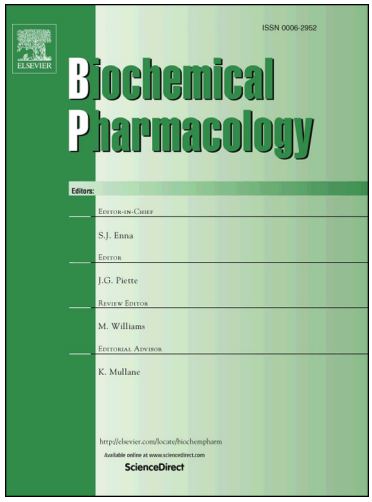


New insights about the peculiar role of the 28-38 C-terminal segment and some selected residues in PACAP for signaling and neuroprotection

Mathilde Poujol de Molliens, Myriam Létourneau, Dominic Devost, Terence E. Hébert, Alain Fournier, David Chatenet

PII: S0006-2952(18)30167-9
DOI: https://doi.org/10.1016/j.bcp.2018.04.024
Reference: BCP 13133

To appear in: *Biochemical Pharmacology*

Received Date: 20 February 2018
Accepted Date: 24 April 2018

Please cite this article as: M.P. de Molliens, M. Létourneau, D. Devost, T.E. Hébert, A. Fournier, D. Chatenet, New insights about the peculiar role of the 28-38 C-terminal segment and some selected residues in PACAP for signaling and neuroprotection, *Biochemical Pharmacology* (2018), doi: https://doi.org/10.1016/j.bcp.2018.04.024

New insights about the peculiar role of the 28-38 C-terminal segment and some selected residues in PACAP for signaling and neuroprotection

Mathilde Poujol de Molliens[1,2], Myriam Létourneau[1,2], Dominic Devost[3], Terence E. Hébert[3], Alain Fournier[2], David Chatenet[1*]

[1]INRS – Institut Armand-Frappier, Groupe de Recherche en Ingénierie des Peptides et en Pharmacothérapie (GRIPP), Université du Québec, Ville de Laval, QC, Canada

[2]INRS – Institut Armand-Frappier, Laboratoire d'études moléculaires et pharmacologiques des peptides (LEMPP), Université du Québec, Ville de Laval, QC, Canada

[3]Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada

Corresponding author.

[*] Pr. David Chatenet, INRS – Institut Armand-Frappier, Groupe de Recherche en Ingénierie des Peptides et en Pharmacothérapie (GRIPP), Université du Québec, Ville de Laval, QC, Canada, H7V 1B7

Tel: +1-450-687-5010; Fax: +1-450-686-5446; E-mail address: david.chatenet@iaf.inrs.ca

Abbreviations:

BRET, bioluminescence resonance energy transfer; ACN, acetonitrile; BBB, blood-brain barrier; Bip, biphenylalanine ; Boc, tert-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate; CHO cells, Chinese hamster ovary cells; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's medium; DMF, dimethylformamide; FBS, foetal bovine serum; Fmoc, fluorenylmethyloxycarbonyl; GFP 10, green fluorescent protein 10; GPCR, G protein-coupled receptor; HEK 293 cells, human embryonic kidney 293 cells; Hyp, hydroxyproline; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PAC1, pituitary adenylate cyclase-activating polypeptide receptor 1; PACAP, pituitary adenylate cyclase-activating polypeptide; RlucII, *Renilla* luciferase II; RP-HPLC, reverse phase-high performance liquid chromatography.

Abstract

The pituitary adenylate cyclase-activating polypeptide (PACAP), which exists in two isoforms of 27 and 38 amino acids, can induce neuronal protection *in vitro* and *in vivo* following the activation of PAC1, a class B G protein-coupled receptor (GPCR). With its potent neuroprotective and anti-inflammatory effects, this peptide represents a promising avenue for the development of therapeutic strategies to potentially cure or at least slow the progression of neurodegenerative disorders. Beyond the canonical G protein signal effectors, GPCRs are also coupled to a multitude of intracellular signaling pathways that can be independently activated by biased ligands, thereby expanding vastly the potential for discovering new drugs. Interestingly, some studies have demonstrated distinct signaling features for the PACAP isoforms. With this observation in mind, we assessed the impact of chemical and structural modifications introduced into specific regions of the PACAP isoforms on their neuroprotective effects, and determined the role played by these physico-chemical and structural features on their signaling signatures. Each compound was also evaluated for its ability to bind the PACAP receptors, promote cell survival in a cellular model of Parkinson's disease and stimulate the signaling partners associated with PAC1 activation, including G_s and G_q, as well as β-arrestin 1 and 2. Our results demonstrate that PACAP38 and its related analogs exert a more potent neuroprotective action than their 27-counterparts and that this neuroprotective effect is dependent on both G_q and G_s-dependent signaling. This study will definitely improve our understanding of the molecular and cellular mechanisms associated with PACAP neuroprotection.

Keywords:

PACAP, PAC1 receptor, Parkinson's disease, neuroprotection, BRET-based biosensor, biased agonism.

1. Introduction

The prevalence of Parkinson's disease (PD) has increase by more than 15% between 1990 and 2015, making it one of the most common neuronal disorder with about 4.6 million people affected worldwide (13). This condition is characterized by a cognitive syndrome that is often accompanied with tremors, bradykinesia and muscle rigidity (21). The main cause of these symptoms is the loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) leading to a reduction of dopamine release (10). Many studies have shown that neurodegenerative disorders such as PD involved an inflammatory process and an increase in oxidative stress, two phenomena linked to neuronal apoptosis (15). To date, many treatments aimed at reducing PD symptoms have been developed but no cure has yet been discovered (34). In particular, among the challenges encountered when designing a drug developed for an eventual chronic use, there is the need to efficiently cross the blood-brain barrier (BBB) and to prevent long-term desensitization associated with G protein-coupled receptor kinases (GRK) activation and β-arrestins recruitment (42).

In the past years, the pituitary adenylate cyclase-activating polypeptide (PACAP) has been shown to display neuroprotective effects in *in vitro* and *in vivo* models of Alzheimer's (17, 44) and Parkinson's diseases (7, 28, 45, 57). For instance, it was demonstrated that PACAP reduced inflammatory responses induced by salsolinol or a cocktail of LPS and interferon-γ in SH-SY5Y neuroblastoma cells (7, 8). *In vivo*, PACAP not only prevents dopamine-based neurodegeneration in rat and snail parkinsonian models (33) but also reduces neuronal loss and motor deficits (47). Hence, PACAP represents an attractive therapeutic framework to modify the course of

neurodegenerative disorders, including PD, especially considering its ability to cross the BBB (38).

Isolated in 1989 from ovine hypothalamus on the basis of its capacity to stimulate adenylyl cyclase in rat pituitary cells (37), this peptide exists in two endogenous isoforms termed PACAP38 and PACAP27. The former is a 38-amino acid C-terminally α-amidated molecule, whereas the latter is the C-truncated 27-residue amidated form of PACAP38 (1). Pharmacological studies have demonstrated that both PACAP isoforms bind to three distinct G protein-coupled receptors, *i.e.* PAC1, VPAC1 and VPAC2, through which different actions are mediated (53). On the one hand, PAC1 is widely distributed in the central nervous system and its activation by both PACAP isoforms produce antiapoptotic actions. On the other hand, triggering VPAC1 induces anti-inflammatory processes (11, 46), whereas VPAC2 stimulation mostly leads to peripheral effects such as vasodilation and tachycardia that could be detrimental in the context of chronic treatment (9, 58).

Various studies have been carried out to develop selective PAC1 ligands but to date, only PAC1/VPAC1 selective peptides have been obtained (12, 28). Nonetheless, these compounds revealed important properties including *in vitro* and *in vivo* neuroprotective actions without potent cardiovascular side-effects (28). PACAP structure-activity analyses carried out with various analogs revealed the importance of specific features, such as a N-terminal Asx-turn motif encompassing the first four amino acids, a N-capping molecular arrangement involving amino acids 6 to 11, and a helical structure spanning from residues 8 to 21, for the peptide structural stability, binding affinity and activity (5, 12, 20, 59). For instance, the presence of an Asx-turn, a β-turn-like peptide configuration stabilized by hydrogen bonds, is supported by the production of potent agonists following incorporation of constrained amino acids at position 2 (L-proline and L-hydroxyproline) (4, 12). The N-capping motif, characteristic of all class B GPCR ligands,

appears to be strengthened by hydrophobic interactions mimicking those between Phe^{6} and Tyr^{10} in PACAP (12). Following structural studies, it was recently hypothesized that different N-capping motifs in PACAP could explain, at least in part, the differences in binding affinity observed between PACAP receptors (39). Also, after additional investigations, it was concluded that serine-2, phenylalanine-6, threonine-7 and tyrosine-22 represented key residues for PACAP molecular stabilization and pharmacological properties (14). Notably, Ala-substitutions at position 2, 6, 7 or 22 decreased the affinity and/or the activity toward VPAC2 (4, 43). Also, Ala-scan and Lys-scan carried out on PACAP27 showed the importance of Tyr^{22} for stabilization of the α-helix (4, 5). Further, the addition of a CH_2-NH bond between Lys^{21} and Tyr^{22}, a modification that impaired the helical structure, yielded inactive analogs (3). Finally, for class B GPCR, it has been postulated that there is a two-step process leading to receptor activation: first, the C-terminal segment of the hormone binds to the extracellular domain of the receptor, and second, the N-terminal portion of the peptide ligand is brought into the binding pocket located inside the core of the receptor transmembrane domain (18). Interestingly, and as recently observed with the glucagon receptor, the first extracellular loop undergoes major conformational changes in secondary structure during peptide binding, forming key interactions with the peptide (62). When assessing the binding features of both PACAP isoforms, it appears that the presence of the 28-38 C-terminal segment in PACAP38 modifies the interaction of the ligand with the receptor (50). Given the importance of the C-terminal portion of the ligand for class B GPCR binding, this disparity between the structures of the PACAP isoforms probably results in distinct effects on the receptor activation process and therefore distinct signaling outcomes. Such differences in intracellular signaling between both PACAP isoforms were already highlighted. For instance, Spengler *et al.* demonstrated that PACAP38 is more potent than PACAP27 in stimulating inositol triphosphate (IP_3) production while this order is reversed for cyclic adenosine

monophosphate (cAMP) (48). The neuroprotective action of PACAP has been linked to various pathways, including G_s that leads to the cAMP-dependent protein kinase (PKA) (26), and G_q that yields to protein kinase C (PKC) stimulation (56). Actually, PACAP-induced neuroprotection has primarily been studied in the context of G_s activation (35).

