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Design and *in vitro* characterization of PAC1/VPAC1-selective agonists with potent neuroprotective effects

Ngoc-Duc Doan [a,b], Steve Bourgault [a,b], Agnieszka Dejda [a,b], Myriam Létourneau [a,b], Michel Detheux [c], David Vaudry [b,d], Hubert Vaudry [b,d], David Chatenet [a,b], Alain Fournier [a,b,*]

[a] *Institut Armand-Frappier, Institut National de la Recherche Scientifique, Université du Québec, 531 boulevard des Prairies, Ville de Laval, Québec H7V 1B7, Canada*
[b] *Laboratoire International Associé Samuel de Champlain INSERM – INRS, France, Canada*
[c] *Euroscreen S.A., 47 Rue Adrienne Bolland, B6041 Gosselies, Belgium*
[d] *INSERM-U982, Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, IFRMP 23, Université de Rouen, 76821 Mont-Saint-Aignan, France*

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide that exerts a large array of actions in the central nervous system and periphery. Through the activation of PAC1 and VPAC1, PACAP is able to exert neuroprotective, as well as anti-inflammatory effects, two phenomena involved in the pathogenesis and the progression of neurodegenerative diseases. The aim of the current study was to provide insights into the molecular arrangement of the amino terminus of PACAP and to develop new potent and selective PAC1/VPAC1 agonists promoting neuronal survival. We have synthesized a series of PACAP derivatives and measured their binding affinity and their ability to induce intracellular calcium mobilization for each receptor, *i.e.* PAC1, VPAC1, and VPAC2. Ultimately, analogs with an improved pharmacological profile were evaluated in an *in vitro* model of neuronal loss. Results showed that introduction of a hydroxyproline or an alanine moiety, respectively, at position 2 or 7 generated derivatives without significant VPAC2 agonistic activity. Moreover, the structure–activity relationship study suggests the presence of common (Asx-turn like) and distinct (different N-capping type) secondary structures that might be responsible for receptor recognition, selectivity and activation. Finally, evaluation of the neuroprotective activity of [Ala^7]PACAP27 and [Hyp^2]PACAP27 demonstrated their ability to protect potently human dopaminergic SH-SY5Y neuroblasts against the toxicity of MPP^+, in pre- and co-treatment experiments. These new pharmacological and structural data should prove useful for the rational design of PACAP-derived compounds that could be putative therapeutic agents for the treatment of neurodegenerative diseases.

1. Introduction

Neurodegenerative disorders such as Alzheimer's disease (AD), the most common type of dementia, and Parkinson's disease (PD), the most frequent movement disorder, are morphologically characterized by progressive neuronal loss. At the moment, available drugs are unable to stop the progression of the disease and provide only modest benefit on cognitive, behavioral and functional symptoms. Recently, apoptosis, a specific form of gene-directed programmed cell death, and inflammation, through the action of cytotoxic factors (TNF-α and IL-1), have been implicated as a general mechanism in the degeneration of selective neuronal populations [1–3]. Neuroprotective treatments are nowadays at the forefront of neurodegenerative research, and several therapeutic agents, aiming at protecting neurons from cell death and reducing the progression of the disease, are currently under investigation or already clinically evaluated [4]. However, the blood–brain barrier (BBB), which represents a major obstacle for CNS drugs, has so far precluded the use of many of these compounds. Consequently, drug candidates exhibiting anti-apoptotic and anti-inflammatory properties that have also the ability to cross the BBB are of high interest. In this regard, the neuroprotective and neurotrophic pituitary adenylate cyclase-activating polypeptide (PACAP), with its unique ability to cross the BBB, could be a promising candidate to safely reverse or slow the course of disabling neurological illnesses.

PACAP, a neurohormone belonging to the VIP family, is able to interact with three distinct GPCRs, namely VPAC1 and VPAC2 that

Abbreviations: CNS, central nervous system; MPP^+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

* Corresponding author at: INRS – Institut Armand-Frappier, 531 boulevard des Prairies, Ville de Laval, Québec H7V 1B7, Canada. Tel.: +1 450 687 5010; fax: +1 450 686 5566.
E-mail addresses: alain.fournier@iaf.inrs.ca, alain.fournier@adm.inrs.ca (A. Fournier).

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recognize both PACAP and VIP with similar affinity and PAC1 that shows high affinity for PACAP and low affinity for VIP [5]. Consistent with its widespread distribution in the CNS and in most peripheral organs, PACAP, which exists in two isoforms (PACAP27 and PACAP38), participates in the regulation of numerous physiological processes [5]. In particular, PACAP exerts potent neuroprotective effects, through the activation of PAC1, by reducing apoptosis, both *in vivo* and *in vitro*, in many experimental models including Alzheimer's, Huntington's, and Parkinson's diseases as well as in cerebral ischemia, traumatic brain and spinal cord injuries [5–7]. Besides, PACAP is able to modulate the inflammatory response, associated with most of the neurodegenerative diseases, by inhibiting both chemokine production and NFkB binding through specific activation of VPAC1 receptors [8]. *In vivo* studies in knockout PACAP mice suggested its critical involvement in the carefully controlled immune response that is necessary for proper nerve regeneration after injury [9]. Meanwhile, avoiding the activation of the VPAC2 receptor, mostly involved in peripheral actions, could improve not only the effectiveness of the treatment but also minimize the putative side effects associated with its activation, such as vasodilation [10], insulin secretion and water retention [11].

Since the discovery of PACAP, structure–activity relationship (SAR) studies have been carried out to identify the molecular determinants responsible for the recognition and activation of PACAP-related receptors [7]. PACAP activates its receptors, belonging to the B1 subtype of GPCRs, following a two-step binding model in which the C-terminal segment of the ligand binds to the first extracellular domain, which then may position the amino-terminal portion of the peptide hormone in close proximity to the transmembrane regions of the receptor to initiate signalling. Accordingly, deletion in the C-terminal domain of PACAP reduces drastically the binding affinity without altering the propensity of the analogs to activate the receptor [12,13]. Conversely, SAR studies have indicated that the PACAP-associated biological activity and selectivity may be dependent on the structure adopted by the disordered N-terminal portion of the molecule [13,14]. In fact, N-terminal deletion, leading to a potent PAC1/VPAC2 antagonist, *i.e.* PACAP(6–38) [15], as well as chemical modifications in this N-terminal random coil region resulted in significant changes in biological activity and receptor specificity [16,17]. As a matter of fact, when bound to the PAC1 receptor, the segment 3–7 of PACAP displays an unusual coil made up of consecutive type II′ (residues 3–6) and type I (residues 4–7) β-turns [18]. Although the structural requirements for high affinity interaction of PACAP and VIP with their receptors have been extensively studied, it is still unclear which amino acids or secondary structure distinctive of VIP and PACAP confer receptor selectivity [7].

