 1.5</td>
<td>4.7 ± 1.3</td>
<td>60.1 ± 17.4a</td>
<td>200.5 ± 61.0a</td>
<td>2.9 ± 1.4</td>
<td>6.7 ± 1.7</td>
<td>87.5 ± 35.4a</td>
<td>260.4 ± 70.2a</td>
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<tr>
<td>EpiAO</td>
<td>0.08 ± 0.02</td>
<td>0.07 ± 0.02</td>
<td>0.07 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.07 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.02</td>
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<tr>
<td>NEAO</td>
<td>0.16 ± 0.02</td>
<td>0.20 ± 0.05</td>
<td>0.23 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.22 ± 0.03</td>
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<td>MAP</td>
<td>157.6 ± 5.5</td>
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<td>142.3 ± 6.8</td>
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<td>139.8 ± 6.3</td>
<td>141.9 ± 6.7</td>
<td>143.9 ± 6.2</td>
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<tr>
<td>HR</td>
<td>156.3 ± 2.6</td>
<td>159.8 ± 5.2</td>
<td>159.7 ± 5.1</td>
<td>156.8 ± 4.8</td>
<td>158.8 ± 4.8</td>
<td>158.2 ± 4.9</td>
<td>157.2 ± 5.0</td>
<td>157.2 ± 5.0</td>
</tr>
<tr>
<td>BFAV</td>
<td>8.1 ± 1.1</td>
<td>8.3 ± 1.0</td>
<td>8.9 ± 1.0a</td>
<td>10.1 ± 1.1a</td>
<td>7.3 ± 1.1</td>
<td>7.4 ± 1.1</td>
<td>8.2 ± 1.1a</td>
<td>9.4 ± 1.2a</td>
</tr>
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### Responses to a severe hypotension

In the group receiving the acute infusion of SNP alone, the administration of SNP caused an immediate and marked decrease in mean arterial pressure (Fig. 2, A and B). This increase in catecholamine output was rapid, and the peak response was observed during the infusion. The increased catecholamine output returned to the corresponding control levels ~10 min after the cessation of infusion. Catecholamine responses to PACAP-27 during severe hypotension. An important fall in mean arterial pressure occurred immediately after the bolus intravenous injection of SNP after a similar profile to that obtained in dogs receiving SNP alone. The adrenal catecholamine output increased markedly in response to severe hypotension with a concomitant local infusion of PACAP-27 (Fig. 2, A and B). Aortic catecholamine concentrations and heart rate also increased, whereas adrenal venous blood flow did not change significantly (Table 2).

The changes in the net catecholamine responses, defined as delta (Δ) catecholamine output, observed during the infusion of the vehicle alone, the SNP-induced hypotension, the PACAP-27 alone, and the SNP-induced hypotension combined with the local PACAP-27 infusion are summarized in Fig. 3. Those catecholamine responses were calculated from the peak responses obtained during the first 5 min after SNP injection and/or PACAP-27 infusion. In the presence of PACAP-27, the maximum increase in catecholamine secretion during SNP-induced hypotension was significantly greater than that observed in the group receiving either SNP or PACAP-27 alone (Fig. 3). Furthermore, a two-way analysis of variance performed on these maximum responses revealed the presence of a significant interaction between the SNP- and the PACAP-mediated catecholamine responses (P = 0.005 for epinephrine; P = 0.039 for norepinephrine), indicating a synergistic interaction between these two factors.

### Responses to severe hypotension in dogs with left adrenal denervation

In the group receiving the acute left adrenal denervation, a bolus intravenous injection of SNP caused an immediate and marked decrease in mean arterial pressure (Fig. 2, A and B). This increase in catecholamine output was rapid, and the peak response was observed during the infusion. The increased catecholamine output returned to the corresponding control levels ~10 min after the cessation of infusion. Catecholamine responses to PACAP-27 during severe hypotension. An important fall in mean arterial pressure occurred immediately after the bolus intravenous injection of SNP after a similar profile to that obtained in dogs receiving SNP alone. The adrenal catecholamine output increased markedly in response to severe hypotension with a concomitant local infusion of PACAP-27 (Fig. 2, A and B). Aortic catecholamine concentrations and heart rate also increased, whereas adrenal venous blood flow did not change significantly (Table 2).

