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S. Lamouche and N. Yamaguchi

*Am J Physiol Regulatory Integrative Comp Physiol*, February 1, 2001; 280 (2): R510-R518.

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# Modulation of adrenal catecholamine release by PACAP in vivo

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**Lamouche, Stéphane, Daniel Martineau, and Nobuharu Yamaguchi.** Modulation of adrenal catecholamine release by PACAP in vivo. *Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R162–R170, 1999.*—The aim of the present study was to investigate whether pituitary adenylate cyclase-activating polypeptide-(1–27) (PACAP27) can modulate the adrenal catecholamine (CA) secretion induced by splanchnic nerve stimulation (SNS) and by exogenous acetylcholine (ACh) in anesthetized dogs. Plasma CA concentrations in adrenal venous and aortic blood were quantified by a high-performance liquid chromatography coupled with electrochemical detection. Adrenal venous blood flow was measured by gravimetry. Local infusion of PACAP27 (0.5, 5, and 50 ng) to the left adrenal gland via the adrenolumbar artery resulted in an increase in CA output, reaching a significant level at the highest dose tested. Either direct SNS (2 Hz) or local infusion of ACh (0.5 µg) to the left adrenal gland produced significant increases in CA output to an extent similar to that obtained with 50 ng of PACAP27 alone. In the presence of PACAP27 (50 ng), CA responses to either SNS or exogenous ACh were significantly potentiated by approximately four- and sixfold, respectively, compared with those obtained in response to each stimulus alone. However, the enhanced CA responses to ACh were not significantly different from those to SNS. The results indicate that the increase in adrenal CA secretion, induced by either direct SNS or exogenous ACh, is synergistically enhanced by PACAP27. The study suggests that the enhanced CA secretion may result from the activation of a PACAP-mediated facilitatory mechanism(s) localized presumably at the postsynaptic level in the canine adrenal medulla in vivo, although the possible involvement of presynaptic mechanisms cannot completely be ruled out in the present study.

pituitary adenylate cyclase-activating polypeptide-(1–27); splanchnic nerve; acetylcholine; dog; potentiation; medullary secretion

PITUITARY ADENYLATE cyclase-activating polypeptide (PACAP) is a member of the family of peptides involving vasoactive intestinal polypeptide (VIP), secretin, and glucagon. It was first isolated by Miyata et al. in 1989 (13) from ovine hypothalamic tissues on the basis of its ability to stimulate adenylate cyclase. There exist two isoforms composed of 27 and 38 amino acids residues (PACAP27 and PACAP38), both of which are widely distributed in the peripheral and central nervous systems (1). PACAP has a variety of biological activities, including hormone production and secretion in the pituitary gland (6, 13), thyroid gland (2), and

pancreas (26). More recently, the presence of PACAP has also been demonstrated in the rat adrenal gland (1, 20), in which the expression of PACAP type I receptor is the most pronounced (14, 19, 20). In cultured porcine (8) and rat (24) adrenal chromaffin cells and rat adrenal gland in vivo (25), PACAP has been shown to increase the basal catecholamine secretion. More recently, we have also demonstrated that, in anesthetized dogs, both PACAP27 and PACAP38 locally administered to the adrenal gland resulted in a dose-dependent increase in the basal catecholamine secretion (5). Furthermore, PACAP immunoreactive nerve fibers ending on chromaffin cells have been identified in the rat adrenal gland (3, 8, 14). PACAP was also shown to be released in response to splanchnic nerve stimulation (SNS) in isolated, perfused rat adrenal gland (22). These observations suggest that PACAP may play a role of cotransmitter released along with acetylcholine (ACh) during stimulation of the splanchnic nerve. However, the potential interaction of PACAP with the splanchnic nerve activation and the resulting catecholamine secretion in the adrenal gland remains mostly unknown under in vivo conditions. The specific aim of the present study was to investigate whether the presence of PACAP27 could modulate the adrenal catecholamine secretion induced either by direct electrical stimulation of the splanchnic nerve or by exogenously administered ACh in anesthetized dogs.

## METHODS

*Preparation of animals.* Adult male 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; Harvard, South Natick, MA). The rectal temperature was monitored and kept constant at  $37.5 \pm 0.5^\circ\text{C}$  by means of a thermoregulator (model 74; Yellow Springs Instruments, Yellow Springs, OH) connected to a heating pad. Both 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 of direct SNS.* After a median laparotomy and a left flank incision, the left splanchnic nerve was dissected free from surrounding tissues, firmly double ligated ~2 cm from the adrenal gland, and protected from dryness by applying mineral oil. Direct electrical stimulation was applied to the distal ligated end of this nerve. All of 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 stimulation of the left splanchnic nerve (4, 29). This denervation procedure was also applied in the second series of experiments in which the secretagogue effect of

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exogenous ACh was tested in the absence and presence of PACAP27.

*Preparation of local intra-arterial drug infusion to the left adrenal gland.* The experimental model used in this study has previously been validated in our laboratory and reported in full detail elsewhere (27). Briefly, after the left adrenal denervation, the left adrenolumbar artery was dissected free from surrounding tissues and cannulated in a retrograde manner, i.e., from the peripheral end toward the gland and aorta. The catheter (PE-90) was then advanced so that the tip of the catheter reached underneath the gland or to a level close to the adrenolumbar arterial-aortic junction. The volume of the catheter was fixed to be 0.5 ml, and the catheter was connected to an infusion pump (model 1140-001; Harvard). For the second series of experiments, a smaller size of catheter (PE-50) was used, but the volume of this catheter was also fixed to be 0.5 ml, and the catheter was connected to a double-infusion pump (model 55-2226; Harvard).

