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Pituitary adenylate cyclase activating polypeptide (PACAP) is localized in human dermal neurons and causes histamine release from skin mast cells

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Abstract. *Objective and Design:* Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide homologous with vasoactive intestinal polypeptide (VIP) which is known to induce histamine release in human skin mast cells. PACAP has not been detected in human skin. The purposes of the study were to investigate the occurrence of PACAP in human skin and to evaluate the histamine releasing activity of the two common pro-PACAP products, PACAP-27 and PACAP-38.
Material: Fourteen human surgical skin samples were obtained. PACAP and VIP were visualized by immunohistochemistry. A microdialysis technique was used to measure histamine release in intact skin samples following intradermal injections of the peptides.
Results: PACAP and VIP were localized in dermal nerves in connection with sweat glands. Intradermal injection of 3 or 10 μm PACAP significantly released histamine. Kinetics of histamine release showed peak release 2–4 min after skin challenge. Ten μm of PACAP-27, VIP and somatostatin caused histamine release with similar efficacy, whereas PACAP-38 was less effective. Substance P was twice as efficient as PACAP-27, whereas calcitonin gene-related peptide did not release histamine.
Conclusions: PACAP is found in human skin and is capable of releasing histamine from skin mast cells.

Key words: Human – Immunohistochemistry – Mast cells – Microdialysis – Neuropeptides – Skin

Introduction

The immune and the nervous systems appear to be functionally interconnected [1–4]. In many tissues, a morphological association between mast cells and neuropeptides has been demonstrated [5]. Furthermore, functional evidence suggests that neuropeptides may play a role in the regulation of the secretory functions of mast cells. Thus, mast cells release histamine in response to substance P, vasoactive intestinal polypeptide (VIP) and somatostatin [6]. However, mast cells are heterogenous in respect to degranulation responsiveness to regulatory peptides. In the rat, peritoneal mast cells respond to a large range of neuropeptides, including the peptides mentioned above, whereas only substance P had a secretory effect on mucosal mast cells [6]. In humans, only mast cells of the skin, not those from lung, intestine or from most other anatomical localizations, release histamine when challenged with these peptides [6,7]. The reasons for this inter-species and intra-species mast cell heterogeneity still remain to be clarified. Several neuropeptides have been suggested to play a role in skin inflammation [3,8], but the exact contribution of each peptide in human inflammatory skin remains to be defined.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the secretin/glucagon/VIP family of peptides [9-11]. PACAP has been demonstrated in the central nervous systems, in posterior pituitary, peripheral nerves and adrenal gland of mammalian species including man, in germ cells of rat testis and in steroidogenic cells of rat ovary [10-13]. So far, PACAP has not been demonstrated in human skin. Two biological active forms of PACAP, PACAP-38 and PACAP-27, are derived from a single 176-amino acid precursor. PACAP-38 is the predominant form in the tissues [11, 12].

PACAP-27 shows 68% sequence homology with VIP [11] which has been shown to be a potent mast cell degranulation agent of human mast cells in vitro [7, 14] as well as in vivo [15]. In rodents, PACAP causes histamine release from mast cells [16]. The purposes of the present study were to investigate if PACAP is localized in human

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skin and to examine its possible histamine releasing activity in intact human skin ex vivo by microdialysis technique.

Materials and methods

Tissue

Skin specimens were taken from the breast or the lower leg in patients undergoing surgery for breast cancer or amputation. Tissue specimens from 14 donors were immediately immersed in ice cold saline and kept at approximately 5 °C. Microdialysis was performed within 6 h and biopsies for immunohistochemistry were immersed in fixative within 2 h after surgery. Informed consent was obtained from each subject before surgery, and the study was approved by the local Ethics Committee.

Skin microdialysis technique

Dialysis fibers with an outer diameter of 216 μm, 2,000 Dalton molecular weight cut off, a length of 15 mm available for diffusion, and an 30 mm outlet were constructed from dialysis fibers as previously described [17, 18]. The skin specimens were cut into pieces allowing insertion of up to six dialysis fibers arranged in parallel in each piece. The dialysis probes were inserted intradermally using 23 gauge guide cannulae. The distance between each cannula was 10–15 mm. Previous studies in vivo have shown that histamine release occurs exactly at the site of stimulus, histamine diffusion within the skin being minimal [19].

