

PACAP Immunoreactivity in Human Malignant Tumor Samples and Cardiac Diseases

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Abstract Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic and multifunctional neuropeptide having important roles in various physiological processes. Recent trends in PACAP research point to the clinical introduction of PACAP or its analogs/fragments possibly in the near future. Recently, we have shown the presence of PACAP in human plasma, milk, placenta, and follicular fluid samples. However, relatively few data are available on PACAP in human tissues from patients with different disorders. The aim of the present study was to determine, by

radioimmunoassay, the tissue level of PACAP38-like immunoreactivity (LI) and PACAP27-LI in different primary non-small cell lung cancer, colon tumor samples, and in cardiac muscle samples from patients suffering from ischemic heart disease and valvular disorders. We also labeled the PAC1 receptors in human cardiac cells. All samples showed significantly higher PACAP38-LI compared with PACAP27-LI. We found significantly lower levels of PACAP38-LI and PACAP27-LI in tumoral and peripheral samples compared with normal healthy tissue in both lung and colon cancers. Further investigations are necessary to describe the exact function of PACAP in oncogenesis. We showed that PACAP38-LI and PACAP27-LI are significantly higher in ischemic heart diseases compared with valvular abnormalities, suggesting that PACAP might play a role in ischemic heart disorders.

Keywords PACAP · Colon cancer · Lung cancer · Heart disorder · PAC1 receptor

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was isolated from ovine hypothalamus in 1989, based on its activating effect on adenylate cyclase in the pituitary gland. PACAP is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family and exists in 27 and 38 amino acid form, the latter being the predominant peptide in mammalian tissues. PACAP is a pleiotropic and multifunctional neuropeptide having important roles in various physiological processes (Vaudry et al. 2009). Since its discovery, a large number of studies have shown its neurotrophic, neuroprotective, general cytoprotective, and anti-inflammatory effects both in vitro and in vivo (Delgado and Ganea 2001;

Somogyvari-Vigh and Reglodi 2004; Gasz et al. 2006a,b; Botia et al. 2007; Racz et al. 2007; Dejda et al. 2008; Szakaly et al. 2008; Vaudry et al. 2009; Atlasz et al. 2010; Bourgault et al. 2011; Reglodi et al. 2011; Seaborn et al. 2011).

Recent trends in PACAP research point to the clinical introduction of PACAP or its analogs/fragments possibly in the near future. Recently, we have shown the presence of PACAP in human plasma, where elevated PACAP levels were measured during pregnancy (Borzsei et al. 2009; Reglodi et al. 2010). We have also detected the presence of PACAP38-like immunoreactivity (LI) in the human milk (Borzsei et al. 2009), placenta, and follicular fluid samples (Brubel et al. 2010, 2011; Koppan et al. 2012). However, relatively few data are available on PACAP in human tissues from patients with different disorders.

PACAP has important functions in the regulation of cell growth, proliferation, differentiation, and apoptosis (Dejda et al. 2008; Vaudry et al. 2009; Seaborn et al. 2011). It acts via three receptors, the specific PAC1 receptor and the VPAC1 and VPAC2 receptors, the latter two binding VIP and PACAP with equal affinity (Vaudry et al. 2009). PACAP receptors are expressed in the vast majority of the frequently occurring human tumors, including primary lung and colon cancers (Reubi 2000; Reubi et al. 2000; Busto et al. 1999, 2000, 2003). PACAP influences expression of various factors that play a role in tumor growth (Zia et al. 1995; Moody et al. 2002, 2012; Le et al. 2002) and both anti- and proapoptotic effects have been described in different tumors (Vaudry et al. 2009) suggesting a possible involvement of PACAP in oncogenesis. The aim of the first part of the study was to determine the tissue level of PACAP38-LI and PACAP27-LI in different primary non-small cell lung cancer (NSCLC) and colon tumor samples with radioimmunoassay (RIA) examination.

PACAP has diverse regulatory functions in the nervous, endocrine, gastrointestinal, immune, and cardiovascular system (Vaudry et al. 2009). PACAP and its receptors (PAC1, VPAC1, and VPAC2) have been shown in the heart of different mammals (Wei and Mojsov 1996; Calupca et al. 2000; DeHaven and Cuevas 2002; Ushiyama et al. 2006), and it is able to modulate cardiac functions (Naruse et al. 1993; Parsons et al. 2000; Hirose et al. 2001; Hardwick et al. 2006). Recently, our research group has shown the cardioprotective effect of PACAP in vitro (Gasz et al. 2006a, b; Racz et al. 2008, 2010; Roth et al. 2009). Mori and coworkers (2010) provided in vivo evidence for cardioprotective effect of PACAP on doxorubicin-induced cardiomyopathy in mice. Although, these results suggest that this peptide might be a potential treatment of cardiac diseases in the future; there are no data about PACAP and PAC1 receptors from patients with cardiac disorders. Therefore, in the second part of this study, our aim was to examine the PACAP38-LI and PACAP27-LI with RIA in cardiac muscle

samples from patients suffering from ischemic heart disease and valvular disorders and to label PAC1 receptors with immunohistochemistry in the myocardium.

