Pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal polypeptide and their receptors: distribution and involvement in the secretion of Podarcis sicula adrenal gland

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Abstract

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are regulatory neuropeptides of the hypothalamus–hypophyseal–adrenal axis, acting via the common receptors VPAC1 and VPAC2 and the selective PACAP receptor PAC1. In the adrenal glands of the Italian wall lizard, Podarcis sicula, the presence of VIP in chromaffin cells, and the VIP-stimulated release of catecholamine and aldosterone in vivo, was previously shown. To examine the localization of both peptides and receptors and their mRNAs in the adrenal gland of P. sicula, immunohistochemistry and in situ hybridization were performed: PACAP and its mRNA were detected in chromaffin cells, VPAC1 was found associated with steroidogenic tissue, VPAC2 and PAC1 with chromaffin tissue. Using ‘far western blot’ technique, we showed the presence of specific binding sites for VIP/PACAP in the adrenal glands of the lizard. The effects of both VIP and PACAP on the adrenal cells of the lizard were examined in vitro in adrenal cell co-cultures: both VIP and PACAP enhanced catecholamine, corticosterone and aldosterone release from adrenal cell co-culture in a time- and dose-dependent manner. The catecholamine release was inhibited by PAC1 antagonist and in VPAC2 immunoneutralized adrenal cells. The effects of VIP and PACAP on aldosterone secretion were counteracted by VPAC1 antagonist administration in vitro. Corticosterone secretion elicited by VIP was not blocked by VPAC1 antagonist, while the PACAP-induced release of corticosterone was blocked by the antagonist. Overall, our investigations indicate that these neuropeptides of the secretin superfamily can act not only as neurotransmitters but also as autocrine and paracrine regulators on chromaffin and cortical cells, being important mediators of the non-cholinergic system in the lizard adrenal gland.


Introduction

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) belong to a well-known regulatory peptide family that includes peptides such as secretin and glucagon. VIP and PACAP are known to be regulators within the hypothalamus–pituitary–adrenal axis (Nussdorfer & Malendowicz 1998, Vaudry et al. 2000). The presence of both peptides in the chromaffin cells of several species, the selective distribution of their common receptors VPAC1 and VPAC2 and the PACAP exclusively receptor PAC1, in the steroidogenic and chromaffin tissues forming adrenals of vertebrates (Vaudry et al. 2000, Laburthe et al. 2002, Conconi et al. 2006) strongly suggest that these neuropeptides can directly stimulate adrenal glands.

It has been demonstrated that PACAP is able to enhance the expression of tyrosine hydroxylase, dopamine β-hydroxylase and phenylethanolamine N-methyltransferase, the enzymes involved in the synthesis of catecholamines (Tonshoff et al. 1997, Choi et al. 1999, Corbitt et al. 2002). Moreover, it has been shown that VIP and PACAP stimulate catecholamine secretion from rat chromaffin cells in dose-dependent manner and aldosterone secretion from rat and human adrenal cortex (Nussdorfer & Malendowicz 1998, Mazzocchi et al. 2002). In addition, it has been demonstrated that PACAP-induced catecholamine secretion from the rodent adrenal gland occurs through the activation of adenylyl cyclase and increased cytosolic calcium levels (Przywara et al. 1996). PACAP also induces catecholamine secretion from chromaffin cells both in Ondatra zibethicus (Montpetit & Perry 2000) and in human adrenal cortex (Mazzocchi et al. 2002).

PACAP-induced corticosterone and aldosterone secretion has been shown in rat adrenal slices, but not on dispersed adrenocortical cells, suggesting that chromaffin and steroid cells...
must be present together to have a functional response (Andreas et al. 1995). PACAP-induced secretion of corticosterone and aldosterone from dispersed interrenal cells of *Rana ridibunda* and from perfused frog adrenal explants has been demonstrated (Nussdorfer & Malendowicz 1998, Yon et al. 2001); moreover, it raises cyclic adenosine monophosphate in bovine glomerulosa cells (Botard et al. 1997) and stimulates calcium mobilization in frog adrenocortical cells (Yon et al. 1994).

Despite this large amount of information about vertebrates, the investigations about these peptides in reptiles are scant, although this class has a crucial role in vertebrate evolution and the study of their endocrine system could suggest new insights into vertebrate endocrinology. The presence of VIP in chromaffin tissue, and VIP-stimulated release of catecholamines and aldosterone *in vivo* was previously shown in the Italian wall lizard *Podarcis sicula* (LaForgia et al. 1999). It has also been demonstrated that VIP administration enhances the shift from noradrenaline to adrenaline production in the chromaffin cells (De Falco et al. 2003); further, we cloned lizard PACAP and showed that it is produced and widely expressed by *P. sicula* brain (Valiante et al. 2007).

Hence, to address the hypothesis of local control by neuropeptides in *P. sicula* adrenal glands, our present investigations concerned the localization of VIP, PACAP and their receptors in the adrenal gland of *P. sicula*, immunohistochemically and by *in situ* hybridization. Further, to better understand the signalling pathways existing in the regulation of adrenals by these regulatory peptides, we investigated VPAC<sub>1</sub>, VPAC<sub>2</sub> and PAC<sub>1</sub> receptor expressions. Moreover, we studied the effects of VIP and PACAP administration, alone or in presence of receptor antagonists, on *in vitro* cell co-cultures in order to clarify the role of these peptides in the crosstalk between chromaffin and steroidogenic cells.

