

Neuropeptides, Growth Factors, and Cytokines: A Cohort of Informational Molecules Whose Expression Is Up-Regulated by the Stress-Associated Slow Transmitter PACAP in Chromaffin Cells

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Abstract Pituitary adenylate cyclase-activating polypeptide (PACAP) is a co-transmitter with acetylcholine at the adrenomedullary synapse, mediating sustained hormone secretion and regulation of cellular plasticity in response to stress at the level of gene transcription. Here we have extended our investigation of PACAP-regulated neuroendocrine cell-specific genes from PC12 cells to PC12 cells expressing physiological levels of the PAC1hop receptor found on chromaffin cells *in vivo*. PACAP induces in these PC12_bPAC1hop cells an additional cohort of genes, compared to PC12 cells, enriched in informational molecules including cytokines, neuropeptides, and growth factors. Using two newly developed microarray platforms for expressed bovine transcripts, we further examined PACAP-induced genes in bovine chromaffin cells during a period of exposure (6 h) corresponding to a period of prolonged metabolic or psychogenic stress *in vivo* during which PACAP is released from the splanchnic nerve onto chromaffin cells. As in PC12_bPAC1hop cells, PACAP induced in bovine chromaffin cells a cohort of genes encoding

secretory proteins, identified by tiling for cellular localization using Ingenuity Pathway Analysis, which were highly enriched in informational molecules (secreted proteins acting at extracellular receptors). These included cytokines, growth factors and hormones, as well as converting enzymes, or protease inhibitors modulating converting enzyme function. Several neuropeptide prohormone transcripts not previously shown to be PACAP-regulated in chromaffin cells, such as thyrotropin-releasing hormone, and tachykinin precursor 1, were identified. Identification of this cohort of informational molecule-encoding transcripts suggests a wider, more integrative role for PACAP as a co-transmitter specific to stress transduction in the adrenal medulla.

Keywords Adrenal medulla · Chromaffin cell · Cytokine · Growth factor · Hormone · Microarray · Neuropeptide · PACAP · PC12 · Stress

Introduction

Chromaffin cells of the adrenal medulla are derived from the neural crest, as are sympathetic neurons. Along with catecholamines, several neuropeptides are stored in and secreted from chromaffin cells, and their expression is increased by splanchnic nerve firing during metabolic stress (Fischer-Colbrie et al. 1992; Stroth et al. 2007). Splanchnic nerve terminals in the adrenal medulla, like preganglionic sympathetic inputs, contain and release pituitary adenylate cyclase-activating polypeptide (PACAP), while chromaffin cells (and post-ganglionic sympathetic neurons) express the high affinity PACAP receptor PAC1 (Hamelink et al. 2002a). The bovine chromaffin cells (BCCs) express a single isoform of PACAP receptor, bPAC1hop coupled to intracellular Ca^{2+} mobilization and cAMP generation (Mustafa et al. 2007).

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Prolonged PACAP exposure results in a robust persistent catecholamine release from PC12 cells (Taupenot et al. 1999) and isolated adrenal chromaffin cells (Watanabe et al. 1992; Wakade 1998; Tornøe et al. 2000). PACAP also regulates the expression of the neuropeptides enkephalin (Hahm et al. 1998; Monnier and Loeffler 1998), neuropeptide Y (NPY; Colbert et al. 1994), galanin (GAL; Anouar et al. 1999), secretogranin II (SCG2; Turquier et al. 2001), and vasoactive intestinal polypeptide (VIP; Lee et al. 1999), which are presumably co-released with catecholamines in vivo upon splanchnic nerve stimulation of chromaffin cells. PACAP is also a regulator of neuropeptide expression in cultured rat sympathetic neurons (Braas et al. 2007).

Messenger RNAs encoding VIP and galanin are up-regulated in adrenal glands in a PACAP-dependent manner after treatment with lipopolysaccharide (Ait-Ali et al. 2010). These neuropeptides may act to modulate the inflammatory response by enhancing steroidogenesis in the neighboring adrenal cortex (Ehrhart-Bornstein et al. 1991; Bornstein et al. 1996; Andreis et al. 2007), or altering the function of immune cells trafficking the gland (Fischer-Colbrie et al. 2005; Goetzl et al. 2008; Yadav and Goetzl 2008). Thus, the function of PACAP at the adrenomedullary synapse includes control of the release and biosynthesis of both catecholamines and neuropeptides. Additional peptides and growth factors are present in the adrenal medulla, but their regulation during stress has never been investigated. PACAP is released at the adrenomedullary synapse only during high-frequency splanchnic nerve stimulation as occurs during stress (Kuri et al. 2009). Comprehensive microarray examination of chromaffin cells exposed to PACAP in culture could reveal additional stress-related informational molecules potentially regulated by PACAP in vivo. We have, therefore, extended our previous microarray investigation of PACAP gene regulation from PC12 cells to PC12 cells expressing specifically the PAC1hop receptor at levels similar to those found on chromaffin cells in vivo. In addition, recently developed bovine microarray platforms allowed us to examine PACAP regulation of primary bovine chromaffin cells directly upon treatment with PACAP for periods similar to continuous splanchnic nerve stimulation during metabolic or psychogenic stress (Hamelink et al. 2002b; Stroth and Eiden 2010). In both cases, in addition to neuropeptide up-regulation, transcripts encoding hormones, cytokines, and growth factors were up-regulated by exposure to PACAP.