*Preparation of an extracorporeal adrenal venous circuit.* A polyethylene catheter (PE-240), one end of which was specially shaped to allow an easy cannulation, was inserted in the left adrenolumbar vein through the left femoral vein. The catheter was tied at the adrenoabdominal vena caval junction to prevent dilution of adrenal venous blood with abdominal vena caval blood. The left adrenolumbar vein distal to the gland was ligated to obtain actual adrenal venous blood. Venous blood from the gland was drained in a small blood reservoir filled with physiological saline. The volume of the catheter was fixed to be 1.5 ml. Blood volume in the reservoir was kept as small as possible with the use of an automatic blood level controller connected to a perfusion pump (Masterflex model 7016-52; Cole-Parmer Instrument, Chicago, IL). This served to return adrenal venous blood through a catheter inserted in the right femoral vein at a perfusion rate adjusted as closely as possible to a stabilized initial venous blood flow (27). After all surgical procedures were completed, sodium heparin (200 U/kg iv) was administered, followed by 100 U/kg every hour thereafter. The dog was 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 were simultaneously sampled into graded, chilled tubes for catecholamine analyses. Adrenal venous blood flow was determined by a gravimetric method at each sampling time point (27). Hematocrit was measured in all adrenal venous blood samples. Blood (1.5 ml) was transferred to a centrifuge tube containing 30  $\mu$ l of preservative solution (pH 6.5) consisting of EGTA (95 mg/ml) and glutathione (60 mg/ml). 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 assay. 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 "Coulochem II" (model 5200; ESA, Bedford, MA; see Ref. 27). At the end of each experiment, the left adrenal gland was removed and weighed. Adrenal catecholamine data were expressed in net catecholamine output calculated as follows: net output of adrenal catecholamine ( $\text{ng} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ) =  $([\text{CA}]_{\text{AV}} - [\text{CA}]_{\text{AO}}) \times \text{BF}_{\text{AV}} \times (1 - \text{Hct}_{\text{AV}}) / \text{wet weight of gland}$ , where  $[\text{CA}]_{\text{AV}}$  is plasma catecholamine concentration in adrenal venous blood,  $[\text{CA}]_{\text{AO}}$  is plasma catecholamine concentration in aortic blood,  $\text{BF}_{\text{AV}}$  is adrenal venous blood flow, and  $\text{Hct}_{\text{AV}}$  is adrenal venous blood hematocrit.

*Experimental protocol.* The present study consisted of two series of experiments. The first series was to investigate the effect of PACAP27 on the catecholamine secretion in response to a direct SNS. The second series was to evaluate whether the PACAP27-induced adrenal catecholamine release can be modified in the presence of exogenous ACh.

The first series involved four groups. The first group ( $25.6 \pm 1.9$  kg,  $n = 6$ ) received a vehicle and sham stimulation and served as the control group. One minute after the initial control sample was taken, a vehicle (saline 0.9%, pH 7.38) was infused into the left adrenolumbar artery at a rate of 0.5 ml/min. The dead volume of the adrenal arterial (0.5 ml) and venous (1.5 ml) catheter was taken into account in relation to the infusion rate and adrenal venous blood flow, respectively. Therefore, the infusion period was for 2 min: the first minute for flushing the existing saline previously filled in the catheter and the second minute for infusing the vehicle or drug (the net infusion). An adrenal venous blood sample was obtained during this net infusion period (0-1 min) with a delay depending on the venous blood flow, followed by sample collections during 1-2, 2-3, 5-6, 10-11, and 15-16 min after the onset of net 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 collections at these sampling time points. Sample obtained 15 min after the vehicle infusion served as control for the second administration of the vehicle, and then the same protocol was repeated for the second, third, and fourth administration of the vehicle.

The second group ( $26.1 \pm 0.7$  kg,  $n = 6$ ) received the vehicle and three different doses of PACAP27 (0.5, 5, and 50 ng with concentrations of 1, 10, and 100 ng/ml, respectively; Sigma Chemical, St. Louis, MO) along with sham stimulation with an interval of 15 min. 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 the first three samples of the adrenal venous blood obtained during and after the net infusion, the remaining blood was not returned to the dog to prevent potential systemic hemodynamic changes due to the released catecholamines. This precaution was also applied in the group receiving either the vehicle, ACh, and SNS.

In the third group ( $31.5 \pm 2.3$  kg,  $n = 6$ ), the distal end of the tightly ligated left splanchnic nerve was stimulated with bipolar platinum electrodes by rectangular pulses of 2-ms duration and supramaximal voltage (12 V) at a fixed frequency of 2 Hz for 1 min by means of an electronic stimulator (model SEN-3301; Nihon Kohden). The SNS was repeated four times at 15 min-intervals. After taking initial controls, blood samples were taken during SNS (0-1) followed by sample collections during 1-2, 2-3, 5-6, 10-11, and 15-16 min after the onset of stimulation. The vehicle (saline) was infused simultaneously during SNS following exactly the same procedure described in the first group. This procedure was repeated for the second, third, and fourth stimulations.

The fourth group ( $25.9 \pm 1.6$  kg,  $n = 6$ ) received SNS in the same way as described for the third group. After the infusion of the vehicle (saline) along with the first stimulation, 0.5, 5, and 50 ng of PACAP27 was administered along with the second, third, and fourth stimulation, respectively, following the same protocol described for the third group. The doses of PACAP27 and the frequency of stimulation used in this study were selected on the basis of our previous observations obtained under similar experimental conditions (4, 5).