**Materials and Methods**

**Animals**

The animals (number = 50; 14–16 g body weight each) were housed in soil-filled terrarium, *fed ad libitum* and exposed to natural photoperiod (16 h light:8 h darkness) and temperature (25 °C day:18 °C night). Captivity lasted 20 days to reverse capture-related stress (Manzo et al. 1994). The experiments were performed in accordance with the guidelines of institutional committees (Italian Ministry of Health) and the European Communities Council to minimize the number of animals used. All results were obtained through three independent experiments.

**Experimental procedure**

Lizards (325 μg/g body weight) were anaesthetized using ketamine hydrochloride (Parke-Davis, Berlin, Germany) and killed; adrenals were excised and fixed for 24 h in Bouin’s solution or 4% paraformaldehyde in PBS (137 mM NaCl, 2-7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>) pH 7-4. Adrenal samples were then dehydrated and embedded in paraffin wax. Paraffin-embedded glands were then sectioned at 5–7 μm and sections lifted onto baked superfrost glass slides (Menzel-Glaser, Braunschweig, Germany). Slides were then dried at 37°C overnight and stored at 4°C until use. Consecutive slides were utilized in immunohistochemistry and *in situ* hybridization procedures.

Some adrenals were incubated 48 h in complete F-10 Ham medium (see below) with VPAC<sub>1</sub> antagonist (Ac–His<sup>1</sup>, d-Phe<sup>2</sup>, Lys<sup>15</sup>, Arg<sup>16</sup>) VIP-(3–7), growth hormone-releasing factor–(8–27)–NH<sub>2</sub> (VPAC<sub>1</sub>–A) 10<sup>−7</sup> M (Phoenix Pharmaceuticals, Belmont, CA, USA) and then fixed and embedded in paraffin wax.

**Immunohistochemistry**

The immunohistochemistry procedure was described elsewhere (Valiante et al. 2007). Briefly, antigen unmasking was carried out in 10 mM citrate buffer (pH 6-0) in a microwave oven for 2×10 min at 400 W. Sections were then washed 3×5 min in 0·1 M phosphate buffer (pH 7·4), treated for 30 min with 0·3% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous peroxidases and with 1:100 normal goat serum to block non-specific sites.

Overnight incubation was performed at 4°C with a rabbit anti-PACAP27 antibody (Phoenix Pharmaceuticals) diluted 1:300 in normal goat serum or anti-VPAC<sub>1</sub>, anti-VPAC<sub>2</sub> and anti-PAC<sub>1</sub> receptor expressions. Moreover, we studied the effects of VIP and PACAP administration, alone or in presence of receptor antagonists, on *in vitro* cell co-cultures in order to clarify the role of these peptides in the crosstalk between chromaffin and steroidogenic cells.

**In situ hybridization**

For this study, we used digoxigenin-labelled RNA probes synthesized with DIG RNA labelling kit (Roche, Mannheim, Germany). They were complementary to the lizard PACAP (Valiante et al. 2007), human VPAC<sub>2</sub> and mouse VPAC<sub>1</sub>. cDNA were kindly provided by P Robberecht and M S O’Doriso respectively (Svoboda et al. 1994, Karacay et al. 2001). Briefly, after transfection of JM109 cells, plasmids were recovered with Ultraclean miniplasmid kit (MoBIO Labs, Carlsbad, CA, USA) and used for an *in vitro* transcription
reaction to synthesize both sense and antisense RNA probes of 300 bp each. In situ hybridization procedure was performed as described elsewhere (Valiante et al. 2007). All solutions used in in situ hybridization were made with bidistilled water treated with 0-1% diethylpyrocarbonate (Sigma) to inactive RNAses and then autoclaved. After dewaxing, the sections were permeabilized in 1 M Tris–HCl, 0.5 M EDTA containing 10 μg/ml proteinase K, post-fixed in 4% paraformaldehyde in PBS and acetylated in 0.1 M triethanolamine (pH 8.0) with 0-25% acetic anhydride. Slides were immersed for 2 h at 70 °C in pre-hybridization buffer containing 50% deionized formamide (Applichem, Darmstadt, Germany), SSC 5× (Applichem), Denhardt solution 1× (Sigma), denatured salmon sperm DNA 100 μg/ml (Applichem), tRNA 100 μg/ml (Roche), 20% dextran sulphate (Sigma) and then immersed overnight at 70 °C in the same buffer added with 2 ng/μl of appropriate sense or antisense riboprobes. After post-hybridization washes in 0.5× SSC and 20% formamide (Sigma), slides were immersed in NTE (0.5 M NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA), containing R.Nase A 10 μg/ml (Applichem) and then blocked with 2% blocking reagent (Roche), 10% normal sheep serum (NSS, Sigma) in 100 mM maleic acid buffer. To reveal the reaction, an anti-DIG alkaline phosphatase–conjugated antibody (Roche) was used and BM Purple or NBT/BCIP (Roche) colorimetric reaction was performed following manufacturer’s instruction. Sections were counterstained with nuclear Fast Red (Vector, Burlingame, CA, USA).

Images elaboration

Both in situ and immunohistochemical signals were analysed with Axioskop System (Zeiss, Oberkochen, Germany) under light conditions and images acquired by KS 300 2.0 software (Zeiss).