Materials and Methods

PC12_bPAC1hop Cell Culture and Microarray Analysis

PC12 cells (PC12-G clonal line, Rausch et al. 1988) were stably transfected with a vector expressing bovine PAC1hop

under the control of the cytomegalovirus promoter as described previously (Mustafa et al. 2007), and this cell line (PC12-G, clone#9; referred to here as PC12_bPAC1hop) was treated with 100 nM PACAP-38 (Phoenix Pharmaceuticals, Burlingame, CA) for 6 h and RNA was extracted using RNeasy Mini Spin Columns (QIAGEN, Valencia, CA) according to standard procedures. Linear amplification of RNA was performed (Amino Allyl MessageAmp II aRNA Amplification Kit, Ambion, Austin, TX) prior to labeling with Cy3 and Cy5 fluorescent dyes (GE Healthcare UK, Chalfont, Buckinghamshire, England), and microarray hybridization to oligonucleotides arrays consisting of the Qiagen mouse MV3 oligo set with oligonucleotide length generally ~ 70 nucleotides. General methods for data extraction, normalization, and filtration for quality and dye bias have been summarized in detail previously (Samal et al. 2007). Here data from three microarrays were analyzed in the NCI mAdb microarray databasing system after standard deArray M-value correction with allowed quality ratio of 0.3. Significance of expression was evaluated using statistical analysis of microarray (SAM) at a false discovery rate of $\sim 10\%$. Data were filtered for expression ratios ≥ 2 in 100% of all arrays, and resulting gene list was exported to MS Excel for further manipulation.

BCC Culture and Drug Treatments

Primary cultures of BCCs were obtained after retrograde perfusion of bovine adrenal glands with 0.1% collagenase (Worthington Biochemical, Lakewood, NJ) and 30 U/ml DNase (Sigma-Aldrich), followed by dissociation of the digested adrenal medulla. The cells were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 5% fetal calf serum and 100 U/ml penicillin–streptomycin, 2 mM glutamine, 10 $\mu\text{g}/\text{ml}$ cytosine β -D-arabinofuranoside, and 100 U/ml nystatin. Chromaffin cells were purified by differential plating as described previously (Anouar et al. 1994). Cells were then plated in the same medium as above at a density of 1×10^6 cells/ml, 2 ml/well, in poly-D-lysine-coated 6-well plates and treated with PACAP-38 (100 nM, Phoenix Pharmaceuticals, Burlingame, CA), and at a density of 0.75×10^6 cells/well, 1.5 ml/well, in poly-D-lysine-coated 12-well plates and treated with PACAP-38 (100 nM) with or without pharmacological inhibitors U0126 (10 μM , Calbiochem) or H89 (10 μM , Calbiochem). The inhibitors were dissolved in dimethylsulfoxide (U0126) or water/ethanol (1:1, v:v) (H89), and added 30 min before the start of PACAP treatment.

Bovine Chromaffin Cell RNA Extraction, Microarray Procedure and Analysis

Total RNA was isolated from cultured BCCs using RNeasy Mini Spin Columns (QIAGEN) and quantified by

spectrophotometry. RNA was processed for Agilent and Affymetrix array after Bioanalyzer (Agilent, Inc) determination of quantity and quality (RNA integrity number greater than 7.3). Affymetrix GeneChip Bovine Genome Array (900562) and Agilent Bovine Gene Expression Microarray (design ID 015354) arrays were used for hybridization.

For Affymetrix microarray chips processing samples were prepared according to Affymetrix protocols (Affymetrix, Inc). 5 µg of total RNA was used per labeling reaction, in conjunction with the Affymetrix-recommended protocol ‘One-Cycle Target Labeling and Control Reagents’. The hybridization cocktail containing the fragmented and labeled cDNAs was added to the Bovine Genome Array Affymetrix GeneChip. The chips were washed and stained by the Affymetrix Fluidics Station using the standard format and protocols as described by Affymetrix. The probe arrays were stained with streptavidin phycoerythrin solution (Molecular Probes, Carlsbad, CA) and enhanced by using an antibody solution containing 0.5 mg/ml of biotinylated antistreptavidin (Vector Laboratories, Burlingame, CA). An Affymetrix Gene Chip Scanner 3000 was used to scan the probe arrays.

Agilent microarray slides were processed as follows: 1 µg of either treated or untreated total RNA samples were reverse transcribed with an oligo dT-T7 and amplified using the Ambion MessageAmpTM II Kit (Ambion, Austin, TX). The generated aminoallyl UTP-labeled RNAs were then coupled with either Cy3 (untreated) or Cy5 (PACAP treated) mono NHS ester CyDyes from GE Healthcare (Piscataway, NJ). After purification, the labeled antisense RNAs were hybridized overnight to the Agilent Bovine (V2) Gene Expression Microarray, 4 × 44 K oligo chips using the Agilent two-color hybridization protocol (Version 5.7). The slides were then washed according to the Agilent two-color buffers and temperatures. A laser confocal Agilent Technologies Scanner G2505A US14702390 (Agilent Technologies, Palo Alto, CA) was used to scan the hybridized Cy3 and Cy5 probes on the chips. The fluorescent intensities at the target locations on the array were extracted using Agilent Feature Extraction Software (Version 9.5.1.1). The expression values were normalized using the default linear-Lowess normalization.

On the Affymetrix platform technical repeats were performed with three sets of arrays with labeled RNA from untreated samples and samples treated with PACAP. For Agilent arrays, Cy5-labeled RNA from PACAP-treated samples were combined with Cy3-labeled RNA from the untreated samples for hybridization in triplicates.

The Affymetrix CEL files were analyzed using Partek Genomic suite following the vendor’s recommendation. ‘robust multichips analysis’ (RMA) normalization was used followed by Student’s *t* test to determine significantly

up- or down-regulated genes. A subset of genes was selected whose expression was altered by PACAP at least or greater than twofold ($P \leq 0.05$, Student’s *t* test, $n = 3$ for each group) and annotated using the latest annotation files from <http://www.affymetrix.com>.

