PACAP-(1–38) as neurotransmitter in the porcine adrenal glands

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PACAP-(1–38) as neurotransmitter in the porcine adrenal glands. Am J Physiol Endocrinol Metab 279: E1413–E1425, 2000.—The concentration of pituitary adenylyl cyclase-activating polypeptide [PACAP-(1–38)] in porcine adrenal glands amounted to 14 ± 3 pmol/g tissue. PACAP immunoreactive (PACAP-IR) fibers innervated adrenal chromaffin cells (often co-localized with choline acetyltransferase). Subcapsular fibers traversed the cortex-innervating endocrine cells and blood vessels [some co-storing mainly calcitonin gene-related peptide but also vasoactive intestinal polypeptide (VIP)]. PACAP-IR fibers were demonstrated in the splanchic nerves, whereas IR adrenal nerve cell bodies were absent. In isolated, vascularly perfused adrenal gland, splanchic nerve stimulation (16 Hz) and capsaicin (10 M) increased PACAP-(1–38) release (1.6-fold and 6-fold respectively, P = 0.02). PACAP-(1–38) dose-dependently stimulated cortisol (2 × 10−10 M; 24-fold increase, P = 0.02) and chromogranin A fragment (2 × 10−9 M; 15-fold increase, P = 0.05) secretion. Both were strongly inhibited by the PAC1/VPAC2 receptor antagonist PACAP-(6–38) (10−7 M). PACAP-(6–38) also inhibited splanchic nerve (10 Hz)-induced cortisol secretion but lacked any effect on splanchic nerve-induced pancreastatin secretion. PACAP-(1–38) (2 × 10−10 M) decreased vascular resistance from 5.5 ± 0.6 to 4.6 ± 0.4 mmHg-min-ml−1. PACAP-(6–38) had no effect on this response. We conclude that PACAP-(1–38) may play a role in splanchic nerve-induced adrenal secretion and in afferent reflex pathways.

Pituitary adenylyl cyclase-activating polypeptide receptor antagonist; pancreastatin; catecholamines; cortisol

Pituitary adenylyl cyclase-activating polypeptide [PACAP-(1–38)] and [PACAP-(1–27)] are neuropeptides belonging to the vasoactive intestinal polypeptide (VIP)-secretin-glucagon family of peptides and were originally isolated from the ovine hypothalamus on the basis of their effect on hypophysial AMP production (28, 29). The peptides are both COOH-terminally amidated and are derived from the same precursor protein.

Three PACAP receptors have been isolated, all belonging to the VIP-secretin-glucagon subfamily of the seven transmembrane-spanning G protein-coupled receptors (20). The PACAP-preferring PAC1 receptor has 1,000 times higher affinity for PACAP than for VIP, whereas the VPAC1 and the VPAC2 receptors bind PACAP and VIP with equal affinities.

Since their discovery in 1989, the PACAPs have been found to have widespread distributions and effects (1). In rat (8, 13, 21, 30) and frog adrenal glands (43), PACAP immunoreactivity has been localized to nerve fibers. Furthermore, PACAP has been reported to be a potent secretagogue of catecholamine and cortisol secretion in various preparations and cell lines of rat (16, 42), frog (44), calf (9), pig (27), and human adrenal glands (31), presumably via PAC1 and VPAC2 receptors, which have been located in the gland (30, 33, 38). These findings suggest that PACAP may act as a neurotransmitter in the adrenal gland. However, the contributory role of neuronal PACAP to the adrenal secretory and vascular effects of splanchic nerve stimulation has never been elucidated.

This contributory role was investigated in the present study by use of the isolated, perfused porcine adrenal gland with preserved nerve and vascular supply. The investigation included quantification and immunohistochemical localization of PACAP in the porcine adrenal gland. Co-localization with choline acetyltransferase (CHAT) and calcitonin gene-related peptide (CGRP) was used as markers for efferent sympathetic nerve fibers and sensory nerve fibers, respectively. Furthermore, the contribution of PACAP to the cortical (cortisol), medullary, and vascular responses to nerve stimulation was studied by use of the receptor antagonist PACAP-(6–38). Pancreastatin (a processing fragment of chromogranin A) (26), epinephrine, and norepinephrine were used as indexes of medullary se-
cretion. Last, the expression of the mRNAs encoding the different PACAP receptors was analyzed.

MATERIALS AND METHODS

Animals

Danish LYY strain pigs (15–20 kg) were fasted for 24 h but allowed free access to drinking water. After premedication with midazolam (15 mg, Dormicum; Roche, Basel, Switzerland) and ketamine (250 mg, Ketalar; Parke-Davis Scandinavia, Solna, Sweden) and induction with pentobarbital sodium (250 mg, Tiomebumal-natrium; SAD, Denmark), animals were anesthetized with intravenous α-chloralose (1 g; Merck, Darmstadt, Germany) and ventilated with intermittent positive pressure (N₂O-O₂). The animal experiments were performed according to the principles of laboratory animal care (Law on Animal Experiments in Denmark, Publication no. 382 of June 10, 1987).