VPAC receptor far western blot

Protocol for membrane preparation was described elsewhere (Cao et al. 1995) and modified as follows: to isolate membrane proteins, P. sicula adrenals were quickly removed, immersed in 5 mM HEPES (pH 7.4), 1 mM EDTA and homogenized in ice cold potter. Samples were centrifuged at 4000 g at 4 °C for 20 min to eliminate nuclei and cell debris; supernatant, containing membranes, was recovered and centrifuged at 40 000 g at 4 °C for 20 min; the pellet was resuspended in 20 mM HEPES (pH 7.4), 1 mM EDTA, 0-5 mM phenylmethylsulphonyl fluoride, 0-1 mg/ml bacitracin, 0-1 mg/ml soybean trypsin inhibitor, 5 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin and re-homogenized to extract cell membranes proteins. After a centrifugation at 40 000 g at 4 °C for 20 min, the pellet, containing membrane proteins, was resuspended in the previous buffer, dialysed overnight at 4 °C and protein content assessed by the Pierce method (Pierce, Rockford, IL, USA) in about 2 μg/μl. SDS-PAGE was performed using a 10% acrylamide gel under non-reducing conditions (Laemmli 1970); 12.5 μl medium containing 20 μg protein and the molecular standard markers (Sigma) were loaded in separate lanes. Proteins were transferred on nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), which was blocked with 5% non-fat milk (Bio–Rad) in TBS (10 mM Tris–HCl (pH 7.5), 50 mM NaCl). ‘Far western blot’ technique to display-binding protein was carried out (Hall 2004). The membrane was washed in TBS with 0-1% Tween 20 and 1% BSA (washing buffer), and incubated with VIP or PACAP27 (Phoenix Pharmaceuticals) 1:100 in washing buffer for 1 h at room temperature, then with rabbit anti-VIP or anti–PACAP27 antibodies (Phoenix Pharmaceuticals) 1:300 in washing buffer overnight at 4 °C; after removing the antibody, excess membrane was incubated with anti-rabbit biotinated polyclonal secondary antibody (Pierce) for 1 h at room temperature. The reaction was revealed using an avidin–biotin peroxidase complex (Pierce) following the manufacturer’s instructions, DAB (Sigma) as chromogen in 0.03% of H2O2 in TBS (pH 7.5). The controls were carried out by omitting VIP, PACAP or anti–VIP and anti–PACAP incubation.

VIP and PACAP immunoprecipitation and dot blot peptide analysis

To estimate the VIP and PACAP section in the co-culture medium, dot blot assay was performed as described elsewhere (Nezlin 2000). Complete F–10 HAM (see below) was collected from control co-culture after 24 h. Immunoprecipitation with anti–VIP or anti–PACAP27 antibodies linked to protein–A acrylic beads conjugated (Sigma) was carried out overnight at 4 °C. Peptide–antibody beads complexes were then separated by centrifugation at 12 000 g for 5 min. Pellets were resuspended in PBS, heated at 95 °C for 5 min, to remove protein–A antibody complex, supernatants collected by centrifugation at 12 000 g for 5 min. One microlitre per sample, containing the purified secreted peptides, was spotted on a nitrocellulose filter (Schleicher and Schuell). Standard PACAP and VIP 0-1 M were diluted 3, 10, 30, 100 and 1000 times and used to generate a calibration curve. To determine the unspecific reaction between the medium and antibodies, fresh sterile F–10 HAM was spotted at the same peptide dilution. Distilled water was used as negative control. Filter was blocked for 1 h in 5% non-fat dry milk (Bio–Rad), 0-2% Tween–20 in TBS to avoid unspecific binding, then incubated 1 h with the appropriate primary antibody, 1:300 diluted in the same buffer. After washing in TBS–TWEEN, biotinylated secondary antibody (Vector) was added for 30 min. Filters were then incubated for 30 min in a solution of streptavidin peroxidase complex and DAB (Vector) was used to detect peptides, following manufacturer’s instruction. The acquisition of filters was carried out as previously described (Bjornsson 1998). Filters were acquired using Epson Perfection 1660 photo (Epson, Cinisello Balsamo, Italy) scanner with reflectance mode, γ–curve set at 1-0 and images saved in RGB.
mode. Densitometric computer analysis of spots was then performed with Scion Image software (Scion Corporation, Frederick, MA, USA) according to Malagoli et al. (2004). Optical values of secreted peptides were compared with optical values of known dilutions of pure peptides and the evaluation of VIP and PACAP content in the medium was obtained.

P. sicula cell co-culture

All solutions were 0.22 μm filtered, autoclaved and UV irradiated overnight. Adrenals were immersed in sterile cold physiologic solution for reptiles (0.75% NaCl) and transferred in the enzymatic solution made of F-10 HAM (Sigma), 0.7% collagenase I (Sigma), 0.1 mg/ml DNase I (Sigma), 0.16% dispase (Sigma), 2 mM L-glutamine, 10% FBS heat inactivated, 100 U/ml penicillin, 100 μg/ml streptomycin and 40 μg/ml gentamycin (Invitrogen). The digested solution was centrifuged at 400 g for 10 min at 4°C and the pellet resuspended in complete F-10 HAM medium containing 2 mM L-glutamine, 10% FBS heat inactivated, 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamycin and 0.2% BSA (Applichem). Cell viability assessed by trypan blue staining was >90%. Finally, cells were incubated at 25°C with 5% of CO2 for 24 h at a concentration of 2 × 10⁵ cells/well, medium collected and used as control for both basal hormone and peptide evaluation (baseline).