The second series consisted of two separate groups. The first group ( $33.6 \pm 3.0$  kg,  $n = 5$ ) received two identical doses of ACh (0.5  $\mu$ g with concentration of 1.0  $\mu$ g/ml; Sigma

Table 1. Plasma catecholamine concentrations in adrenal venous and aortic blood, adrenal venous blood flow, adrenal venous hematocrit, mean aortic pressure, and heart rate in the group receiving PACAP27 during sham stimulation

Parameters	C1	SA 1 min	C2	PACAP (0.5 ng) 1 min	C3	PACAP (5 ng) 1 min	C4	PACAP (50 ng) 1 min
Epi <sub>AV</sub> , ng/ml	6.9 ± 2.4	6.0 ± 1.9	6.5 ± 2.1	8.2 ± 3.0	11.4 ± 5.4	18.5 ± 7.0	16.3 ± 9.1	147.5 ± 51.3*†
NE <sub>AV</sub> , ng/ml	0.9 ± 0.3	0.8 ± 0.2	1.0 ± 0.3	1.0 ± 0.3	1.6 ± 0.7	2.1 ± 1.0	2.0 ± 1.1	13.3 ± 4.1*†
Epi <sub>AO</sub> , ng/ml	0.24 ± 0.09	0.23 ± 0.12	0.23 ± 0.08	0.21 ± 0.08	0.30 ± 0.09	0.18 ± 1.23	0.28 ± 0.11	0.33 ± 0.14
NE <sub>AO</sub> , ng/ml	0.29 ± 0.04	0.28 ± 0.04	0.27 ± 0.02	0.24 ± 0.02	0.29 ± 0.04	0.28 ± 0.04	0.31 ± 0.05	0.32 ± 0.03
BF <sub>AV</sub> , ml/min	3.4 ± 0.7	3.7 ± 0.7*	3.0 ± 0.6	3.5 ± 0.7*	2.9 ± 0.5	3.3 ± 0.6*	2.7 ± 0.4	3.4 ± 0.7*
Hct <sub>AV</sub> , %	51.7 ± 2.1	47.7 ± 2.2	51.7 ± 2.5	48.2 ± 2.2	52.0 ± 2.3	48.7 ± 2.2	52.5 ± 2.3	49.0 ± 2.4
MAP, mmHg	122.3 ± 3.65	123.3 ± 5.3	121.9 ± 6.0	122.2 ± 5.2	119.0 ± 5.6	121.1 ± 7.1	116.0 ± 7.0	118.8 ± 7.2
HR, beats/min	147.7 ± 4.5	147.0 ± 4.5	149.3 ± 6.2	149.7 ± 5.8	152.0 ± 5.6	151.0 ± 5.1	149.0 ± 6.7	147.7 ± 5.5

Values are means ± SE. PACAP, pituitary adenylate cyclase-activating polypeptide; Epi<sub>AV</sub>, adrenal venous epinephrine; NE<sub>AV</sub>, adrenal venous norepinephrine; Epi<sub>AO</sub>, aortic epinephrine; NE<sub>AO</sub>, aortic norepinephrine; BF<sub>AV</sub>, adrenal venous blood flow; Hct<sub>AV</sub>, adrenal venous hematocrit; MAP, mean aortic pressure; HR, heart rate. \* $P < 0.05$  versus corresponding control values (C1–C4) observed immediately before the infusion of saline (SA) or PACAP27 along with sham stimulation. † $P < 0.05$  vs. 3 preceding responses observed at 1 min.

Chemical) with an interval of 15 min between each infusion. One minute after the initial control sample was taken, the vehicle (saline) and ACh were infused simultaneously in the left adrenolumbar artery at a rate of 0.25 ml/min to obtain, when combined together, a final rate of 0.5 ml/min following the similar protocol described in the first series of experiments. Adrenal venous and aortic blood samples were similarly obtained following the same procedure described for the third group of the first series. Samples obtained 15 min after the onset of vehicle infusion served as control for the second administration of the vehicle and ACh. The second group (29.8 ± 1.9 kg,  $n = 5$ ) received a first infusion of ACh (0.5 µg) along with the vehicle (saline) and, 15 min later, received a second infusion of ACh (0.5 µg) combined with an infusion of PACAP27 (50 ng). The protocols for local drug administrations and blood sampling were exactly the same as described for the vehicle control group of this series. The dose of ACh was determined according to several dose-finding experiments with doses ranging from 0.05 to 5 µg. The dose of 0.5 µg was selected because the catecholamine responses to this dose were similar in magnitude to those induced by SNS at 2 Hz.

The experimental protocol has been approved by the animal research committee of the Université de Montréal. The animals used in this study have been cared for and used in accordance with the principles of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

**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 Dunnett's method. Comparisons of catecholamine responses to ACh before and after PACAP27 administration were made using the paired  $t$ -test. A two-way analysis of variance was conducted to assess possible interactions between PACAP27 and SNS as well as PACAP27 and exogenous ACh. When applicable, a preliminary logarithmic transformation was used to satisfy the condition of a normal distribution of variances (23). All results are expressed as means ± SE, and a  $P < 0.05$  was considered statistically significant.

## RESULTS

**Catecholamine secretion in response to PACAP27.** Control values observed during resting periods within

a given experimental period remained relatively stable, and the observed variations within the same subjects were not statistically significant in any group tested. Local infusions of the vehicle (saline) with an interval of 15 min into the left adrenolumbar artery did not significantly affect the basal output of adrenal catecholamine during a given experimental period of ~60 min. The basal plasma concentrations as well as the basal output of adrenal epinephrine and norepinephrine increased in response to the local administration of PACAP27, with doses of 0.5, 5, and 50 ng administered within an interval of 15 min. However, both epinephrine and norepinephrine responses to PACAP27 reached a statistically significant level only at the highest dose tested (Table 1 and Fig. 1A). The onset of catecholamine response to PACAP27 was rapid, and the increased catecholamine output returned to the corresponding control levels by ~10 min after the cessation of PACAP27 infusion (Fig. 1A). Plasma concentrations of epinephrine and norepinephrine in aortic blood, mean aortic pressure, heart rate, and adrenal venous hematocrit remained statistically unchanged throughout the experiment (Tables 1–3).

**Catecholamine secretion in response to SNS.** Both adrenal epinephrine and norepinephrine output were significantly elevated in response to the first stimulation period at a frequency of 2 Hz. The catecholamine responses to SNS were rapid and short lasting, so that the increased output returned to its prestimulation control value in ~3 min after the cessation of SNS (Fig. 1B). The increases in epinephrine and norepinephrine output in response to SNS were reproducible upon repetition of the subsequent three stimulations given with an interval of 15 min at the same frequency (Fig. 1B). The fourth catecholamine responses were slightly smaller in magnitude than those to the first stimulation, but there was no statistical difference among them.