Therefore, the impact of various chemical and structural modifications at the N-terminus of the neuropeptide PACAP was investigated with an emphasis on the development of selective PAC1/VPAC1 analogs. Our results suggest some important differences not reported previously in the key pharmacophores responsible for the specificity of PACAP toward its different receptors that need to be considered. Moreover, we identified two PAC1/VPAC1 selective agonists and demonstrated their ability to protect human neuronal SH-SY5Y cells against the toxicity of MPP^+.

2. Experimental procedures

2.1. Materials and peptide synthesis

$Na^{125}I$ was purchased from Perkin Elmer (Montreal, QC, CAN). Resins, amino acid derivatives and coupling reagents used to produce PACAP analogs were from ChemImpex International (Wood Dale, IL, USA). All other chemicals and cell culture media were from Sigma Aldrich (Mississauga, ON, CAN) and Fisher Scientific (Nepean, ON, CAN) except Coelenterazine H from Promega (Madison, WI, USA), and digitonin from Calbiochem (La Jolla, CA, USA).

The synthesis and characterization of PACAP analogs described herein were previously reported [14]. However, it is worth to mention that the data for PAC1 presented in this paper were obtained with CHO cells stably co-expressing PAC1 receptors and a mitochondrial apo-aequorin protein. All peptides were synthesized on Rink-amide AM resin using the standard Fmoc/tBu chemistry. After acidic cleavage and purification, all peptides were characterized by mass spectrometry and RP-HPLC. Fractions containing the desired product were pooled, lyophilized and kept at −20 °C until use (purity > 95%).

2.2. Cell culture

Transfected CHO cells (Perkin Elmer, AequoScreen) co-expressing the human PAC1, VPAC1 or VPAC2 receptor and a mitochondrial apo-aequorin protein were maintained in Ham-F12 medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 UI/mL each of penicillin and streptomycin, 400 μg/mL G418 and 250 μg/mL zeocin (Invitrogen, Burlington, ON, CAN). These cell lines were used in binding and calcium mobilization assays.

SH-SY5Y cells, a neuroblastic subclone expressing constitutively PAC1 and VPAC2 receptors [19], were cultured in a 1:1 mixture consisting of Ham's F12 Nutrient mixture and Minimum Essential Eagle medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 UI/mL each of penicillin and streptomycin, and 10% FBS. The loss of neuronal characteristics has been described with increasing passage numbers. Therefore, the presence of specific characteristics such as neuronal marker (GAP-43) was verified regularly by Western blot analysis.

All cell lines were maintained as a monolayer at 37 °C in a humidified atmosphere of 5% CO_2/95% air and passages were performed by trypsinization when confluence was reached.

2.3. Binding assay

Acetylated PACAP27 was radioiodinated using the chloramine-T technique [20] and purified on a Sep-Pak C18 cartridge (Waters, Milford, MA, USA). CHO cells expressing one of the PACAP-related receptors were seeded at a density of 125,000 cells per well in 24-well plates. After 24 h, the culture medium was removed, and cells were first incubated at room temperature for 10 min in binding buffer (0.1% BSA, 25 mM Tris–HCl, 25 mM $MgCl_2$, and 5 μg/L bacitracin, pH 7.4) and then exposed to increasing peptide concentrations in the presence of 0.05 nM ^{125}I-Ac-PACAP27. After 2 h at room temperature, cells were washed twice with binding buffer, lysed (0.1 M NaOH), and the cell-bound radioactivity was quantified using a γ-counter (1470 Automatic Gamma Counter, Perkin Elmer). Results were expressed as the percentage of the specific binding of ^{125}I-Ac-PACAP27 obtained in the absence of competitive ligands. Non-specific binding was determined in the presence of 10 μM PACAP38 and averaged at 5–10% of total binding.

2.4. In vitro calcium mobilization assay

Cells in mid-log phase, grown in media without antibiotics 18 h prior to the test, were detached with PBS-EDTA, recovered by centrifugation and re-suspended at 5×10^6 cells/mL in DMEM/F12 supplemented with 10 mM HEPES and 0.1% BSA. The resulting suspension was placed in a beaker wrapped in aluminum foil and,

after addition of Coelenterazine H at a final concentration of 5 μM, was gently agitated with a magnetic stirrer for 4 h at room temperature. Cells were then diluted 10-fold and incubated another hour under the same condition. In the meantime, 50 μL of peptide ligands, at final concentrations varying from 10^{-12} M to 10^{-5} M, were distributed in a 96-well plate. Finally, cells were equally distributed (25,000 cells per well) using a MLX Microtiter® Plate Luminometer (Dynex Technologies Inc.) and the emitted light was recorded over a 20 s period. Digitonin, at a final concentration of 25 μM, was used as a positive control to ensure the formation of the aequorin complex. Integrated values of emitted light for each dose were compared with that of PACAP38 (10^{-5} M) and the resulting percentages were used to construct the concentration–response curve related to each analog.

2.5. Cell survival

SH-SY5Y cells were seeded into 96-well plates at a density of 3×10^4 cells/well and incubated 48 h to ensure cell adhesion. Cells were first treated with various concentrations of MPP^+ in order to identify the dose generating 50% cell death after 24 h. In pre-treatment experiments, peptides (100 nM final concentration) were added into the wells containing a serum free medium and incubated for 4 h prior to MPP^+ treatment (2 mM final concentration). In co-treatment experiments, both peptides (100 nM) and MPP^+ (2 mM) were added simultaneously in a serum free medium. Cell viability was assessed 24 h after MPP^+ treatments by means of a MTT assay kit (Promega, Madison, WI, USA) using a microplate reader (MTX^{TC} Revelation, Dynex Tech., VA, USA) to determine the optical density related to the conversion of MTT into purple-colored water-soluble formazan. Positive control (MPP^+-treated cells) and negative control (non-treated cells) were included in each experiment.