Extraction and Chromatography

Porcine adrenal glands were carefully dissected from chloralose-anesthetized pigs and immediately frozen for later extraction by means of an acid-ethanol technique (22). The extracts were subjected to gel permeation chromatography on a Sephadex G50 (fine grade) column (1,000 g; Merck, Darmstadt, Germany) and ventilated with intermitted positive pressure (N₂O-O₂). The animal experiments were performed according to the principles of laboratory animal care (Law on Animal Experiments in Denmark, Publication no. 382 of June 10, 1987).

Porcine cDNA was subjected to reverse-phase HPLC on a Sephadex G50 (fine grade) column (1,000 g; Merck, Darmstadt, Germany) and ventilated with intermitted positive pressure (N₂O-O₂). The animal experiments were performed according to the principles of laboratory animal care (Law on Animal Experiments in Denmark, Publication no. 382 of June 10, 1987).

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In Situ Hybridization Histology

Porcine spinal cord specimens were obtained from medullary thoracic segment T₉–T₁₂ and rapidly frozen on dry ice. Twelve-micrometer-thick sections were cut in a cryostat, dried, and stored at −80°C until hybridization. Hybridization was carried out using [³²P]UTP-labeled antisense and sense rat PACAP cRNA probes prepared by in vitro transcription by use of T₇ (sense) and SP6 (antisense) RNA polymerase. The template was a plasmid containing cDNA encoding the whole PACAP sequence (19). The plasmid (pcDNA3, Invitrogen) was linearized with Hind III for antisense or Apa I for the sense probe. Transcription was performed at 37°C for 2 h in 20 µl containing 5 × transcription buffer (Roche, Hvidovre, Denmark), 25 mM dithiotreitol (DTT), 20 U RNasin (Amersham-Pharmacia, Birkerod, Denmark), 1.5 mM NTP-mix (Roche), 40 U polymerase T₇ (Stratagene) or SP6 (Roche), and 2 µM [³²P]UTP (3,000 mCi, Amersham-Pharmacia). After removal of the DNA template by addition of 1 µl RNasin (30–40 units), 2 µl tRNA (10 µg/µl), and 1 µl DNase (Roche), followed by incubation for an additional 15 min at 37°C, the probes were purified by water-phenol extraction, followed by chloroform-isomyl alcohol extraction, and finally, ammonium acetate-ethanol precipitation. The labeled product was fragmented by incubation in hydrolysis buffer for 60 min at 60°C and was used in a concentration of 1 × 10⁻⁷ cm⁻³.

In situ hybridization was performed as previously described (17). Briefly, after hybridization overnight at 53°C, the sections were washed in 4 × saline sodium citrate (4 × SSC = 0.6 M NaCl and 0.06 M sodium citrate) and 4 mM EDTA for a few minutes at room temperature, followed by RNase treatment for 30 min (RNase A buffer; Sigma, Vallensbæk Strand, Denmark). After being washed in 2 × SSC and 2 mM EDTA at room temperature for 60 min, followed by being

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PACAP, pituitary adenylyl cyclase-activating polypeptide; VIP, vasoactive intestinal polypeptide; CGRP, calcitonin gene-related peptide; CHAT, choline acetyltransferase; FITC, fluorescein isothiocyanate.
Identification of PACAP Receptors

Adrenal glands were isolated, thinly sliced, and immediately frozen on dry ice. The slices were separated macroscopically into cortex and medulla and were then further frozen in liquid nitrogen and stored at –80°C until analysis. RNA was extracted by the acid-guanidinium thiocyanate-phenol-chloroform method (7). Integrity of the RNA was controlled by running RT-PCR using human primers for β-actin (Stratagene) on 50 ng total RNA, and the Titan One Tube RT-PCR System (Roche). Temperature profile was as follows: 1 cycle at 55°C for 30 min and 94°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s; last cycle at 68°C for 10 min and 5°C for 10 min. The assay for PAC1 was done with primers matching the human cDNA sequence and located upstream of the HIP-HOP splice variant region identified in the rat (34). Sense primer was CCTGTGTCACTCAAAGTAGT and antisense primer was GACATGGGAGTGGTAGAG. Two hundred nanograms total RNA were amplified in each reaction using the Titan One Tube RT-PCR System. RT-PCR was as follows: 1 cycle at 50°C for 30 min and 94°C for 2 min; 10 cycles of amplification at 94°C for 30 s, 50°C for 30 s, and 68°C for 45 s; 25 cycles of amplification at 94°C for 30 s, 50°C for 30 s, and 68°C for 45 s + 5 s/cycle; last cycle at 68°C for 10 min and 5°C for 10 min. The 550-bp product formed by the use of these primers was verified by sequencing with a Thermo Sequanase radiolabeled terminator cycle sequencing kit (Amersham-Pharmacia-Biotech). Primers matching the human VPAC1 cDNA sequence were used for detection of VPAC1 RNA, whereas both rat and human primers were used for detection of VPAC2 RNA. VPAC1-sense was AGCAGGACACATGTCTAT, and antisense was GTATG-TACCCCCCAGTAGTAC. For VPAC1, the RT-PCR profile was as for PAC1. The 380-bp product formed by use of these primers was verified by sequencing as above. Rat VPAC2 sense was CACTATGTAGTGGTGTCGCG and antisense was GCCACTGAGAACTCAGGCGCATG, and human VPAC2 sense was TAGAGTGTAGTGGGTCCAG and antisense was CCAGAAAGATGGGCCCCATG. RT-PCR profiles were as those for PAC1; however, both the reverse transcription reaction and the annealing temperature in the amplification were 50°C when using rat primers and 45° and 50°C when using human primers. All PCR products were visualized on ethidium bromide containing 1% agarose gels.