**Effects of PACAP27 on SNS-induced catecholamine secretion.** The adrenal catecholamine responses to SNS during saline infusion were similar to those observed in the vehicle control group (Fig. 1C). The SNS-induced catecholamine responses during the simultaneous infu-

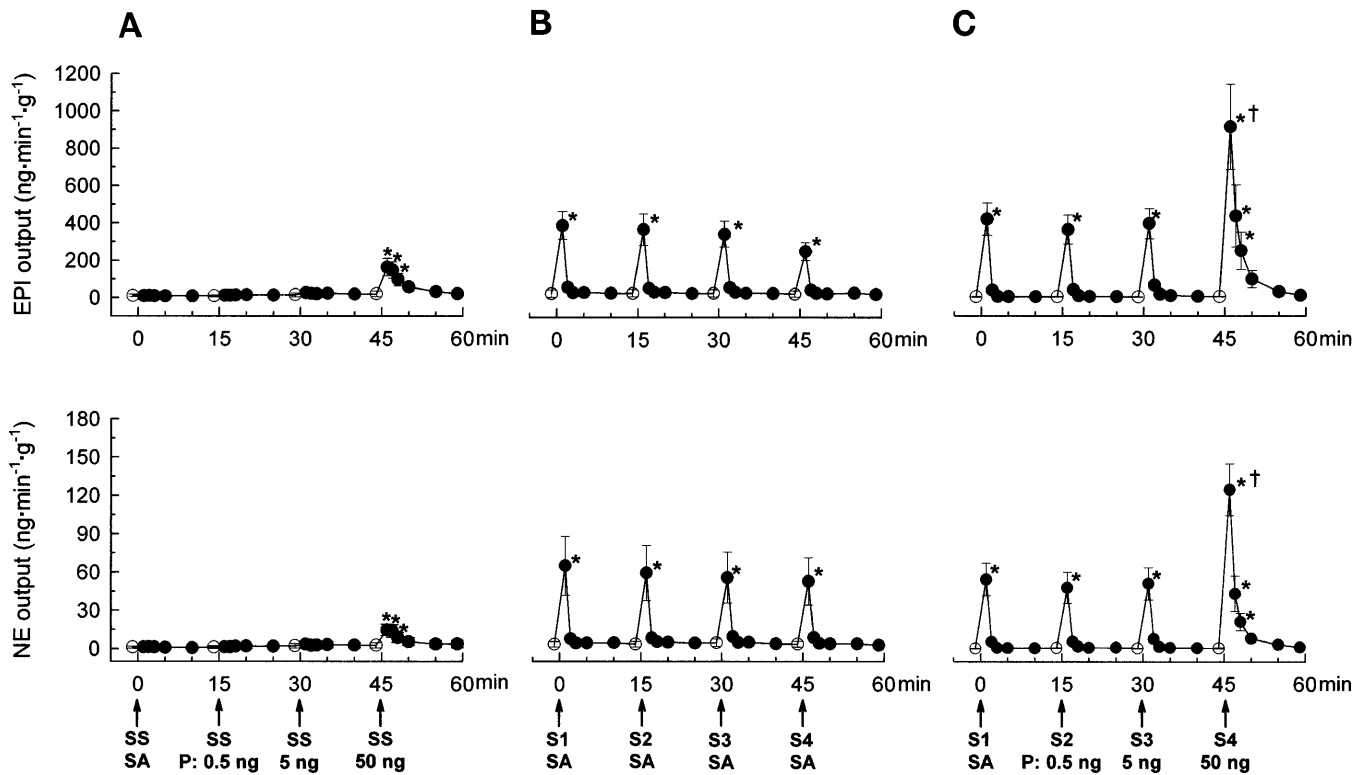


Fig. 1. Adrenal epinephrine (Epi) and norepinephrine (NE) output in response to the administration of pituitary adenylate cyclase-activating polypeptide-(1–27) [PACAP27 (P): 0.5, 5, and 50 ng] with sham stimulation (SS; A); to repetitive direct splanchnic nerve stimulation at 2 Hz, given at 0, 15, 30, and 45 min (S1–S4) in the group receiving the vehicle (saline, SA; B); and to the same stimulation in the group receiving PACAP27 (0.5, 5, and 50 ng) as indicated underneath arrows (C). Open circles indicate control values taken immediately before stimulation and administration of either saline or PACAP27. \*  $P < 0.05$  vs. corresponding control values; †  $P < 0.05$  vs. 3 preceding responses.

sion of PACAP27 with a dose of 0.5 and 5 ng followed a pattern similar to that observed in the SNS-saline control response. However, during the infusion of PACAP27 at a dose of 50 ng, the peak catecholamine responses of both epinephrine and norepinephrine output to SNS were significantly enhanced (Fig. 1C). These enhanced catecholamine output responses to SNS in the presence of PACAP27 were significantly higher than those observed during the three preceding stimulations (Fig. 1C). This holds true for changes in

plasma concentrations of both epinephrine and norepinephrine in adrenal venous blood, despite the fact that adrenal venous blood flow increased significantly (Table 2). The net changes in maximum catecholamine output during the infusion of saline alone, PACAP27 (50 ng) alone, the SNS (2 Hz) alone, and the SNS (2 Hz) plus PACAP27 (50 ng) infusion are summarized in Fig. 2. A two-way analysis of variance revealed that the SNS-induced catecholamine secretion in the presence of PACAP27 was significantly greater than that observed

Table 2. Plasma catecholamine concentrations in adrenal venous and aortic blood, adrenal venous blood flow, adrenal venous hematocrit, mean aortic pressure, and heart rate in the group receiving PACAP27 during splanchnic nerve stimulation