2.6. DAPI staining

SH-SY5Y cells, plated at a density of 1×10^5 cells per well, were cultured for 48 h on glass coverslips coated with poly-L-lysine. Following medium removal, serum-free medium containing peptide (100 nM) was added and incubated for 4 h before MPP^+ treatment (0.5 mM; 24 h). Then, cells were washed twice with PBS, fixed with acetone:methanol (1:1) mixture for 15 min at −20 °C, and finally incubated at room temperature with DAPI (1 μg/mL, 10 min). The chromatin condensation was visualized by epifluorescence using the ImagePro Plus computer software (Media Cybernetics, Silver Spring, MD, USA).

2.7. DNA content analysis

Evaluation of nuclear morphological events following MPP^+ exposure with or without PACAP analogs was performed by the analysis of internucleosomal DNA fragmentation using cell cycle analysis for G1/G0 subdiploid peak quantification. The presence of cells with DNA stainability lower than that of G_1-cells was investigated by propidium iodide staining and flow cytometry. SH-SY5Y cells were seeded at a density of 2.5×10^5 cells per well in 12-well plates. After 72 h of incubation (~80% confluence), medium was changed for serum-free medium containing peptide (10^{-7} M) and cells were incubated for 4 h before being treated with MPP^+ (0.5 mM) for another 24 h. Harvested cells were resuspended in PBS and fixed with acetone/methanol (1:1) at −20 °C for 1 h. Fixed cells were washed twice with PBS and were finally resuspended at 1×10^6 cells/mL in Krishan Buffer (0.1% sodium citrate, 0.3% NP-40, 20 μg/mL RNase and 50 μg/mL PI). Cells were then incubated at 37 °C for 1 h, and analyzed by flow cytometry on a FACScan® (Becton Dickinson, Oakville, ON, CAN). The appearance of sub-diploid DNA peak represents a specific marker of apoptosis. Analyses were performed using live gates to discriminate between doublets and cells exhibiting reduced DNA content corresponding to the sub G1/G0 diploid peak, which were defined as apoptotic cells [21].

2.8. Statistical analysis

Binding and functional experiments (calcium and cell survival assays) were performed at least in quadruplicate and data, expressed as mean ± SEM, were analyzed with the Prism software (Graphpad Software, San Diego, CA). The pIC_{50}, pEC_{50}, and maximal efficacy values were determined from the concentration–response curves using a sigmoidal dose–response fit with variable slope. Statistical comparisons were performed by ANOVA using a Dunnett's multiple comparison test, and differences were considered significant where $P < 0.05$.

3. Results

3.1. Pharmacological evaluation of PACAP-substituted analogs

Binding affinity and biological activity of PACAP, VIP and PACAP analogs were measured using CHO cell lines co-expressing the human PAC1, VPAC1 or VPAC2 receptors and a mitochondrial aequorin protein. Data are collected in Tables 1 and 2 and depicted in Figs. 1 and 2. In our assays, both PACAP isoforms (PACAP27 and PACAP38) exhibited similar affinities and potencies for the different receptors (analogs 1 and 2). As expected, VIP, which is able to specifically bind and activate VPAC1 and VPAC2 receptors in a concentration-dependent manner, is a weak agonist of the PAC1 receptor (analog 3).

3.1.1. Ala-scan

The contribution of each residue side-chain in the biological activity of the peptide was investigated by systematic substitution of each amino acid of PACAP27 with an Ala moiety. Pharmacological profiles obtained for Ala-substituted PACAP27 analogs indicated that the imidazole ring of His^1, the carboxylic group of Asp^3 and the phenyl moiety of Phe^6 are crucial for binding affinity and biological activity of the peptide to all three receptor types, whereas replacement of Gly^4 with Ala had no significant effect (analogs 4, 6, 7, 9). Substitution of Ser^2 with Ala did not affect the properties of the PACAP27 derivative toward PAC1 and VPAC1 but slightly decreased its affinity and potency toward VPAC2 (analog 5). A moderate loss of binding affinity and biological activity for all three receptors was observed following the replacement of Ile^5 with Ala (analog 8). Interestingly, we observed that [Ala^7]PACAP27 is characterized by a slight reduction of its affinity toward PAC1 ($pIC_{50} = 7.78 \pm 0.16$) and VPAC1 ($pIC_{50} = 7.51 \pm 0.21$), but a significant loss toward VPAC2 ($pIC_{50} = 5.80 \pm 0.12$) (analog 10). Although this analog behaves as a full agonist for PAC1 and VPAC1 receptors, [Ala^7]PACAP27 had almost no VPAC2-related activity at 10^{-6} M.

3.1.2. D-scan and N-methyl scan

In order to explore the importance of the orientation of the amino acid side-chains in the biological activity of the peptide, a series of D-isomer-substituted peptides was synthesized (analogs 11–16). Similarly, we introduced concomitantly N-methyl substitution on each N-terminal α-amino acid (analogs 17–22) in order to restrict the amide bond, eliminate hydrogen-bond donating ability, affect backbone torsional angles and allow the formation of a *cis* peptide bond. Indeed, both modifications (D-scan and N-methyl scan) can be used to evaluate to which extent a backbone conformational restriction affects biological activity. Substitution of His^1, Ser^2, and Asp^3 with their corresponding D-counterparts resulted in a slight increase of selectivity toward PAC1 receptor without affecting profoundly the biological activity of the peptide

Table 1
Binding affinity of PACAP analogs.