All peptides and antagonists were dissolved in complete medium to the required concentrations. To assess the action of VIP and PACAP, a range of concentrations from 10⁻¹¹ M to 10⁻⁸ M were used and medium gathered after 1 h. To determine the time-course curve, VIP or PACAP 10⁻⁷ M was added to the medium and after 1, 3, 6 and 24 h medium was collected and stored at −20°C until hormone assay. To study the receptor pathways involved in the peptide-induced adrenal cell response, several receptor antagonists were added in combination: VPAC₁-A 10⁻⁶ M antagonist of VPAC₁ receptor and/or PACAP 6–38 10⁻⁶ M (Phoenix Pharmaceuticals) selective antagonist of PAC₁ receptor and propanolol (Sigma) antagonist of β-adrenoceptors; 30 min later, cells were incubated with VIP or PACAP 10⁻⁷ M and the medium was collected after 1 h. To block the VPAC₂ pathway, immunoneutralization according to Reed et al. (1999) was performed. Lizards were i.p. injected daily with VPAC₂ antisierum 10 μl/10 g (Santacruz Biotechnology) or an equal volume of NSS (controls). On day 10 of injection (2 h after injection), adrenals were removed, cells dispersed, placed in complete F-10 HAM and then immediately treated with antagonists as described above.

Hormone assay

Corticosterone was measured, using a sensitive and highly specific radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA, USA), as previously described (De Falco et al. 2004). Corticosterone titres in picograms per millilitre were calculated using the standard curve generated in the assay with known amounts of radio-inert corticosterone purchased from Amersham; sensitivity was 176 pg/ml. Cross-reactivity of the corticosterone antiserum with other steroids was given by the manufacturer. Inter- and intra-assay coefficients of variation were 3.4 and 5.8% respectively. Aldosterone was measured using a sensitive and highly specific radioimmunoassay kit (ALDO-RIACT, Schering, France) following the manufacturer's instructions; sensitivity was 70 pg/ml. Cross-reactivity of the aldosterone antiserum with other steroids was given by the manufacturer. Inter- and intra-assay coefficients of variation were 7.7 and 8.4% respectively.

As previously described, 200 μl catecholamines (noradrenaline and adrenaline) were extracted from the medium by the alumina adsorption method (De Falco et al. 2004). Catecholamine levels were measured by high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC incorporates a Varian Star 9012 solvent delivery system (Varian, Walnut Creek, CA, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ, USA). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide as an internal standard in all determinations, with a detection limit of 0.1 nmol/l. Catecholamine concentration was calculated as the sum of noradrenaline and adrenaline values and expressed in pg/ml.

Statistical analysis

All data were expressed as means ± s.e.m. The non-parametric Kruskal–Wallis analysis with Bonferroni method was performed to test whether peptides have a significant effect on basal levels of aldosterone, corticosterone and catecholamine release. The non-parametric Kendall coefficient of correlation was used to estimate the linearity of relation between the arbitrary optic units and the concentrations of peptides in the dot blot assay.

Results

Chromaffin and steroidogenic tissues show a distinctive histology in the adrenal glands of P. sicula. In contrast to mammals, chromaffin cells form a dorsal ribbon placed in the outer region of the adrenal surrounding inner steroidogenic ribbons (Fig. 1a); in addition, scattered adrenal cells are found between steroidogenic cells (Fig. 1a).

Immunohistochemistry and in situ hybridization

PACAP PACAP immunoreactivity was localized at a strong level of expression in both noradrenaline and adrenaline chromaffin cells of adrenal ribbon; moreover, a strong immunoreactivity was localized in the adrenal islets too (Fig. 1b). Furthermore, a weaker PACAP immunoreactivity was also found in steroidogenic cells near to the plasmatic membrane (Fig. 1b). This steroidogenic PACAP immunopositivity was
Figure 1  PACAP and its messenger in the adrenal gland of Podarcis sicula. The 20 mm scale bar corresponds to: (a, d and g) 50 µm; (b, c and f) 16.5 µm; (e) 20 µm. (a) The histology of the adrenal gland of Podarcis sicula is evidenced by Mallory's staining by which noradrenaline cells (NE) and adrenaline cells (E) are orange and red coloured respectively. NE and E cells are arranged in a dorsal ribbon lying on steroidogenic tissue (ST). Several groups of adrenaline cells (black arrows) are dispersed between the steroidogenic tissue. (b) Immunohistochemistry for PACAP: section treated with PACAP antibody. Adrenaline islets immunoreactive to PACAP antibody (black arrows). A weak labelling occurs in steroidogenic tissue (ST). Two blood vessels (black arrow head) containing red blood cells without signal indicate the specificity of the immunohistochemical reaction. (c) Section of an adrenal gland pre-incubated with VPAC₁-A 10⁻³ M and then with PACAP antibody. Steroid cells lack of PACAP immunoreactivity. (d) Immunohistochemistry for PACAP: control section pre-incubated with PACAP 10⁻⁶ M shows no labelling. Chromaffin tissue (CT). (e) In situ hybridization for PACAP mRNA: section treated with antisense riboprobe showing specific labelling of chromaffin cells and the lack of signal in the steroidogenic tissue. (f) In situ hybridization for PACAP mRNA: adrenaline cells, dispersed between steroidogenic tissues, are labelled for PACAP messenger. (g) In situ hybridization for PACAP mRNA: control section hybridized with sense riboprobe shows no labelling. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-07-0127.
reversed after pre-treatment with VPAC1-A 10^{-3} M (Fig. 1c). Controls treated by pre-incubating antibody with PACAP 10^{-9} M (Fig. 1d) or by omitting primary antibody (data not shown) showed no labelling in both tissues.