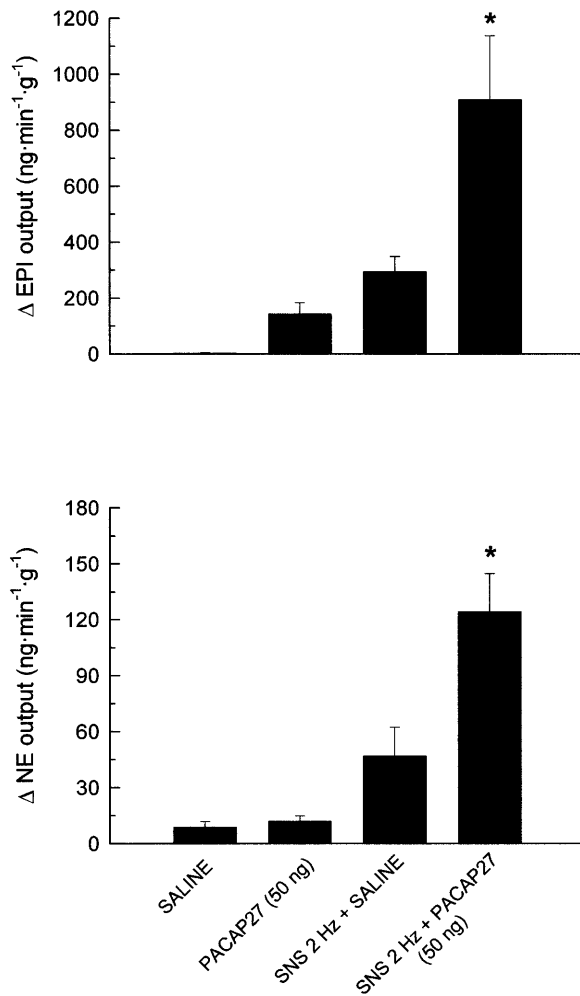
Parameters	C1	S1 + SA 1 min	C2	S2 + PACAP (0.5 ng) 1 min	C3	S3 + PACAP (5 ng) 1 min	C4	S4 + PACAP (50 ng) 1 min
Epi <sub>AV</sub> , ng/ml	4.1 ± 1.0	265.5 ± 70.5*	4.8 ± 1.3	244.8 ± 61.9*	3.8 ± 0.9	280.6 ± 68.8*	5.8 ± 1.8	639.2 ± 156.7*†
NE <sub>AV</sub> , ng/ml	0.7 ± 0.2	34.2 ± 9.5*	1.0 ± 0.2	30.6 ± 8.9*	0.8 ± 0.2	34.3 ± 9.1*	0.9 ± 0.2	82.2 ± 11.9*†
Epi <sub>AO</sub> , ng/ml	0.17 ± 0.03	0.11 ± 0.01	0.19 ± 0.05	0.08 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.15 ± 0.04	0.11 ± 0.01
NE <sub>AO</sub> , ng/ml	0.27 ± 0.06	0.27 ± 0.05	0.26 ± 0.05	0.22 ± 0.06	0.24 ± 0.07	0.46 ± 0.13	0.26 ± 0.08	0.33 ± 0.08
BF <sub>AV</sub> , ml/min	2.8 ± 0.3	3.9 ± 0.3*	2.8 ± 0.2	3.8 ± 0.2*	2.5 ± 0.1	3.6 ± 0.2*	2.5 ± 0.2	3.5 ± 0.2*
Hct <sub>AV</sub> , %	47.2 ± 3.1	45.3 ± 3.2	49.3 ± 3.0	46.0 ± 3.2	49.7 ± 3.0	45.8 ± 3.2	49.7 ± 3.0	46.0 ± 3.2
MAP, mmHg	115.5 ± 25.1	115.7 ± 24.2	114.7 ± 24.1	118.8 ± 24.9	116.0 ± 24.6	118.6 ± 25.2	118.3 ± 24.2	115.9 ± 24.4
HR, beats/min	178.8 ± 12.1	176.4 ± 11.2	181.8 ± 12.2	180.2 ± 11.5	182.6 ± 14.9	180.8 ± 15.3	183.8 ± 15.2	182.6 ± 16.2

Values are means ± SE. \*  $P < 0.05$  vs. corresponding control values (C1–C4) observed immediately before infusion of saline (SA) or PACAP27 along with splanchnic nerve stimulation (S1–S4). †  $P < 0.05$  vs. 3 preceding responses observed at 1 min.

**Table 3.** Plasma catecholamine concentrations in adrenal venous and aortic blood, adrenal venous blood flow, adrenal venous hematocrit, mean aortic pressure, and heart rate in the group receiving PACAP27 during ACh administration

Parameters	C1	ACh (0.5 $\mu$ g) + SA		ACh (0.5 $\mu$ g) + PACAP (50 ng)	
		1 min	C2	1 min	
Epi <sub>AV</sub> , ng/ml	8.6 $\pm$ 2.8	128.8 $\pm$ 25.1*	10.2 $\pm$ 5.4	659.2 $\pm$ 300.1*†	
NE <sub>AV</sub> , ng/ml	2.4 $\pm$ 1.0	36.3 $\pm$ 8.2*	2.8 $\pm$ 1.2	180.5 $\pm$ 56.6*†	
Epi <sub>AO</sub> , ng/ml	0.13 $\pm$ 0.04	0.11 $\pm$ 0.01	0.13 $\pm$ 0.04	0.13 $\pm$ 0.03	
NE <sub>AO</sub> , ng/ml	0.20 $\pm$ 0.05	0.21 $\pm$ 0.04	0.25 $\pm$ 0.07	0.19 $\pm$ 0.03	
BF <sub>AV</sub> , ml/min	3.3 $\pm$ 0.5	5.1 $\pm$ 0.5*	3.1 $\pm$ 0.5	5.1 $\pm$ 0.4*	
Hct <sub>AV</sub> , %	46.1 $\pm$ 3.2	42.1 $\pm$ 2.6	46.4 $\pm$ 3.3	42.8 $\pm$ 2.9	
MAP, mmHg	136.3 $\pm$ 8.2	137.9 $\pm$ 8.3	137.4 $\pm$ 8.0	137.5 $\pm$ 8.1	
HR, beats/min	159.2 $\pm$ 8.4	160 $\pm$ 8.9	160.2 $\pm$ 9.1	161.8 $\pm$ 9.7	

Values are means  $\pm$  SE. \*  $P < 0.05$  compared with corresponding control values (C1–C2). †  $P < 0.05$  vs. response to ACh in the absence of PACAP27.