No	Compounds	MW (Da)		Binding affinity (pIC_{50})[a]		
		(Observed)	(Calculated)	PAC1	VPAC1	VPAC2
1	PACAP27	3147.9	3147.6	8.34 ± 0.07	8.23 ± 0.07	7.99 ± 0.16
2	PACAP38	4534.5	4535.3	8.21 ± 0.08	8.17 ± 0.09	7.76 ± 0.15
3	VIP	3326.1	3325.8	5.47 ± 0.12	8.37 ± 0.08	7.88 ± 0.11
4	[Ala^1]PACAP27	3081.6	3081.5	6.42 ± 0.19	6.41 ± 0.10	6.17 ± 0.09
5	[Ala^2]PACAP27	3132.0	3131.6	8.33 ± 0.12	8.15 ± 0.07	7.16 ± 0.11
6	[Ala^3]PACAP27	3105.4	3103.6	5.99 ± 0.12	5.85 ± 0.12	5.79 ± 0.13
7	[Ala^4]PACAP27	3161.6	3161.6	8.03 ± 0.16	8.53 ± 0.09	7.76 ± 0.09
8	[Ala^5]PACAP27	3106.3	3105.6	7.30 ± 0.17	7.28 ± 0.10	6.84 ± 0.12
9	[Ala^6]PACAP27	3077.7	3076.5	5.88 ± 0.13	5.84 ± 0.35	5.83 ± 0.15
10	[Ala^7]PACAP27	3117.8	3117.6	7.78 ± 0.16	7.51 ± 0.21	5.80 ± 0.12
11	[D-His^1]PACAP27	3147.5	3147.6	8.19 ± 0.15	7.35 ± 0.11	7.23 ± 0.07
12	[D-Ser^2]PACAP27	3147.9	3147.6	8.11 ± 0.13	7.47 ± 0.11	7.37 ± 0.11
13	[D-Asp^3]PACAP27	3148.0	3147.6	8.04 ± 0.20	7.62 ± 0.09	7.17 ± 0.08
14	[D-Ile^5]PACAP27	3148.7	3147.6	6.37 ± 0.11	7.19 ± 0.17	6.85 ± 0.08
15	[D-Phe^6]PACAP27	3148.1	3147.6	5.64 ± 0.13	5.72 ± 0.21	5.67 ± 0.16
16	[D-Thr^7]PACAP27	3147.7	3147.6	5.85 ± 0.19	5.15 ± 0.36	6.17 ± 0.06
17	[N-Me-Ser^2]PACAP27	3162.7	3161.6	7.82 ± 0.12	7.08 ± 0.06	7.14 ± 0.10
18	[N-Me-Asp^3]PACAP27	3161.6	3161.6	7.01 ± 0.14	7.20 ± 0.17	7.09 ± 0.08
19	[Sar^4]PACAP27	3161.9	3161.6	5.98 ± 0.11	<5	6.01 ± 0.24
20	[N-Me-Ile^5]PACAP27	3162.0	3161.6	5.62 ± 0.18	<5	5.72 ± 0.34
21	[N-Me-Phe^6]PACAP27	3161.3	3161.6	5.50 ± 0.10	5.21 ± 0.33	<5
22	[N-Me-Thr^7]PACAP27	3162.9	3161.6	5.69 ± 0.14	<5	<5
23	[Pro^2]PACAP27	3158.2	3157.7	8.11 ± 0.21	7.79 ± 0.13	6.67 ± 0.11
24	[D-Pro^2]PACAP27	3157.9	3157.7	6.33 ± 0.14	6.04 ± 0.13	5.65 ± 0.10
25	[Hyp^2]PACAP27	3174.6	3173.7	8.07 ± 0.18	7.40 ± 0.11	6.89 ± 0.07
26	[Aib^2]PACAP27	3146.8	3146.6	8.02 ± 0.13	7.68 ± 0.17	7.66 ± 0.11
27	[Aib^4]PACAP27	3176.3	3175.6	7.12 ± 0.12	7.64 ± 0.11	7.48 ± 0.09
28	[Pro^4, Gly^5]PACAP27	3132.1	3131.5	5.42 ± 0.16	<5	<5
29	[D-Pro^4, Gly^5]PACAP27	3131.9	3131.5	5.56 ± 0.10	<5	<5
30	[γ-lactam^{4,5}]PACAP27	3175.7	3174.9	5.72 ± 0.12	<5	<5
31	[Ind^6]PACAP27	3147.0	3145.6	5.71 ± 0.16	<5	<5
32	[Tic^6]PACAP27	3157.6	3157.6	5.66 ± 0.12	5.67 ± 0.09	<5
33	[Disc^6]PACAP27	3158.5	3157.6	5.45 ± 0.13	<5	<5
34	[Tiq^6]PACAP27	3145.9	3145.6	5.59 ± 0.16	<5	<5
35	[Tyr^6]PACAP27	3164.4	3163.6	8.10 ± 0.18	7.34 ± 0.15	7.27 ± 0.27
36	[Cha^6]PACAP27	3153.7	3153.6	7.97 ± 0.14	7.85 ± 0.12	7.02 ± 0.11
37	[Bip^6]PACAP27	3224.8	3224.6	8.36 ± 0.17	7.85 ± 0.15	7.64 ± 0.11
38	[Nal^6]PACAP27	3198.2	3197.6	8.19 ± 0.16	7.30 ± 0.14	7.74 ± 0.14

[a] Negative log concentration producing 50% of inhibition of specific binding of ^{125}I-Ac-PACAP27.

(analogs 11–13). However, replacement of Ile with D-Ile at position 5 resulted in a slightly improved selectivity toward VPAC1 for this PACAP derivative (analog 14) with a concomitant loss in biological activity for PAC1 and VPAC2 receptors. Incorporation of D-amino acids at position 6 or 7 was deleterious for all three receptor types (analogs 15–16).

Such a drastic loss for all three receptors was also observed with the substitution of residues Gly^4, Ile^5, Phe^6 or Thr^7 by their N-methylated counterparts (analogs 19–22). However, even though it altered the ability of the peptide to induce calcium mobilization, no dramatic changes in the binding properties were noticed following the replacement of Ser^2 or Asp^3 (analogs 17–18). Indeed, [N-Me-Ser^2]PACAP27 was found to be a partial agonist for VPAC2 receptor (pEC_{50} = 6.42 ± 0.17 and E_{max} = 71 ± 6%).

3.1.3. Conformational constraints

3.1.3.1. Position 2. Results obtained from D-scan and N-methyl scan suggested that the His^1-Ser^2-Asp^3 sequence may play a crucial role in the interaction and activation of PACAP/VIP receptors by adopting a specific conformation. As such, modifications at position 2 were achieved in order to get further information about the bioactive conformation of the N-terminal segment. Incorporation of structural restrictions such as turn inducers, *i.e.* Pro or Hyp, led to a weak loss of VPAC2 binding properties while keeping a high affinity and biological activity for PAC1 and VPAC1 (analogs 23, 25). However, these analogs, [Pro^2]PACAP27 and [Hyp^2]PACAP27, produced only a weak activation of VPAC2 receptors in our intracellular calcium mobilization assay ([Hyp^2]-PACAP27, E_{max} = 23 ± 5% and [Pro^2]PACAP27, E_{max} = 87 ± 7% at 10^{-5} M). The substitution of Ser^2 with D-Pro (analog 24) reduced dramatically the affinity of the analog for the three receptors with $pIC_{50} > 6$ (Table 1) and gave rise to an almost inactive compound, [D-Pro^2]PACAP27 being inactive up to 10^{-6} M on all receptors (Table 2). Interestingly, introduction of an Aib moiety at this position did not affect the pharmacological profile of the peptide (analog 26).