The in situ hybridization performed at high-stringency conditions showed the presence of PACAP mRNA exclusively in both noradrenaline and adrenaline chromaffin cells, while steroidogenic cells did not express PACAP messenger (Fig. 1e). In addition, a strong signal was found in the adrenaline cells that compose islets scattered in the steroidogenic tissue (Fig. 1f). Chromaffin and steroidogenic tissues hybridized with sense probe were lacking hybridization signal (Fig. 1g).

**VIP/PACAP receptors**

Using VPAC2 riboprobes on adrenal sections, the chromaffin ribbon was extensively labelled (Fig. 2a and c). Labelling for VPAC2 receptor mRNA occurred in the cytoplasmic portion of chromaffin cells but not in the nuclei (Fig. 2c). In contrast to the strongly labelled chromaffin cells, complete lack of hybridization signal in the steroidogenic tissue was found, clearly evident at higher magnification (Fig. 2a and c).

To localize VPAC1 mRNA, we used a VPAC1 riboprobe. Intriguingly, we observed a different distribution pattern compared with VPAC2. VPAC1 was exclusively localized within the cytoplasmic portion of steroidogenic cells (Fig. 2d). To test the specificity of our in situ hybridization experiments, two sorts of control were used: 1) a negative control performed with sense riboprobes did not show labelling in both tissues (Fig. 2b) and 2) a positive control performed using rat brain sections that certainly express VIP receptors (Usdin et al. 1994; data not shown).

VPAC2 immunoreactivity was associated with the chromaffin tissue (Fig. 2e). The labelling extensively occurs in both noradrenaline and adrenaline cells (Fig. 2e). Immunoreactivity for VPAC3 was found in the steroidogenic cells (Fig. 2f). Interestingly, it localizes in the cytoplasm near the nuclei, between them and the membranes which are in proximity of blood vessels (Fig. 2f).

Immunoreactivity for PAC1 is restricted to the chromaffin cells exclusively (Fig. 2g). Further, not all chromaffin cells express labelling for PAC1, but immunoreactivity seems to be found only in adrenaline cells, which are located closer to the steroidogenic tissue (Fig. 2h).

Negative controls for VIP and PACAP receptor immunohistochemistry show no immunoreactivity (Fig. 2i–l).

**VIP/PACAP receptor far western blot**

Far western blot experiments performed with VIP/anti-VIP showed two bands that represent binding sites for VIP approximately at 45 and 55 kDa (Fig. 3a) respectively. Moreover, the far western blot performed with PACAP/anti-PACAP revealed an additional band at 60 kDa (Fig. 3b). Controls, obtained omitting peptides (Fig. 3c and d) or appropriate antibodies (data not shown), showed no bands.

**Effects of VIP on adrenal cell co-cultures**

Steroidogenic and chromaffin cells co-cultured in the presence of VIP (10^{-11} M–10^{-5} M) increased their secretion of catecholamines, corticosterone and aldosterone (Fig. 4). Catecholamine secretion by chromaffin cells was strongly increased in a dose-dependent manner with a maximum effect at 10^{-7} M (Fig. 4). Also, corticosterone secretion by steroidogenic cells was affected by VIP (10^{-9} M–10^{-7} M) in a dose-dependent manner increasing about 1.7-fold at 10^{-7} M (Fig. 4). By contrast, aldosterone secreted by steroidogenic cells was weakly enhanced by VIP reaching the maximum effect at 10^{-7} M (Fig. 4). Further, desensitization to VIP occurs at 10^{-5} M since the dose is 100- to 1000-fold higher than physiological levels of VIP (Fig. 4).

Once the maximum effective VIP dose was established at 10^{-7} M, we decided to verify whether VIP can stimulate steroidogenic and chromaffin cell co-cultures in a time-dependent manner. We showed that catecholamine secretion was determined in a time-dependent manner with a strong increase after 1 h and reaching the maximum level after 6 h (Fig. 5a). This VIP-induced enhancement was only weakly affected by contemporary administration of VIP (10^{-7} M) plus VPAC1-A (10^{-6} M; Fig. 5a). Corticosterone was strongly increased by VIP in a time-dependent manner by about twofold after 6 h (Fig. 5b). VPAC1-A (10^{-6} M) administration did not modify the corticosterone response to VIP (10^{-7} M; Fig. 5b). Aldosterone secretion was faintly...