**Fig. 2.** Maximum net increases ( $\Delta$ ) in epinephrine (Epi) and norepinephrine (NE) output in the group receiving saline, PACAP27 (50 ng), direct splanchnic nerve stimulation (SNS; 2 Hz), and SNS in the presence of PACAP27 (50 ng). Maximum net response in each group was calculated with the data obtained from the first series of experiments. \*  $P < 0.05$  vs. all 3 other groups.

in response to each stimulus alone, thus indicating a synergistic interaction between the two factors (Fig. 2).

**Catecholamine secretion in response to exogenous ACh.** The simultaneous administration of saline during ACh (0.5  $\mu$ g) infusion increased significantly the basal output of adrenal epinephrine and norepinephrine (Fig. 3A). The catecholamine responses were rapid and short lasting. The responses returned to the corresponding control value in  $\sim 3$  min after the cessation of the infusion (Fig. 3A). The increasing responses of adrenal catecholamine output induced by ACh were reproducible upon the second administration of ACh at the same dose with an interval of 15 min (Fig. 3A).

**Effect of PACAP27 on ACh-induced catecholamine secretion.** The catecholamine responses to the first administration of ACh in the presence of saline were similar to those obtained in the control group (Fig. 3B). In the presence of PACAP27 (50 ng), however, the ACh-induced increases in catecholamine output were significantly enhanced compared with those observed in response to ACh in the absence of PACAP27 (Fig. 3B). Similar increasing responses were obtained in plasma concentrations of both epinephrine and norepinephrine in adrenal venous blood, despite the significant increase in adrenal venous blood flow (Table 3). The net catecholamine responses during the infusion of saline alone, PACAP27 alone, ACh alone, and ACh in the presence of PACAP27 are summarized in Fig. 4. The net increases in ACh-induced catecholamine secretion were significantly potentiated by PACAP27 compared with those obtained in response to each stimulus alone, showing the presence of interaction between exogenous ACh and PACAP27 (Fig. 4). However, the potentiated catecholamine responses to SNS in the presence of PACAP27 were not significantly different from those to ACh administered along with PACAP27.

## DISCUSSION

The present results demonstrate that the local infusion of 50 ng of PACAP27 in the left adrenolumbar artery resulted in a significant increase in the basal secretion of catecholamines from the canine adrenal gland *in vivo*. Furthermore, adrenal catecholamine responses to either SNS or exogenous ACh were reproducible upon the repetition of each stimulus alone with an interval of 15 min. The data indicate that the increase in catecholamine secretion induced by either SNS or ACh was significantly enhanced in the presence of PACAP27 in a synergistic manner. These observations suggest that PACAP27 may play a role of neuro-modulator, facilitating cholinergic neurotransmission in the canine adrenal *in vivo*.

In the present study, adrenal medullary stimulation locally applied with either PACAP27, SNS, or ACh alone resulted in significant increases in basal catecholamine output from the gland without significant changes in systemic parameters such as circulating catecholamine levels in aortic blood, mean aortic pressure, and heart rate. It should be noted that plasma catecholamine concentration in adrenal venous blood

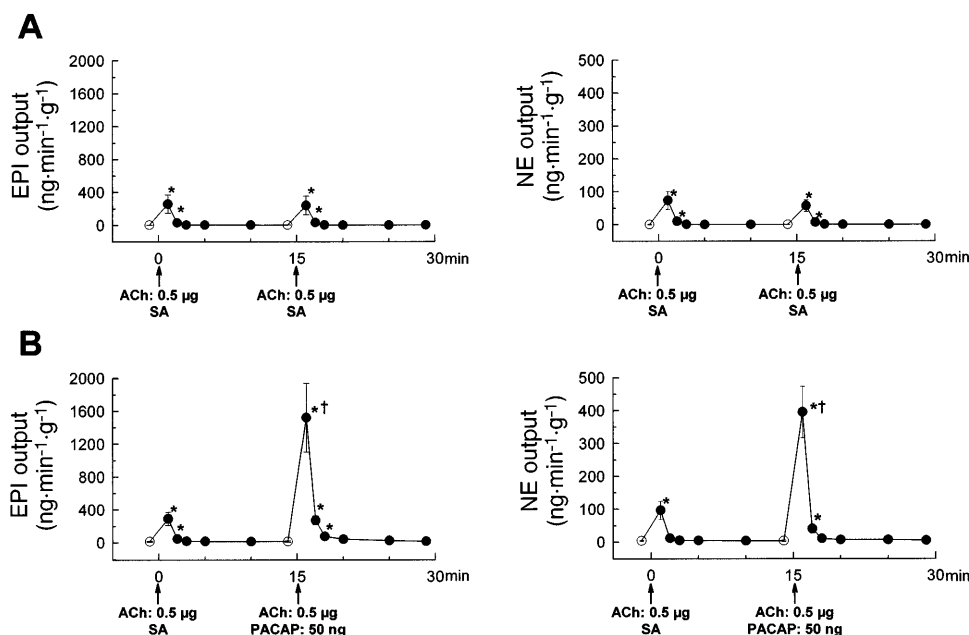


Fig. 3. Adrenal epinephrine (Epi) and norepinephrine (NE) output in response to repeated administrations of ACh (0.5 µg) given at 0 and 15 min in the group receiving vehicle (saline, SA; A) and PACAP27 (50 ng; B) as indicated underneath arrows. Open circles indicate control values taken immediately before drug administration. \* $P < 0.05$  vs. corresponding control values; † $P < 0.05$  vs. preceding response.

also increased in response to those stimuli despite the simultaneous increase in adrenal venous blood flow. These observations are consistent with the view that the increase in catecholamine output induced by those stimuli resulted from an actual increase in adrenal catecholamine release and not from secondary indirect effects of either systemic or local hemodynamic changes, in agreement with our recent findings with PACAP27 and PACAP38 obtained under the similar experimental conditions (5).