3.1.3.2. Position 4. Recently, the NMR analysis of PACAP21 bound to PAC1 receptor has shown that the segment 3–7 of the peptide adopts a unique bioactive conformation [18]. Several modifications, aiming at replacing Gly^4 or the Gly^4-Ile^5 dipeptide, were incorporated in order to stabilize the putative β-turn. Replacement of the achiral Gly moiety by the quaternary achiral amino acid Aib (analog 27) induced a significant reduction of affinity for PAC1 and a moderate decrease on VPAC receptors. Moreover, introduction of a conformational constraint at positions 4 and 5, such as the Pro-Gly and D-Pro-Gly dipeptides, or a Gly-Val Freidinger's γ-lactam, resulted in a drastic loss of binding affinity and biological activity for the three receptor subtypes (analogs 28–30).

3.1.3.3. Position 6. Substitution of the Phe^6 residue, known to be potentially involved in the N-capping phenomenon, with conformationally constrained amino acids produced analogs completely devoid of any biological activity (analogs 31–34).

Table 2
Agonist activity of PACAP analogs.

No	Compounds	PAC1		VPAC1		VPAC2	
		pEC_{50}[a]	E_{max} (%)[b]	pEC_{50}[a]	E_{max} (%)[b]	pEC_{50}[a]	E_{max} (%)[b]
1	PACAP27	9.21 ± 0.09	99 ± 2	8.13 ± 0.08	106 ± 4	7.60 ± 0.05	104 ± 2
2	PACAP38	8.34 ± 0.09	100 ± 4	7.81 ± 0.08	101 ± 3	7.59 ± 0.07	102 ± 2
3	VIP	5.42 ± 0.12	82 ± 11	7.44 ± 0.09	102 ± 3	7.70 ± 0.10	106 ± 2
4	[Ala^1]PACAP27	6.39 ± 0.10	98 ± 5	6.41 ± 0.08	97 ± 4	<5	48 ± 6[c]
5	[Ala^2]PACAP27	8.42 ± 0.11	100 ± 2	7.60 ± 0.11	10 ± 4	6.91 ± 0.07	109 ± 3
6	[Ala^3]PACAP27	<5	71 ± 7[c]	<5	70 ± 5 [c]	Inactive[d]	
7	[Ala^4]PACAP27	8.76 ± 0.05	100 ± 1	8.11 ± 0.06	102 ± 2	7.72 ± 0.07	108 ± 3
8	[Ala^5]PACAP27	7.74 ± 0.06	104 ± 2	7.40 ± 0.09	103 ± 3	6.48 ± 0.09	99 ± 4
9	[Ala^6]PACAP27	6.12 ± 0.08	84 ± 7	<5	35 ± 6 [c]	<5	41 ± 6 [c]
10	[Ala^7]PACAP27	7.57 ± 0.09	102 ± 2	7.54 ± 0.06	105 ± 5	<5	41 ± 5 [c]
11	[D-His^1]PACAP27	7.78 ± 0.15	82 ± 4	6.40 ± 0.13	99 ± 3	6.46 ± 0.13	95 ± 6
12	[D-Ser^2]PACAP27	7.35 ± 0.12	107 ± 5	7.56 ± 0.07	101 ± 2	6.64 ± 0.09	97 ± 4
13	[D-Asp^3]PACAP27	8.06 ± 0.08	99 ± 3	7.39 ± 0.10	112 ± 4	6.78 ± 0.09	109 ± 4
14	[D-Ile^5]PACAP27	6.62 ± 0.13	98 ± 5	7.24 ± 0.07	98 ± 3	6.13 ± 0.11	89 ± 5
15	[D-Phe^6]PACAP27	<5	52 ± 1 [c]	Inactive[d]		Inactive[d]	
16	[D-Thr^7]PACAP27	6.02 ± 0.10	72 ± 1	6.26 ± 0.22	79 ± 9	Inactive[d]	
17	[N-Me-Ser^2]PACAP27	7.60 ± 0.06	102 ± 2	7.35 ± 0.1	106 ± 5	6.42 ± 0.17	71 ± 6
18	[N-Me-Asp^3]PACAP27	6.66 ± 0.06	102 ± 3	7.11 ± 0.07	109 ± 3	6.38 ± 0.13	109 ± 7
19	[Sar^4]PACAP27	<5	55 ± 14 [c]	Inactive[d]		Inactive[d]	
20	[N-Me-Ile^5]PACAP27	<5	34 ± 11 [c]	Inactive[d]		Inactive[d]	
21	[N-Me-Phe^6]PACAP27	<5	62 ± 3 [c]	Inactive[d]		Inactive[d]	
22	[N-Me-Thr^7]PACAP27	5.67 ± 0.07	79 ± 2	<5	36 ± 4 [c]	Inactive[d]	
23	[Pro^2]PACAP27	8.47 ± 0.06	100 ± 2	8.05 ± 0.09	98 ± 3	<5	87 ± 7 [c]
24	[D-Pro^2]PACAP27	<5	75 ± 17 [c]	Inactive[d]		Inactive[d]	
25	[Hyp^2]PACAP27	8.21 ± 0.05	96 ± 2	7.03 ± 0.09	97 ± 3	<5	23 ± 5 [c]
26	[Aib^2]PACAP27	8.36 ± 0.12	98 ± 3	7.76 ± 0.05	106 ± 2	7.14 ± 0.07	111 ± 3
27	[Aib^4]PACAP27	7.50 ± 0.08	101 ± 3	7.53 ± 0.07	106 ± 2	7.15 ± 0.13	88 ± 5
28	[Pro^4, Gly^5]PACAP27	<5	57 ± 4[c]	Inactive[d]		Inactive[d]	
29	[D-Pro^4, Gly^5]PACAP27	<5	34 ± 5[c]	Inactive[d]		Inactive[d]	
30	[γ-lactam^{4,5}]PACAP27	5.77 ± 0.07	85 ± 7	Inactive[d]		Inactive[d]	
31	[Ind^6]PACAP27	Inactive[d]		Inactive[d]		Inactive[d]	
32	[Tic^6]PACAP27	<5	61 ± 5[c]	Inactive[d]		Inactive[d]	
33	[Disc^6]PACAP27	Inactive[d]		Inactive[d]		Inactive[d]	
34	[Tiq^6]PACAP27	Inactive[d]		Inactive[d]		Inactive[d]	
35	[Tyr^6]PACAP27	8.72 ± 0.14	97 ± 3	7.67 ± 0.06	99 ± 2	6.42 ± 0.10	109 ± 6
36	[Cha^6]PACAP27	7.71 ± 0.07	102 ± 3	7.19 ± 0.12	106 ± 5	6.43 ± 0.11	96 ± 5
37	[Bip^6]PACAP27	9.23 ± 0.16	100 ± 4	7.33 ± 0.09	104 ± 4	7.09 ± 0.06	104 ± 3
38	[Nal^6]PACAP27	8.70 ± 0.15	104 ± 4	7.53 ± 0.08	105 ± 3	6.69 ± 0.11	96 ± 5