The changes in the net catecholamine responses, defined as delta (Δ) catecholamine output, observed during the infusion of the vehicle alone, the SNP-induced hypotension, the PACAP-27 alone, and the SNP-induced hypotension combined with the local PACAP-27 infusion are summarized in Fig. 3. Those catecholamine responses were calculated from the peak responses obtained during the first 5 min after SNP injection and/or PACAP-27 infusion. In the presence of PACAP-27, the maximum increase in catecholamine secretion during SNP-induced hypotension was significantly greater than that observed in the group receiving either SNP or PACAP-27 alone (Fig. 3). Furthermore, a two-way analysis of variance performed on these maximum responses revealed the presence of a significant interaction between the SNP- and the PACAP-mediated catecholamine responses (P = 0.005 for epinephrine; P = 0.039 for norepinephrine), indicating a synergistic interaction between these two factors.
However, both outputs and plasma concentrations of epinephrine and norepinephrine increased only slightly in response to the severe hypotension (Fig. 4A and B, and Table 3). The maximum net catecholamine outputs during SNP-induced hypotension were significantly attenuated by ~85% (P = 0.045 for epinephrine; P = 0.021 for norepinephrine) in the group receiving the acute left adrenal denervation compared with those obtained in dogs with the normal left adrenal innervation (Fig. 5A and B). Aortic epinephrine and norepinephrine concentrations and heart rate also increased in response to severe hypotension (Table 3). Adrenal venous blood flow decreased significantly during the SNP challenge (Table 3).

The aims of the present study were to evaluate if endogenous PACAP could actually be released into the adrenal venous effluent during direct splanchnic nerve stimulation in vivo and to determine whether the presence of exogenous PACAP in the adrenal gland could locally modulate the adrenomedullary function during sympathoadrenal reflex activation in anesthetized dogs. Direct splanchnic nerve stimulation caused significant increases in the release of both adrenal catecholamines and PACAP-38-ir in a frequency-dependent manner, showing strong correlations between the output of catecholamines and PACAP-38-ir. The results also indicate that the adrenal catecholamine response to the SNP-induced severe hypotension was significantly enhanced in a synergistic manner in the presence of PACAP-27 administered locally to the adrenal gland, showing a facilitating interaction between these two factors.

In the present study, we measured endogenous PACAP-38-ir in the local adrenal venous blood under the mean arterial pressure (Fig. 4C).
basal conditions as well as during direct splanchnic nerve stimulation, because the tissue distribution of endogenous PACAP-38 predominates over the other isomer (2) and because immunoreactive PACAP has been localized to splanchnic nerve fibers innervating chromaffin cells in the adrenal medulla (6, 12, 26, 27).

Our present data show for the first time in vivo that endogenous PACAP-38 could be released into the adrenal venous effluent during direct splanchnic nerve stimulation following a frequency-dependent manner.

### Table 2. Changes in plasma catecholamine concentrations and hemodynamic parameters in dogs receiving either SNP or PACAP-27 alone or a combination of both treatments

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Basal</strong></td>
<td><strong>Peak change (0–5 min)</strong></td>
<td><strong>Basal</strong></td>
<td><strong>Peak change (0–5 min)</strong></td>
</tr>
<tr>
<td>EpiAV</td>
<td>12.1 ± 3.9</td>
<td>7.1 ± 3.0</td>
<td>16.0 ± 9.3</td>
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<tr>
<td>NEAV</td>
<td>1.9 ± 0.9</td>
<td>1.2 ± 0.5</td>
<td>1.9 ± 0.7</td>
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<tr>
<td>EpiAO</td>
<td>0.09 ± 0.06</td>
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<td>0.20 ± 0.02</td>
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<td>MAP</td>
<td>131.2 ± 12.0</td>
<td>128.6 ± 8.0</td>
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<tr>
<td>HR</td>
<td>167.7 ± 7.3</td>
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<td>BFAV</td>
<td>3.4 ± 0.5</td>
<td>3.9 ± 0.4</td>
<td>3.8 ± 0.4</td>
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</tbody>
</table>

Values are means ± SE. Gr, group; n, number of dogs in each group; SNP, sodium nitroprusside; EpiAV, ng/ml; NEAV, norepinephrine ng/ml; EpiAO, ng/ml; NEAO, ng/ml; MAP, mmHg; HR, beats/min; BFAV, ml/min. *P < 0.05 compared with corresponding control values.