Agilent files with processed signals from red to green channels were also analyzed using Partek Genomic suite. After log 2 transformation and quantile normalization, a filter was applied to remove the transcripts with very low fluorescence signal (<200). A list of significantly regulated transcripts was created, as described above, using Student’s *t* test. Annotation was carried out using the latest information from Agilent (<http://earray.chem.agilent.com>). Additional annotation was performed using tools from NCBI (<http://www.ncbi.nlm.nih.gov/>).

The primary microarrays data from this study have been deposited in MIAME format in the NCBI gene expression omnibus (GEO) data repository under accession number GSE24070.

Bioinformatic Analysis of Protein-Encoding Genes Up-Regulated by PACAP in PC12_bPAC1hop and Bovine Chromaffin Cells and Released into the Extracellular Space

Cellular locations of named transcripts up-regulated 2-fold or more by PACAP in PC12_bPAC1hop or bovine chromaffin cells were identified using ingenuity knowledge environment (IPA). Gene ontology (GO) analysis was then employed to assign function to these genes.

Q-RT-PCR

Approximately 2 µg of total RNA was submitted to DNase I (RNase-free; Invitrogen) digestion and reverse transcribed using random hexamers pdN₆ (Invitrogen) and SuperScript II RNase H⁻ reverse transcriptase (Invitrogen). Splign (<http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form>) was used to identify the exon and intron boundaries. Then Gene-specific forward and reverse primers, listed in supplementary ‘Primer table’ were designed using Primer 3 input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) across exon–exon junctions. Real-time PCR (Q-RT-PCR) was performed in a pre-made reaction mix in the presence of the transcribed cDNA and 10 nM of each specific primers, using the SYBR green chemistry and an iCycler real-time detection system (Bio-Rad, Hercules, CA). Fold changes in mRNA levels were determined by normalizing against a non-variable control transcript, CGA, using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001).

Results and Discussion

PACAP Up-Regulates Informational Molecules in PC12_bPAC1hop Cells

We noticed in our previous studies that secretory proteins known to be present, and in some cases induced by PACAP, in primary chromaffin cells were neither expressed, nor induced by PACAP in the PC12 cell line (Vaudry et al. 2002; Samal et al. 2007; Eiden et al. 2008; Ghzili et al. 2008). The PC12 cell response to PACAP is a compound one—the initial phase of growth arrest and neurite extension takes the cells to a neuronal phenotype, followed by terminal differentiated into a final neurochemical phenotype (Rausch et al. 1988). We noted in our 2008 study that secretory proteins were generally not induced by PACAP in PC12 cells: only when transfecting PC12 cells with a ‘physiological’ level of PAC1 (PC12_bPAC1hop cells) was one of these more ‘fully differentiated’ marker, tachykinin precursor 1 (Tac1), expressed (Eiden et al. 2008). We examined the entire cohort of secretory protein-encoding transcripts in the PC12_bPAC1hop cell transcriptome induced by 6 h of treatment with PACAP-38 (100 nM). Among named transcripts (169 identified transcripts) shown to be up-regulated by PACAP in PC12_bPAC1hop cells, all potential extracellular (secreted) molecules were identified by tiling according to their cellular location using IPA. 21 proteins

were assigned to the extracellular space, and fell into six groups: cytokines (4), growth factors (4), peptidases (2), transporters (1), kinases (1) and ‘other’ (10). GO analysis, which cannot be used to tile gene lists, but does identify functional, locational, and process attributes of transcripts, was then used to (i) confirm extracellular localization and (ii) confirm the functional assignments above, and assign function to the 10 genes in the ‘other’ category. This resulted in the list shown in Table 1: the 21 extracellularly located proteins whose mRNAs were up-regulated by PACAP clustered as cytokines (4), growth factors (4), hormone activity (1), integrin binding (2), neuropeptide activity (1), neuropeptide hormone activity (4), peptidase (2), peptidase inhibitor (1), sugar binding (1) and unknown (1). The assignments ‘transporter’ for prodynorphin, and ‘kinase’ for stanniocalcin 1 were re-curated to ‘neuropeptide hormone activity’ and ‘hormone activity’ (Table 1).

PACAP Up-Regulates a Cohort of Informational Molecules in Bovine Chromaffin Cells Similar to that Induced in PC12_bPAC1hop Cells

Two different platforms, Affymetrix GeneChip® Bovine Genome Array and Agilent Bovine Gene Expression Microarray were employed for this analysis. Using the criteria described in [Materials and Methods](#), 376 PACAP-up-regulated transcripts were identified as known genes

Table 1 PACAP-responsive transcripts representing secreted molecules in PC12_bPAC1hop cells

Gene name	Gene symbol	Ratio ($n = 3$)	Function
Nicotinamide phosphoribosyltransferase	NAMPT	7.18	Cytokine
Chemokine (C–C motif) ligand 2	CCL2	3.03	Cytokine
Chemokine (C–C motif) ligand 7	CCL7	12.05	Cytokine
Interleukin 11	IL11	5.93	Cytokine
Fibroblast growth factor 2 (basic)	FGF2	3.01	Growth factor
Heparin-binding EGF-like growth factor	HBEGF	4.35	Growth factor
Platelet derived growth factor C	PDGFC	3.81	Growth factor
VGF nerve growth factor inducible	VGF	3.14	Growth factor
Stanniocalcin 1	STC1	3.04	Hormone activity
Laminin, alpha 5	LAMA5	9.62	Integrin binding
Laminin, gamma 2	LAMC2	16.91	Integrin binding
Neurexophilin 3	NXPH3	5.01	Neuropeptide activity
Neuropeptide Y	NPY	3.41	Neuropeptide hormone activity
Prodynorphin	PDYN	2.67	Neuropeptide hormone activity
Prepronociceptin	PNOC	3.68	Neuropeptide hormone activity
Tachykinin, precursor 1	TAC1	3.01	Neuropeptide hormone activity
Matrix metalloproteinase 10 (stromelysin 2)	MMP10	2.64	Peptidase
Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	MMP3	3.81	Peptidase
Serpin peptidase inhibitor, clade E member 1	SERPINE1	13.46	Peptidase inhibitor
Lectin, galactoside-binding, soluble, 9	LGALS9	25.64	Sugar binding
Cysteine-rich secretory protein 1	CRISP1	3.12	Unknown