The development of functionally selective ligands, also termed biased agonists, is critical for understanding the molecular pharmacology of GPCR activation but also for the conception of safer drugs with reduced side-effects (25). Indeed, by antagonizing detrimental GPCR signaling pathways while stimulating beneficial downstream processes, biased ligands could deliver more precise therapeutic benefits with fewer side-effects than current GPCR-targeted drugs (31). For example, as recently reported, TRV0109101, a G protein-biased agonist of the μ-opioid receptor, is unable to promote opioid-induced mechanical allodynia following chronic administration. Such compounds may therefore represent an effective therapeutic avenue for managing chronic pain with reduced propensity for opioid-induced hyperalgesia (27). Similarly, the conception of a G protein biased PAC1 agonist might therefore represents an attractive means to slow down neurodegeneration. Currently, such derivatives do not yet exist, thereby preventing the implementation of definitive studies and their therapeutic validation. Thus, the goal of this study was to evaluate the impact of physico-chemical and structural parameters on PACAP27- and PACAP38-associated signaling signatures and neuroprotective effects. Based on previous structure-activity analyses, selected compounds were evaluated for their capacity to bind PAC1, VPAC1 and VPAC2 receptors, to protect human neuroblastoma cells against 1-methyl-4-phenylpyridinium (MPP^+) toxicity and, using bioluminescence resonance energy transfer (BRET)-based biosensors, to promote G protein (G_s and G_q) and β-arrestin 1/2 activation. Our results reveal the peculiar role of the 28-38 C-terminal segment of PACAP for its neuroprotective

action and suggest that PACAP-induced neuroprotection depends on multiple signaling pathways mostly driven by G_q and G_s activation.

2. Materials and Methods

2.1. Materials.

The fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, the Fmoc-Rink-amide AM-functionalized polystyrene resin and the coupling reagent (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were purchased from Chem-Impex (Wood Dale, IL). Trifluoroacetic acid (TFA), methanol (MeOH), acetonitrile (ACN), diethyl ether, N,N-dimethylformamide (DMF), piperidine, dichloromethane (DCM) and bovine serum albumin (BSA) were obtained from Fisher Scientific (Nepean, ON). $Na^{125}I$ was purchased from Perkin-Elmer (Montreal, QC). Coelenterazine 400a was purchased from Gold Biotechnology, Inc (St. Louis, MO). G418-sulfate was purchased from bioWORLD (Dublin, OH). N,N-Diisopropylethylamine (DIPEA), chloramine-T, 1,2-ethanedithiol (EDT), thioanisol, α-cyano-4-hydroxycinnamic acid, phenol, 1-methyl-4-phenyl pyridinium (MPP^+) and all other chemicals were from Sigma-Aldrich (Mississauga, ON). Ham's F12, MEM medium, DMEM medium, OptiMEM, fetal bovine serum (FBS), L-glutamine, D-glucose, penicillin and streptomycin (P/S) and all other cell culture reagents were from Wisent (St-Bruno, QC).

2.2. Peptide synthesis

All peptides were synthesized using a commercially available Fmoc-Rink-amide AM resin and a Fmoc-based solid phase peptide synthesis procedure. Following a previously published protocol (12), all protected amino acids (3 equiv, based on the substitution of the resin) were coupled using an *in situ* activation step with BOP (3 equiv) and DIEA (5 equiv) in DMF for 45 min. Deprotection of the Fmoc-protecting group was achieved with 20% piperidine in DMF. Each coupling and deprotection step was monitored using a ninhydrin test. Peptide-resins were dried *in vacuo* and then cleaved with reagent K (TFA/water/phenol/thioanisole/EDT; [82.5:5:5:5:2.5]). Peptides were purified by preparative reverse phase HPLC to attain a minimum of 95% purity and the molecular weight of each compound was verified by MALDI-TOF mass spectrometry

using an α-cyano-4-hydroxycinnamic acid matrix (Voyager DE, Applied Biosystems, Foster City, CA). Peptides were lyophilized and kept at -20°C until use.

2.3. Cell culture

Chinese hamster ovary (CHO) cells stably and individually expressing human PAC1, VPAC1 or VPAC2 receptors were grown in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 UI/mL of penicillin and streptomycin (P/S) and 400 µg/mL of G418. The neuroblastoma cell line SH-SY5Y, endogenously expressing the human PAC1 and VPAC2 receptors but not VPAC1 (32), were grown in a 1:1 mixture of MEM medium and Ham's F-12 nutrient mixture containing 15% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 UI/mL of P/S. Human embryonic kidney (HEK) 293 cells were grown in DMEM medium supplemented with 10% FBS, 4.5 g/L D-glucose, 2 mM L-glutamine and 100 UI/mL of P/S. All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passages were performed using trypsinization when cells reached about 80% confluence.

2.4. Radioligand binding assays

Acetylated PACAP27 was radioiodinated using the chloramine-T oxidation technique and purified on a Sep-Pak C_{18} cartridge (Waters, Milford, MA) (19). Binding assays were performed using CHO cells stably transfected with the human PAC1, VPAC1 or VPAC2 receptor isoforms. Cells were seeded at a density of 150,000 cells/well in 24-well plates. The next day, cells were washed with binding buffer (0.1% BSA, 25 mM Tris–HCl, 25 mM $MgCl_2$, pH 7.4) for 10 min at room temperature. The solution was replaced with a fresh solution containing 0.05 nM of ^{125}I-Ac-PACAP27 and increasing concentrations of different competitor peptide (10^{-14} to 10^{-5}M). After 2 h of incubation at room temperature, cells were washed twice with the binding buffer and then

lysed with sodium hydroxide (0.1 M). Cell-bound radioactivity was quantified using a γ-counter (1470 Wizard, Perkin Elmer, Woodbridge, ON). Results were expressed as a percentage of the specific binding of ^{125}I-Ac-PACAP27. Non-specific binding was determined in the presence of PACAP38 (10^{-5}M).

2.5. Cell survival assays

SH-SY5Y cells, seeded into 96-well plates at a density of 30,000 cells/well, were incubated for 48 h to ensure cell adhesion. Cells were starved for 2 h and then incubated at 37°C for 4 h with peptides (10^{-7}M) prior to the addition of 1-methyl-4-phenyl pyridinium (MPP^+; 1.5 mM final concentration). Cell viability was assessed 24 h after the MPP^+ treatment using the cell titer Blue-cell viability assay kit (Promega, Madison, WI) and a microplate reader FlexStation®3 (Molecular Devices, San Jose, CA) to determine fluorescence intensity related to the conversion of resazurin into resoflurin. Positive control (MPP^+-treated cells) and negative control (non-treated cells) were included in each experiment.

2.6. Bioluminescence resonance energy transfer assay

Using bioluminescence resonance energy transfer (BRET) biosensors, the activation of G_q and G_s, as well as those of β-arrestin 1 and 2, was monitored following the stimulation of PAC1. This receptor (pcDNA 3.1_PAC1) and one of the signaling pathway-related biosensors were transiently co-transfected into HEK293 cells using the TransIT-2020 transfection reagent (Mirus Bio, Madison, WI) for pLVXi2H-Polycis_Gα_q-RlucII-GFP$_{10}$-γ1-β1, pcDNA3.1_Gα_s-RlucII and pcDNA3.1_Gγ1-GFP$_{10}$, and Lipofectamine®2000 (Invitrogen, Burlington, ON) for pLVXi2H_GFP10-βarr1-RlucII and pLVXi2H_GPP10-βarr2-RlucII. A 1.5:1 ratio of TransIT-

2020 or Lipofectamine®2000/DNA was used. On the day of the transfection, cells were trypsinized and suspended at a density of 150,000 cells/mL for G_q, and 300,000 cells/mL for G_s, β-arrestin 1 and β-arrestin 2 in DMEM supplemented with 2.5% FBS. DNA and TransIT-2020 or Lipofectamine®2000 were combined in OptiMEM, and the mixture was incubated at room temperature for 30 min before being added to the cells. Transfected cells were then seeded at a density of 15,000 cells/well for G_q, and 30,000 cells/well for G_s, β-arrestin 1 and β-arrestin 2 in 96-well plates. After 24 h, medium was removed and changed with DMEM supplemented with 5% FBS, 400 µg/mL of G418, 100 UI/mL of P/S and incubated for another 24 h. Cells were washed with 120 µL PBS solution supplemented with 0.1 % glucose, and then incubated with 80 µL of this solution for 2 h. Then, 10 µL of a 1/20 dilution (diluted before use) of coelenterazine 400A (stock at 1 mM in ethanol) in PBS solution supplemented with 0.1 % glucose and 10 µL of the 10X appropriate concentration of peptide were added and then incubation was resumed for 30 min at room temperature for G_q experiments, and 10 min at room temperature for G_s and β-arrestin 1. For β-arrestin 2, peptides were added directly after replacement of the media with 80 µL of PBS/0.1% glucose, and the cells were incubated 2 h at 37°C. Filters were set at 410 nm and 515 nm to detect the *Renilla* luciferase II (RlucII, donor) and green fluorescent protein 10 (GFP10, acceptor) light emissions, respectively. BRET signals, measured on a TECAN Infinite 2000 (Morrisville, NC), were monitored for 5 min after co-addition of coelenterazine 400A and ligands. BRET ratios were determined by calculating the ratio of the light emitted by GFP10 over the light emitted by the RlucII. BRET signals were normalized to the maximum signal measured with PACAP27 or PACAP38.

