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A structure–function study of PACAP using conformationally restricted analogs: Identification of PAC1 receptor-selective PACAP agonists

Q1 Irene Ramos-Álvarez[a], Samuel A. Mantey[a], Taichi Nakamura[a], Bernardo Nuche-Berenguer[a], Paola Moreno[a], Terry W. Moody[b], Jerome L. Maderdrut[c], David H. Coy[c], Robert T. Jensen[a,*]

Q2 [a] Digestive Diseases Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892-1804, United States
[b] Center for Cancer Research, Office of the Director, NCI, National Institutes of Health, Bethesda, MD 20892-1804, United States
[c] Peptide Research Laboratory, Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112-2699, United States

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) has widespread physiological/pathophysiological actions and there is increased interest for its use therapeutically, especially in the CNS (neuroprotection). Unfortunately, no selective PACAP-analogs exist for PACAP-preferring PAC1-receptors, primarily because of its high sequence identity to VIP and particularly, because of the inability of structure–function studies to separate the pharmacophore of PAC1-R from VPAC1-R, which has high affinity for PACAP and VIP. The present study attempted to develop PAC1-R-selective agonists primarily by making conformationally restricted PACAP-analogs in positions important for receptor-selectivity/affinity. Forty-six PACAP-related-analogs were synthesized with substitutions in positions 1–4, 14–17, 20–22, 28, 34, 38 and receptor-selectivity determined in PAC1-R,VPAC1-R,VPAC2-R-transfected or native cells from binding or cAMP-generation experiments. Fifteen PACAP-analogs had 6–78-fold higher affinities for PAC1-R than VPAC1-R and 13 were agonists. Although binding-affinities correlated significantly with agonist potency, the degree of receptor-spareness varied markedly for the different PACAP-analogs, resulting in selective potencies for activating the PAC1 receptor over the VPAC1 receptor from 0- to 103-fold. In addition, a number of PACAP-analogs were identified that had high selectivity for PAC1-R over VPAC2-R as well as PACAP-analogs that could prove more useful therapeutically because of substitutions known to extend their half-lives (substitutions at potential sites of proteolysis and attachment of long-chain fatty acids). This study provides for the first time a separation of the pharmacophores for PAC1-R and VPAC1-R, resulting in PACAP-related analogs that are PAC1-R-preferring. Some of these analogs, or their modifications, could prove useful as therapeutic agents for various diseases.

Abbreviations: BSA, bovine serum albumin fraction V; cAMP, cyclic adenosine 3′:5′-monophosphate; CNS, central nervous system; DMEM, Dulbecco's minimum essential medium; DTT, dithiothreitol; EC_{50}, concentration causing half-maximal stimulation; FBS, fetal bovine serum; GRP, gastrin-releasing peptide; GHRH, growth hormone-releasing hormone; IC_{50}, half-maximal inhibitory concentration; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC1-R, PACAP preferring receptor; PACAP27, 27-amino-acid form of PACAP; PACAP38, 38-amino-acid form of PACAP; PBS, phosphate-buffered saline; PLC, phospholipase C; Ro 25-1553, Ac-His-Ser-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Lys-Leu-Arg-Lys-Gln-Nle-Ala-Ala-Lys-cyclo[Lys-Tyr-Leu-Asn-Asp]-Leu-Lys-Lys-Gly-Gly-Thr-NH_2; Sup T1 cells, human Sup-T1 lymphoblastoma cells naturally containing VPAC2-R; T47D cells, human T47D breast cancer cells naturally containing VPAC1-R; VIP, vasoactive intestinal peptide; VPAC1-R, VIP/PACAP receptor, subtype 1 (VIP1 receptor); VPAC2-R, VIP/PACAP receptor, subtype 2 (VIP2 receptor); 3T3, mouse embryonic fibroblast cells.

* Corresponding author at: Digestive Diseases Branch, NIDDK, NIH, Building 10, Room 9C-103, 10 Center Drive, MSC 1804, Bethesda, MD 20892-1804, United States. Tel.: +1 301 496 4201; fax: +1 301 402 0600.
E-mail address: robertj@bdg10.niddk.nih.gov (R.T. Jensen).

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Introduction

Pituitary adenylate cyclase-activating polypeptide, first isolated from hypothalamus, occurs in two forms: either with 38-amino acid residues (PACAP38) or the other, containing only the first 27 residues (PACAP27) [35]. PACAP has a high sequence identity to vasoactive intestinal polypeptide (VIP) with PACAP27 sharing 68% sequence identity with VIP [54,55]. Their actions are mediated by three Class II (secretin-type) G-protein-coupled receptors: PAC1 receptor (PAC1-R), VPAC1 receptor (VPAC1-R) and VPAC2 receptor (VPAC2-R) [20]. PACAP38/27 have high affinities for all three receptors and VIP only has high affinities for the latter two receptors [20,55]. These receptors all signal through cAMP with PAC1-R also activating phospholipase C in many tissues [12,20,55].