The skin specimen was fixed on a temperature regulated plate. The skin temperature was kept within 32–34 °C controlled by a precision thermometer (DM 852, Ellab, Copenhagen, Denmark) with a needle-type sensor. The ex vivo skin microdialysis technique has recently been described in detail [18].

Each dialysis probe was connected to individual syringes mounted in a microinjection pump (CMA/100, CMA/Microdialysis AB, Stockholm, Sweden), each fiber being perfused in parallel at a rate of 3.0 μl/min with isotonic Krebs Ringer bicarbonate buffer (KRB). Dialysate was collected at 2-min intervals (6 μl fractions). Prior to skin challenge, two baseline values of 6 μl were collected over 4 min. Following skin challenge, dialysate was collected at 2 min intervals (6 μl) for 20 min.

All peptides were dissolved in water and further diluted in KRB containing 0,02% human serum albumin as a peptide carrier to working concentration of 0.3–10 μM. All substances were administered as 25 μl aliquots to the skin by intradermal injections [17].

Histamine in dialysates was assayed spectrofluorometrically as previously described [17, 20]. The analytical sensitivity was 25 nM of histamine.

Immunohistochemistry

Two breast skin samples from two patients was fixed in 2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer (pH 7.2) for 20 h. After cryoprotection in 20% sucrose for another 24 h the tissues were cut in 12 μm thick sections and placed on silane coated glass slides.

Immunohistochemistry was performed as described [21, 22] using a mouse monoclonal antibody directed against PACAP (kindly provided by Jens Hannibal, Department of Clinical Biochemistry, Bispebjerg Hospital, Bispebjerg, Denmark) and a rabbit polyclonal antibody against VIP (kindly provided by Jan Fahrenkrug, Department of Clinical Biochemistry, Bispebjerg Hospital, Bispebjerg, Denmark). The specificities of these antibodies have been described previously [21, 22]. A rabbit polyclonal antibody against tryptase was used in dilution 1:1000 (kindly provided by Thorbjørn Bjerke, Department of Respiratory Medicine, Aarhus County Hospital, Aarhus, Denmark). A biotin labeled goat-antimouse antibody (Jackson ImunoResearch Lab.,Inc., West Grove, PA) diluted 1:100 and the avidin-biotin-complex method including tyramide amplification of the signal (Renaissance TSA-Indirect, DuPont NEN Research Products, Boston MA) and streptavidin labeled with Texas Red were used to visualize PACAP. Fluorescein isothiocyanate (FITC) labeled swine anti-rabbit (Dako, code no. F205) diluted 1:40 was used to visualize VIP and tryptase. As control, sections were processed without primary antibody; these controls showed no staining of nerve fibers or mast cell like elements, but unspecific staining of glands were seen.

Statistics

Results are expressed as mean ± SEM. Statistical analyses were performed using Friedmann non-paired analysis of variance. Wilcoxon and Mann Whitney tests were used for paired and non-paired two sample comparisons, respectively. p-values (two-sided) less than 0.05 were considered statistically significant.

Results

Histamine release

In each single experiment peak histamine concentration (nM) and total histamine release in 20 min (pmol/20 min) were calculated, the results being corrected for spontaneous histamine release before skin challenge. Basal histamine was 77 ± 2 nM (n=14). Seven skin samples were challenged with PACAP-27, another seven samples with PACAP-38. In each experiment, the negative control was KBR with 0.02% human albumin and the positive control was 10 μM of VIP. In four of the skin specimens, enough material was available to compare histamine release by the two PACAP peptides and VIP with that of substance P, somatostatin and calcitonin gene related peptide (CGRP).