Methods

RIA Method

In the first part of the study, we examined the PACAP38-LI and PACAP27-LI in tissue samples from patients treated operatively for NSCLC or colon adenocarcinoma with RIA. We collected samples from the central and peripheral parts of lung ($n=9$) and colon tumors ($n=9$) and from healthy lung and colon tissue during surgery. Peripheral samples were taken within 2 cm of cancer margin and histologically confirmed as intact. In the second part of the experiment, we examined cardiac tissue samples collected from patients with ischemic heart disease ($n=11$), mitral valve insufficiency, or aortic stenosis ($n=8$) during heart surgery. We collected samples excised from the right auricle during the insertion of cannula of the extracorporeal circulating system. All human sample collections were carried out according to a protocol approved by the Institutional Ethic Committee (PTE KK 3118, 3936). In all cases, we obtained written consent of the volunteers.

Tissues samples were weighed and homogenized in ice-cold distilled water. The homogenate was centrifuged (12,000 rpm, 4°C, 30 min), and the supernatant was further processed for RIA analysis of PACAP38-LI and PACAP27-LI with a specific and sensitive RIA technique developed in our laboratory (Jakab et al. 2004; Borzsei et al. 2009; Brubel et al. 2010), and immunoreactivity of the peptides was calculated with the help of a calibration. Briefly: *Antiserum*: PACAP38: “88111-3” (working dilution 1:10,000) and PACAP27: “88123” (dilution: 1:45,000). *Tracer*: mono-¹²⁵I-labelled ovine PACAP 24-38 and mono-¹²⁵I-labelled ovine PACAP27 prepared in our laboratory (5,000 cpm/tube). *Standard*: Ovine PACAP38 and PACAP27 were used as a RIA standard ranging from 0 to 1,000 fmol/mg. Assays were prepared in 1 ml phosphate buffer (0.05 mol/l, pH 7.4) containing 0.1 M NaCl, 0.05% NaN₃, 0.25% bovine serum albumin (Sigma). One hundred microliters antiserum (working dilution, 1:100,000), 100 µl RIA tracer (5,000 cpm/tube), and 100 µl standard or unknown samples were measured into polypropylene tubes with assay buffer. After 48–72 h incubation at 4°C, the antibody-bound peptide was separated from the free one by addition of 100 µl separation solution (10 g charcoal, 1 g dextran, 0.2 g commercial fat-free milk powder in 100 ml distilled water). Following centrifugation (3,000 rpm, 20 min, 4°C), the tubes were gently decanted, and the radioactivity of the precipitates was measured in a gamma counter. PACAP38-LI and PACAP27-LI of the

unknown samples were read from a calibration curve. Results are given as femtomoles PACAP38-LI and PACAP27-LI per milligram tissue weight. Differences between PACAP38-LI and PACAP27-LI were analyzed by Student's *t* test and differences between samples were analyzed using ANOVA. In all cases, $p < 0.05$ was considered statistically significant.

Histological Examination

The cardiac tissue samples were dissected in ice-cold phosphate-buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Tissue was then washed in phosphate-buffered saline (PBS) and cryoprotected in 10% sucrose for 1 h and 20% sucrose in PBS overnight at 4°C. For cryostat sectioning, tissues were embedded in tissue-freezing medium (Tissue-Tek, OCT Compound, Sakura Finetech, NL), cut in a cryostat (Leica, Nussloch, Germany) at 10- μ m radial sections. Sections were mounted on chrome–alum–gelatin-coated subbed slides and stored at –20°C until use. One part of the tissue sections was stained with hematoxylin–eosin staining, and the other parts were labeled with PAC1 receptor immunohistochemistry. At the beginning of the immunohistochemistry, tissue sections were rinsed in PBS, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and then incubated with 0.1% bovine serum albumin, 1% normal goat serum, and 0.1% Na-azide in PBS for 1 h to minimize nonspecific labeling. Sections were incubated with anti-PAC1 receptor antibody raised in rabbit (1:100, kind gift from Prof. Seiji Shioda) for overnight at room temperature. After several washes in PBS, sections were incubated for 2 h in the dark with the corresponding Alexa Fluor “568” secondary antibody also raised in rabbit (1:1,000, Southern Biotech). Sections were then washed in PBS and were coverslipped using Fluoromount-G (Southern Biotech). For control experiments, primary antisera were omitted, and specific cellular staining was not found. Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a CCD camera. Images were taken with the Spot software package. Photographs were further processed with the Adobe Photoshop 7.0 program. Images were adjusted for contrast only, aligned, arranged, and then labeled using the functions of the above program.