[a] Negative log concentration producing 50% of the maximum effect.
[b] Percentage of efficacy as compared to the maximal value obtained with PACAP38.
[c] Maximal value obtained at 10^{-5} M.
[d] Inactive at 10^{-6} M.

Modulation of the hydrophobic and/or aromatic characteristics of the side-chain, *i.e.* with the introduction of cyclohexylalanine (Cha), 4,4′-biphenylalanine (Bip), 1-naphthylalanine (Nal), and tyrosine moieties (analogs 35–38), did not dramatically alter the binding affinity and potency of the peptide toward PAC1 receptors but influenced to some extent the specificity of the analogs for the different receptors.

3.2. Protective effect of PACAP derivatives on dopaminergic neurons

3.2.1. MTT assay

Both PACAP isoforms as well as [Ala^7]PACAP27 and [Hyp^2]PACAP27 (both presenting an improved selectivity toward PAC1 and VPAC1) were evaluated in an *in vitro* model of Parkinson's disease for their effectiveness in protecting dopaminergic neurons (SH-SY5Y) against MPP^+, a dopaminergic neurotoxin. Results showed that 2 mM MPP^+ is required to produce 50% cells death after a 24 h treatment (Fig. 3A). Pre-treatment with PACAP38, PACAP27 or their analogs 4 h prior to the MPP^+ treatment (2 mM) was able to produce a significant protection of the cells against the MPP^+ insult. As a matter of fact, both endogenous isoforms increased cell viability up to 82 ± 10% and 70 ± 5%, respectively (Fig. 3B). Comparable neuroprotective effect was observed with [Ala^7]PACAP27 and [Hyp^2]PACAP27. Similarly, co-treatment experiments resulted in an increase of cell viability and demonstrated the effectiveness of PACAP isoforms and analogs to protect significantly dopaminergic neuroblastoma SH-SY5Y cells against the toxicity of MPP^+ (Fig. 3C). For instance, all compounds were able to bring the cell viability up to 60% ($P < 0.05$).

3.2.2. DAPI staining and DNA content analysis

Chromatin condensation, characteristic of apoptotic events [22], as well as an increasing percentage of apoptotic cells (27 ± 3% versus 4 ± 2% for the control) were clearly observed in samples treated with MPP^+ (Fig. 4A and B). Following pre-treatment with PACAP isoforms, a significant reduction of apoptotic cells (14 ± 2% and 13 ± 4%, respectively) and chromatin condensation was observed (Fig. 4A and B). Moreover, these effects were not observed with VIP and were blocked following the concomitant treatment of the cells with PACAP38 (10^{-7} M) and a PAC1 antagonist (PACAP(6–38); 10^{-6} M). Finally, [Ala^7]PACAP27 and [Hyp^2]PACAP27, two PAC1/VPAC1 selective agonists, were able, like PACAP, to reduce by almost 50% the percentage of apoptotic cells and to prevent chromatin condensation following MPP^+ treatment (Fig. 4A and B).

4. Discussion

The consensus two-step model for ligand-binding and signalling of type B1 GPCRs [23] proposes that the C-terminal segment of the ligand binds to the first extracellular domain of the receptor,