2.7. Data analysis

Binding experiments, cell survival assays and G protein and β-arrestin activation assessments were performed at least in quadruplicate. Data, expressed as mean ± S.E.M, were analyzed with Prism software (Graphpad Software, San Diego, CA). Sigmoidal concentration-response fits with variable slope and one-site competition functions were used to determine EC_{50} and IC_{50}, respectively. A decrease in BRET signal was observed at high concentrations of PACAP resulting in bell-shaped curves. This phenomenon has been previously described in the literature and is explained by receptor internalization (23). Thereby, E_{max} of each ligand was normalized with the highest value obtained for PACAP27 or PACAP38. Log τ/K_A was determined using the Black-Leff operational model (52). Statistical comparisons were analyzed by the Student's t-test, and differences were considered significant when $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

3. Results

3.1. Binding affinity of PACAP and related analogs for PAC1, VPAC1 and VPAC2 receptor

Both PACAP isoforms and their related analogs were tested for their capacity to bind PACAP receptors, *i.e.* PAC1, VPAC1 and VPAC2. The binding affinity of PACAP27 and its related analogs has been previously determined by our group (12) and is used for comparison with binding data obtained for PACAP38 and related analogs. As shown in Table 1, replacement of the serine residue at position 2 with an L-alanine, *i.e.* [Ala^2]PACAP38/27, significantly increased the binding affinity of PACAP38 for PAC1 (pIC_{50} = 8.76 ± 0.08, $^{**}P < 0.01$), VPAC1 (pIC_{50} = 9.68 ± 0.18, $^{*}P < 0.05$), and VPAC2 (pIC_{50} = 9.45 ± 0.13, $^{**}P < 0.01$). However, this modification did not change the binding affinity of PACAP27 for the PAC1 or VPAC1 receptors but reduced it significantly for VPAC2 (pIC_{50} = 7.16 ± 0.11, $^{*}P < 0.05$). On the one hand, the substitution of Ser^2 with D-proline, a constrained D-amino acid, reduced significantly the ability of both PACAP isoforms to bind PAC1 ([D-Pro^2]PACAP38; pIC_{50} = 6.75 ± 0.12, $^{***}P < 0.001$ and [D-Pro^2]PACAP27; pIC_{50} = 6.33 ± 0.14, $^{**}P < 0.01$), which was not the case when this residue was substituted with L-hydroxyproline. Additionally, both [D-Pro^2]PACAP isoforms showed an important binding reduction for VPAC1. On the other hand, [D-Pro^2]PACAP38 bound VPAC2 with the same affinity as did the endogenous ligand while this modification significantly decreased the binding affinity of PACAP27 (pIC_{50} = 5.65 ± 0.10, $^{***}P < 0.001$). Moreover, L-hydroxyproline incorporation at this position affected PACAP27 binding affinity towards both VPAC receptors (VPAC1, pIC_{50} = 7.40 ± 0.11, $^{**}P < 0.01$ and VPAC2, pIC_{50} = 6.89 ± 0.07, $^{**}P < 0.01$) but only slightly altered the ability of PACAP38 to bind VPAC1 (pIC_{50} = 7.57 ± 0.20). Replacement of the aromatic and hydrophobic phenylalanine residue at position 6 with a L-biphenylalanine (Bip) did not alter the binding affinity of either PACAP isoform for PAC1, VPAC1 or VPAC2. Additionally, as observed with [Ala^2]PACAP27, introduction of an alanine

moiety at position 7 of PACAP27 gave rise to an analog that exhibited a lower binding affinity to all three PACAP receptors (PAC1, pIC_{50} = 7.78 ± 0.16, *P < 0.05; VPAC1, pIC_{50} = 7.51 ± 0.21, *P < 0.05; VPAC2, pIC_{50} = 5.80 ± 0.12, ***P < 0.001) compared to its PACAP38 counterpart (PAC1, pIC_{50} = 8.52 ± 0.11; VPAC1, pIC_{50} = 7.78 ± 0.24; VPAC2, pIC_{50} = 7.48 ± 0.15). Finally, replacement of Tyr^{22} with L-alanine reduced the capacity of both isoforms to bind PAC1 ([Ala^{22}]PACAP38, pIC_{50} = 7.54 ± 0.16, *P < 0.05 and [Ala^{22}]PACAP27, pIC_{50} = 6.94 ± 0.11, **P <0.01) but interestingly, this modification was the only one among those that we studied that lowered binding to VPAC1 and VPAC2 significantly.

3.2. Survival assays

Each compound was evaluated for its ability to protect the neuroblastoma SH-SY5Y cell line against the neurotoxic effects of MPP^+, which produces neuronal damages very similar to those observed in Parkinson's disease (22). These neuroblastoma, which endogenously express PAC1 and VPAC2 receptors (32), exhibit dopaminergic neuron characteristics (30, 41) and have been largely used as an *in vitro* model of Parkinson's disease (60). Although the SH-SY5Y cells have not been differentiated into a neuronal subtype, several studies have shown the usefulness of this cell line to investigate neuroprotective action (7, 8, 28). With the exception of [Ala^2]PACAP27/38, [Hyp^2]PACAP38, [Bip^6]PACAP27/38, [Ala^7]PACAP27/38, [Ala^{22}]PACAP27/38, all the other analogs were statistically unable to protect neuronal cells, although the binding affinity of [D-Pro^2]PACAP27/38 for PAC1 receptors, and to some extent that of [Hyp^2]PACAP27, were severely altered. Interestingly, [Ala^2]PACAP38, [Hyp^2]PACAP38, [Bip^6]PACAP38, [Ala^7]PACAP38 and [Ala^{22}]PACAP38 seemed to protect neurons against cell death more efficiently than their 27-amino acid counterparts, a feature also observed between native PACAP isoforms (Fig. 1).

3.3. Pharmacological characterization of PACAP38, PACAP27, and their related analogs

PACAP inhibits neuronal apoptosis *via* the stimulation of multiple signaling pathways involving elevation of cAMP and calcium levels, following the activation of distinct G proteins (35). In order to more closely examine the signaling pathways potentially involved in the neuroprotective action of PACAP and its related analogs, all peptides were evaluated, using BRET-based biosensors, for their ability to activate G_q, G_s, β-arrestin 1 and β-arrestin 2 (Table 2, Fig. 2 and 3).

3.3.1 G_q activation

As shown in Table 2, [Ala^2]PACAP38 (pEC_{50} = 7.42 ± 0.18; E_{max} = 95% ± 6) and [Bip^6]PACAP38 (pEC_{50} = 7.41 ± 0.30; E_{max} = 102% ± 10) stimulated G_q with a potency and an efficacy similar to that of PACAP38 (pEC_{50} = 7.03 ± 0.15; E_{max} = 94% ± 7), and these results are in accordance with their neuroprotective effects. In addition, [Hyp^2]PACAP38 and [Ala^7]PACAP38 stimulated G_q with no loss of potency (pEC_{50} = 7.45 ± 0.81 and pEC_{50} = 6.66 ± 0.32, respectively) but with a significant reduction in efficacy (E_{max} = 22% ± 6, ****P* <0.001, and E_{max}= 61% ± 11, **P* <0.05, respectively) compared to PACAP38 (pEC_{50} = 7.03 ± 0.15, E_{max} = 94% ± 7). These analogs could be therefore considered as partial agonists for the G_q activation. Furthermore, [D-Pro^2]PACAP38 failed to activate G_q, while [Ala^{22}]PACAP38 was a full agonist but showed an important loss of potency (pEC_{50} = 6.32 ± 0.12, ***P* <0.01 and E_{max} = 124% ± 11). These results appear to be in agreement with the neuroprotective effects measured with each analog. We next evaluated the impact of these substitutions on PACAP27 isoforms (Table 2, Fig. 3). Except for [Ala^2]PACAP27, all analogs showed a reduction in potency and/or efficacy. For instance, [D-Pro^2]PACAP27 acted as a weak partial agonist (pEC_{50} = 5.82 ± 0.45, ***P* <0.01;

E_{max} = 36% ± 13, ***P <0.001). [Hyp^2]PACAP27 (pEC_{50} = 6.75 ± 0.20) and [Bip^6]PACAP27 (pEC_{50} = 6.74 ± 0.20) stimulated G_q with the same potency as PACAP27 (pEC_{50} = 6.93 ± 0.09) but with a significant loss of efficacy (E_{max} = 57% ± 7, ***P <0.001 and E_{max} = 86% ± 9, *P <0.05, respectively), while [Ala^7]PACAP27 and [Ala^{22}]PACAP27-induced G_q activation was significantly less potent than with PACAP27 (pEC_{50} = 6.26 ± 0.12, **P <0.01 and pEC_{50} = 6.38 ± 0.09, **P <0.01, respectively) but with a comparable efficacy.