Fig. 3. Maximum net increases (Δ) in Epi and NE output obtained during the first 5 min after SNP administration and/or PACAP-27 infusion. Maximum net responses in each group were calculated with the data obtained from the second series of experiments. A significant interaction exists between PACAP-27 and SNP. *P < 0.05, 2-way analysis of variance.

Fig. 4. Adrenal Epi (A) and NE (B) output and MAP (C) in response to a single bolus dose of SNP given intravenously in the groups with either normal innervated (IG; n = 8) or acute denervated left adrenal gland (SNX; ♂ n = 8). Arrow, injection of SNP. *P < 0.05 compared with corresponding initial values for each group (open symbols).
Table 3. Changes in plasma catecholamine concentrations and hemodynamic parameters in dogs with left adrenal denervation receiving SNP

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Peak change (0–5 min)</th>
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<tbody>
<tr>
<td></td>
<td>Epi_{AV}</td>
<td>NE_{AV}</td>
</tr>
<tr>
<td></td>
<td>10.52 ± 0.91</td>
<td>34.14 ± 12.57*</td>
</tr>
<tr>
<td></td>
<td>1.57 ± 0.54</td>
<td>5.47 ± 2.06*</td>
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<tr>
<td></td>
<td>0.07 ± 0.02</td>
<td>0.57 ± 0.19*</td>
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<tr>
<td></td>
<td>0.14 ± 0.29</td>
<td>0.44 ± 0.08*</td>
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<tr>
<td></td>
<td>137.8 ± 6.5</td>
<td>44.8 ± 4.4*</td>
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<tr>
<td></td>
<td>164.0 ± 7.7</td>
<td>195.1 ± 12.0*</td>
</tr>
<tr>
<td></td>
<td>2.9 ± 0.2</td>
<td>1.5 ± 0.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. SNX, left adrenal denervation; n, number of dogs; Epi_{AV}, ng/ml; NE_{AV}, ng/ml; Epi_{AO}, ng/ml; NE_{AO}, ng/ml; MAP, mmHg; HR, beats/min; BF_{AV}, ml/min. *P < 0.05 compared with corresponding control values.

and reaching a statistical significance with the highest frequency (20 Hz) tested. This in vivo finding is consistent with previous in vitro observations in that the release of endogenous PACAP-38 during either transmural or direct nerve stimulation occurred only when the relatively high frequencies (10–16 Hz) were applied in the isolated, perfused rat (30) and porcine (27) adrenal gland. In addition, we also found in this study that the stimulation-induced release of PACAP-38-ir could be closely correlated with that of epinephrine and norepinephrine under in vivo conditions. Although the adrenal catecholamine secretory response is primarily controlled by the classical neurotransmitter ACh (12), the present observation may imply the existence of functional interaction in the adrenal gland between endogenous PACAP and catecholamine secretions by modulating the cholinergic neurotransmission during the sympathoadrenal activation in certain physiological situations (12, 15).

Under certain conditions associated with stress, such as severe hypotension, a reflex activation of the sympathoadrenal system occurs to rapidly restore the cardiovascular homeostasis (16). In the present experiment, the intravenous injection of SNP caused a rapid and severe fall in mean arterial blood pressure associated with an immediate and significant rise in the adrenal catecholamine secretion. It has been well documented that the hypotensive effects of intravenous SNP are due to peripheral vasodilatation and reduction in peripheral resistance as a result of the action of released nitric oxide on the vascular beds (1, 28). The immediate adrenomedullary response to the SNP-induced hypotension, therefore, may have resulted from the baroreflex-mediated sympathoadrenal stimulation, because the left adrenal catecholamine response was diminished by ~85% in dogs receiving the acute surgical denervation of the left adrenal gland. With respect to the validity of the acute surgical denervation used in the present study, we previously showed that the centrally mediated adrenomedullary response to bilateral carotid occlusion was almost completely diminished in the denervated left adrenal gland (35). In the present study, plasma catecholamine concentrations in aortic blood significantly increased during the SNP-induced hypotension in dogs with the denervated left adrenal gland. This increase in circulating catecholamines is due most probably to the increased catecholamine output from the right adrenal gland that has been kept normally innervated. Thus the lack of the catecholamine response in the left denervated adrenal gland during the severe hypotension ensures the functional existence of the splanchnic neural pathway mediating the medullary secretion during the reflex activation of the sympathoadrenal system.