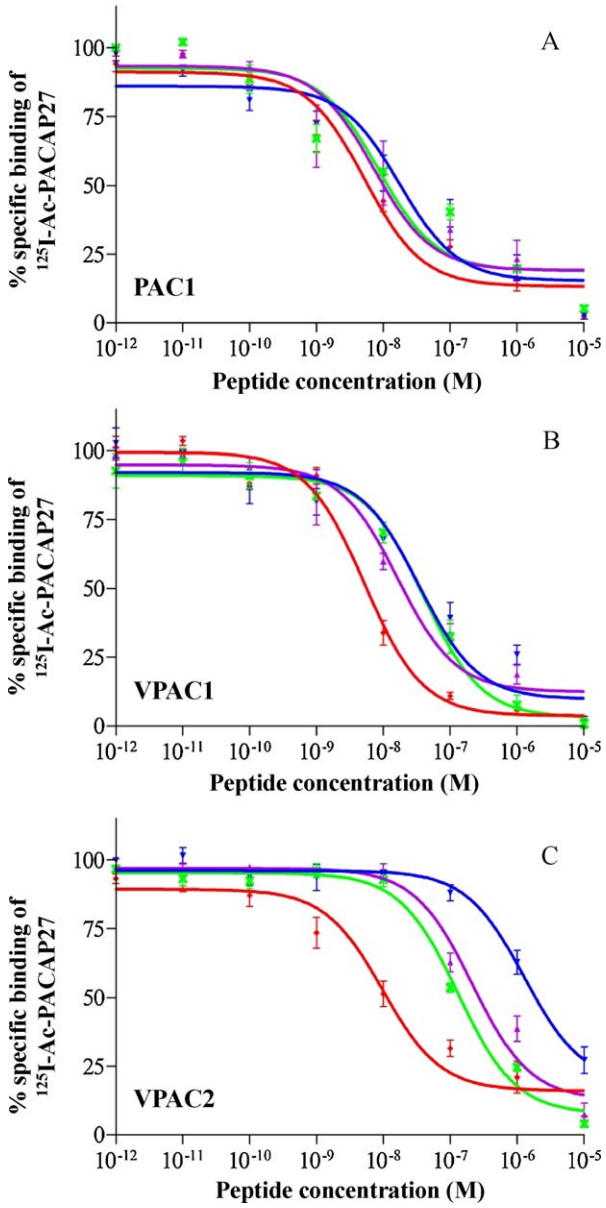

Fig. 1. Inhibition of ^{125}I-Ac-PACAP27 binding to CHO-transfected cells expressing the PAC1 (A), VPAC1 (B) and VPAC2 (C) receptor with increasing concentrations of PACAP27 (♦), [Ala^7]PACAP27 (▼), [Hyp^2]PACAP27 (×), or [Pro^2]PACAP27 (▲). Data are expressed as a percentage of the specific binding of ^{125}I-Ac-PACAP27 in the absence of the competitive ligands. Data represent the mean ± S.E.M. of at least 5 independent assays performed in duplicate.

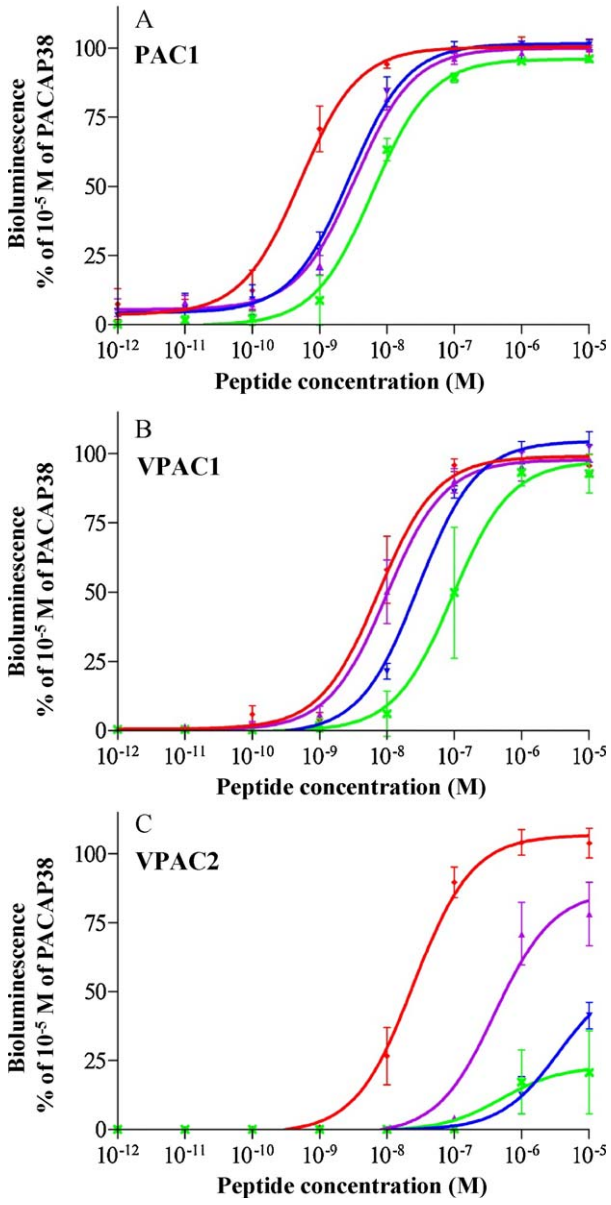

Fig. 2. Effect of PACAP27 (♦), [Ala^7]PACAP27 (▼), [Hyp^2]PACAP27 (×), or [Pro^2]PACAP27 (▲) on Ca^{2+} mobilization using CHO cells co-expressing human PAC1 (A), VPAC1 (B) or VPAC2 (C) receptors and a mitochondrial apo-aequorin protein. Data are expressed as a percentage of the response (bioluminescence) obtained with 10^{-5} M PACAP38 added to the same 96-well culture plate. Data represent the mean ± S.E.M. of at least 4 independent assays performed in duplicate.

which then may position the amino-terminal portion of the peptide hormone in close proximity to the external regions of the receptor to initiate signalling. Thus, specific spatial positioning of N-terminal amino acid side-chains is likely to govern the pharmacological specificity of each class B GPCR ligand. Therefore, the effects of chemical modifications within the N-terminal domain in terms of receptor selectivity and biological activity were assessed and our results outline specific structures enabling receptors of the VIP/PACAP family to discriminate between ligands.

4.1. Residues 1–3: Asx-turn like structure

Sequential replacement of N-terminal amino acids with alanine revealed the critical role of the side-chain of residue His^1 and Asp^3 in the recognition and activation processes to all receptors. Supporting the importance of the His residue, it was shown that its deletion or its replacement with Phe or 3-Me-His residues produced a drastic loss of binding affinity and biological activity [13,24]. Interestingly, methylation at position 1 of this imidazole ring did not affect the potency of PACAP analogs suggesting a distinct contribution of the two nitrogen atoms of the His side-chain [14]. In addition, it was previously reported that the deprotonated state of the imidazole ring was a major determinant for an optimal binding of PACAP [13]. Such a critical role was also described for VIP, in which the replacement of His with Ala abolished the ability of the corresponding analog to bind to both VPAC receptors, without an apparent change in its overall conformation [25]. Altogether, these results suggest that this residue is likely to participate in the formation of a hydrogen bond before (as for PACAP) or upon receptor binding (as for VIP). The importance of the carboxylic group in position 3 was also highlighted in the literature since the replacement of Asp^3 with