3.3.2 G_s activation

Since the neuroprotective effect of PACAP is known to involve activation of G_s, peptides were also evaluated for their ability to activate this G protein. All PACAP38 related analogs retained the capacity to stimulate G_s. In particular, [D-Pro^2]PACAP38, which was inactive for G_q stimulation assay, stimulated G_s with no significant reduction in potency or efficacy (pEC_{50} = 6.52 ± 0.42 and E_{max} = 59% ± 14) compared to PACAP38 (pEC_{50} = 7.58 ± 0.27 and E_{max} = 76% ± 7). Furthermore, [Ala^2]PACAP38, [Hyp^2]PACAP38 and [Ala^{22}]PACAP38 acted as full agonists and appeared to be more efficacious than PACAP38 (E_{max} = 76% ± 7) to activate G_s (E_{max} = 103% ± 10, *P <0.05; E_{max} = 120% ± 12, **P <0.01; and E_{max}= 114% ± 11, *P <0.05, respectively). Finally, [Bip^6]PACAP38 (pEC_{50} = 8.09 ± 0.43) and [Ala^7]PACAP38 (pEC_{50} = 8.01 ± 0.43) stimulated G_s with a higher potency than PACAP38 (pEC_{50} = 7.58 ± 0.27) but with a comparable efficacy (Table 2). Similar experiments were also performed with PACAP27 and its related analogs (Table 2). The replacement of Ser^2 with L-alanine had no significant effect on G_s activation. However, the substitution at this position with constrained amino acids such as D-proline or L-hydroxyproline revealed a significant impact. [D-Pro^2]PACAP27 acted as a partial agonist with a pEC_{50} < 5 and a maximal efficacy of 44% (**P <0.01), while [Hyp^2]PACAP27,

which exhibited only a weak reduction of its potency (pEC_{50} = 7.50 ± 0.22), stimulated G_s with a striking efficacy (E_{max} = 175% ± 10, ***P <0.001). A similar observation was made with [Ala^7]PACAP27, for which the potency to activate the G_s pathway was reduced significantly (pEC_{50} = 7.22 ± 0.38, *P < 0.05) but with an efficacy that was undeniably increased (E_{max} = 139% ± 15, **P < 0.01). Additionally, the increase of bulkiness, hydrophobicity and aromaticity at position 6 through introduction of a biphenylalanine moiety, *i.e.* [Bip^6]PACAP27, did not alter G_s activation. Finally, [Ala^{22}]PACAP27 exhibited a significant reduction of potency for G_s activation (pEC_{50} = 7.00 ± 0.23, **P < 0.01) (Table 2).

3.3.3 β-arrestin 1 and 2 activation

Analogs were also tested for their capacity to stimulate β-arrestins. As shown in Table 2, except for [D-Pro^2]PACAP38, all analogs activated β-arrestin 1. More precisely, the treatment of HEK293 cells transiently transfected with PAC1 with [Ala^2]PACAP38 (pEC_{50} = 6.71 ± 0.16, E_{max} = 109% ± 8) or [Ala^{22}]PACAP38 (pEC_{50} = 6.44 ± 0.13, E_{max} = 139% ± 11) produced a β-arrestin 1 activation similar to that of PACAP38 (pEC_{50} = 6.76 ± 0.12, E_{max} = 121% ± 8). Furthermore, [Hyp^2]PACAP38 displayed the same propensity to activate the β-arrestin 1 pathway but with a reduced efficacy (E_{max} = 89% ± 9, *P <0.01) compared to PACAP38 (E_{max} = 121% ± 8). Notably, [Bip^6]PACAP38 (pEC_{50} = 7.44 ± 0.15, ***P <0.001) and [Ala^7]PACAP38 (pEC_{50} = 7.13 ± 0.11, **P <0.01) exhibited a higher tendency to activate β-arrestin 1 relative to PACAP38. Also, [Ala^7]PACAP38 was able to reach 147% ± 6 of the efficacy associated with PACAP38-mediated β-arrestin 1 activation. As for β-arrestin 1 activation, only [D-Pro^2]PACAP38 did not activate the β-arrestin 2 pathway. Also, on the one hand, [Ala^2]PACAP38 (pEC_{50} = 6.92 ± 0.18) and [Hyp^2]PACAP38 (pEC_{50} = 7.23 ± 0.41) activated β-arrestin 2, as did the endogenous ligand

(pEC_{50} = 6.80 ± 0.22), while, on the other hand, [Bip^6]PACAP38, [Ala^7]PACAP38 and [Ala^{22}]PACAP38 stimulated β-arrestin 2 recruitment with greater efficacies (E_{max} = 148% ± 11, $^{**}P < 0.01$; E_{max} = 220% ± 15, $^{***}P < 0.001$; E_{max} = 233% ± 22, $^{***}P < 0.001$, respectively) than PACAP38 (E_{max} = 92% ± 10). As shown in Table 2, all PACAP27 analogs activated the β-arrestin 1 pathway with various potencies. In particular, [Ala^{22}]PACAP27 exhibited rather low potency (pEC_{50} = 6.23 ± 0.13, $^{*}P < 0.05$) to activate β-arrestin 1 while exhibiting similar efficacy (E_{max} = 133% ± 14) than that of PACAP27 (pEC_{50} = 6.56 ± 0.07, E_{max} = 118% ± 5). Also, as for [D-Pro^2]PACAP38, its PACAP27 counterpart compound failed to activate β-arrestin 1, while [Bip^6]PACAP27 (E_{max} = 152% ± 9) activated β-arrestin 1 with an efficacy significantly higher than that of PACAP27 but with a similar potency.

The 27-amino acid analogs were also tested for their capacity to stimulate the β-arrestin 2 pathway. Surprisingly, [D-Pro^2]PACAP27 (pEC_{50} = 5.67 ± 0.30) exhibited a reduced ability to promote β-arrestin 2 activation and was only able to achieve around 55% of PACAP27-associated recruitment, revealing itself as a partial agonist for this pathway. Also, [Ala^7]PACAP27 activated the β-arrestin 2 signaling with a pEC_{50} = 6.42 ± 0.11, thus markedly lower than the value measured with the endogenous ligand, but with an efficacy (E_{max} = 134% ± 8) significantly higher than that of PACAP27. Finally, the other analogs were equipotent to the endogenous ligand at activating β-arrestin 2 (Table 2).

4. Discussion

Biological responses to hormonal stimuli are encoded during the initial interactions between a ligand and its cognate receptor(s). Since downstream signaling is tightly coupled to specific ligand/receptor interactions, structurally different ligands might therefore modulate downstream signaling events differentially (16). Over the years, evidence for functional

selectivity or biased agonism following activation of PAC1 by its two endogenous ligands has accumulated (48, 55). While most descriptions of biased agonism have been centered on differential effects of synthetic drugs, there are several important G protein-coupled receptor (GPCR) families that bind to multiple endogenous agonists such as the somatostatin and opioid receptor families. Although this has been traditionally attributed to the redundancy of some biological systems, the existence of multiple ligands for the same receptor could represent an added layer of control to engender finely tuned physiological responses through biased agonism/functional selectivity (51). For instance, it has been shown that PACAP27 is more potent and efficacious than PACAP38 to induce cAMP production while this order is reversed for inositol triphosphate production (48). More recently, it has been demonstrated that PACAP38, but not PACAP27, was able to trigger $ERK_{1/2}$ phopshorylation in glial cells, while both isoforms stimulated cellular cAMP production, albeit with different potencies (55). Induced PAC1 signaling in response to PACAP38 or PACAP27 could therefore lead to distinct effects for each peptide in the pathogenesis and progression of PAC1-associated diseases (53, 55). Hence, advances in our understanding and treatment of PAC1-associated diseases will emerge from an evolving comprehension of ligand-receptor interactions, along with their associated downstream effectors and divergent signaling pathways.

To gain some insight regarding the particular molecular pharmacology of each PACAP isoform and also, in order to probe specific structural and/or physico-chemical determinants that might explain their distinct activation profile, we examined the binding affinity as well as the ability of PACAP38, PACAP27 and some related analogs to trigger multiple signaling pathways, including different G proteins and β-arrestin isoforms. As described above, point substitutions were introduced within key pharmacophoric elements of the PACAP isoforms, *i.e.* the Asx-turn motif (position 2), the N-capping domain (positions 6 and 7), and the α-helical region (position

22) (28). These analogs were selected based on their ability to bind preferentially PAC1 and VPAC1 ([Ala^2]PACAP27, [D-Pro^2]PACAP27, [Hyp^2]PACAP27, [Ala^7]PACAP27, and [Ala^{22}]PACAP27/38) (12, 43) or to behave as a superagonist ([Bip^6]PACAP27) (4). In addition, because the C-terminal 28-38 region is an integral part of the physico-chemical and/or structural parameters regulating PAC1 signaling pathways, we evaluated the contribution of this region on the neuroprotective action of the peptide molecules of this study by synthesizing analogs according to both PACAP isoform scaffolds.

With respect to their binding affinity, it was noticed that most PACAP38 isoforms interacted more efficiently with PAC1 and VPAC2 than their PACAP27 counterparts. Accordingly, it has been previously shown that the 28-38 segment forms a short helix that facilitates the binding of PACAP38 through an electrostatic interaction involving basic C-terminal residues and an acidic patch located on the N-terminal ectodomain of PAC1 (49). Because both PACAP isoforms are well known for their anti-apoptotic actions, using SH-SY5Y cells, each analog was tested for its ability to prevent MPP^+-induced apoptosis. Out of the 12 analogs, only 9 derivatives, *i.e.* [Ala^2]PACAP27/38, [Hyp^2]PACAP38 [Bip^6]PACAP27/38, [Ala^7]PACAP27/38 and [Ala^{22}]PACAP27/38, protected SH-SY5Y cells against MPP^+ toxicity despite the fact that all analogs bound to PAC1. Interestingly, PACAP38 and its derivatives were better neurorotective agents than their PACAP27 counterparts. For instance, while [Ala^2]PACAP27 and [Ala^2]PACAP38 bound with a similar affinity to PAC1, [Ala^2]PACAP38 exerted a significantly stronger neuroprotective action than its parent derivative (Fig. 1, Table 1). A similar pattern was also observed with [Hyp^2]PACAP38, [Bip^6]PACAP38, [Ala^7]PACAP38, and [Ala^{22}]PACAP38. For [Hyp^2]PACAP38 and the last two analogs, their PACAP27 counterparts were almost devoid of any protective action (Fig. 1). Regarding the analogs unable to provide any protection, it appeared that the replacement in PACAP38 or PACAP27 of serine-2

with a D-proline, or substitution of tyrosine-22 with L-alanine, induced a substantial reduction of their binding affinities for all PACAP receptors, a condition that probably explains their lack of efficacy. However, this justification is not sufficient to explain the behavior of [Hyp^2]PACAP27, which is inactive despite a binding affinity similar to those of the endogenous peptides. As previously described, structurally different analogs might stabilize different receptor conformations and lead to subtle bias in signaling pathways associated with the activation of a given receptor. While we know the impact of the C-terminal domain of PACAP38, as well as that of a few modifications on binding affinity and anti-apoptotic function, we have currently no insights regarding the pharmacophore contribution to PAC1-associated signaling.