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**Figure 2** VPAC mRNAs in the adrenal gland of Podarcis sicula. The 20 mm scale bar corresponds to: a) 100 μm; b) 83 μm; c) 25 μm; d) 25 μm; e) 25 μm; f) 20 μm; g) 20 μm; h) 13 μm; i) 20 μm; j) 25 μm; k) 20 μm; l) 20 μm. (a) Podarcis sicula adrenal gland section hybridized with antisense riboprobe complementary to VPAC2 mRNA. The labelling of chromaffin cells (arrows) is evident while steroidogenic cells lack labelling. (b) VPAC2 mRNA in situ hybridization occurred in cytoplasmic portion of chromaffin cells that possess nuclear region without signal (black arrow heads). (c) VPAC2 riboprobe labelling occurs in cytoplasmic portion of chromaffin cells that possess nuclear region, which lacks hybridization signal. (c) VPAC2 riboprobe labelling occurs in cytoplasmic portion of chromaffin cells, complete lack of hybridization signal in the steroidogenic tissue was found, clearly evident at higher magnification. (e) Immunohistochemistry for VPAC1 shows that this receptor is widely expressed in chromaffin cells. Steroidogenic cells lack of labelling. (f) VPAC1 immunohistochemistry is found in the cytoplasm of steroid cells exclusively. (g) PAC1 receptor immunoreactivity is observed in chromaffin cells. (h) Higher magnification of PAC1 immunohistochemistry that shows only few chromaffin cells are labelled. Steroidogenic cells lack labelling. (i) VPAC1 in situ hybridization negative control obtained using sense probe. No signal is evident. Sections were counterstained with Mayer’s hemalum. (j, k and l) VPAC2, VPAC1 and PAC1 immuno histochemistry negative controls respectively. Sections were counterstained with Mayer’s hemalum. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-07-0127

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enhanced by VIP reaching a maximum increase after 24 h. Intriguingly, aldosterone secretion by steroidogenic cells was completely inhibited by VPAC1-A (10⁻⁶ M) administration (Fig. 5b). This dataset strongly suggests that multiple receptor pathways are involved in the adrenal control by VIP. Hence, to identify the receptor pathways implicated in the VIP-induced secretion in adrenal cell co-cultures, we performed several experiments blocking all known receptors involved but one at a time (see materials and methods). The administration of VIP in co-cultures where only the VPAC2 pathway was allowed to be active evokes a catecholamine release of +44% on the basal secretion, but not steroid secretion (Fig. 5c). When VIP was added to co-cultures where only VPAC1 pathway was available, catecholamines did not rise and steroid secretion increased by +33 and +75% for corticosterone and aldosterone respectively (Fig. 5d).

Effects of PACAP on adrenal cell co-cultures

Steroidogenic and chromaffin cells co-cultured in the presence of PACAP (10⁻¹¹–10⁻⁵ M) secreted increasing levels of corticosterone, aldosterone and catecholamines (Fig. 6). PACAP dose dependently increased catecholamines to a very low amount (10⁻⁹ M) showing maximal effect at 10⁻⁷ M (Fig. 6). PACAP (10⁻¹¹–10⁻⁷ M) elevated corticosterone and aldosterone levels with highest values, +106 and +74% respectively, at concentration of 10⁻⁷ M (Fig. 6).

Once the maximum effective PACAP dose was established at 10⁻⁷ M, we decided to verify the time-dependent effects of PACAP. A time-course curve showed that PACAP enhanced catecholamine secretion (+27%) as early as 1 h, with a maximum stimulatory effect at 24 h (Fig. 7a). Intriguingly, we observed a slight decrease of the long-term releasing by chromaffin cells (6–24 h) contemporarily adding VPAC1-A and PACAP (Fig. 7a). Time-dependent curves showed a strong release of corticosterone and aldosterone under PACAP stimulation within 6 h, keeping high levels up to 24 h (Fig. 7b). In particular, in vitro PACAP administration had a strong effect on the corticosterone, increasing its secretion of 119% with respect to baseline after 24 h (Fig. 7b). After 24 h, aldosterone reached its peak value of 50% above basal secretion (Fig. 7b). The PACAP-induced corticosterone, and aldosterone secretion was strongly inhibited by VPAC1-A (Fig. 7b).

Similarly to VIP, to detect the receptor pathways involved in the PACAP-induced secretion of adrenal cells, we carried out several experiments; the administration of PACAP in co-cultures where both VPAC1 and VPAC2 are available provokes a large catecholamine release and a still considerable steroid secretion (Fig. 7c). When only PAC1 pathway was available, PACAP induces a massive release of catecholamines (+61%), whereas steroids were not influenced (Fig. 7d). The PACAP-induced response through the VPAC2 pathway also induces an increase of catecholamines release (+55%), whereas it did not significantly affect corticosterone and aldosterone secretion (Fig. 7e). The PACAP-induced response through the VPAC1 pathway enhances the secretion of corticosterone and aldosterone but only a 6% increase in catecholamine secretion after 24 h (Fig. 7f).

Dot blot peptide analysis

The release of PACAP in the culture medium was 0.03 pmol/ml and VIP 0.16 pmol/ml, after 24 h of co-culture (Fig. 8). Controls did not show immunoreaction (data not shown).

Discussion

A growing body of evidence indicates that VIP and PACAP are contained in numerous peripheral tissues where they have been found to exert their pleiotropic effects through the interaction with three receptors termed PAC1, which is coupled by PACAP exclusively, VPAC1, and VPAC2, which are common receptors with relative affinity for these peptides (Vaudry et al. 2000, Conconi et al. 2006).

Previous immunohistochemical investigations on adrenal glands of the Italian wall lizard Podarcis sicula showed a VIP immunopositivity localized in chromaffin cells, ganglion cells and nerve fibres (Laforgia et al. 1999), suggesting a possible role
of VIP in the adrenal control as occurs in other vertebrates (Nussdorfer & Malendowicz 1998). Furthermore, recent studies have shown that the in vivo administration of exogenous VIP acts on adrenal glands of *P. sicula*, enhancing adrenaline production (De Falco et al. 2003). Further, the evolutionary conservation of PACAP in *P. sicula* (Valiante et al. 2007) suggests an important role of these neuropeptides in the physiology of reptiles.