Table 2 PACAP-responsive transcripts representing secreted molecules in bovine chromaffin cells

Gene name	Gene symbol	Q-RT-PCR fold increase (SEM)	Function	Expressed in chromaffin cells	PACAP-regulated in chromaffin cells
Follistatin	FST	9.75 (1.23)	Activin/inhibin activity ^a	Schneider et al. (2000) (AG)	Reported here
Chemokine (C-X-C motif) ligand 14	CXCL14	43.16 (6.00)	Cytokine	Reported here	Reported here
Nicotinamide phosphoribosyltransferase	NAMPT	3.03 (1.02)	Cytokine	Reported here	Reported here
Secretogranin II	SCG2	9.85 (1.39)	Cytokine	Fischer-Colbrie et al. (1995)	Turquier et al. (2001)
Tumor necrosis factor superfamily 8	TNFSF8	9.19 (0.57)	Cytokine	Reported here	Reported here
Bone morphogenetic protein 1	BMP1	5.27 (1.26)	Growth factor	Reported here	Reported here
Brain-derived neurotrophic factor	BDNF	8.37 (1.90)	Growth factor	Reported here	Reported here
Fibroblast growth factor 1 (acidic)	FGF1	3.06 (0.87)	Growth factor	Reported here	Reported here
Fibroblast growth factor 12	FGF12	2.19 (0.37)	Growth factor	Reported here	Reported here
Fibroblast growth factor 2 (basic)	FGF2	6.82 (1.26)	Growth factor	Grothe and Meisinger (1997)	Reported here
Inhibin, beta A	INHBA	13.24 (1.76)	Growth factor	Schneider et al. (2000) (AG)	Reported here
Transforming growth factor, beta 2	TGFB2	8.90 (1.13)	Growth factor	Reported here	Samal et al. (2007)
VGF nerve growth factor inducible	VGF	3.79 (0.51)	Growth factor	Laslop et al. (1994)	Reported here
Stanniocalcin 1	STC1	8.84 (0.93)	Hormone activity	Reported here	Reported here
Adenylate cyclase-activating polypeptide 1	ADCYAP1	8.54 (1.67)	Neuropeptide hormone activity	Conconi et al. (2006)	Reported here
Calcitonin-related polypeptide beta	CALCB	34.91 (7.47)	Neuropeptide hormone activity	Reported here	Reported here
Cholecystokinin	CCK	10.34 (3.04)	Neuropeptide hormone activity	Ohmori (1998)	Reported here
Galanin prepropeptide	GAL	5.77 (0.71)	Neuropeptide hormone activity	Laslop and Mahata (2002)	Anouar et al. (1999)
Neuropeptide Y	NPY	10.09 (0.34)	Neuropeptide hormone activity	Murabayashi et al. (2007)	Reported here
Thyrotropin-releasing hormone	TRH	6.65 (1.65)	Neuropeptide hormone activity	Montagne et al. (1999) (AG)	Reported here
Vasoactive intestinal peptide	VIP	605.63 (82.91)	Neuropeptide hormone activity	Laslop and Mahata (2002)	Lee et al. 1999
Tachykinin, precursor 1	TAC1	341.38 (38.98)	Neuropeptide hormone activity ^a	Murabayashi et al. (2007)	Reported here
Tachykinin 3	TAC3	24.17 (2.13)	Neuropeptide hormone activity ^a	Reported here	Reported here
Proprotein convertase subtilisin/kexin type 1	PCSK1	7.35 (1.29)	Peptidase	Guillemot et al. (2006)	Reported here
Proprotein convertase subtilisin/kexin type 5	PCSK5	2.67 (1.22)	Peptidase	Mercure et al. (1996) (AG)	Reported here
Plasminogen activator, tissue	PLAT	5.63 (0.38)	Peptidase	Reported here	Reported here
Plasminogen activator, urokinase	PLAU	6.77 (2.02)	Peptidase	Reported here	Reported here
Serpin peptidase inhibitor, clade E, member 1	SERPINE1	2.83 (0.45)	Peptidase inhibitor	Reported here	Reported here
Serpin peptidase inhibitor, clade E, member 2	SERPINE2	1.51 (0.21)	Peptidase inhibitor	Reported here	Reported here
Tissue factor pathway inhibitor 2	TFPI2	6.53 (1.90)	Peptidase inhibitor	Reported here	Reported here

Note Braas et al. (2007) reported up-regulation of FST, BDNF, VGF, PACAP, NPY, TAC1, TRH, PCSK1, and PLAT in SCG cultures after PACAP treatment; CCK secretion is stimulated by PACAP in STC-1 cells (Chang et al. 1996); Reichenstein et al. (2008) reported up-regulation of BDNF by PACAP in cortical neurons AG adrenal gland; ND

^a This function reassigned by author curation (correction of GO lack of assignment)

using both platforms (down-regulated genes were not considered in this analysis).