Results

In the first part of the study, we investigated the level of PACAP38-LI and PACAP27-LI in normal, tumoral, and peritumoral (peripheral) lung tissue samples. Our results showed significantly lower level of PACAP38-LI and PACAP27-LI in tumoral and peripheral samples compared

with normal healthy tissue. Significantly higher PACAP38-LI was found in normal and tumoral tissue samples compared with PACAP27-LI (Fig. 1).

The RIA examination of colon tissue samples showed similar results: both PACAP38-LI and PACAP27-LI were significantly lower in tumoral and peripheral samples compared with normal healthy tissue, and PACAP38-LI was significantly higher in all samples compared with PACAP27-LI in the same tissue (Fig. 2).

In the second part of the study, we collected samples from the heart during cardiac surgery. We compared the PACAP38-LI and PACAP27-LI in heart tissue samples from ischemic heart disorder and valvular abnormalities (aorta stenosis or mitral insufficiency). PACAP38-LI was significantly higher in all samples compared with PACAP27-LI. PACAP38-LI and PACAP27-LI were significantly higher in patients, where cardiac surgery was indicated for ischemic heart disease compared with those who were suffering from valvular dysfunction (Fig. 3).

In the lung, we have a rich capillary meshwork around the alveoli, therefore, the relatively high amount of blood in the lung samples can lead to higher PACAP38-LI and PACAP27-LI in the lung tissue compared with colon and heart samples (Figs. 1, 2, and 3). The baseline quantitative data are therefore not compared between samples originated from different tissues.

In order to investigate the expression of PAC1 receptors in the heart muscle, we obtained heart muscle samples during cardiac surgery. The samples were excised from the heart muscle wall during the insertion of cannula of extracorporeal circulating system to the right atrium of patients with ischemic cardiac disease. We used hematoxylin–eosin staining to orientate the heart muscle cells in cross- and longitudinal sections. In parallel slides, PAC1 receptor immunohistochemistry showed receptor expression in the heart muscle cells. The endocardial connective tissue remained PAC1-receptor-negative, but the non-specific binding of the red blood cells was detectable as an auto-fluorescence between the heart muscle cells (Fig. 4).

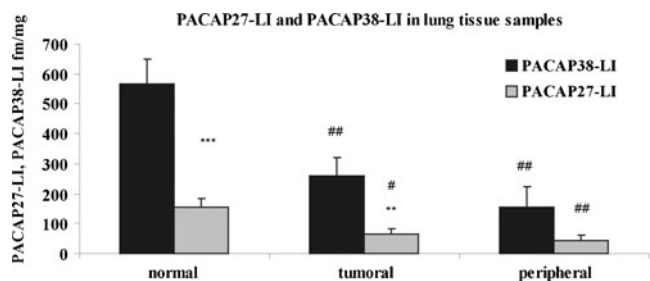


Fig. 1 PACAP38-like immunoreactivity (LI) and PACAP27-LI determined by RIA from normal, tumoral, and peripheral lung tissue samples. Data are means \pm SEM. ** $p < 0.01$; *** $p < 0.001$ vs. PACAP38-LI; # $p < 0.05$; ## $p < 0.01$ vs. normal tissue samples

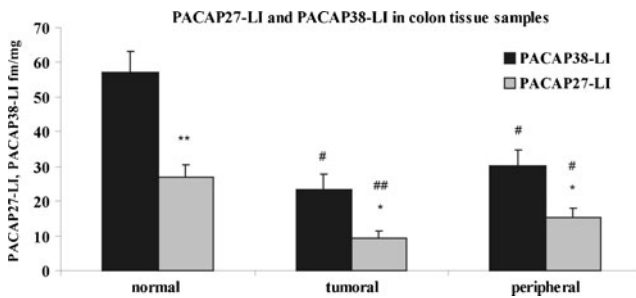


Fig. 2 PACAP38-like immunoreactivity (LI) and PACAP27-LI determined by RIA from normal, tumoral, and peripheral colon tissue samples. Data are means±SEM. * p <0.05; ** p <0.01 vs. PACAP38-LI; # p <0.05; ## p <0.01 vs. normal tissue samples