Isolated Perfused Adrenal Gland

Porcine adrenal glands with preserved splanchnic nerves were isolated and perfused as previously described (23). Preservation of splanchnic innervation was ascertained from the effect of electrical nerve stimulation on vascular resistance. In separate experiments in which the splanchnic nerves were crushed with a forceps distal to the position of the electrode, nerve stimulation was without effect on vascular resistance or adrenal secretion. The adrenal glands were placed in a thermostat-controlled bath (containing Ringer's lactate) and perfused in a single-pass system using a Krebs-Ringer bicarbonate solution supplemented with 0.1% human serum albumin (Behringwerke), 5% Dextran T 70 (Pharmacia), glucose (5 mmol/l), a mixture of essential and nonessential amino acids (total concentration 5 mmol/l; Vamin; Kabi Pharmacia, Uppsala, Sweden) and 10–15% fresh, carefully washed bovine erythrocytes, as previously described (25). The perfusion medium was gassed with a 95% O₂-5% CO₂ mixture. Because of the presence of erythrocytes, indomethacin (ConfortiD; Dunex, Copenhagen, Denmark) was added to the perfusion medium to a concentration of 5 mg/l to prevent formation of prostaglandins. The perfusion flow was kept constant at 12 ml/min, which resulted in a perfusion pressure of ~60 mmHg. The venous effluent was collected for 1-min intervals into low-adsorption tubes (Minisorp, Nunc, Life Technologies, Roskilde, Denmark) and was centrifuged within a few minutes at 4°C. The supernatant was stored at –20°C until radioimmunoassay.

Experimental Protocol

The adrenal gland preparation was perfused for 30 min before the first nerve stimulation or peptide infusion period. All antagonist experiments consisted of three 5-min periods of nerve stimulation or peptide infusion separated by 15-min rest periods, with an additional infusion of the PACAP receptor antagonist PACAP 6–38 5 min before and during the second stimulation or infusion.

The splanchnic nerves were threaded through a bipolar tunnel electrode. Electrical stimulations were carried out by means of 10-mA square-wave impulses of 4-ms duration at 10 Hz (pancreastatin and cortisol secretion) and at 16 Hz [PACAP-(1–38) release and vascular response] for 5 min.

Synthetic PACAP(1–38) and VIP (codes 8920 and 7161, Peninsula, Merseyside, UK) were dissolved in 0.5 M acetic acid containing in addition 1% human serum albumin (Behringwerke) and were diluted further in perfusion medium. PACAP (6–38) (code 8980, Peninsula) was diluted in perfusion medium. Captopin (code M-2028, Bie and Berntsen, Rødovre, Denmark) was dissolved in a small amount of ethanol (96%) and was diluted in perfusion medium as above. The peptides were added to the arterial line to give perfusate concentrations of 10⁻⁷ M [PACAP(6–38)], 2 × 10⁻¹¹ M-2 × 10⁻⁹ M [PACAP(1–38)], 2 × 10⁻¹⁰ M (VIP) and 10⁻⁷ M (captopin).

In some experiments, hexamethonium (code H-2138, Sigma, St. Louis, MO) was added to the perfusate to a concentration of 3 × 10⁻⁵ M at least 15 min before first nerve stimulation, a concentration known to block all nicotinic activity (24).

Analyses

Chromatographic separations of adrenal extracts were analyzed for PACAP(1–27) and PACAP(1–38) as described (37). PACAP(1–38), VIP, and pancreastatin in the effluent from the adrenal glands were measured with radioimmunoassays against porcine standards (6, 12, 37). Cortisol and catecholamines were measured by radioimmunoassay with commercially available kits (Coat-A-Count, Kingo Diagnostic, Praesto; and KatCombi, code #KE29291, Biotech-IgG, Copenhagen, Denmark). For cortisol measurements, effluents from the following time points were pooled: minutes –2 and –1, 1, 2, and 3, 5 and 6, 8, and 9, and 13 and 14, where minutes 1–5 were the duration of splanchnic nerve stimulation or peptide infusion. For catecholamine measurements, effluents from the following time points were pooled: minutes –3–1 and 5–7.

Statistical Evaluation

Because of variation between the adrenal gland preparations with respect to basal and stimulated outputs, responses...
to peptide infusions and nerve stimulations are presented as 5-min average fractional changes from baseline secretion in percentages. The average output in the prestimulatory 5-min period ([pancreastatin, PACAP-(1–38), VIP], prestimulatory 2-min period (cortisol), or 3-min period (catecholamines) was defined as 100%. All results are expressed as means ± SE.