PACAP, both the 27 and the 38 amino acid form, released histamine in intact human skin ex vivo. The kinetics of histamine release by VIP, PACAP-27 and PACAP-38 are shown in Fig. 1. In most cases, peak histamine values were found in samples collected from 2–4 min after injection of all three peptides. The histamine concentrations after 4 min was significantly higher than basal levels using all three

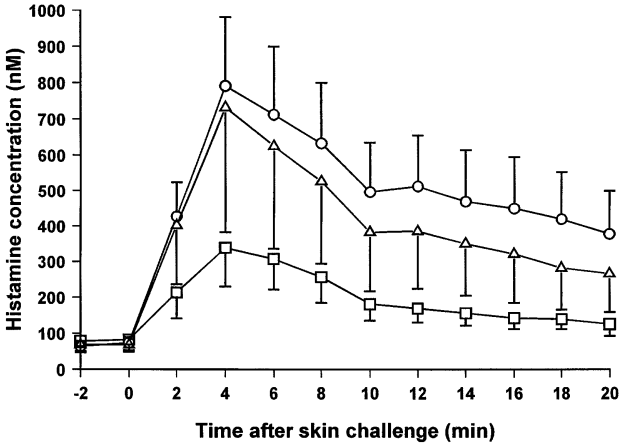

Fig. 1. Kinetics of histamine release by 10 μM VIP (circles), 10 μM PACAP-27 (triangles) and 10 μM PACAP-38 (squares). Values are mean of seven experiments (VIP, 14 experiments), SEM is shown by bar. The histamine concentrations induced after 4 min was significantly higher than basal levels using all three peptides.

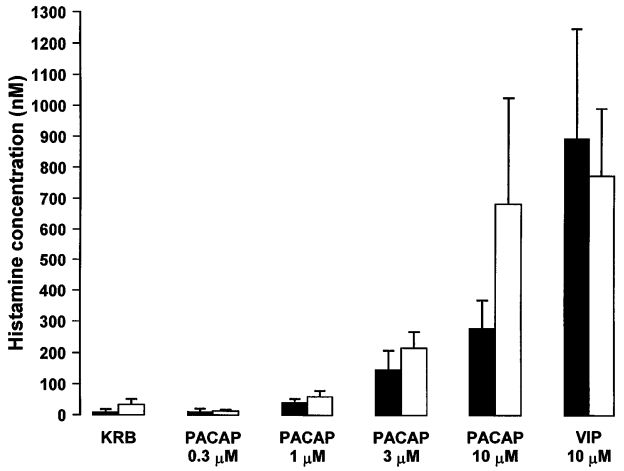

Fig. 2. Dose-response diagram showing peak histamine release in two series of experiments injecting PACAP-38 (filled bars) or PACAP-27 (open bars) in the concentration range 0.3 - 10 μM. Samples were collected in the 2-4 min interval after skin challenge. Histamine release by buffer (KRB) or 10 μM VIP in each series of experiments are also shown (filled bars, PACAP-38 experiments; open bars, PACAP-27 experiments). All values are mean (n=7), SEM is shown by bar. Histamine concentrations induced by 3 and 10 μM PACAP-27 as well as PACAP-38 was significantly higher than basal levels.

peptides. The kinetics of histamine release was independent on the magnitude of histamine release induced by various concentrations of peptides (data not shown). The histamine release induced by VIP and PACAP 27 was comparable whereas histamine release by PACAP 38 was significantly lower than that of PACAP-27 and VIP.

Fig. 2 shows peak histamine release using different concentrations of PACAP-27 and PACAP-38. Control values obtained with KRB and VIP in the two series of experiments are also shown. Significant histamine release, compared with buffer values, was obtained with 3 μM and 10 μM concentrations of PACAP-27 and PACAP-38. Peak release obtained with 10 μM PACAP-27 was similar to the release by VIP, whereas the release by 10 μM PACAP-38 was significantly lower.

The cumulative histamine release in the first 20 min after injection of 10 μM PACAP-27 was 20.0 $\pm$ 9.7 pmol comparable to 26.7 $\pm$ 9.8 pmol induced by 10 μM VIP. With 10 μM PACAP-38 the value was 7.3 $\pm$1.8 pmol. This was significantly lower than the 23.7 $\pm$ 7.7 pmol histamine released using 10 μM VIP in the same skin samples.

The relative magnitude of response of various neuropeptides (all tested in 10 μM concentrations) was investigated in four skin samples (Table1). Substance P was nearly twice as potent as PACAP-27, VIP and somatostatin, whereas PACAP-38 was less potent. CGRP caused virtually no histamine release. Due to the number of experiments, no statistical calculations were made.