Among 376 identified transcripts shown to be up-regulated 2-fold or more in either Agilent or Affymetrix arrays, 39 potential extracellular (secreted) molecules were identified using IPA. GO analysis confirmed the extracellular location of 35/39 transcripts while 4/39 were Ingenuity locational misassignments (proteins facing but not in the extracellular space). The 35 confirmed extracellular transcripts were further tiled according to their function using IPA and fell into five tiled groups: cytokines (4), growth factors (9), peptidases (5), kinases (1) and ‘other’ (16). GO

was used again to confirm the functional assignments above, and assign function to the 16 genes in the ‘other’ category. This resulted in the list shown in Table 2: 30 of the 35 transcripts were confirmed as true positives by Q-RT-PCR and clustered as activin/inhibin activity (1) cytokines (4), growth factors (8), hormone activity (1), neuropeptide hormone activity (9), peptidases (4) and peptidase inhibitors (3). The assignment ‘kinase’ for stanniocalcin 1 was re-curated to ‘hormone activity’, in Table 2. The expression of the additional neuropeptides, cytokines and growth factors as well as intracellular and extracellular processing enzymes points to potential roles for PACAP at the

adrenomedullary synapse in addition to enhanced catecholamine and steroidogenic peptide secretion and synthesis.

About a third of these transcripts coded for neuropeptide-containing prohormones. Neuropeptide genes are among those most dynamically regulated by PACAP in bovine chromaffin cells (Babinski et al. 1996; Hamelink et al. 2002a). In addition, ‘neuropeptide hormone activity’ as a GO category is highly enriched among the transcripts up-regulated by PACAP (GO Tree Machine analysis of set of 376 up-regulated transcripts: <http://bioinfo.vanderbilt.edu/gotm/>). Neuropeptides, including GAL and VIP, among others are also consistently up-regulated in adrenal medulla in vivo following splanchnic nerve stimulation. Most of the neuropeptides in this transcriptome analysis are previously shown to be PACAP-regulated in chromaffin cells, either in vivo, such as GAL and VIP (Ait-Ali et al. 2010) or in cell culture (Table 2). Novel PACAP-regulated neuropeptides identified here include NPY, TAC1, thyrotropin-releasing hormone (TRH), CCK, and TAC3 (Table 2).

Transcripts encoding not only neuropeptides (NPY, TAC1, TRH), but growth factors (BDNF, VGF) and bioactive peptide-generating peptidases (PCSK1, PLAT) have been reported to be up-regulated by PACAP in superior cervical ganglion cultures (SCG; Braas et al. 2007). We have also found several growth factors and bioactive peptide-generating peptidases, including those above, to be regulated after 6 h of treatment by PACAP. Follistatin a molecule displaying an activin/inhibin activity and playing an essential role in reproduction but also non-reproductive function such as tissue repair and inflammation (Schneider et al. 2000) is up-regulated by PACAP in chromaffin cells, as are transcripts encoding the growth factors FGF1 and FGF12. Interestingly, in addition to the informational molecules themselves, both processing enzyme- and peptidase inhibitor-encoding transcripts (PCSK1, PCSK5, PLAT, and PLAU, and SERPINE1, SERPINE2, and TFPI2, respectively) are also regulated by PACAP in chromaffin cells (Table 2).

Cytokines are perhaps least expected among informational molecules to be up-regulated specifically by PACAP in chromaffin cells. NAMPT, for example, is widely induced by inflammatory stimuli in cells involved in innate immunity as well as in epithelial and endothelial cells (Luk et al. 2008). SCG2 and TNFSF8 both play major roles in innate immunity (Brogden et al. 2005; Kennedy et al. 2006). The chemokine CXCL14 is also known for its antimicrobial activity (Maerki et al. 2009). Regulation of these molecules by PACAP points toward a role for the adrenal medulla in host defense mechanisms in stress/inflammation conditions. However, it is not known if cytokines and neuropeptides are secreted from the same large dense-core vesicles, or from different secretory compartments, in chromaffin cells.

In addition to previously identified neuropeptides, we show here that there is a concerted up-regulation of

expression of several additional peptides, as well as growth factors. Previous studies have shown that dysregulated expression or function of peptides such as CCK and VGF, involved in behavioral and energy homeostasis (Jethwa and Ebling 2008; Szelenyi 2010), as well as NPY and TAC1, can be involved in the etiology of major neuropathologies such as schizophrenia or depression (De Wied and Sigling 2002). TRH, a hypothalamic hypophysiotropic neuropeptide, might have antidepressant effects (De Wied and Sigling 2002), but can also play an important role in homeostatic regulation of immune function (Kamath et al. 2009). These informational molecules, whose production and probable secretion is enhanced by PACAP, may also be enhanced in vivo in a PACAP-dependent manner, during the stress response, providing additional integrative functions for the adrenal medulla as an organismic stress transducer.

The Effect of PACAP on TAC1 and VIP Gene Expression is Dependent on PKA or ERK 1/2 Activation in BCCs

PACAP signaling through the PAC1 receptor present on chromaffin cells is known to involve a wide variety of signal transduction pathways by enhancement of calcium influx, mobilization of sequestered intracellular calcium to the cytosol, and elevation of cyclic AMP (cAMP) levels (Mustafa and Eiden 2006, Gerdin and Eiden 2007). We wished to investigate whether PACAP employs only one or several of these pathways to regulate genes encoding informational molecules. We therefore examined the involvement of cAMP-dependent protein kinase (PKA) and the MAP kinases ERK 1/2, in the stimulatory effect of PACAP on neuropeptide gene expression (Fig. 1). Application of the p42/44 ERK 1/2 MAPK inhibitor U0126 (10 μ M) to primary cultures of bovine chromaffin cells strongly inhibited the effect of PACAP on TAC1 and VIP mRNA levels (Fig. 1a, b). However, the PKA inhibitor H89 (10 μ M) had a differential effect on induction of the two targets, suppressing the effect of PACAP on TAC1 mRNA levels (Fig. 1a) without altering the stimulatory effect of PACAP on VIP mRNA expression (Fig. 1b). It has been previously reported that PACAP acts independently of PKA on VIP biosynthesis in bovine chromaffin cells (Hamelink et al. 2002a). PACAP was also described to affect neurite-genesis through a cAMP-dependent, PKA-independent pathway proceeding through ERK (Ravni et al. 2008). Our data indicate that neuropeptide biosynthesis in chromaffin cells is regulated by PACAP via multiple pathways.