The statistical evaluations were carried out with the use of the Friedman analysis of variance for repeated measures. When the former allowed rejection of the null hypothesis, Wilcoxon’s Matched Pairs Test was used for the dose-response experiments.

For the antagonist experiments, Wilcoxon’s Matched Pairs Test was used. The cumulated incremental endocrine responses were expressed as fractional changes (as above) because of a general tendency toward decreasing rates of secretion throughout the experiments. The mean of the control periods (the first and third splanchnic nerve stimulation-peptide infusion periods) was compared with the period of antagonism (the second splanchnic stimulation-peptide infusion period). Wilcoxon’s Matched Pairs Test was also used for comparison of the basal release of PACAP-(1–38) and the release of PACAP-(1–38) induced by splanchnic nerve stimulation and capsaicin, respectively. An additional Matched Pairs Test was used for comparing the PACAP-, VIP-, and splanchnic nerve-induced vascular response to basal conditions.

Linear regression analysis was used for testing the relationship between the secretion of catecholamines and pancreastatin induced by PACAP-(1–38). P values < 0.05 were considered significant.

RESULTS

Tissue Concentrations and Chromatographic Profiles

Figure 1 shows the chromatographic profiles of IR-PACAP-(1–27) (A) and PACAP-(1–38) (B) in extracts of porcine adrenal glands subjected to gel permeation chromatography (n = 7). Both appeared as single homogeneous peaks at elution positions corresponding to the respective synthetic peptides. The concentrations of PACAP-(1–27) and PACAP-(1–38) calculated as the sum of eluted IR material amounted to 0.15 ± 0.02 and 14 ± 3 pmol/g tissue, respectively. Upon HPLC analysis of a pool of extracts of adrenal glands, IR-PACAP-(1–38) eluted at the same position as synthetic PACAP-(1–38) (Fig. 1C). Due to the very small amounts of IR-PACAP-(1–27) in the extracts, no further immunochemical characterization was done for this peptide.

Immunohistochemistry

PACAP-IR was localized to beaded nerve fibers in all parts of the adrenal gland (Fig. 2), but no PACAP-IR nerve cell bodies were observed. Immunoreactive nerve fibers were also found in the splanchnic nerves at the diaphragmatic level (Fig. 2A).

In the medulla, a delicate, dense network of PACAP-IR nerve fibers was located in close apposition to chromaffin cells, presumably innervating them (Fig. 2B). Many of these co-stored CHAT-IR (Fig. 2, B and C). Some of the PACAP-IR nerve fibers also co-stored VIP-IR (not

Fig. 1. Gel filtration and HPLC profiles of the pituitary adenylate cyclase-activating polypeptides (PACAPs). A: elution position of PACAP-(1–27) in ethanol extracts of pig adrenal glands subjected to gel filtration (n = 7). Arrow at coefficient of distribution (Kd) 0.63 indicates the elution position of synthetic PACAP-(1–27). B: elution position of PACAP-(1–38) in acid ethanol extracts of pig adrenal glands subjected to gel filtration (n = 7). Arrow at Kd 0.58 indicates the elution position of synthetic PACAP-(1–38). C: reverse-phase HPLC showing elution position of immunoreactive PACAP-(1–38) in extracts of pig adrenal glands. Arrow at a concentration of acetonitrile (AcN) at 29% indicates the elution position of synthetic PACAP-(1–38). The concentration profile of AcN is indicated by dashed line.
shown); however, the number of PACAP-IR nerve fibers exceeded the number of VIP-IR nerve fibers. No co-localization between PACAP-IR and CGRP-IR was observed in the medulla.

In the cortex, PACAP-IR nerve fibers were demonstrated in the subcapsular tissue ramifying into the cortical cell layers (Fig. 2, D and F). Most of the cortical PACAP-IR fibers contained CGRP-IR and innervated endocrine cells and blood vessels (Fig. 2, D and E), but endocrine and vascular PACAP-IR nerve fibers without CGRP-IR were also observed (Fig. 2D). Some cortical PACAP-IR nerve fibers co-stored VIP-IR (Fig. 2, F and G). These nerve fibers, which were also found near endocrine cells and blood vessels, were few. The number of cortical VIP-IR nerve fibers exceeded the number of PACAP.
**In Situ Hybridization**

By in situ hybridization, PACAP mRNA was localized to nerve cell bodies in the intermediolateral column of the lateral horn (IML) at the level of the medullary thoracic segment T9-T12 (Fig. 3, A and B).

Control hybridization with sense probes showed no labeling (Fig. 3C).

**PACAP Receptor Identification**

Expression of mRNA encoding the three PACAP receptors was studied by RT-PCR in extracts of the medulla and the cortex. PAC1 PCR fragments of 550 bp were detected exclusively in the medulla (Fig. 4A), whereas the 380-bp PCR fragments generated with VPAC1 primers were found in both medulla and cortex (Fig. 4B). The VPAC2 primers were supposed to generate a ~380-bp fragment. However, no fragment of this size was detected in either the adrenal cortex or the medulla by either human or rat specific primers.