Localization of PACAP

Immunohistochemical staining demonstrated PACAP in human skin. PACAP appeared to occur in nerves also containing VIP (Fig. 3). The mast cells seemed to be randomly distributed in the skin but was sometimes closely associated with PACAP containing nerves (Fig. 4). This pattern was constantly found in separate sections in two skin specimens.

Table 1. Rank order of histamine release by intradermel injection of 10 μM concentrations of various peptides in human skin ex vivo. Values are mean $\pm$ SEM of four experiments.

	Peak histamine concentration (nM)	Cummulative histamine release (pmol/20 min)
Substance P	1723 $\pm$ 416	55.8 $\pm$ 13.7
Somatostatin	990 $\pm$ 286	35.3 $\pm$ 12.3
VIP	804 $\pm$ 244	27.8 $\pm$ 8.4
PACAP-27	688 $\pm$ 156	23.6 $\pm$ 6.0
PACAP-38	226 $\pm$ 83	6.2 $\pm$ 2.0
CGRP	79 $\pm$ 17	0.9 $\pm$ 1.1

VIP = Vasoactive intestinal polypeptide; PACAP = Pituitary adenylate cyclase activationg polypeptide; CGRP = Calcitonin gene-related peptide.

Discussion

A high number of neuropeptides appear to be present in peripheral sensory nerves in the skin. In this study we showed that PACAP is also present in skin of humans. The functional roles of neuropeptides in the skin remain largely unknown. However, several lines of evidence have suggested that neuropeptides are involved in neuroimmunological reactions [5].

Neuropeptides with basic amino acid residues like substance P and VIP have been shown to induce histamine release from dispersed mast cells [16, 23, 24] and in intact human skin as measured by microdialysis technique [15, 17, 18, 25]. In this study it was demonstrated for the first time that PACAP released histamine in human skin. In regard to histamine release, substance P showed the highest efficacy followed by somatostatin, VIP, PACAP-27 and PACAP-38. CGRP caused virtually no histamine release. These findings on non-PACAP peptides are largely in agreement with results in dispersed human skin mast cells [23, 26].

The kinetics of histamine release by PACAP and VIP were rapid, peak histamine release occurred from 2–4 min after intradermal injection of peptides. This pattern of histamine release by peptides in human skin is similar to that induced by non-immunological mast cell activators in dispersed skin mast cells in vitro [7] and in intact human skin ex vivo [18] as well as in vivo [17, 19]. Standard deviations of the results shown here are similar to previous studies [18]. The main component is inter-individual variations ranging from 50–70%, intraindividual variations being only in the range 15-20% [18].

Histamine release occurred at concentrations of 3 μM or higher. These findings are comparable with previous findings in vitro as well as in vivo where rather high concentrations (1–30 μM) of neuropeptides are needed to induce histamine release in skin mast cells [17, 23]. The histamine releasing activity requires a basic N-terminal region and a lipophilic C-terminal sequence in some peptides [6, 23]. In other