It will be of great interest to examine the signaling pathways employed by PACAP in regulation of cytokines and growth factors, as well as neuropeptides, in chromaffin cells. PC12 and PC12_bPAC1hop cells differ mainly in the greatly enhanced calcium influx obtained in the latter cells

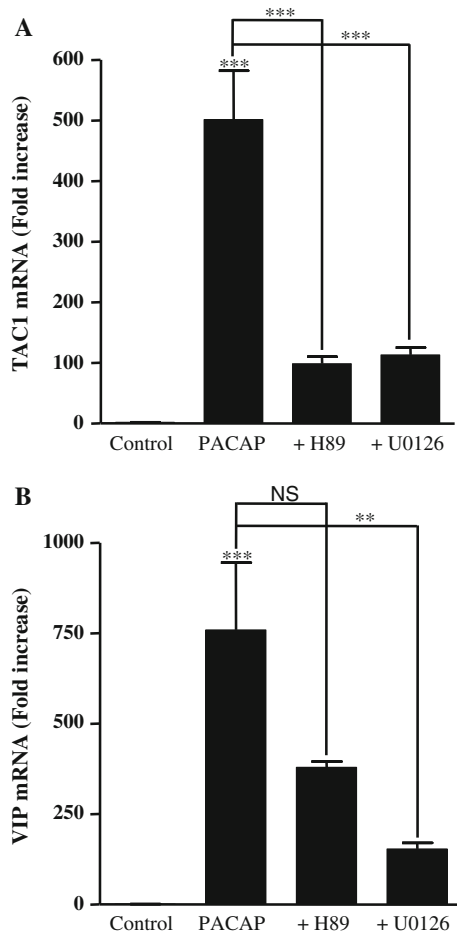


Fig. 1 Effect of inhibiting MEK or PKA on PACAP induction of neuro peptide gene expression. Chromaffin cells were incubated for 6 h in control conditions or with 100 nM PACAP, in the absence or presence of 10 μ M U0126 or 10 μ M H89 (**a**, **b**) as described in “Materials and Methods”. TAC1 (**a**) and VIP (**b**) mRNA levels, determined by Q-RT-PCR, are expressed as fold increase over corresponding control values and represent means \pm SEM of three or four determinations for each condition from one experiment representative of three different experiments. ** $P < 0.05$; *** $P < 0.001$ versus the corresponding control (one-way ANOVA test, Bonferroni posttest). NS not significant

upon exposure to PACAP (Mustafa et al. 2007). Since PC12_bPAC1hop cells also express a similar cohort of informational molecules (neuropeptides as well as cytokines and growth factors) as do bovine chromaffin cells after exposure to PACAP, this unique transcriptomic ‘stress signature’ of PACAP signaling may be due to a concerted regulation by cAMP and calcium, unique to PAC1hop-dependent receptor activation, for this neuropeptide first messenger.

Conclusion

Transcriptome analysis reveals a class of gene targets for PACAP signaling that can be conceptualized as

informational molecules: peptide hormones, neuropeptides, growth factors, cytokines, and enzymes responsible for hormone processing or the activation of extracellular enzymatic or hormonal cascades. That these are regulated as a cohort by PACAP, and presumably released at the adrenomedullary synapse selectively during stress, may point toward a role for the adrenal medulla as a stress transducer whose role extends beyond elaboration of catecholamines and steroidogenic neuropeptides to include the episodic production of these additional blood-borne informational molecules, providing further integration of the stress response.

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References

- Ait-Ali D, Stroth N, Sen JM, Eiden LE (2010) PACAP-cytokine interactions govern adrenal neuropeptide biosynthesis after systemic administration of LPS. *Neuropharmacology* 58:208–214
- Andreis PG, Tortorella C, Ziolkowska A, Spinazzi R, Malendowicz LK, Neri G, Nussdorfer GG (2007) Evidence for a paracrine role of endogenous adrenomedullary galanin in the regulation of glucocorticoid secretion in the rat adrenal gland. *Int J Mol Med* 19:511–515
- Anouar Y, MacArthur L, Cohen J, Iacangelo AL, Eiden LE (1994) Identification of a TPA-responsive element mediating preferential transactivation of the galanin gene promoter in chromaffin cells. *J Biol Chem* 269:6823–6831
- Anouar Y, Lee HW, Eiden LE (1999) Both inducible and constitutive activator protein-1-like transcription factors are used for transcriptional activation of the galanin gene by different first and second messenger pathways. *Mol Pharmacol* 56:162–169
- Babinski K, Bodart V, Roy M, De Lean A, Ong H (1996) Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. *Neuropeptides* 30:572–582
- Bornstein SR, Haidan A, Ehrhart-Bornstein M (1996) Cellular communication in the neuro-adrenocortical axis: role of vasoactive intestinal polypeptide (VIP). *Endocr Res* 22:819–829
- Braas KM, Schutz KC, Bond JP, Vizzard MA, Girard BM, May V (2007) Microarray analyses of pituitary adenylate cyclase activating polypeptide (PACAP)-regulated gene targets in sympathetic neurons. *Peptides* 28:1856–1870
- Brogden KA, Guthmiller JM, Salzet M, Zasloff M (2005) The nervous system and innate immunity: the neuropeptide connection. *Nat Immunol* 6:558–564
- Chang CH, Chey WY, Braggins L, Coy DH, Chang TM (1996) Pituitary adenylate cyclase-activating polypeptide stimulates cholecystokinin secretion in STC-1 cells. *Am J Physiol* 271:G516–523
- Colbert RA, Balbi D, Johnson A, Bailey JA, Allen JM (1994) Vasoactive intestinal peptide stimulates neuropeptide Y gene expression and causes neurite extension in PC12 cells through independent mechanisms. *J Neurosci* 14:7141–7147