Although the two PACAP isoforms are very potent secretagogues in the adrenal medulla both in vitro and in vivo (29), we previously showed that under the similar experimental conditions, the amplitude of adrenal catecholamine response to PACAP-27 was at least three times greater than that obtained with PACAP-38 on the same molar basis in anesthetized dogs (11). In the present experiments, we therefore used PACAP-27 to ensure the adrenal catecholamine response to PACAP. In dogs receiving PACAP-27 alone, the local administration of this peptide to the normally innervated adrenal gland resulted in marked increases in the catecholamine secretions without significantly affecting the mean arterial blood pressure. The catecholamine response to PACAP-27 resulted most probably from its specific action on the PAC_{1} receptor, as the presence of PACAP-6–27, a selective

Fig. 5. Maximum net increases in Epi and NE output obtained during the first 5 min after SNP administration. Maximum net response in the group with either IG or SNX left adrenal gland was calculated with the data obtained from the 2nd and 3rd series of experiments, respectively. *P < 0.05 between IG and SNX.
PAC1 receptor antagonist (22, 23), in the adrenal gland almost completely blocked the PACAP-27-induced catecholamine response under similar experimental conditions in anesthetized dogs (18). Even if PACAP-6–27 may have certain affinity for VPAC2 receptor as in the case of PACAP-6–38 (13), this possibility does not seem to have direct relevance to the adrenal gland in which the PAC1 receptor is almost exclusively expressed (19). Moreover, the observation that arterial blood pressure did not significantly change during PACAP-27 infusion is consistent with the view that the PACAP-27-induced increase in catecholamine secretion resulted from its local action on the adrenal medulla and not from secondary reflexive effects due to hypotension that could also be produced by PACAP when administered intravenously (24, 25).

Adrenal venous blood flow remained statistically unchanged during the concomitant administration of PACAP-27 and SNP, whereas it increased with PACAP-27 alone and decreased with SNP alone. It has been demonstrated that in anesthetized dogs, SNP-induced hypotension with a decrease of mean arterial pressure of ~55% caused a significant reduction of blood flow in the ascending aorta, celiac, superior mesenteric, renal, and iliac arteries (3). In the light of these observations, the severe hypotension (~60% of control) provoked in the group receiving SNP alone might have induced a similar reduction of blood flow in the abdominal region, ultimately resulting in a diminished blood supply to the adrenal gland. On the other hand, PACAP is also known to be a potent vasodilator (24, 25). In the present study, however, PACAP-27 was locally administered to the adrenal gland without producing any significant systemic effects. Because PACAP is such a potent vasodilator, the elevation in adrenal blood flow during a single infusion of PACAP-27 alone may have resulted from its local vasodilating effect. Therefore, when both SNP and PACAP-27 were administered concomitantly through their respective routes (intravenously and local arterial infusion, respectively), it is most likely that PACAP-27 evoked a local adrenal vasodilatation that counteracted a decrease in adrenal blood supply due to the SNP-induced severe hypotension. The net result of both effects is finally illustrated by a null gain in the adrenal venous blood flow.

The synergistic effect of PACAP-27 on the adrenal catecholamine secretion, observed in the present study, resulted most probably from the local interaction of PACAP-27 with the operative splanchnic neurotransmission largely mediated by neural ACh in the adrenal medulla during the SNP-induced hypotension. In this context, we previously showed that in a similar experimental setup, locally applied PACAP-27 synergistically enhanced the adrenal catecholamine secretion induced by either direct splanchnic nerve stimulation or the local administration of ACh to the adrenal gland in anesthetized dogs (17). Taken together, the previous and present observations suggest that the presence of PACAP in the adrenal medulla enhances adrenal catecholamine secretion either by facilitating the release of neural ACh or by postsynaptic multiple intracellular interactions with various second messengers (31). Although a potential PACAP-induced presynaptic facilitation of ACh release has been suggested by the enhanced cardiac parasympathetic cholinergic neurotransmission in anesthetized dogs (14, 25), the site of interaction in the canine adrenal gland cannot precisely be determined in the present study.