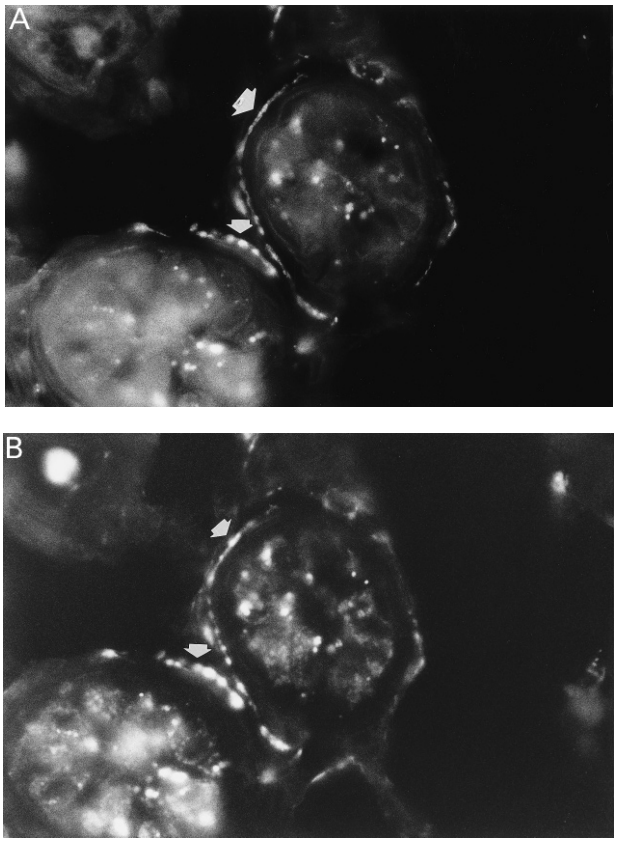

Fig. 3. Immunohistochemical double-staining of VIP and PACAP in human skin. (A) VIP immunoreactivity (rabbit polyclonal antibody). (B) PACAP immunoreactivity (mouse monoclonal antibody). VIP and PACAP was detected in identical nervefibers around sweat glands (arrows). Light microscopy ×400.

peptides the only characteristic is an abundance of basic amino acids [24]. Further, the activity is mediated via direct interaction with G-proteins [24]. VIP, PACAP-27 and PACAP-38 all contain a basic amino acid in the N-terminus. PACAP-27 has close amino acid homology with VIP [11], both containing a lipophilic C-terminal sequence, whereas PACAP-38 has a C-terminal extension (28–38) containing several basic amino acid residues including the C-terminal amino acid. Since the efficacy of PACAP-38 was lower that of PACAP-27 and VIP, it may suggest that the C-terminal sequence interfered with the stimulation of histamine release induced by N-terminal parts of the molecule. In line with this idea, it has been observed, that a single C-terminal basic amino acid reduced the potency of C3a (24).

The fact that PACAP-38 showed less efficacy than PACAP-27 was in contrast to the findings in rat mast cells where the opposite was found [16]. The discrepancy may be due to species variations of mast cells.

VIP (PACAP 2) receptor mRNA has been found in a human mast cell line [27]. However, it is unlikely that the effect of PACAP or VIP is mediated through a receptor for two reasons. First, micromolar concentrations are necessary to induce histamine release. Second, PACAP-38 and PACAP-27 bind to VIP/PACAP-receptors with similar affinity [25, 28], but their histamine releasing properties differed significantly. PACAP-38, containing both a basic N-terminal and C-terminal, showed the lowest efficacy.

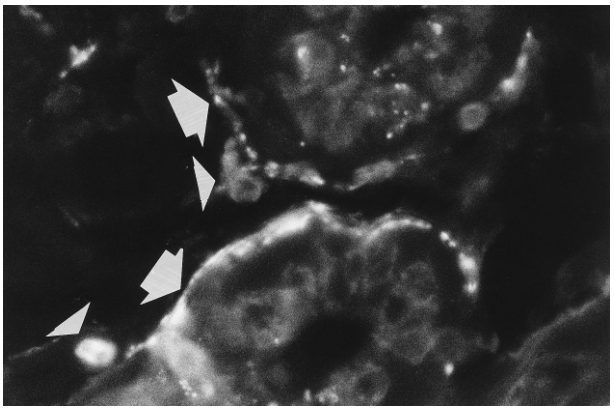

Fig. 4. Immunohistochemical double-staining of PACAP and tryptase in human skin. PACAP (mouse monoclonal antibody) was localized in nervefibers around sweat glands (arrows) now and then in close association with mast cells (arrow heads). Light microscopy ×630.

Consequently, it seems reasonable to assume that the histamine releasing effect of PACAP on mast cells is mediated via direct interaction with G-proteins as shown for other neuropeptides [24].

Previous studies indicated that VIP is present in all human skin regions with the highest concentration in axilla [29]. By immunohistochemistry, VIP nerves have been shown almost exclusively to innervate sweat glands [30]. In agreement with these observations, we found VIP only in nerve fibers around sweat glands. PACAP appeared to co-localize with VIP similar to the findings in human vagina [31]. The fact that PACAP containing fibers was seen in close contact with mast cells may suggest physiological interactions. Alternatively, and more likely, it may be a matter of coincidence. Recent studies have failed to show release of substance P in neurogenic inflammation in human skin [32, 33]. It therefore appears that the role of VIP and PACAP in human neurogenic inflammation should be examined more closely in order to clarify the physiological implications of the histamine releasing ability of these peptides.

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