Discussion

In the first part of our experiment, we examined PACAP38-LI and PACAP27-LI in human samples from normal and cancerous lung tissues. We found significantly lower levels of PACAP27-LI and PACAP38-LI in tumoral and peripheral tissues compared with normal samples. In humans, PACAP-positive nerve fibers are found in the tracheal and bronchial wall, situated among smooth muscle bundles, and around glands and small blood vessels (Luts et al. 1993). Numerous experiments investigated the presence of different PACAP receptors in normal and tumoral human lung tissue samples using different methods (Reubi 2000; Reubi et al. 2000; Busto et al. 1999, 2000, 2003). PACAP and PACAP receptors have been described both in small cell lung cancer (SCLC) and NSCLC lines (Moody et al. 1993, 2000; Zia et al. 1995). PACAP affects the growth and proliferation of lung cancer cells; for example, the protooncogene *c-fos* plays a role in cell proliferation and differentiation, and PACAP is able to increase *c-fos* mRNA in SCLC cells (Draoui et al. 1996). The PACAP antagonist PACAP6-38 reverses the increase in *c-fos* mRNA and inhibits SCLC growth in vitro and in vivo (Draoui et al. 1996) and NSCLC growth in vivo (Zia et al. 1995). PACAP also stimulates the MAPK and EGF receptor tyrosine phosphorylation and vascular endothelial cell growth factor mRNA in lung

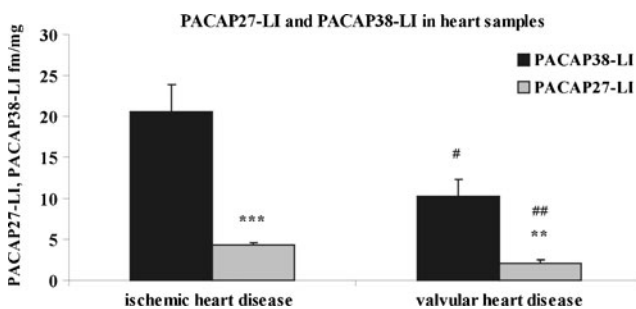


Fig. 3 PACAP38-like immunoreactivity (LI) and PACAP27-LI determined by RIA from heart samples. Data are means±SEM. ** p <0.01; *** p <0.001 vs. PACAP38-LI; # p <0.05; ## p <0.01 vs. normal tissue samples

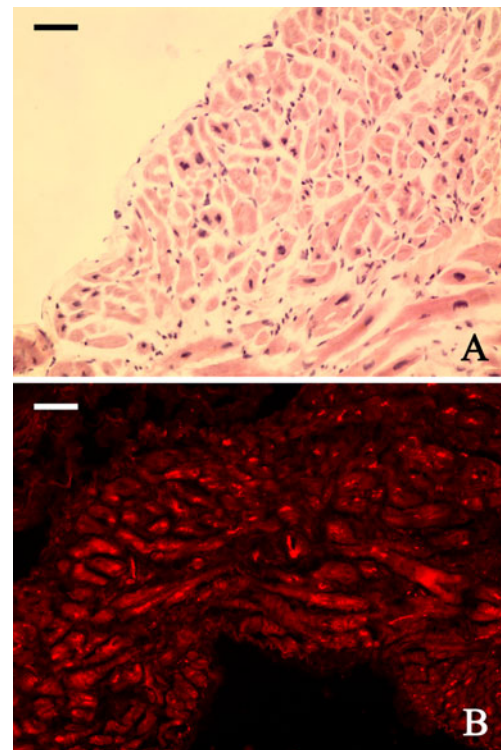


Fig. 4 Representative pictures of human heart muscle stained by hematoxylin–eosin (a) and PAC1 receptor immunohistochemistry (b). Scale bar, 20 μ m

cancer cell lines (Moody et al. 2002, 2012). As PACAP is an autocrine growth factor for lung cancer, the relatively lower amount of PACAP38-LI and PACAP27-LI in tumoral and peritumoral tissues may suggest the overuse of the peptide by tumoral cells. On the other hand, the significantly lower immunoreactivity in the tumor might be caused by the degeneration of the structures containing high amount of PACAP such as smooth muscle and endocrine cells.