With respect to a potentially functional role of either endogenous PACAP-27 or PACAP-38, both peptides would theoretically play a similar role of facilitating the catecholamine secretion, because both peptides have the same binding affinity to PAC1 receptor (13). The relative potency, however, as judged from the catecholamine releasing effects of exogenous PACAP-27 and PACAP-38 on the canine adrenal gland (11), must be greater with PACAP-27 if the latter peptide can be actually released along with PACAP-38. Nevertheless, it has been reported that, in the porcine adrenal gland in vitro, the tissue content of PACAP-27 was found to be only 1% of that of PACAP-38 and that, perhaps more importantly, PACAP-27 could not be detected in the adrenal venous perfusate after direct splanchnic nerve stimulation (27). As endogenous PACAP-38 could be released into the venous effluent in the porcine adrenal in vitro (27) as well as in the canine adrenal in vivo during direct splanchnic nerve stimulation as observed in this study, it is conceivable that PACAP-38 rather than PACAP-27 may play a role of a neuromodulator to facilitate the sympathoadrenal activity in a certain physiopathological situation. Nevertheless, a possible implication of endogenous PACAP-27 in the canine adrenal gland in vivo cannot completely be excluded in the present study.

Under the present experimental conditions, however, the magnitude of adrenal catecholamine release during the SNP-induced hypotension is roughly equivalent to or even smaller than that obtained at 2 Hz in the group receiving direct splanchnic nerve stimulation. On the basis of this rather over-simplified comparison, it appears unlikely that a significant amount of endogenous PACAP can be released from splanchnic nerve endings during severe hypotension. On the other hand, it must be noted that the concentration of endogenous PACAP-38 found in this study was obtained in the adrenal venous effluent, leaving a possibility that its intrasynaptic concentration at the adrenomedullary synapse may be much more elevated to an extent sufficient to modulate the cholinergic neurotransmission. Also, it has been well documented that the general anesthesia, including the one with pentobarbital sodium, considerably blunts peripheral responses to the baroreceptor-mediated reflexes (5). Under a less suppressive anesthesia, the SNP-induced hypotension may have generated a greater sympathoadrenal response, a condition under which endogenous PACAP may be preferentially released from the splanchnic nerves.
In conclusion, the present study demonstrates that endogenous PACAP-38 can be released in the adrenal medulla during direct splanchnic nerve stimulation at a preferably high frequency under in vivo conditions. The study also indicates that the presence of exogenous PACAP-27 in the adrenal gland potentiates in a synergistic manner the catecholamine secretion during SNP-induced severe hypotension. Taken together, these results are compatible with the view that PACAP may play a functional role as a local neuromodulator in facilitating or sustaining adrenal catecholamine secretion during severe activation of the sympathoadrenal system. However, the actual pathophysiological implication of endogenous PACAP-38 remains to be further investigated under various reflex-induced sympathoadrenal stimulations.

Perspectives

In the present study, we used SNP as a pharmacological tool to induce a rapid and severe hypotension that provoked a strong baroreflex-mediated activation of the sympathoadrenal system. As the SNP-induced hypotension results from a decrease in peripheral resistance due to the vasodilating effect of nitric oxide released from the SNP molecule (1), it is conceivable that the released nitric oxide may affect the multiple mechanisms controlling the adrenal catecholamine secretion. In this context, it has been shown that in the isolated, perfused canine adrenal gland, endogenous nitric oxide inhibited the nicotine-induced catecholamine release (4). In contrast, however, nitric oxide produced a large stimulation of the basal catecholamine secretion as well as the evoked catecholamine release induced by low concentrations of nicotine in cultured bovine chromaffin cells (21). Therefore, parallel to the present study, we tested the direct in vivo effect on the adrenal gland of SNP locally administered in the same way as in the case of PACAP-27 infusion. Within the dose range of SNP (0.065–6.5 μg per adrenal gland) there was no significant direct effect on the basal adrenal catecholamine secretion in anesthetized dogs. With the highest dose tested (6.5 μg per adrenal gland), however, the basal catecholamine secretion increased slightly due to the indirect, baroreflex-induced activation of the sympathoadrenal system. Thus nitric oxide released from the SNP molecule does not directly affect the basal catecholamine secretion in the canine adrenal gland under in vivo conditions. This observation is consistent with a previous study that found, in the canine adrenal gland in vivo, another nitric oxide donor, NOC-7, had no direct effect on the basal adrenal catecholamine secretion (20). Nevertheless, either the potentially facilitating or inhibiting effect of SNP-derived nitric oxide on the PACAP-27-induced adrenal catecholamine secretion remains to be determined under conditions where the operative sympathoadrenal system is absent to define whether the observed synergism results from the interaction either with the neural factors or with the SNP-derived nitric oxide.

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