**PACAP-(1–38) Release**

In all of seven perfused adrenal glands, splanchnic nerve stimulation (16 Hz) caused a release of PACAP-(1–38), amounting to 160 ± 15.6% of basal release (Fig. 5A, P = 0.02, basal release = 24 ± 4 pmol/l). This effect...
PACAP-(1–38) increased cortisol secretion again to coinfusion of PACAP-(6–38). A postantagonist infusion of PACAP-(6–38) (10⁻⁶ M) was studied in three experiments. Infusion of VIP increased cortisol secretion to 3,727 ± 1,794% of basal secretion (213 ± 174 nmol/l), and this was reduced to 519 ± 289% of basal secretion (153 ± 83 nmol/l) by coinfusion of PACAP-(6–38). A postantagonist VIP control infusion increased cortisol secretion to 4,914 ± 2,201% of basal secretion (basal secretion = 51 ± 24 nmol/l). Compared with the mean response to VIP, PACAP-(6–38) significantly reduced the incremental VIP-induced cortisol secretion to 7 ± 4% (Fig. 7C, P < 0.005 by Student’s t-test for paired data).

Due to the known release of VIP during stimulation of the splanchnic nerves and the effect of VIP on cortisol secretion (10), the influence of PACAP-(6–38) (10⁻⁷ M) on VIP-induced cortisol secretion (2 × 10⁻¹⁰ M) was studied in three experiments. Infusion of VIP increased cortisol secretion to 3,727 ± 1,794% of basal secretion (213 ± 174 nmol/l), and this was reduced to 519 ± 289% of basal secretion (153 ± 83 nmol/l) by coinfusion of PACAP-(6–38). A postantagonist VIP control infusion increased cortisol secretion to 4,914 ± 2,201% of basal secretion (basal secretion = 51 ± 24 nmol/l). Compared with the mean response to VIP, PACAP-(6–38) significantly reduced the incremental VIP-induced cortisol secretion to 7 ± 4% (Fig. 7C, P < 0.005 by Student’s t-test for paired data).

**Cortical Secretion**

PACAP-(1–38) caused an increase in pancreatic secretion (Fig. 8A, n = 7) with a significant effect being observed at 2 × 10⁻⁹ M (P = 0.02, basal secretion = 128 ± 30 pmol/l).
The effect of PACAP-(6–38) (10\(^{-7}\) M) on PACAP-(1–38) (2 \(	imes\) 10\(^{-9}\) M)-induced pancreastatin secretion was studied in six experiments. PACAP-(6–38) alone had no effect on the pancreastatin secretion. PACAP-(1–38) increased pancreastatin secretion to 1,388 ± 784% of basal secretion (basal secretion = 389 ± 119 pmol/l), which was reduced to 166 ± 45% of basal secretion (basal secretion = 1,599 ± 446 pmol/l) by an additional infusion of PACAP-(6–38). A postantagonist infusion of PACAP-(1–38) increased the PACAP-(1–38)-induced pancreastatin secretion again to 358 ± 106% of basal secretion (basal secretion = 682 ± 136 pmol/l). Compared with the mean response to PACAP-(1–38), PACAP-(6–38) significantly reduced the mean incremental PACAP-(1–38)-induced pancreastatin secretion to 26 ± 18%. (Fig. 8B, P < 0.05).

Splanchnic nerve stimulation (10 Hz, n = 7) significantly increased basal pancreastatin secretion to 14,745 ± 9,044% of basal secretion (P < 0.02, basal secretion = 76 ± 21 pmol/l), and this was unaffected by PACAP-(6–38) at 10\(^{-7}\) M (not shown). During the addition of hexamethonium (3 \(	imes\) 10\(^{-5}\) M, n = 3), ...
splanchnic nerve stimulation (10 Hz) increased the secretion of pancreastatin to only 322 ± 71% of basal secretion (P = 0.09, Student's t-test for paired data, n = 3, data not shown). There was no effect of PACAP-(6–38) (10^{-7} M) on this response (data not shown).

Catecholamine secretion. To study the relationship between PACAP-(1–38)-induced pancreastatin, epinephrine, and norepinephrine secretion, catecholamines were measured in selected effluent samples of a few perfusion experiments. With respect to epinephrine, PACAP-(1–38) significantly increased epinephrine secretion to 1,221 ± 324% of basal secretion (P < 0.05, Student's t-test for paired data, basal secretion = 29.8 ± 22 ng/ml, n = 4). This increase was reduced by coinfusion of PACAP-(6–38) (10^{-7} M) to 89 ± 29% of basal secretion (basal secretion = 125 ± 100 ng/ml). A postantagonist infusion of PACAP-(1–38) increased the secretion of epinephrine to 476 ± 145% of basal secretion (basal secretion = 41 ± 18 ng/ml). Compared with the mean response to PACAP-(1–38), PACAP-(6–38) inhibited the mean incremental PACAP-(1–38)-induced epinephrine secretion to 2.5 ± 1.4% (P < 0.0001, Student's t-test for paired data, data not shown).