Figure 5 Effects of VIP $10^{-7}$ M administration on co-cultured adrenal cells. Data are expressed in pg/ml ± S.E.M. PACAP 6–38 (10$^{-6}$ M) was added, where appropriate, to avoid endogenous PACAP stimulation. (a) Time course of catecholamine after VIP (●) or VIP + VPAC₁-A (10$^{-6}$ M) (○) administration. *P<0.01 versus baseline. (b) Time course of corticosterone (squares) and aldosterone (triangles) after VIP (black) or VIP + VPAC₁-A (empty) administration. *P<0.05; **P<0.01; ***P<0.001 versus baseline. **P<0.01 when VIP is compared with VIP plus antagonist for aldosterone. (c) VIP effects on catecholamine and steroids through VPAC₂ receptor exclusively. To block simultaneously VPAC₁, β-adrenoceptors and PAC₁, samples were treated with VIP + VPAC₁-A (10$^{-6}$ M) + Propanolol (10$^{-6}$ M) + PACAP 6–38 (10$^{-6}$ M) respectively. *P<0.05. (d) VIP effects on catecholamine and steroids through VPAC₂ receptor exclusively. To block PAC₁, β-adrenoceptors and VPAC₂ pathways, VPAC₂ immunoneutralized adrenals were treated with VIP + Propanolol (10$^{-6}$ M) + PACAP 6–38 (10$^{-6}$ M). *P<0.05.

Figure 6 Adrenal cell co-culture dose–response to PACAP administration. Effect of increasing concentration of PACAP on catecholamine (●), aldosterone (▲) and corticosterone (■) secretion respectively. The lowest concentration that caused a significant increase of catecholamine, aldosterone and corticosterone release was $10^{-11}$ M. Values are expressed in pg/ml and means ± S.E.M.
steroidogenic cells did not produce PACAP mRNA, in accordance with what other authors have reported (Vaudry et al. 2000, Mazzocchi et al. 2002). Thus, it is conceivable that the PACAP immunoreactivity found in steroidogenic tissue may be due to the PACAP secreted by chromaffin cells and bound to receptors exposed on the membrane of steroidogenic cells, since the immunoreactive signal was very close to the plasma membrane of steroidogenic cells, and it disappears when sections of VPAC₁-A-treated adrenals were used.

Secondly, we evaluated the expression of membrane proteins able to bind VIP and/or PACAP using SDS-PAGE in non-reducing condition that allows unaltered receptors to bind ligand (Guse-Behling et al. 1992). We showed, in the lizard adrenals, the presence of two membrane proteins that bind VIP and PACAP ranging between 45 and 55 kDa and a third membrane protein at 60 kDa, which binds PACAP exclusively. Since their molecular weights approximately correspond to the known molecular weights of vertebrate VPAC₁, VPAC₂ and PAC₁ receptors.


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Peptides and reptile adrenal regulation · S VALIANTE, M PRISCO and others

respectively, the hypothesis of a complete receptor set expression in the reptile adrenal, as occurs in other vertebrates, is conceivable. Moreover, performing in situ hybridization and immunohistochemistry, we showed a different distribution of the three receptor subtypes: PAC1 receptor is expressed in a few chromaffin cells exclusively and this is in agreement with data available in literature (Vaudry et al. 2000); VPAC1 mRNA and the polypeptide receptor were expressed in the steroidogenic cells, while VPAC2 receptor and its mRNA were found in the chromaffin cells. This is in contrast with the mammalian distribution pattern, since in the rat adrenals there is an opposite association pattern (Usdin et al. 1994) and in humans both receptors are expressed in the zona glomerulosa and in the adrenal medulla (Mazzocchi et al. 2002). This peculiar spatial distribution of chromaffin cells in reptile adrenal, which could be associated with the characteristic pattern of receptor expression, remains to be determined and is an intriguing hypothesis.

Several lines of evidence suggest that chromaffin and steroidogenic cells strongly interact through a crosstalk in autocrine/paracrine manner (Wong et al. 1995, Haidan et al. 1998, Shepherd & Holzwath 2001). These interactions are mediated by catecholamines and further by several peptides which are secreted by chromaffin cells, acting through specific receptors exposed on both types of tissue (Vaudry et al. 2000). Since it has been previously shown that intra-adrenal communication between adrenocortical and chromaffin cells is essential for the complete hormonal response (Haidan et al. 1998), we performed cell co-culture experiments to preserve the intercellular cross-talking mechanisms.

We demonstrated that the administration of VIP and PACAP affected the secretion of both steroidogenic and chromaffin tissues, enhancing the production of corticosterone, aldosterone and catecholamines respectively. We showed that this was a dose- and time-dependent effect. Our data are consistent with our previous in vivo studies on the adrenal glands of P. sicula (De Falco et al. 2003) and in good agreement with that reported in the literature (Mazzocchi et al. 2002).

Co-culturing chromaffin and adrenocortical cells in the presence of VIP and its receptor antagonist VPAC1-A, we showed that aldosterone secretion was under the influence of the VIP/VPAC1 pathway, given that its secretion was decreased by VPAC1-A. This is consistent with the localization of VPAC1 receptor and its mRNA in steroidogenic cells.