To understand the impact of each modification and to pinpoint specific signaling pathways associated with their anti-apoptotic actions, using BRET-based biosensors, we evaluated the propensity of each analog to activate G_q and G_s, as well as β-arrestin 1 and 2 (Table 2). At position 2, replacement of the serine residue with an alanine did not produce an activation profile different from that observed for PACAP27 (Fig. 3). However, this modification, in combination with the presence of the 28-38 segment, improved significantly β-arrestin 2 and G_s activation (Table 2, Fig. 2). While it is well known that the G_s pathway is involved in the neuroprotective effect of both PACAP isoforms, the participation of β-arrestins in neuroprotection has not yet been definitely confirmed. Nonetheless, their involvement was suspected as receptor internalization processes have been linked to its neuroprotective action (36). To date, only one study has shown that PACAP38-sustained $ERK_{1/2}$ signaling was linked to β-arrestin 1 activation (6), and that this mechanism, *i.e.* $ERK_{1/2}$ phosphorylation, might be involved in PACAP-associated neuroprotection (24, 54, 56). Interestingly, the absence of the hydroxyl group at position 2, produced by substituting the serine residue with an alanine, did not

reduce the ability of the related analog to efficiently activate G_s and G_q. While demonstrating an impact of the C-terminal region of the ligand on PAC1 activation profile, this data also confirms that the hydroxyl group of serine-2 is not critical for PAC1 receptor activation and its subsequent neuroprotective action. In addition, introduction of a conformational restriction at this position *via* insertion of a hydroxyproline residue, which generated the selective PAC1/VPAC1 agonist [Hyp^2]PACAP27 (12), strongly promoted G_s activation and produced a β-arrestin 1 and 2 activation similar to that of PACAP27, but triggered only partial G_q activation (Fig. 3, Table 2). The same substitution in PACAP38 further decreased the efficiency of [Hyp^2]PACAP38 to activate G_q activation but promoted G_s activation strongly, as did the PACAP27 counterpart, and reduced the efficacy to activate β-arrestin 1 while leaving β-arrestin 2 signaling unaffected. Surprisingly, while minor differences between the activation profile of [Hyp^2]PACAP38 and [Hyp^2]PACAP27 were noted, only [Hyp^2]PACAP38 was able to significantly protect neuroblastoma cells. Those differences were observed with respect to G_q and β-arrestin 1 signaling with [Hyp^2]PACAP38 being more potent than [Hyp^2]PACAP27 to activate G_q but less efficient than [Hyp^2]PACAP27 to activate β-arrestin 1. Hence, these data suggest that β-arrestin 1 is not involved in the neuroprotective action of PACAP.

As previously noted, binding of the cationic C-terminal tail of PACAP38 with an acidic patch located in the N-terminal domain of PAC1 promotes, within the activation pocket of the receptor, a particular positioning of the N-terminal portion of the 38-amino acid ligand (49). As reported for the glucagon receptor, such an initial interaction promotes major conformational modifications at the receptor level and facilitates additional contacts between the peptide and the first extracellular loop (61, 62). Therefore, the N-terminal segment of PACAP38, encompassing the Asx-turn, is probably oriented in the receptor cavity in a slightly different manner from the

one that occurs with PACAP27, which does not possess the basic C-terminal stretch (49). Hence, these results support the presence of specific chemical determinants/interactions within the PACAP/PAC1 complex that modulate distinct aspect of downstream cellular signaling. Thus, it can be postulated that the introduction of a modification in either PACAP27 or PACAP38 does not imply a transposition of the signaling signature and pharmacological effects on both isoforms. Analysis of the activation profile of the active [Ala^2]PACAP27 analog and the inactive [Hyp^2]PACAP27 derivative suggests that the ability of the analog to activate G_q and/or β-arrestin 1 influences its propensity to protect SH-SY5Y cells against the MPP^+ toxicity (Figs. 1, 2 and 3, Table 2). Accordingly, it was demonstrated that G_q-associated effectors, and more particularly Akt, are linked to the neuroprotective action of PACAP through PAC1 (36). Similarly, when looking at the signaling signature of [Bip^6]PACAP38 and [Bip^6]PACAP27, a small but significant reduction of G_q efficacy was observed for [Bip^6]PACAP27 (Fig. 2 and 3), which also appears to be less neuroprotective than its PACAP38 counterpart (Fig.1). Also, while both [Ala^7]PACAP38 and [Ala^7]PACAP27 protected SH-SY5Y neuroblastoma cells, [Ala^7]PACAP38 exerted a trend to a better neuroprotective action than its PACAP27-related analog. An overview of their activation profiles revealed significant variations in their ability to potently and efficiently activate G_q, β-arrestin 2, and G_s. The latter is positively linked to different cAMP-regulated protein kinases that mediate the neuroprotective effects of PACAP (35). A similar observation was also made with the pair [Ala^{22}]PACAP27/38 since the PACAP38 derivative appears to activate G_s with a higher potency than the PACAP27 counterpart, which could again explain, at least in part, their differential neuroprotective actions. Overall, most of the time, the 38-amino acid PACAP isoforms promoted a greater activation of β-arrestin 1, β-arrestin 2, or both than their PACAP27 counterparts. This observation further supports the role of the C-

terminal portion of PACAP38 for the activation of β-arrestins, and in particular β-arrestin 2. Whether or not this phenomenon is only dependent on the presence of the 28-38 C-terminal segment remains to be determined. Indeed, this observation could also be linked to differential positioning of the N-terminal Asx-turn of PACAP27 and PACAP38 in the receptor environment, thereby stabilizing or disrupting specific microswitches involved in β-arrestin activation (29). Altogether, it appears that neuroprotective actions cannot be linked to a single signaling pathway but more likely would be the result of multiple events that would lead to a neuroprotective effect , upon reaching a particular threshold. Also, this data supports the idea that the binding and activation determinants between PACAP27 and PACAP38 are not the same. Further, it has been proposed that PACAP binds to PAC1 *via* a two-step activation process and that the dissimilar C-terminal segments of PACAP isoforms bind to the receptor extracellular domains through distinct electrostatic, hydrophobic and hydrogen bond interactions allowing the N-terminal portion of the peptide to access the transmembrane core and induce receptor activation (40). These differential interactions characterizing each PACAP isoform and the N-terminal region of PAC1 probably activate switches distinctively and induce the activation of unique sets of intracellular effectors. Notably, it has been recently demonstrated that PAC1 transits through several conformational states, spreading from the extracellular domain to the second messenger binding sites (29). Finally, a recent study reported the importance of the first extracellular domain of the human glucagon receptor. Especially, this study demonstrated that the β-hairpin formation plays a role in stabilizing the ligand-receptor interaction (62). Our results improve the understanding of activation of class B GPCRs and bring some insights about the pharmacological differences observed between PACAP27 and PACAP38.

In conclusion, we showed that the introduction of alike modifications in both PACAP isoforms generated analogs with distinct signaling signatures. This observation suggested a role for the C-terminal 28-38 segment in the binding and activation processes, and more acutely in the β-arrestins activation. Interestingly, the study revealed that introduction of a specific modifications at position 6, 7 or 22 in PACAP27 and PACAP38 consistently improved their potency and efficiency to drive β-arrestin activation, but left G protein stimulation almost unaffected. Among the different modifications introduced in PACAP27 and PACAP38, the presence of D-Pro at position 2 has been the only one that drastically and negatively affected β-arrestins recruitment. We also observed that the neuroprotective action of the PACAP27- and PACAP38-related analogs was multiparametric and necessitated the activation of both G_q and G_s and their associated downstream effectors. Over the years, both isoforms have been used to study their neuroprotective potential of PACAP in various diseases. However, characteristics such as a short *in vivo* half-life, especially for PACAP38, or their lack of selectivity, PAC1 versus VPAC receptors, have prevented their use as therapeutic agents (3). However, the ability to cross the BBB, either passively (PACAP27) or through a transporter-mediated mechanism (PACAP38), makes these peptides highly interesting as chemical templates for peptide-based drug design (2). While PACAP38 seems more suitable due to its higher *in vitro* neuroprotective effects, its use could be hampered by its ability to hyperactivate β-arrestins, a feature that could lead to receptor desensitization. Altogether, PACAP27, with its higher plasma stability, its ability to diffuse passively throughout the BBB and most importantly, its relative inability to overstimulate β-arrestins appears so far as a better template than PACAP38 for the development of a peptide-derived drug designed to treat neurodegenerative diseases such as PD. Although the data are still preliminary, the present study showed that the introduction of various modifications within key

pharmacophores of PACAP was able to perturb signaling signatures compared to endogenous ligands. A more comprehensive library screening is therefore necessary to clearly highlight the specific physico-chemical and/or structural elements modulating PACAP27 signaling.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

We thank the Canadian Institutes of Health Research (CIHR), the Fonds de recherche du Québec – Nature et Technologies and the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding.

Uncategorized References

1. Arimura A. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol. 1998;48(5):301-31.

2. Banks WA, Kastin AJ, Komaki G, Arimura A. Passage of pituitary adenylate cyclase activating polypeptide1-27 and pituitary adenylate cyclase activating polypeptide1-38 across the blood-brain barrier. The Journal of pharmacology and experimental therapeutics. 1993;267(2):690-6.