Norepinephrine secretion was also significantly increased by PACAP-(1–38) to 903 ± 206% of basal secretion (P < 0.05, Student's t-test for paired data, basal secretion = 58.3 ± 41 ng/ml, n = 4), and again, infusion of PACAP-(6–38) reduced this response to 242 ± 111% of basal secretion (basal secretion = 227 ± 174 ng/ml). A control infusion of PACAP-(1–38) increased norepinephrine secretion again to 361 ± 103% of basal secretion (basal secretion = 87 ± 34 ng/ml). Compared with the mean response to PACAP-(1–38), PACAP-(6–38) significantly reduced the mean incremental PACAP-(1–38)-induced norepinephrine secretion to 5.7 ± 4.2% (P < 0.0005, Student's t-test for paired data, data not shown). PACAP-(6–38) alone had no effect on either epinephrine or norepinephrine secretion.

Determination of the correlation between pancreastatin and epinephrine/norepinephrine was carried out on 14 samples from 7 pigs. The correlation coefficient for PACAP-(1–38) (2 × 10^{-9} M)-induced pancreastatin and epinephrine secretion was 0.77 (P < 0.005). The correlation coefficient for PACAP-(1–38) (2 × 10^{-9} M)-induced pancreastatin and norepinephrine secretion was 0.76 (P < 0.005). Determination of the correlation between epinephrine and norepinephrine was also carried out on 14 samples from 7 pigs. The correlation coefficient for PACAP-(1–38) (2 × 10^{-9} M)-induced epinephrine and norepinephrine secretion was 0.94 (P < 0.0001).

Perfusion Pressure

During PACAP-(1–38) (2 × 10^{-10} M) infusion, vascular resistance fell from 5.5 ± 0.6 to 4.6 ± 0.4 mmHg·min·ml^{-1} (Fig. 9A, P = 0.03, n = 11). PACAP-(6–38) at 10^{-7} M had no effect on PACAP-(1–38)-induced vascular resistance (n = 6, data not shown).

Stimulation of the splanchnic nerves (16 Hz) significantly increased vascular resistance from 5.6 ± 0.38 to 5.9 ± 0.4 mmHg·min·ml⁻¹ (data not shown, P = 0.02, n = 21). After addition of hexamethionium (3 × 10⁻⁵ M) to the perfusion medium, splanchnic nerve stimulation (16 Hz) significantly reduced vascular resistance from 5.3 ± 0.4 to 4.9 ± 0.3 mmHg·min·ml⁻¹ (Fig. 9B, P = 0.008, n = 9). PACAP-(6–38) (10⁻⁷ M) had no effect on either splanchnically-induced vasoconstriction or vasodilation (n = 6 and 3, respectively, data not shown).

Due to the supposed localization of the VPAC₁ receptors to adrenal blood vessels (38) and the release of VIP during splanchnic nerve stimulation, the effect of VIP on vascular resistance was studied. VIP at 2 × 10⁻¹⁰ M significantly reduced vascular resistance from 5.9 ± 1.1 to 4.8 ± 0.9 mmHg·min·ml⁻¹ (Fig. 9C, P < 0.05, n = 5). PACAP-(6–38) (10⁻⁷ M) had no effect on VIP-induced vasodilation (n = 3, data not shown).

**VIP Release**

PACAP-(1–38) (2 × 10⁻⁹ M) did not cause a measurable release of VIP (n = 3, data not shown). Stimulation of the splanchnic nerves (16 Hz) caused a 855 ± 391% increase in the release of VIP compared with basal release (P = 0.01, basal release = 7.6 ± 1.3 pmol/l, data not shown, n = 8), which is in agreement with a previous report (10). Splanchnic nerve stimulation (16 Hz) after the addition of hexamethionium (3 × 10⁻⁵ M) to the perfusion medium also caused a significant release of VIP, amounting to 3,015 ± 2,449% of basal release (P = 0.03, basal release = 5.3 ± 3.6 pmol/l, data not shown, n = 6).

**DISCUSSION**

In this study, we investigated the role of PACAP-(1–38) as a neurotransmitter in porcine adrenal glands, in particular its role in splanchnic nerve-induced adrenal responses.

The main findings were as follows. 1) Relatively high concentrations of PACAP-(1–38) are present in the porcine adrenal glands. 2) PACAP is localized to nerve fibers in the medulla and cortex, and PACAP message is localized to cell bodies in the lateral horns of the spinal cord. 3) PAC₁ receptor mRNA was present in the medulla, and VPAC₁ receptor mRNA was present in both the medulla and cortex; VPAC₂ receptor message was undetectable. 4) Electrical splanchnic nerve stimulation and capsaicin both released PACAP-(1–38) from isolated perfused adrenal glands, and hexamethionium abolished nerve-stimulated release. 5) PACAP infusion, as well as splanchnic nerve stimulation, caused a release of both cortical (cortisol) and medullary (pancreastatin and catecholamines) secretory products. 6) The PAC₁ and VPAC₂ receptor antagonist PACAP-(6–38) strongly inhibited the cortisol but not pancreastatin response to nerve stimulation. 7) Nerve stimulation caused a hexamethionium-resistant release of the VPAC₁ and VPAC₂ receptor agonist VIP, and VIP-induced cortisol secretion was inhibited by PACAP-(6–38). 8) PACAP and VIP both decreased vascular resistance of the adrenal glands, but PACAP-(6–38) influenced neither peptide- nor nerve stimulation-induced changes in vascular resistance.