- Conconi MT, Spinazzi R, Nussdorfer GG (2006) Endogenous ligands of PACAP/VIP receptors in the autocrine-paracrine regulation of the adrenal gland. *Int Rev Cytol* 249:1–51
- De Wied D, Sigling HO (2002) Neuropeptides involved in the pathophysiology of schizophrenia and major depression. *Neurotox Res* 4:453–468
- Ehrhart-Bornstein M, Bornstein SR, Scherbaum WA, Pfeiffer EF, Holst JJ (1991) Role of the vasoactive intestinal peptide in a neuroendocrine regulation of the adrenal cortex. *Neuroendocrinology* 54:623–628
- Eiden LE, Samal B, Gerdin MJ, Mustafa T, Vaudry D, Stroth N (2008) Discovery of pituitary adenylate cyclase-activating polypeptide-regulated genes through microarray analyses in cell culture and in vivo. *Ann N Y Acad Sci* 1144:6–20
- Fischer-Colbrie R, Eskay RL, Eiden LE, Maas D (1992) Transsynaptic regulation of galanin, neurotensin, and substance P in the adrenal medulla: combinatorial control by second-messenger signaling pathways. *J Neurochem* 59:780–783
- Fischer-Colbrie R, Laslop A, Kirchmair R (1995) Secretogranin II: molecular properties, regulation of biosynthesis and processing to the neuropeptide secretoneurin. *Prog Neurobiol* 46:49–70
- Fischer-Colbrie R, Kirchmair R, Kahler CM, Wiedermann CJ, Saria A (2005) Secretoneurin: a new player in angiogenesis and chemotaxis linking nerves, blood vessels and the immune system. *Curr Protein Pept Sci* 6:373–385
- Gerdin MJ, Eiden LE (2007) Regulation of PC12 cell differentiation by cAMP signaling to ERK independent of PKA: do all the connections add up? *Sci STKE* 2007:pe15
- Ghzi H, Grumolato L, Thouennon E, Tanguy Y, Turquier V, Vaudry H, Anouar Y (2008) Role of PACAP in the physiology and pathology of the sympathoadrenal system. *Front Neuroendocrinol* 29:128–141
- Goetzl EJ, Chan RC, Yadav M (2008) Diverse mechanisms and consequences of immunoadoption of neuromediator systems. *Ann N Y Acad Sci* 1144:56–60
- Grothe C, Meisinger C (1997) The multifunctionality of FGF-2 in the adrenal medulla. *Anat Embryol (Berl)* 195:103–111
- Guillemot J, Barbier L, Thouennon E, Vallet-Erdtmann V, Montero-Hadjadje M, Lefebvre H, Klein M, Muresan M, Plouin PF, Seidah N, Vaudry H, Anouar Y, Yon L (2006) Expression and processing of the neuroendocrine protein secretogranin II in benign and malignant pheochromocytomas. *Ann N Y Acad Sci* 1073:527–532
- Hahm SH, Hsu CM, Eiden LE (1998) PACAP activates calcium influx-dependent and -independent pathways to couple met-enkephalin secretion and biosynthesis in chromaffin cells. *J Mol Neurosci* 11:43–56
- Hamelink C, Lee HW, Chen Y, Grimaldi M, Eiden LE (2002a) Coincident elevation of cAMP and calcium influx by PACAP-27 synergistically regulates vasoactive intestinal polypeptide gene transcription through a novel PKA-independent signaling pathway. *J Neurosci* 22:5310–5320
- Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, Eiden LE (2002b) Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proc Natl Acad Sci USA* 99:461–466
- Jethwa PH, Ebling FJ (2008) Role of VGF-derived peptides in the control of food intake, body weight and reproduction. *Neuroendocrinology* 88:80–87
- Kamath J, Yarbrough GG, Prange AJ Jr, Winokur A (2009) The thyrotropin-releasing hormone (TRH)-immune system homeostatic hypothesis. *Pharmacol Ther* 121:20–28
- Kennedy MK, Willis CR, Armitage RJ (2006) Deciphering CD30 ligand biology and its role in humoral immunity. *Immunology* 118:143–152
- Kuri BA, Chan SA, Smith CB (2009) PACAP regulates immediate catecholamine release from adrenal chromaffin cells in an activity-dependent manner through a protein kinase C-dependent pathway. *J Neurochem* 110:1214–1225
- Laslop A, Mahata SK (2002) Neuropeptides and chromogranins: session overview. *Ann N Y Acad Sci* 971:294–299
- Laslop A, Mahata SK, Wolkersdorfer M, Mahata M, Srivastava M, Seidah NG, Fischer-Colbrie R, Winkler H (1994) Large dense-core vesicles in rat adrenal after reserpine: levels of mRNAs of soluble and membrane-bound constituents in chromaffin and ganglion cells indicate a biosynthesis of vesicles with higher secretory quanta. *J Neurochem* 62:2448–2456
- Lee HW, Hahm SH, Hsu CM, Eiden LE (1999) Pituitary adenylate cyclase-activating polypeptide regulation of vasoactive intestinal polypeptide transcription requires Ca^{2+} influx and activation of the serine/threonine phosphatase calcineurin. *J Neurochem* 73:1769–1772
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408
- Luk T, Malam Z, Marshall JC (2008) Pre-B cell colony-enhancing factor (PBEF)/visfatin: a novel mediator of innate immunity. *J Leukoc Biol* 83:804–816
- Maerki C, Meuter S, Liebi M, Muhlemann K, Frederick MJ, Yawalkar N, Moser B, Wolf M (2009) Potent and broad-spectrum antimicrobial activity of CXCL14 suggests an immediate role in skin infections. *J Immunol* 182:507–514
- Mercure C, Jutras I, Day R, Seidah NG, Reudelhuber TL (1996) Prohormone convertase PC5 is a candidate processing enzyme for prorenin in the human adrenal cortex. *Hypertension* 28:840–846
- Monnier D, Loeffler JP (1998) Pituitary adenylate cyclase-activating polypeptide stimulates proenkephalin gene transcription through AP1- and CREB-dependent mechanisms. *DNA Cell Biol* 17:151–159
- Montagne JJ, Ladram A, Nicolas P, Bulant M (1999) Cloning of thyrotropin-releasing hormone precursor and receptor in rat thymus, adrenal gland, and testis. *Endocrinology* 140:1054–1059
- Murabayashi H, Kuramoto H, Kawano H, Sasaki M, Kitamura N, Miyakawa K, Tanaka K, Oomori Y (2007) Immunohistochemical features of substance P-immunoreactive chromaffin cells and nerve fibers in the rat adrenal gland. *Arch Histol Cytol* 70:183–196
- Mustafa T, Eiden LE (2006) Secretin superfamily: PACAP, VIP, and related neuropeptides. S. US, *Handbook of neurochemistry and molecular neurobiology neuroactive proteins and peptides*, Springer, Heidelberg, pp 463–498
- Mustafa T, Grimaldi M, Eiden LE (2007) The hop cassette of the PAC1 receptor confers coupling to Ca^{2+} elevation required for pituitary adenylate cyclase-activating polypeptide-evoked neurosecretion. *J Biol Chem* 282:8079–8091
- Ohmori Y (1998) Localization of biogenic amines and neuropeptides in adrenal medullary cells of birds. *Horm Metab Res* 30:384–388
- Rausch DM, Iacangelo AL, Eiden LE (1988) Glucocorticoid- and nerve growth factor-induced changes in chromogranin A expression define two different neuronal phenotypes in PC12 cells. *Mol Endocrinol* 2:921–927
- Ravni A, Vaudry D, Gerdin MJ, Eiden MV, Falluel-Morel A, Gonzalez BJ, Vaudry H, Eiden LE (2008) A cAMP-dependent, protein kinase A-independent signaling pathway mediating neurogenesis through Egr1 in PC12 cells. *Mol Pharmacol* 73:1688–1708
- Reichenstein M, Rehavi M, Pinhasov A (2008) Involvement of pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors in the mechanism of antidepressant action. *J Mol Neurosci* 36:330–338