PACAP and its receptors have widespread distributions in both the central nervous system (CNS) and peripheral tissues with wide-ranging activities in different tissues, both physiologically and pathophysiological [12,20,43,55]. PACAP has numerous functions in the CNS (release of oxytocin/vasopressin, circadian rhythm, behavioral changes, neurotrophic-development, glial cell activity, pituitary cell function), the cardiovascular system (vasorelaxant effects on vascular tone, direct cardiac effects), immune system (monocyte differentiation, regulatory of inflammatory process), the urogenital and respiratory systems (smooth muscle activity, mucus secretion, urinary bladder function), gastrointestinal tract (alters secretion [salivary, pancreatic, gastric], proliferation) and endocrine glands (adrenal, thyroid, islets), as well as the gonads (regulates gonadal activity) [12,20,54,55]. In pathologic processes, PACAP has been shown to have a number of beneficial effects, especially related to its neuroprotective and neurotrophic actions manifested by its ability to stimulate the migration, proliferation, differentiation, and survival of neural cells [20,54]; its ability to exert potent immuno-modulatory actions that are primarily anti-inflammatory [57]; its ability to enhance insulin release and ameliorate islet injury [1] and its ability to ameliorate renal failure/injury [3,31]. Primarily due to these actions, PACAP has been shown to have beneficial effects in models of stroke (focal cerebral ischemia), traumatic brain/spinal injury, various CNS/neurological diseases (Parkinson's disease, Huntington's chorea, Alzheimer's disease, schizophrenia) [14,33,46,47,57], diabetes [1], kidney failure due to various diseases (myeloma, diabetic nephropathy, contrast-induced nephropathy) [3,30,31] and to prevent the toxic effects of numerous neurotoxins (glutamate, lipopolysaccharide, ethanol, oxidative stress, β-amyloid) [7,20,37,43,54]. Furthermore, PAC1-R is frequently overexpressed by various human tumors and PACAP has been shown to have both stimulatory and inhibitory effects on the growth of various tumors leading to the proposal that development of PAC1-R selective agonists or antagonists could give rise to useful tools for the treatment of cancers [36,48,55].

Most of the beneficial effects of PACAP are mediated via PAC1-R and, therefore, there is considerable interest in identifying PAC1-R selective agonists for possible therapeutic effects [32]. At present, no suitable VIP/PACAP-analog exists and the only selective PAC1 agonist, maxadilan, is a 61-amino-acid peptide with no sequence similarity to PACAP [29] and is rarely used. The development of PAC1 selective agonists has been difficult because PACAP38, which is the predominant form in both the CNS and peripheral tissues, is rapidly degraded [4,54]; has high sequence identity to VIP, and interacts with high affinities with the other VIP-PACAP family of receptors (VPAC1-R and VPAC2-R) [20,54]. To identify VIP/PACAP-analogs with selectivity for PAC1-R over VPAC1-R has proven particularly difficult, and none have been described.

The purpose of this study was to attempt to identify, using information from previous structure–function and conformations studies of PACAP/VIP, VIP/PACAP-analogs with selectivity for PAC1-R over VPAC1-R. To accomplish this goal, 46 PACAP-analogs (primarily PACAP38 analogs) were synthesized with substitutions that were conformationally restricting to attempt to identify analogs with preferential binding/activation of PAC1-R. Using this approach, we identified for the first time PACAP-analogs that preferentially bound to PAC1-R and/or activated PAC1-R over VPAC1-R, and also identified strategies that could be used to reduce interactions with VPAC2-R.

Materials and methods

Materials

NIH 3T3 cells, PANC-1 cells and Sup-T1 cells were from American Type Culture Collection (ATCC), Rockville, MD; Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), G418 sulfate, fetal bovine serum (FBS), RPMI 1640 medium, penicillin, streptomycin and sodium pyruvate from Gibco Life Technology (Grand Island, NY); bacitracin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX), formic acid, ammonium formate, disodium tetraborate, and alumina were obtained from Sigma–Aldrich (St. Louis, MO); iodine-125 (100 mCi/ml) and [2,8-^{3}H]adenine were from Perkin Elmer Life Sciences (Boston, MA); 1,2,4,6-tetrachloro-3α-6α-diphenylglycouril (Iodo-Gen) from Pierce Chemical Co. (Rockford, IL); AG 1-X8 resin from Bio-Rad (Richmond CA). Standard protected amino acids and other synthetic reagents were obtained from Bachem Bioscience Inc. (King of Prussia, PA).

Cell culture

VPAC1 stably transfected into PANC-1 cells (VPAC1-R/PANC-1 cells), VPAC2 stably-transfected into PANC-1 cells (VPAC2-R/PANC-1 cells) [23,25,42] and PAC1-R stably-transfected into NIH 3T3 cells (PAC1-R cells) [15,42,45] were used because they are well characterized and behave similar to wild type receptors. PAC1-R cells were a gift from Dr. J.R. Pisegna, UCLA. VPAC1, VPAC2 PANC-1 cells and PAC-1R cells were grown in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 300 mg/l of G418 sulfate. T47D breast cancer cells naturally containing VPAC1-R (T47D cells) [23] were grown in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 1 mM sodium pyruvate. Sup-T1 lymphoblastoma cells (Sup-T1 cells) naturally expressing VPAC2-R [23] were grown in RPMI supplemented with 10% FBS and 100 U/ml of penicillin, 100 mg/ml of streptomycin. All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Preparation of peptides

PACAP-analogs were synthesized with solid-phase methods as described previously [23–25]. Briefly, solid-phase syntheses of peptide amides were carried out using Boc chemistry on methylbenzhydrylamine resin (Advanced ChemTech, Louiville, KY) followed by HF-cleavage of free peptides amides. The crude peptides were purified on 92.5 cm × 50 cm columns of Vydac C18 silica (10 μm), which was eluted with linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Homogeneity of the peptides was assessed by analytical reverse-phase high-pressure liquid chromatography (HPLC) and purity was usually 97% or higher. Amino acid analysis (only amino acids with primary amino acid groups were quantitated) gave the expected amino acid ratios. Peptide molecular masses were obtained by matrix-assisted laser desorption mass spectrometry (Finegan Lasermat) and all corresponded well with calculated values.

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