Our findings that PACAP-(1–38) is by far the predominant molecular form of the PACAPs in the porcine adrenal gland is in agreement with findings in previous studies in rat and frog (2, 13, 14, 44). Tissue concentrations of PACAP-(1–27) amounted to only 1/100 of those of PACAP-(1–38), and PACAP-(1–27) could not be detected in the adrenal effluent.

The immunohistochemical demonstration of the adrenal distribution pattern of PACAP-IR nerve fibers is similar to that found in rats (8, 13, 21, 30) and suggests that PACAP-(1–38) has an important function as modulator of cortical and medullary secretion. Whereas the capsular area is believed to be the site of control of adrenal blood flow (40), the capsular and vascular localization of PACAP-(1–38) may, in addition, suggest that PACAP-(1–38) is a modulator of the adrenal perfusion. Our finding that PACAP-(1–38) very potently stimulates the secretion of cortisol and pancreastatin/catecholamines and lowers vascular resistance in porcine adrenals is consistent with these suggestions.

The lack of PACAP-IR nerve cell bodies in the porcine adrenal glands, which is in agreement with studies in rats (13, 21, 30), and the localization of PACAP-IR fibers to the splanchnic nerves suggests an extrinsic origin of the PACAP-IR nerve fibers. Using a highly specific radioimmunoassay against PACAP-(1–38) (37), we were able to demonstrate a release of PACAP-(1–38) during electrical stimulation of the splanchnic nerves. Electrical stimulation at 16 Hz was chosen because preliminary experiments had indicated a large release of PACAP-(1–38) at this frequency compared with that at 10 Hz. Using field stimulation of rat adrenal slices, Wakade et al. (41) also found a release of PACAP-(1–38). It is not possible from our studies to conclude whether or not the release represents a release of PACAP-(1–38) from efferent nerves. However, in the medulla, the co-localization between PACAP and CHAT, which is in accord with a previous study (21), and the lack of co-localization between PACAP and CGRP, believed to occur mainly in sensory nerve fibers, suggests that PACAP-(1–38) occurs in pre-synaptic sympathetic efferents. This is further supported by our localization, by in situ hybridization histochemistry, of PACAP-mRNA in nerve cell bodies in the IML T₉–T₁₂. The release of PACAP-(1–38) was abolished by the addition of hexamethionium to the perfusion medium. This may indicate that acetylcholine acting on nicotinic receptors is involved in the release of PACAP-(1–38). Whereas we were unable to localize PACAP-IR nerve cell bodies that could possess such nicotinic receptors, it seems that the peptide is derived from presynaptic efferents as a result of acetylcholine acting on presynaptic excitatory nicotinic receptors (3, 35). Dun et al. (8), however, reported PACAP staining of cell bodies in some sections of the rat adrenal medulla. This raises the possibility that rapid centrifugal axonal transportation of newly synthesized PACAP could explain our inability to detect...
PACAP-containing cell bodies in the porcine medulla. If true, part of the PACAP response to nerve stimulation could be due to nicotinic activation of such medullary cells. Our finding that acute administration of capsaicin, which is known to activate primary sensory nerve fibers, also caused a release of PACAP-(1–38) may indicate that at least a part of the PACAP-(1–38) release could be due to antidromic stimulation of afferent fibers in the splanchnic nerves. The cortical and subcapsular co-localization between PACAP and CGRP also support the suggestion that the PACAP-containing nerve fibers in these regions represent sensory afferents. This interpretation would be in agreement with observations in rats (8), where most cortical PACAP nerve fibers disappeared after subchronic capsaicin treatment (believed to destroy sensory neurons). In further support, some thoracic dorsal root ganglionic nerve cell bodies were found to stain positively for PACAP (8).

The generally low degree of co-localization between PACAP and VIP, in contrast to other porcine tissues examined [the pancreas (36) and the antrum (unpublished studies)], suggests that the two peptides most likely have individual functions in the pig adrenal gland. It is not possible from our studies to conclude anything about the origin of the nerve fibers sharing this coexistence.

PACAP-(1–38) increased the secretion of cortisol dose dependently, which is in agreement with findings in frogs (43, 44) and calves (9), but not with findings in human adrenal slices/dispersed cells (31). However, the negative result of the latter study may be due to the in vitro technique and/or may imply that a more complex pathway is involved in the PACAP-induced cortisol secretion.

PACAP-(1–38) also stimulated the secretion of pancreastatin. Pancreastatin is an abundant processing product derived from the glycoprotein chromogranin A (corresponding to residues 240–288 of the amino acid sequence) and is released in parallel with chromogranin A from adrenal glands (4, 26). It has previously been shown in pigs that the production of epinephrine and norepinephrine takes place in different subpopulations of chromaffin cells (39), whereas pancreastatin is synthesized in both epinephrine- and norepinephrine-producing cells (5). Pancreastatin secretion, therefore, reflects the secretion of most, if not all, medullary endocrine cells.