- Samal B, Gerdin MJ, Huddleston D, Hsu CM, Elkahloun AG, Stroth N, Hamelink C, Eiden LE (2007) Meta-analysis of microarray-derived data from PACAP-deficient adrenal gland in vivo and PACAP-treated chromaffin cells identifies distinct classes of PACAP-regulated genes. *Peptides* 28:1871–1882
- Schneider O, Nau R, Michel U (2000) Comparative analysis of follistatin-, activin beta A- and activin beta B-mRNA steady-state levels in diverse porcine tissues by multiplex S1 nuclease analysis. *Eur J Endocrinol* 142:537–544
- Stroth N, Eiden LE (2010) Stress hormone synthesis in mouse hypothalamus and adrenal gland triggered by restraint is dependent on pituitary adenylate cyclase-activating polypeptide signaling. *Neuroscience* 165:1025–1030
- Stroth N, Hamelink C, Eiden LE (2007) PACAP-dependent cellular plasticity in the mouse adrenal gland. *FASEB J* 21:A1249–A1250
- Szelenyi Z (2010) Cholecystokinin: role in thermoregulation and other aspects of energetics. *Clin Chim Acta* 411:329–335
- Taupenot L, Mahata M, Mahata SK, O'Connor DT (1999) Time-dependent effects of the neuropeptide PACAP on catecholamine secretion: stimulation and desensitization. *Hypertension* 34:1152–1162
- Tornøe K, Hannibal J, Jensen TB, Georg B, Rickelt LF, Andreasen MB, Fahrenkrug J, Holst JJ (2000) PACAP-(1–38) as neurotransmitter in the porcine adrenal glands. *Am J Physiol Endocrinol Metab* 279:E1413–E1425
- Turquier V, Yon L, Grumolato L, Alexandre D, Fournier A, Vaudry H, Anouar Y (2001) Pituitary adenylate cyclase-activating polypeptide stimulates secretoneurin release and secretogranin II gene transcription in bovine adrenochromaffin cells through multiple signaling pathways and increased binding of pre-existing activator protein-1-like transcription factors. *Mol Pharmacol* 60:42–52
- Vaudry D, Chen Y, Ravni A, Hamelink C, Elkahloun AG, Eiden LE (2002) Analysis of the PC12 cell transcriptome after differentiation with pituitary adenylate cyclase-activating polypeptide (PACAP). *J Neurochem* 83:1272–1284
- Wakade AR (1998) Multiple transmitter control of catecholamine secretion in rat adrenal medulla. *Adv Pharmacol* 42:595–598
- Watanabe T, Masuo Y, Matsumoto H, Suzuki N, Ohtaki T, Masuda Y, Kitada C, Tsuda M, Fujino M (1992) Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. *Biochem Biophys Res Commun* 182:403–411
- Yadav M, Goetzl EJ (2008) Vasoactive intestinal peptide-mediated Th17 differentiation: an expanding spectrum of vasoactive intestinal peptide effects in immunity and autoimmunity. *Ann N Y Acad Sci* 1144:83–89