We also observed that corticosterone was only partially affected by VPAC1-A, implying that an additional signalling pathway should be involved: corticosterone release could be under the paracrine control of catecholamines via β-adrenoceptors expressed on steroidogenic cells, since it is well known that catecholamines stimulate corticosteroid secretion (Nussdorfer & Malendowicz 1998): to address this hypothesis, we blocked VPAC1 and β-adrenoceptors, showing that corticosterone release did not arise; hence, the corticosterone increase in VPAC1-A–treated adrenal cells should be due to the VIP-evoked release (via VPAC2, which is still active in VPAC1-A–treated cells) of catecholamines by chromaffin cells in co-cultures, which bind β-adrenoceptors on the steroidogenic cells.

Catecholamines were not inhibited by VPAC1-A but their secretion was completely abolished when VPAC2 immunoneutralized adrenal cells were used, showing that catecholamine release is VPAC2 modulated. This is consistent with the presence of mRNA encoding the VPAC2 receptor in the chromaffin cells solely as shown in our in situ hybridization and immunohistochemistry experiments.

Moreover, we demonstrated that PACAP was able to influence both chromaffin and steroidogenic cells even at very low concentrations (10^{-9} M) suggesting the presence of an effective regulation system in the adrenal gland based on this peptide, as already demonstrated in other species (Yon et al. 1994, Mazzocchi et al. 2002). The dose-dependent stimulating activity of PACAP, within a wide range of concentrations (10^{-9} M–10^{-7} M), was highest at 10^{-7} M. This is consistent with pharmacokinetics of mammals since it has been demonstrated that PACAP exerts its biological functions between 10^{-8} M and 10^{-7} M (Nussdorfer & Malendowicz 1998, Mazzocchi et al. 2002).

The stimulation with PACAP produced a secretion pattern with a characteristic time course: a slow increase after just 1 h and a maximal effect between 6 and 24 h. This time course suggested that PACAP had a long-lasting impact on adrenal cells since after 24 h its stimulatory activity was enduring for adrenal hormones. This long-term action on both chromaffin and steroidogenic cells could be due to the absence in co-cultured cells of a central negative feedback and/or to the presence of paracrine interactions amongst chromaffin and steroidogenic cells, as previously demonstrated in vitro (Guse-Behling et al. 1992, Haidan et al. 1998, Shepherd & Holzwath 2001).

Through the administration of PACAP in co-cultures where only VPAC2 or PAC1 were active, we demonstrated that the VPAC2 and PAC1 receptors are both involved in the PACAP-induced catecholamine secretion and that the PACAP binding to VPAC2 increases catecholamine levels immediately in contrast...
to PAC₁, which has a slower effect in time but reaches higher levels. This conclusion is reinforced by the data showing that the VPAC₂ receptor and its mRNA were localized in chromaffin cells, while only a small number of chromaffin cells express PAC₁. In addition, we showed that PACAP affected steroidogenic cells through interaction with the VPAC₁ receptor, since VPAC₁-A almost completely abolished corticosterone and aldosterone secretion elicited by PACAP and that in VPAC₂ immunoneutralized adrenal cells, PACAP is able to increase steroid secretion exclusively. These data further strengthen the significance of VPAC₁ presence in steroidogenic cells. Furthermore, unlike VIP, PACAP seems to act on steroid secretion via VPAC₁ directly, without the intervention of β-adrenoceptors, since no PACAP-induced corticosterone release was evident when only VPAC₁-A was used; if this is a consequence of peptides, different affinity towards the same receptor remains unknown.

Therefore, in the present paper, we showed that chromaffin cells of *P. sicula* synthesize and release not only VIP, as previously demonstrated (Laforgia *et al.* 1999), but also PACAP. Specifically, we demonstrated that a basal secretion of both VIP and PACAP occurs *in vitro* and VIP secretion was fivefold higher than PACAP: it is noteworthy that an up-regulation of VIP expression by PACAP has been reported (Vaudry *et al.* 2000). Drawing from this background, we can affirm that VIP and PACAP are able to influence both chromaffin and steroidogenic cell hormone secretion, by interacting with different receptors. A direct effect of VIP and PACAP on chromaffin cells occurs through VPAC₂ and PAC₁ receptors, whereas steroidogenic cells can be stimulated through the VPAC₁ receptor; whether this stimulation occurs via intracellular different signalling transduction pathways remains to be determined, but what is remarkable is the tissue-specific distribution of these receptors. The presence of PACAP in chromaffin cells allows us to hypothesize that PACAP released from chromaffin cells, either via splanchnic nerve or autocrine stimulation, may influence adrenal cells in a paracrine manner; this statement may be strengthened given that in *P. sicula* steroidogenic and chromaffin tissues are intimately intermingled and thus anatomically situated for such effective ‘neural–autocrine–paracrine’ regulation (Laforgia *et al.* 1991). The present investigation can be considered a fascinating base in order to clarify the VIP and PACAP roles in reptile endocrinology, since it establishes that, through the existence of multiple receptor pathways, a fine modulation of the lizard adrenal physiology occurs, suggesting that non-cholinergic control is important in the regulation of the lizard adrenocortical and adrenochromaffin cell functions.

Acknowledgements

We gratefully acknowledge Profs P Robberecht and M S O’Dorisio for the kind gift of plasmids. In memory of Mr Giuseppe Falcone who always taught us to look beyond the difficulties. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 11 October 2007

Accepted 13 November 2007

Made available online as an Accepted Preprint 13 November 2007