It has been shown that the administration of PACAP resulted in a severe bradycardia along with an increase in the release of tritiated ACh in isolated, spontaneously beating guinea pig atria (17). PACAP has also been shown to potentiate the cardiac slowing induced by vagal stimulation in anesthetized dogs (16). The similar stimulating effect of PACAP on the cardiac parasympathetic component has been suggested in the dog model both in vivo and in vitro (7, 31). Furthermore, PACAP has been found to produce a concentration-dependent increase in the release of tritiated ACh in the guinea pig ileum in vitro (9). These previous observations obtained from both in vitro and in vivo studies strongly suggest that PACAP may interact with certain presynaptic mechanisms involved in cholinergic neurotransmission in the peripheral autonomic nervous system. However, the present finding that PACAP27 significantly enhanced SNS-induced adrenal catecholamine release may result, at least, from two distinct processes, presynaptic and postsynaptic mechanisms.

In accordance with those previous studies (7, 9, 16, 17, 31), it is plausible that the potentiated catecholamine response to SNS in the presence of PACAP27 could result, at least in part, from an enhanced release of neural ACh from splanchnic nerve endings, because the principal innervation to chromaffin cells in the adrenal medulla is predominantly preganglionic cholin-

ergic in nature. If PACAP27 stimulates specific PACAP receptor presumably localized on splanchnic nerve terminals, the ongoing SNS-induced ACh release might be enhanced, resulting in the potentiation of catecholamine response. Although the underlying mechanism by which PACAP27 enhances SNS-induced ACh release remains to be defined in the adrenal medulla, a  $Ca^{2+}$ -dependent, tetrodotoxin-sensitive mechanism has been suggested to be involved in the negative chronotropic response induced by PACAP38 in the dog heart in vivo (7) as well as in the PACAP-induced release of tritiated ACh from the guinea pig ileum in vitro (9). A presynaptic PACAP receptor-mediated mechanism may thus be involved in the enhanced catecholamine response to SNS in the presence of PACAP27. The potential implication of the presynaptic mechanism could further be corroborated by the existence of preganglionic PACAP-positive fibers containing choline acetyltransferase (8) and specific PACAP type I receptor (14, 18–20) in the adrenal medulla. Nevertheless, the presynaptic hypothesis such as this is not fully compatible with the observation that PACAP directly increases basal catecholamine secretion independent of the cholinergic pathway (25). This inconsistent issue, however, can be accounted for by a possibility that the postulated presynaptic action of PACAP27 may become functional only when the splanchnic nerve firing rate is significantly elevated above the basal resting condition. The interpretation such as this is compatible with the previous finding that, in anesthetized dogs, the vagal stimulation-induced bradycardia was potentiated more effectively at higher stimulation frequencies, whereas the basal resting heart rate did not decrease in the presence of PACAP (16).

Under the present experimental conditions, the enhanced catecholamine response to SNS was also affected by a direct postsynaptic action of PACAP27 on

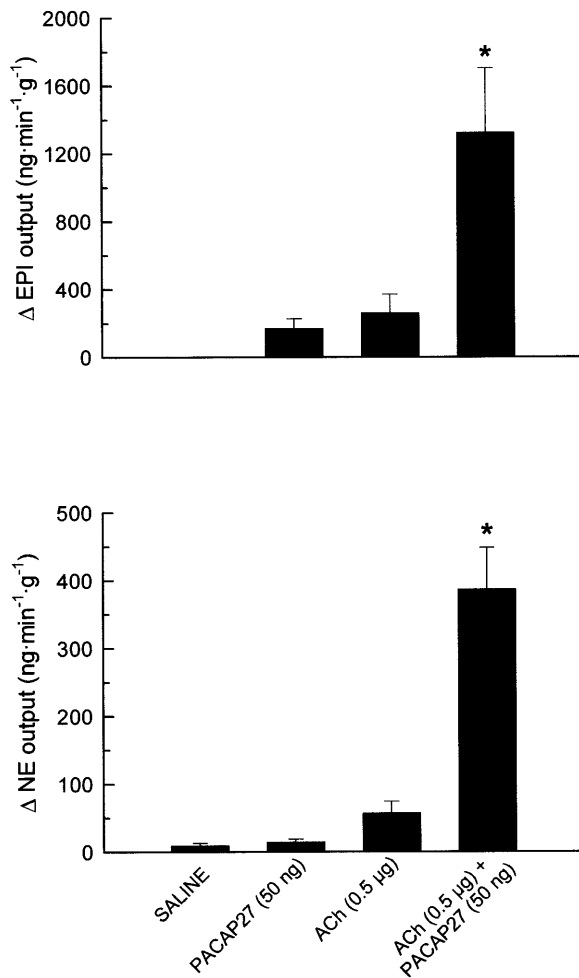


Fig. 4. Maximum net increases ( $\Delta$ ) in epinephrine (Epi) and norepinephrine (NE) output in the group receiving saline, PACAP27 (50 ng), ACh (0.5  $\mu$ g), and the combined infusion of PACAP27 (50 ng) and ACh (0.5  $\mu$ g). Data for calculating the maximum net response in each group were obtained from the groups receiving saline alone and PACAP27 alone in the first series as well as from those receiving ACh alone and ACh plus PACAP27 in the second series of experiments. \*  $P < 0.05$  vs. all 3 other groups.

adrenal chromaffin cells, as clearly indicated by the observation that exogenous ACh-induced catecholamine release was significantly potentiated in the presence of PACAP27. As PACAP is devoid of a direct cholinergic effect on the adrenal medulla in vivo (25), the present observation suggests that the enhancing effect of PACAP27 on ACh-induced catecholamine release may be indirectly mediated via, most likely, specific type I PACAP receptor localized on the surface of adrenal chromaffin cells. In addition to the direct noncholinergic action of PACAP27 on catecholamine secretion, stimulation of the PACAP receptor may result in an upregulation of cholinceptors on the chromaffin cells, resulting in the synergistic enhancement of catecholamine release in response to either endogenous (SNS) or exogenous ACh. In support of this interpretation, it has been demonstrated that, in chick ciliary ganglion in vitro, both PACAP27 and PACAP38 enhanced the sensitivity of cholinceptors to the neural ACh, through a cAMP-dependent mechanism, result-

ing from the stimulation of specific PACAP type I receptors (12). This possibility, however, may be less likely to occur under the present experimental conditions, because PACAP27 was simultaneously administered with either ACh or SNS in the present study, whereas the reported upregulation occurred  $\sim 10$  min after the incubation with PACAP (12). The latter observation suggests the need of either continuous or frequent stimulation of the PACAP receptor for at least 10 min before the application of endogenous (SNS) or exogenous ACh to significantly upregulate surface membrane cholinceptors on the chromaffin cells.