Indeed, PACAP-(1–38)-induced pancreastatin secretion was highly correlated with the secretion of both epinephrine and norepinephrine, which were mutually correlated. Consequently, the pancreastatin analysis was used collectively to gauge medullary secretion in the majority of experiments. A stimulatory effect of PACAP-(1–38) on medullary secretion has also been observed in rat (16, 42), calf (9), and humans (31). At 2 × 10⁻¹⁰ M, PACAP-(1–38) significantly stimulated cortisol secretion, whereas a higher concentration (2 × 10⁻⁹ M) was required to elicit a secretion of pancreastatin. This may be explained by differences in receptor affinity for PACAP-(1–38) in the cortical and medullary tissues, the cortical PACAP receptors having a higher affinity for PACAP-(1–38) than do the medullary receptors. In fact, our results indicate that different receptors are expressed in the cortex and the medulla (see below). Whether this can explain the difference in sensitivity cannot be settled presently.

To elucidate the role of PACAP-(1–38) in the response to nerve stimulation, we used a PACAP receptor antagonist that is known to have a higher affinity for the PAC1 and VPAC2 receptors than for the VPAC1 receptor (15). The strong inhibitory effect of PACAP-(6–38) on PACAP-(1–38)-induced cortisol secretion would suggest that PAC1 and/or VPAC2 receptors are activated; however, mRNAs for these receptors were not found in the cortex. This may be explained either by a complex pathway involving medullary PAC1 receptors or by an interaction with a new, as yet unidentified, cortical PACAP receptor sensitive to PACAP-(6–38). The latter theory is supported by the potent effect of VIP on the secretion of cortisol and seems to exclude involvement of PAC1 receptors. We were unable to detect VPAC2 receptor mRNA by RT-PCR by use of either human or rat specific primers, as previously reported by Usdin et al. (38). The PCR technique should ensure adequate sensitivity, but very weak signals may have been missed in the nucleotide staining step. However, with the same methodology, we were able to detect the VPAC2 receptor in porcine antral muscular layers by means of both human and rat primers (unpublished study). The inhibitory effect of PACAP-(6–38) on splanchnic nerve-induced cortisol secretion suggests that PACAP-(1–38) is involved. However, PACAP-(6–38) also nearly abolished the VIP-induced cortisol secretion. Because VIP is released during stimulation of the splanchnic nerves, as previously described (10) and as confirmed in this study, it is therefore not possible to decide which peptide has the most important function in splanchnic nerve-induced cortisol secretion. The lower cortisol response to PACAP observed after termination of the antagonist administration probably reflects a tendency toward decreased secretory responses of the preparation with time, perhaps as a consequence of the absence of ACTH in the perfusion medium. In unpublished studies in the isolated perfused pancreas, the receptor blockade by PACAP-(6–38) is known to be lifted shortly after termination of infusion.

The strong inhibitory effect of PACAP-(6–38) on PACAP-(1–38)-induced secretion of pancreastatin, epinephrine, and norepinephrine is most likely explained by an interaction with medullary PAC1 receptors as identified in this study. Several groups have also reported the localization of the PAC1 receptor to rat chromaffin cells (30, 32, 33). The lack of effect of PACAP-(6–38) on splanchnic-induced pancreastatin secretion does not exclude a possible role of PACAP-(1–38) in splanchnic-induced medullary secretion, because this may be hidden in the large cholinergic splanchnic response (4). To unmask the noncholinergic medullary response to splanchnic nerve stimulation, nicotinic receptors were blocked by hexamethonium. In
these experiments, PACAP-(6–38) was also without effect on splanchnic nerve-induced pancreastatin secretion, supporting our hypothesis that PACAP-(1–38) release is dependent on presynaptic nicotinic receptor activation.

The lack of effect of PACAP-(6–38) on PACAP-(1–38)-induced vasodilation suggests that the effect is transmitted via VPAC1 receptors, in agreement with our finding of mRNA encoding this receptor in both the cortex and medulla. Usdin et al. (38) also reported a localization of VPAC1 receptors to rat adrenal blood vessels, supporting our data. VIP also caused a decrease in vascular resistance, which was insensitive to PACAP-(6–38). Splanchnic nerve stimulation caused vasocostriction, which was reversed to vasodilation upon addition of hexamethonium, suggesting that a noncholinergic component is involved in the latter response. PACAP-(6–38) affected neither splanchnic nerve stimulation-induced vasocostriction nor vasodilation. Because the splanchnic nerve-induced release of PACAP-(1–38) is abolished by hexamethonium, these findings seem to exclude PACAP as the responsible transmitter. Rather VIP, which in this study was also shown to be a potent vasodilator and which is released by nerve stimulation even in the presence of hexamethonium, could be responsible for the nerve stimulation-induced vasodilation observed during nicotinic receptor blockade. This obviously does not exclude the possibility of PACAP-(1–38) playing a role in the regulation of adrenal perfusion as also indicated by the innervation of blood vessels by PACAP-IR nerve fibers.

In conclusion, PACAP-(1–38) may modulate splanchnic nerve-induced medullary secretion and, furthermore, PACAP-(1–38) and VIP may play a role in splanchnic nerve-induced cortisol secretion and vasodilation. PACAP-(1–38) may also be involved in local or peripheral afferent pathways.

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REFERENCES