Similarly to the lung samples, we also showed significant difference between the normal and tumoral colon tissue samples, where PACAP38-LI and PACAP27-LI were also significantly lower in tumoral and peripheral tissue samples compared with normal tissue. The presence of PACAP in the gastrointestinal tract is well-known (Portbury et al. 1995; Hannibal et al. 1998), where it regulates secretion and motility (Lauff et al. 1999). Similarly to lung tissue, Reubi and coworkers have shown the predominance of VPAC1 receptors in healthy gastrointestinal mucosa and colorectal carcinoma with specific autoradiography (Reubi 2000; Reubi et al. 2000). PACAP and PAC1 receptor immunoreactivities have been shown in nerve fibers of the mucosa, muscles, and myenteric plexus of the stomach and distal colon of the rat where intensive staining has been detected in the ganglionic nerve cells (Sundler et al. 1992; Portbury et al. 1995; Hannibal et al. 1998; Miampamba et al. 2002). PACAP and PAC1 receptor expression have also been detected in

HCT8 human colon tumor cells. PACAP38 increases the number of viable HCT8 cells via the regulation of Fas-R expression in human colon cancer cell line, indicating possible role of PACAP in the regulation of colon cancer growth (Le et al. 2002). PACAP plays a suppressive role in the development of inflammation-associated colon cancer, because PACAP-deficient mice showed more aggressive colorectal tumor growth upon induction of colitis (Nemetz et al. 2008). Similarly to our results, PACAPergic nerve fibers in the submucosal and myenteric plexus decreased significantly in pathological sections from sigmoid and rectum cancer compared with sections from uninvolved intestinal wall (Godlewski and Lakomy 2010). The significant decrease in the PACAP immunoreactivity of the tumoral samples may be caused by the degeneration of the myenteric plexus and the dysfunction in the innervation of colon in tumoral and peritumoral part.

In the second part of the study, we investigated cardiac muscle samples collected from patients with ischemic heart disease, mitral valve insufficiency, or aortic stenosis during heart surgery. We demonstrated that PACAP38-LI and PACAP27-LI were significantly higher in heart muscle of patients with ischemic heart disorder compared with valvular abnormalities. The occurrence of PACAP has been demonstrated in the cardiac muscle as well as in intracardial plexus and cardiac ganglia of different species (Schoenfeld et al. 2000; Chang et al. 2005). PACAP is suggested to act as a cardioprotective factor (Dvorakova 2005). Our research group has shown that PACAP is able to protect the cardiomyocytes against oxidative stress-, ischemia/reperfusion- and doxorubicin-induced apoptosis involving different apoptotic pathways (Gasz et al. 2006a, b; Racz et al. 2008, 2010; Roth et al. 2009). Sano and coworkers (2002) have found that PACAP reduces cardiac fibrosis, and the cardioprotective effect of PACAP is also supported by the observed pulmonary hypertension and heart failure in PAC1-receptor-deficient mice (Otto et al. 2004). Recently, Mori and coworkers (2010) have shown that PACAP-deficient mice display an increased vulnerability to doxorubicin-induced cardiomyopathy in an *in vivo* experiment. In the present study, we found significantly higher level of PACAP38-LI and PACAP27-LI in the ischemic heart tissue, suggesting its possible protective function during ischemic disorders. The protective effect of PACAP against different toxic agents can be attenuated by PACAP6-38, indicating that the cardioprotective effect of PACAP is mostly mediated by PAC1 receptors. Dominant expression of PAC1 receptors is well known in cultured cardiomyocytes; furthermore, human heart tissue expresses RNA transcripts for all three PACAP receptors (Wei and Mojsov 1996). In our study, we showed PAC1 receptors in human heart muscle from patients with ischemic heart disease with immunohistochemical examination.

It is well known that PACAP38 is the dominant form in the mammalian tissue (Vaudry et al. 2009). In the present study, we also detected higher level of PACAP38-LI compared with PACAP27-LI in all of the clinical samples in different disorders.

Significantly higher PACAP38-LI and PACAP27-LI were measured in the healthy lung and colon tissues compared with the respective tumor samples, but further investigations are necessary to describe the exact function of PACAP in oncogenesis. It is known that VIP antagonist are potent inhibitor lung and colon cancer lines (Levy et al. 2002), and PACAP6-38 also inhibits the effect of PACAP on different cancer lines, suggesting potential therapeutic effect in the clinical practice (Le et al. 2002). We showed that PACAP38-LI and PACAP27-LI are significantly higher in ischemic heart disorders compared with valvular abnormalities, suggesting that PACAP might play a role in ischemic heart disorders.

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