3. Bourgault S, Vaudry D, Botia B, Couvineau A, Laburthe M, Vaudry H, et al. Novel stable PACAP analogs with potent activity towards the PAC1 receptor. Peptides. 2008;29(6):919-32.

4. Bourgault S, Vaudry D, Segalas-Milazzo I, Guilhaudis L, Couvineau A, Laburthe M, et al. Molecular and conformational determinants of pituitary adenylate cyclase-activating polypeptide (PACAP) for activation of the PAC1 receptor. J Med Chem. 2009;52(10):3308-16.

5. Bourgault S, Vaudry D, Guilhaudis L, Raoult E, Couvineau A, Laburthe M, et al. Biological and structural analysis of truncated analogs of PACAP27. J Mol Neurosci. 2008;36(1-3):260-9.

6. Broca C, Quoyer J, Costes S, Linck N, Varrault A, Deffayet PM, et al. beta-Arrestin 1 is required for PAC1 receptor-mediated potentiation of long-lasting ERK1/2 activation by glucose in pancreatic beta-cells. J Biol Chem. 2009;284(7):4332-42.

7. Brown D, Tamas A, Reglodi D, Tizabi Y. PACAP protects against salsolinol-induced toxicity in dopaminergic SH-SY5Y cells: implication for Parkinson's disease. J Mol Neurosci. 2013;50(3):600-7.

8. Brown D, Tamas A, Reglodi D, Tizabi Y. PACAP protects against inflammatory-mediated toxicity in dopaminergic SH-SY5Y cells: implication for Parkinson's disease. Neurotoxicity research. 2014;26(3):230-9.

9. Chang Y, Lawson LJ, Hancock JC, Hoover DB. Pituitary adenylate cyclase-activating polypeptide: localization and differential influence on isolated hearts from rats and guinea pigs. Regul Pept. 2005;129(1-3):139-46.

10. Dauer W, Przedborski S. Parkinson's disease: mechanisms and models. Neuron. 2003;39(6):889-909.

11. Dejda A, Seaborn T, Bourgault S, Touzani O, Fournier A, Vaudry H, et al. PACAP and a novel stable analog protect rat brain from ischemia: Insight into the mechanisms of action. Peptides. 2011;32(6):1207-16.

12. Doan ND, Bourgault S, Dejda A, Letourneau M, Detheux M, Vaudry D, et al. Design and in vitro characterization of PAC1/VPAC1-selective agonists with potent neuroprotective effects. Biochem Pharmacol. 2011;81(4):552-61.

13. Feigin VL. Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet Neurology. 2017;16(11):877-97.

14. Fournier A, Bourgault S, Chatenet D. The Pharmacophoric Determinants of PACAP. In: Reglodi D, Tamas A, editors. Pituitary Adenylate Cyclase Activating Polypeptide — PACAP. Cham: Springer International Publishing; 2016. p. 111-32.

15. Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. N Engl J Med. 2003;348(14):1365-75.

16. Galandrin S, Oligny-Longpre G, Bouvier M. The evasive nature of drug efficacy: implications for drug discovery. Trends Pharmacol Sci. 2007;28(8):423-30.

17. Han P, Tang Z, Yin J, Maalouf M, Beach TG, Reiman EM, et al. Pituitary adenylate cyclase-activating polypeptide protects against beta-amyloid toxicity. Neurobiol Aging. 2014;35(9):2064-71.

18. Hoare SR. Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. Drug Discov Today. 2005;10(6):417-27.

19. Hunter WM, Greenwood FC. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature. 1962;194:495-6.

20. Inooka H, Endo S, Kitada C, Mizuta E, Fujino M. Pituitary adenylate cyclase activating polypeptide (PACAP) with 27 residues. Conformation determined by 1H NMR and CD spectroscopies and distance geometry in 25% methanol solution. Int J Pept Protein Res. 1992;40(5):456-64.

21. Jankovic J. Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry. 2008;79(4):368-76.

22. Javitch JA, D'Amato RJ, Strittmatter SM, Snyder SH. Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6 -tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurons explains selective toxicity. Proc Natl Acad Sci U S A. 1985;82(7):2173-7.

23. Jorgensen R, Kubale V, Vrecl M, Schwartz TW, Elling CE. Oxyntomodulin differentially affects glucagon-like peptide-1 receptor beta-arrestin recruitment and signaling through Galpha(s). The Journal of pharmacology and experimental therapeutics. 2007;322(1):148-54.

24. Journot L, Villalba M, Bockaert J. PACAP-38 protects cerebellar granule cells from apoptosis. Ann N Y Acad Sci. 1998;865:100-10.

25. Kenakin TP. Biased signalling and allosteric machines: new vistas and challenges for drug discovery. Br J Pharmacol. 2012;165(6):1659-69.

26. Kienlen Campard P, Crochemore C, Rene F, Monnier D, Koch B, Loeffler JP. PACAP type I receptor activation promotes cerebellar neuron survival through the cAMP/PKA signaling pathway. DNA Cell Biol. 1997;16(3):323-33.

27. Koblish M, Carr R, 3rd, Siuda ER, Rominger DH, Gowen-MacDonald W, Cowan CL, et al. TRV0109101, a G Protein-Biased Agonist of the micro-Opioid Receptor, Does Not Promote Opioid-Induced Mechanical Allodynia following Chronic Administration. The Journal of pharmacology and experimental therapeutics. 2017;362(2):254-62.

28. Lamine A, Letourneau M, Doan ND, Maucotel J, Couvineau A, Vaudry H, et al. Characterizations of a synthetic pituitary adenylate cyclase-activating polypeptide analog displaying potent neuroprotective activity and reduced in vivo cardiovascular side effects in a Parkinson's disease model. Neuropharmacology. 2016;108:440-50.

29. Liao C, Zhao X, Brewer M, May V, Li J. Conformational Transitions of the Pituitary Adenylate Cyclase-Activating Polypeptide Receptor, a Human Class B GPCR. Scientific reports. 2017;7(1):5427-.

30. Lopes FM, Schroder R, da Frota ML, Jr., Zanotto-Filho A, Muller CB, Pires AS, et al. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. Brain research. 2010;1337:85-94.

31. Luttrell LM, Maudsley S, Bohn LM. Fulfilling the Promise of "Biased" G Protein-Coupled Receptor Agonism. Mol Pharmacol. 2015;88(3):579-88.

32. Lutz EM, Ronaldson E, Shaw P, Johnson MS, Holland PJ, Mitchell R. Characterization of novel splice variants of the PAC1 receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. Molecular and cellular neurosciences. 2006;31(2):193-209.

33. Maasz G, Zrinyi Z, Reglodi D, Petrovics D, Rivnyak A, Kiss T, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) has a neuroprotective function in dopamine-based neurodegeneration in rat and snail parkinsonian models. Dis Model Mech. 2017;10(2):127-39.

34. Maiti P, Manna J, Dunbar GL. Current understanding of the molecular mechanisms in Parkinson's disease: Targets for potential treatments. Transl Neurodegener. 2017;6:28.

35. Manecka D-L, Boukhzar L, Falluel-Morel A, Lihrmann I, Anouar Y. PACAP Signaling in Neuroprotection. In: Reglodi D, Tamas A, editors. Pituitary Adenylate Cyclase Activating Polypeptide — PACAP. Cham: Springer International Publishing; 2016. p. 549-61.

36. May V, Lutz E, MacKenzie C, Schutz KC, Dozark K, Braas KM. Pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1HOP1 receptor activation coordinates multiple neurotrophic signaling pathways: Akt activation through phosphatidylinositol 3-kinase gamma and vesicle endocytosis for neuronal survival. J Biol Chem. 2010;285(13):9749-61.

37. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun. 1989;164(1):567-74.

38. Mizushima H, Banks WA, Dohi K, Shioda S, Matsumoto H, Matsumoto K. The effect of cardiac arrest on the permeability of the mouse blood-brain and blood-spinal cord barrier to pituitary adenylate cyclase activating polypeptide (PACAP). Peptides. 1999;20(11):1337-40.

39. Neumann JM, Couvineau A, Murail S, Lacapere JJ, Jamin N, Laburthe M. Class-B GPCR activation: is ligand helix-capping the key? Trends Biochem Sci. 2008;33(7):314-9.

40. Pal K, Melcher K, Xu HE. Structure and mechanism for recognition of peptide hormones by Class B G-protein-coupled receptors. Acta Pharmacol Sin. 2012;33(3):300-11.

41. Presgraves SP, Ahmed T, Borwege S, Joyce JN. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotoxicity research. 2004;5(8):579-98.

42. Rajagopal S, Shenoy SK. GPCR desensitization: Acute and prolonged phases. Cell Signal. 2017.

43. Ramos-Alvarez I, Mantey SA, Nakamura T, Nuche-Berenguer B, Moreno P, Moody TW, et al. A structure-function study of PACAP using conformationally restricted analogs: Identification of PAC1 receptor-selective PACAP agonists. Peptides. 2015;66:26-42.

44. Rat D, Schmitt U, Tippmann F, Dewachter I, Theunis C, Wieczerzak E, et al. Neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) slows down Alzheimer's disease-like pathology in amyloid precursor protein-transgenic mice. FASEB J. 2011;25(9):3208-18.

45. Reglodi D, Tamas A, Lubics A, Szalontay L, Lengvari I. Morphological and functional effects of PACAP in 6-hydroxydopamine-induced lesion of the substantia nigra in rats. Regul Pept. 2004;123(1-3):85-94.

46. Shioda S, Ozawa H, Dohi K, Mizushima H, Matsumoto K, Nakajo S, et al. PACAP protects hippocampal neurons against apoptosis: involvement of JNK/SAPK signaling pathway. Ann N Y Acad Sci. 1998;865:111-7.

47. Shivers KY, Nikolopoulou A, Machlovi SI, Vallabhajosula S, Figueiredo-Pereira ME. PACAP27 prevents Parkinson-like neuronal loss and motor deficits but not microglia activation induced by prostaglandin J2. Biochimica et biophysica acta. 2014;1842(9):1707-19.

48. Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, et al. Differential signal transduction by five splice variants of the PACAP receptor. Nature. 1993;365(6442):170-5.

49. Sun C, Song D, Davis-Taber RA, Barrett LW, Scott VE, Richardson PL, et al. Solution structure and mutational analysis of pituitary adenylate cyclase-activating polypeptide binding to the extracellular domain of PAC1-RS. Proc Natl Acad Sci U S A. 2007;104(19):7875-80.

50. Sze KH, Zhou H, Yang Y, He M, Jiang Y, Wong AO. Pituitary adenylate cyclase-activating polypeptide (PACAP) as a growth hormone (GH)-releasing factor in grass carp: II. Solution structure of a brain-specific PACAP by nuclear magnetic resonance spectroscopy and functional studies on GH release and gene expression. Endocrinology. 2007;148(10):5042-59.

51. Thompson GL, Canals M, Poole DP. Biological redundancy of endogenous GPCR ligands in the gut and the potential for endogenous functional selectivity. Front Pharmacol. 2014;5:262.

52. van der Westhuizen ET, Breton B, Christopoulos A, Bouvier M. Quantification of ligand bias for clinically relevant beta2-adrenergic receptor ligands: implications for drug taxonomy. Mol Pharmacol. 2014;85(3):492-509.

53. Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, et al. Pituitary Adenylate Cyclase-Activating Polypeptide and Its Receptors: 20 Years after the Discovery. Pharmacological Reviews. 2009;61(3):283-357.

54. Villalba M, Bockaert J, Journot L. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. J Neurosci. 1997;17(1):83-90.

55. Walker CS, Sundrum T, Hay DL. PACAP receptor pharmacology and agonist bias: analysis in primary neurons and glia from the trigeminal ganglia and transfected cells. Br J Pharmacol. 2014;171(6):1521-33.

56. Wang G, Qi C, Fan GH, Zhou HY, Chen SD. PACAP protects neuronal differentiated PC12 cells against the neurotoxicity induced by a mitochondrial complex I inhibitor, rotenone. FEBS Lett. 2005;579(18):4005-11.

57. Wang G, Pan J, Tan YY, Sun XK, Zhang YF, Zhou HY, et al. Neuroprotective effects of PACAP27 in mice model of Parkinson's disease involved in the modulation of K(ATP) subunits and D2 receptors in the striatum. Neuropeptides. 2008;42(3):267-76.

58. Warren JB, Cockcroft JR, Larkin SW, Kajekar R, Macrae A, Ghatei MA, et al. Pituitary adenylate cyclase activating polypeptide is a potent vasodilator in humans. J Cardiovasc Pharmacol. 1992;20(1):83-7.

59. Wray V, Kakoschke C, Nokihara K, Naruse S. Solution structure of pituitary adenylate cyclase activating polypeptide by nuclear magnetic resonance spectroscopy. Biochemistry. 1993;32(22):5832-41.

60. Xie HR, Hu LS, Li GY. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. Chinese medical journal. 2010;123(8):1086-92.

61. Yin Y, Zhou XE, Hou L, Zhao LH, Liu B, Wang G, et al. An intrinsic agonist mechanism for activation of glucagon-like peptide-1 receptor by its extracellular domain. Cell Discov. 2016;2:16042.

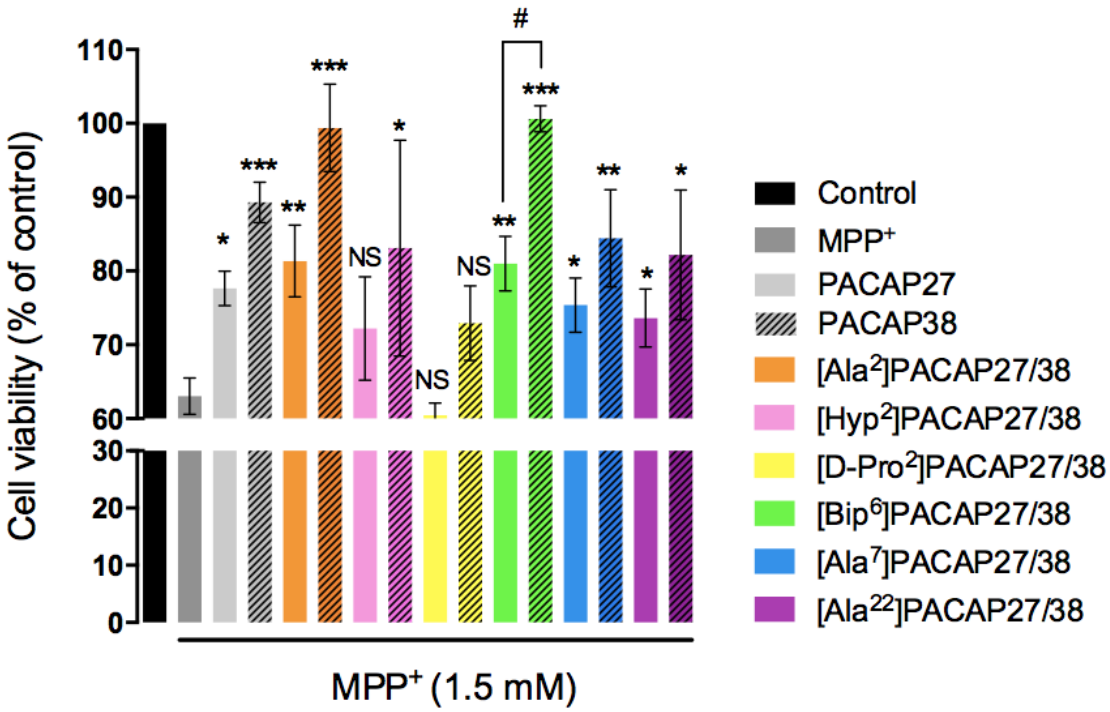
62. Zhang H, Qiao A, Yang D, Yang L, Dai A, de Graaf C, et al. Structure of the full-length glucagon class B G-protein-coupled receptor. Nature. 2017;546(7657):259-64.

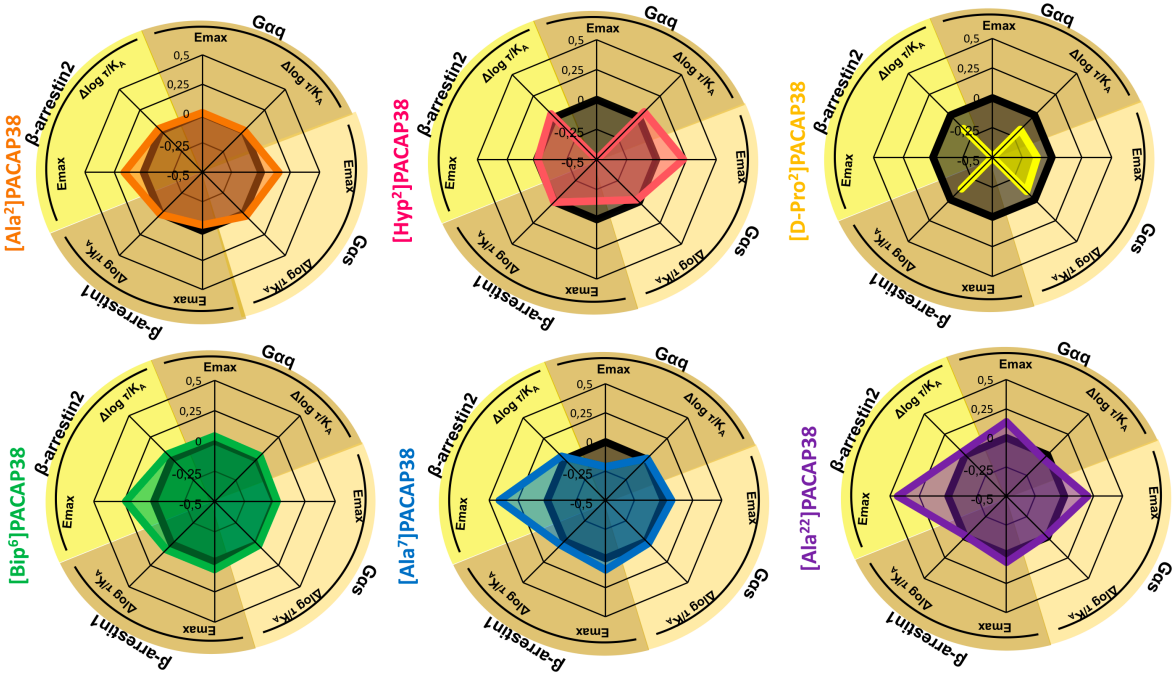
FIGURE LEGENDS

Fig. 1. Effects of MPP^+ (1.5 mM) on SH-SY5Y cell viability after a 4 h pretreatment with PACAP or its related analogs (peptide concentration 10^{-7}M). Results associated with PACAP27 analogs are represented by solid bars while those of PACAP38 and its derivatives are represented by striped bars. Values represent the mean ± S.E.M of 3 to 6 independent experiments performed in sextuplets. Statistical analyses were performed using the Student's t-test versus the percentage of cell viability measured in presence of MPP^+ alone (NS, non-significant; $*P \leq 0.05$; $**P \leq 0.01$ and $***P \leq 0.001$). Statistical analyses between the PACAP27 and PACAP38 isoforms were performed using the Student's t-test ($^{\#}P \leq 0.05$; $^{\#\#}P \leq 0.01$ and $^{\#\#\#}P \leq 0.001$).