Alternatively, certain intracellular interactions between various second messengers at the postsynaptic level may be more likely to be involved in the enhanced catecholamine release in response to cholinergic stimulation in the presence of PACAP. We have previously shown that both cholinergic nicotinic and muscarinic receptors are functionally involved in mediating catecholamine release in response to exogenously administered selective agonists in the canine adrenal in vivo (27, 28). This holds true for the catecholamine secretion induced by exogenous ACh, whereas SNS-induced catecholamine release is likely to be mediated predominantly by the nicotinic receptor (10). On the other hand, it has been shown that, in rat cultured chromaffin cells, PACAP increases  $Ca^{2+}$  influx through a cAMP-mediated mechanism (15). A recent study further indicated that, in bovine adrenal medullary cells, PACAP induces  $Ca^{2+}$  release from ryanodine/caffeine-sensitive stores through a novel intracellular mechanism independent of both inositol trisphosphates and cAMP (21). Moreover, we have recently shown that dihydropyridine-sensitive L-type  $Ca^{2+}$  channel is functionally involved, although to a small extent, in PACAP27-induced adrenal catecholamine release in the canine adrenal in vivo (5). Taken together, these previous observations are compatible with the view that the potentiating effect of PACAP27 on SNS-induced catecholamine release observed in the present study could result from the PACAP-induced increase in intracellular free  $Ca^{2+}$ , through a mechanism either dependent or independent of cAMP, which, in turn, activates protein kinase C, resulting in the amplification of the exocytotic process initiated by the action of neurally released ACh on nicotinic receptor. In support of this interpretation, it has been shown that nicotinic receptor-mediated  $Ca^{2+}$  influx is positively regulated by the activated protein kinase C (11). The similar cascade such as this has previously been postulated for the synergistic interaction between the effects of VIP and ACh on catecholamine release from the isolated, perfused rat adrenal gland (11).

In the course of the present study, we sought the difference of the potentiated catecholamine responses in the presence of PACAP27 between those induced by SNS and those by exogenous ACh, thereby characterizing the effect of PACAP27 on either the pre- or postsynaptic level during SNS. As discussed earlier in this study, the possibility that PACAP27 potentiated the release of neural ACh in response to SNS, resulting in

the enhanced catecholamine secretion, cannot completely be ruled out under the present experimental conditions. The present data indicate that the catecholamine responses to either SNS or exogenous ACh alone were similar in magnitude. Therefore, if the release of neural ACh was actually potentiated by PACAP27 during SNS, the enhanced catecholamine secretion induced by SNS in the presence of PACAP27 ought to be greater than that obtained from the group receiving exogenous ACh and PACAP27. Nevertheless, the potentiated catecholamine responses observed in the group receiving SNS and PACAP27 were not significantly different from those obtained in the group receiving exogenous ACh and PACAP27. This finding suggests that the potentiated catecholamine release in response to SNS in the presence of PACAP27 is less likely to result from the presynaptic facilitatory action of PACAP27 but rather more likely from its action on the postsynaptic level.

In conclusion, the present study was to investigate if PACAP27 can modulate catecholamine secretion in the canine adrenal *in vivo*. The results indicate that locally administered PACAP27 significantly potentiated catecholamine release induced by either SNS or exogenously administered ACh. These results are compatible with the view that PACAP27 potentiates SNS-induced adrenal catecholamine secretion either by facilitating the release of neural ACh or by postsynaptic multiple intracellular interactions between various second messengers. However, the net catecholamine responses potentiated by PACAP27 during SNS were not significantly different from those enhanced by PACAP27 during exogenous ACh infusion. The present study suggests that PACAP27 may play a role of neuromodulator, presumably at the postsynaptic level in the local regulation of catecholamine secretion in the canine adrenal medulla *in vivo*, although the possible involvement of a presynaptic facilitatory effect of PACAP27 cannot totally be ruled out under the present experimental conditions.

The present study clearly indicates that the adreno-medullary response to either endogenous (SNS) or exogenous ACh was significantly enhanced in a synergistic manner in the presence of PACAP27 under *in vivo* conditions. The present observations are compatible with the view that PACAP is functionally involved as a neuromodulator in the local regulation of adreno-medullary secretion. The physiological significance of the present findings is that this concept can be further applied to many pathophysiological situations in which the sympathoadrenal system is functionally involved. In this context, it is conceivable, for example, that the glucose counterregulatory response of the adrenal medulla to insulin-induced hypoglycemia should also be potentiated in the presence of PACAP, so that the increased catecholamine secretion can contribute to rapidly restore the hypoglycemia. This hypothesis has recently been tested in a dog model similar to that used in the present study. The preliminary results indicated that the increased sympathoadrenal activity, as judged by adrenal catecholamine output, during insulin-

induced hypoglycemia, was significantly enhanced in the presence of PACAP27 in accordance with the present observations (30). It is of further interest that this potentiation of catecholamine response became significant only when the hypoglycemic response to insulin reached the maximum level. It is therefore likely that the PACAP-mediated mechanism is functionally involved in the sympathoadrenal system under certain pathophysiological conditions in which the cholinergic mechanism needs to be markedly enhanced or otherwise becomes deficient.

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