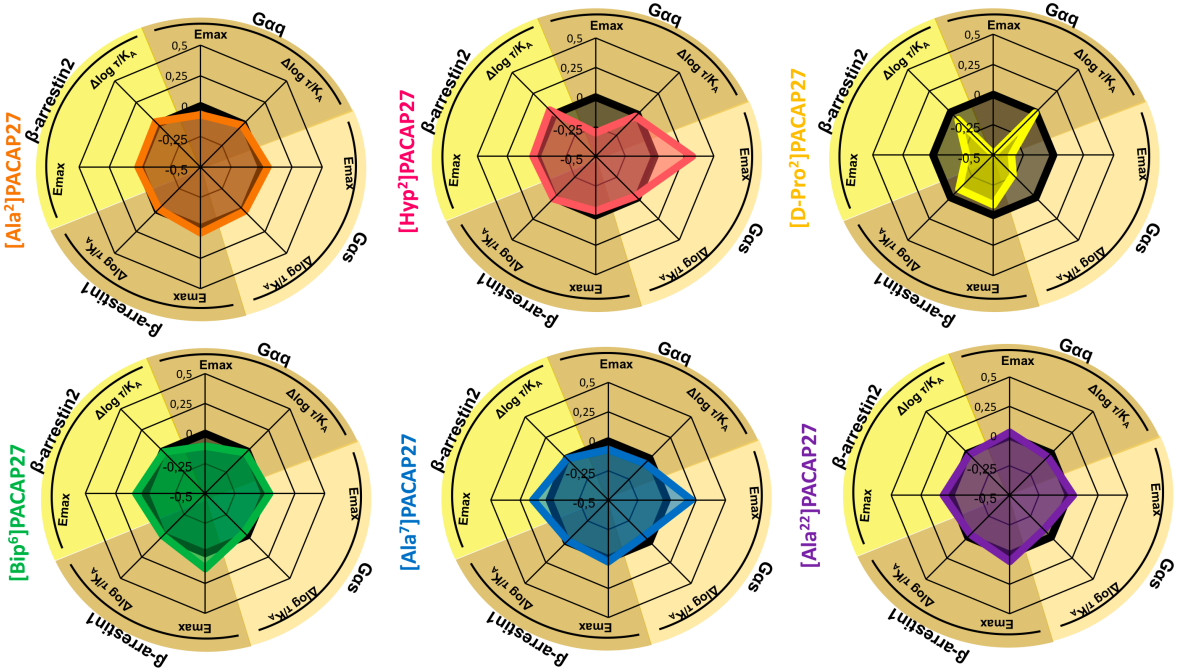
Fig. 2. Kiviat chart of PACAP38 and its related analogs obtained with the responses of the various signaling pathways associated with PAC1 receptor activation. Each Kiviat chart summarizes the maximal effect (E_{max}) and the log τ/K_A of the different ligand-activated signaling pathway. In order to compare activity of each ligand to PACAP38, each E_{max} and Log τ/K_A values were normalized with respect to the value of E_{max} or Log τ/K_A of PACAP38. Data for PACAP38 are represented with the black line.

Fig. 3. Kiviat chart of PACAP27 and its related analogs obtained with the responses of the various signaling pathways associated with PAC1 receptor activation. Each Kiviat chart summarizes the maximal effect (E_{max}) and the log τ/K_A of the different ligand-activated signaling pathway. In order to compare activity of each ligand to PACAP27, each E_{max} and Log τ/K_A values were normalized with respect to the value of E_{max} or Log τ/K_A of PACAP27. Data for PACAP27 are represented with the black line.





Poujol de Molliens et al.
Figure 2



Poujol de Molliens et al.
Figure 3

Table 1. Binding affinity of the PACAP isoforms and their related analogs on human PAC1, VPAC1 and VPAC2 receptors.

Compound	PAC1		VPAC1		VPAC2	
	n	pIC_{50}[a]	n	pIC50	n	pIC_{50}[a]
PACAP38	4	8.19 ± 0.12	3	8.51 ± 0.28	4	7.93 ± 0.21
[Ala2]PACAP38	3	8.76 ± 0.08**	3	9.68 ± 0.18*	3	9.45 ± 0.13**
[D-Pro2]PACAP38	3	6.75 ± 0.12***	3	6.72 ± 0.24**	3	7.98 ± 0.29
[Hyp2]PACAP38	3	8.21 ± 0.15	3	7.57 ± 0.20	3	8.20 ± 0.15
[Bip6]PACAP38	3	8.00 ± 0.16	3	8.06 ± 0.21	3	8.17 ± 0.21
[Ala7]PACAP38	3	8.52 ± 0.11	3	7.78 ± 0.24	3	7.48 ± 0.15
[Ala22]PACAP38	3	7.54 ± 0.16*	3	6.99 ± 0.25*	3	5.60 ± 0.30**
PACAP27[b]	3	8.34 ± 0.07	3	8.23 ± 0.01	3	7.99 ± 0.16
[Ala2]PACAP27[b]	3	8.33 ± 0.12	3	8.15 ± 0.07	3	7.16 ± 0.11*
[D-Pro2]PACAP27[b]	3	6.33 ± 0.14**	3	6.04 ± 0.13***	3	5.65 ± 0.10***
[Hyp2]PACAP27[b]	3	8.07 ± 0.18	3	7.40 ± 0.11**	3	6.89 ± 0.07**
[Bip6]PACAP27[b]	3	8.36 ± 0.17	3	7.85 ± 0.15	3	7.64 ± 0.11
[Ala7]PACAP27[b]	3	7.78 ± 0.16*	3	7.51 ± 0.21*	3	5.80 ± 0.12***
[Ala22]PACAP27	3	6.94 ± 0.11**	4	7.42 ± 0.13**	3	<5***

[a]Negative log concentration producing 50% inhibition of specific binding of ^{125}I-Ac-PACAP27.
[b]Binding values for these analogs were reported elsewhere (24). Stastistical analysis was performed using a Student's t-test versus the value obtained with native PACAP isoforms ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$). Each replicate (n) was conducted on different cell passages.

Table 2. Agonist activity of PACAP isoforms and their related analogs.

Compound	$G\alpha_q$			$G\alpha_s$			β-arrestin1			β-arrestin2		
	n	pEC_{50}[a]	E_{max} (%)[b]	n	pEC_{50}[a]	E_{max} (%)[b]	n	pEC_{50}[a]	E_{max} (%)[b]	n	pEC_{50}[a]	E_{max} (%)[b]
PACAP38	4	7.03 ± 0.15	94 ± 7	10	7.58 ± 0.27	76 ± 7	6	6.60 ± 0.12	121 ± 8	8	6.80 ± 0.22	92 ± 10
[Ala^2]PACAP38	4	7.42 ± 0.18	95 ± 6	5	8.13 ± 0.31	103 ± 10*	3	6.71 ± 0.16	109 ± 8	3	6.92 ± 0.18	129 ± 11
[D-Pro^2]PACAP38	4	inactive		4	6.52 ± 0.42	59 ± 14	5	inactive		3	inactive	
[Hyp^2]PACAP38	4	7.45 ± 0.81	22 ± 6***	4	7.02 ± 0.34	120 ± 12**	3	6.76 ± 0.21	89 ± 9*	4	7.23 ± 0.41	91 ± 12
[Bip^6]PACAP38	4	7.41 ± 0.30	102 ± 10	5	8.09 ± 0.43	79 ± 10	5	7.44 ± 0.15***	137 ± 7	4	7.10 ± 0.21	148 ± 11**
[Ala^7]PACAP38	4	6.66 ± 0.32	61 ± 11*	4	8.01 ± 0.43	87 ± 9	5	7.13 ± 0.11**	147 ± 6*	3	6.67 ± 0.16	220 ± 15***
[Ala^{22}]PACAP38	4	6.32 ± 0.12**	124 ± 11	4	7.40 ± 0.27	114 ± 11*	3	6.44 ± 0.13	139 ± 11	4	6.58 ± 0.20	233 ± 22***
PACAP27	8	6.93 ± 0.09	106 ± 4	9	8.27 ± 0.20	91 ± 5	11	6.56 ± 0.07	118 ± 5	7	6.92 ± 0.10	101 ± 5
[Ala^2]PACAP27	4	6.93 ± 0.11	92 ± 5	4	8.49 ± 0.36	101 ± 9	7	6.48 ± 0.13	128 ± 10	3	7.28 ± 0.29	104 ± 11
[D-Pro^2]PACAP27	3	5.82 ± 0.45**	36 ± 13***	4	<5	44 ± 14**	3	inactive		3	5.67 ± 0.30***	57 ± 16**
[Hyp^2]PACAP27	3	6.75 ± 0.20	57 ± 7***	3	7.50 ± 0.22	175 ± 10***	7	6.64 ± 0.15	109 ± 9	4	7.43 ± 0.26	107 ± 8
[Bip^6]PACAP27	3	6.74 ± 0.20	86 ± 9*	3	7.53 ± 0.41	98 ± 11	4	6.49 ± 0.10	152 ± 9**	4	6.61 ± 0.14	118 ± 8
[Ala^7]PACAP27	3	6.26 ± 0.12**	92 ± 8	3	7.22 ± 0.38*	139 ± 15**	3	6.57 ± 0.16	124 ± 12	3	6.42 ± 0.11*	134 ± 8**
[Ala^{22}]PACAP27	3	6.38 ± 0.09**	113 ± 7	4	7.00 ± 0.23**	98 ± 10	7	6.23 ± 0.13*	133 ± 14	3	6.52 ± 0.16	114 ± 10

[a]Negative logarithm of the concentration producing 50% of maximal effect. [b]Maximum efficacy is expressed as a percentage of activation induced by the related endogenous PACAP. A decrease in BRET signal was observed at high concentrations of PACAP resulting in bell-shaped curves. This phenomenon has been previously described in the literature and is explained by receptor internalization (ref 23). Thereby, E_{max} of each ligand was normalized with the highest value obtained for PACAP27 or PACAP38. Statistical analysis was performed using a Student's t-test versus the value obtained with native PACAP38 (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Each replicate (n) was conducted on different cell passages.

Graphical abstract