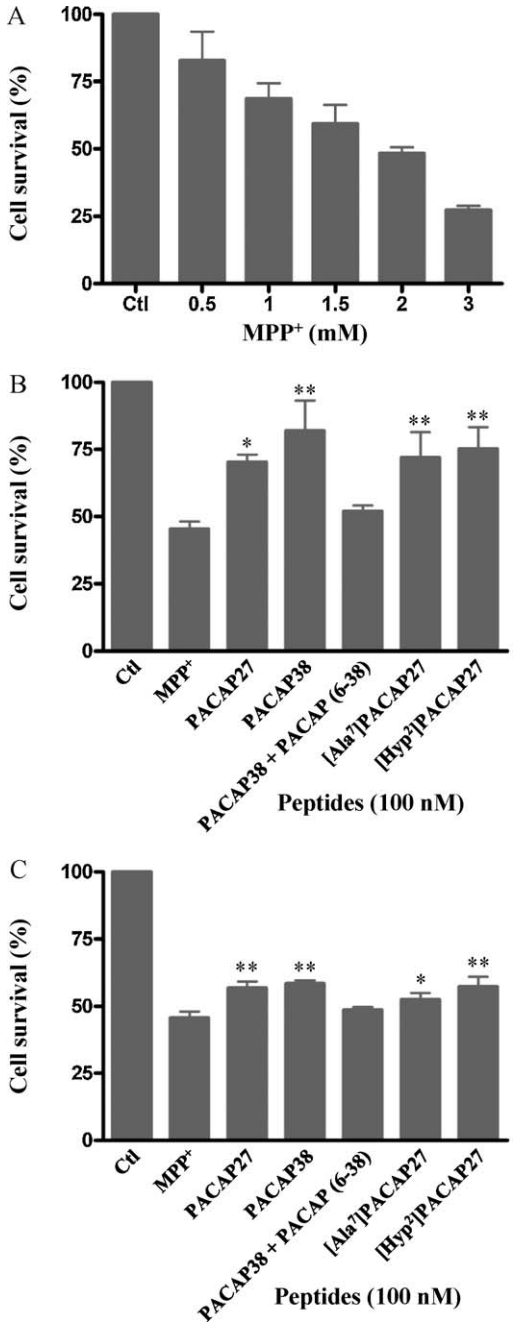

Fig. 3. Neuroprotective effect of PACAP and its analogs in an *in vitro* model of Parkinson's disease. (A) Effect of increasing concentration of MPP^+ on survival of SH-SY5Y cells. (B) Effect of MPP^+ (2 mM) following a 4 h pre-treatment with PACAP or its analogs (100 nM) on SH-SY5Y cell survival. (C) Effect of co-treatment with PACAP or its analogs (100 nM) and MPP^+ (2 mM) on SH-SY5Y cell survival. Each value represents the mean ± S.E.M. of at least 4 independent assays performed in octuplicate. $^*P \leq 0.05$; $^{**}P \leq 0.01$ versus MPP^+-treated cells.

Asn or Lys abrogated the ability of PACAP analogs to bind and activate their cognate receptors [14,25]. Similar behavior was also observed with VIP when Asp was substituted with alanine but was mostly associated with structural changes [25]. In both native peptides (PACAP and VIP), this residue seems to be involved in a direct and mandatory interaction with the receptors. Conversely, substitutions of Ser^2 with Ala, Pro or Hyp residues had no real effect on the binding affinity for PAC1/VPAC1 receptors, while VPAC2 binding was affected. These results suggested that the hydroxyl group located on the residue side-chain is not involved in the recognition process. Indeed, the replacement of Ser^2 with Pro or hydroxyproline (Hyp) gave rise to weak agonists for VPAC2 receptors. NMR studies revealed the presence, in [Pro^2]PACAP27, of a well defined structure, possibly an Asx-turn-like motif (Fig. 5A), that might reflect the bioactive conformation [14]. Such particular turns are similar to H-bonded β-turns since the side-chain of residue (*i*) mimics the residue (*i*) main chain of β-turns. A report demonstrating the ability of the tripeptide His-Pro-X to adopt a defined, compact structure that resembles the Asx-Pro-turn in both its crystalline form and in organic solvent supported the existence of such a structure in PACAP [26]. Moreover, substitution of Gly^4, the residue (*i* + 3) of the postulated Asx-turn, with Sar led to a PACAP analog unable to activate its receptors. These results support the existence of an Asx-turn-like structure. Moreover, the sequence homology existing between PACAP and VIP suggests that this conformation would not only be important for a proper PAC1 interaction but might also be necessary for the binding and activation of VPAC receptors.

4.2. Residues 4–5: encrypted receptor selective dipeptide

As previously demonstrated in protein structures, Asx or Asx-Pro turns are often associated with a regular β-turn [27]. In this regard, the unusual β-coil, observed by Inooka et al. [18], could represent the second structural feature. Swapping of residues at positions 4 and 5, between PACAP27 (Gly-Ile) and VIP (Ala-Val), induced a conformational conversion leading to an inverse impact on biological activity for these analogs toward PAC1 [17]. Supporting this concept, the replacement of the Gly residue with an Ala moiety induced a reduction of binding toward PAC1 but increased the binding for VPAC1. Similarly, replacement of Ile^5 with a less hydrophobic residue reduced its binding for the receptors. These results support the idea that intrinsic characteristics of Gly (achiral; turn inducer) and Ala (helix inducer) residues might reflect the presence of a specific structure (β-turn versus helix) that is able to discriminate PAC1 and VPAC receptors. However, stabilization of such structures with known mimetics, *i.e.* Pro-Gly or D-Pro-Gly dipeptides, which favors type I/II or II′ β-turn arrangements, respectively [28] or a Gly-Val Freidinger's γ-lactam dipeptide, which induces a type II′ β-turn [29], completely abolished the ability of PACAP to bind and activate all receptors. Nevertheless, replacement of Gly^4 with 2-aminoisobutyric acid (Aib), a helix and β-turn inducer, did not induce a drastic decrease in binding affinity and activity toward PACAP/VIP receptors. Consequently, binding specificity could be achieved by the presence of characteristic structural motifs: for PAC1, the presence of a specific β-turn around residues 4-5 whereas an extended α-helix, up to residue 4, might be necessary for high selectivity toward VPAC1. We hypothesize that the inability of VIP to bind and activate efficiently the PAC1 receptor could be partly attributed to its inability to form a stable β-turn in the N-terminal domain.

4.3. Residues 6–10: N-capping motif

As recently suggested, PACAP, VIP and other ligands of the B1 family of GPCRs, seem to adopt an N-capping motif constituting the underlying receptor activation mechanism (Fig. 5B) [30]. As demonstrated, replacement of Phe^6 by Tyr, Cha, Bip or Nal did not alter significantly the binding properties of PACAP analogs but modulated their specificity for the different receptors. Introduction of an alanine moiety at position 6 almost completely abolished the binding to all receptors suggesting that the bulky and hydrophobic features of Phe^6 are more essential than the aromaticity for proper recognition. As reported, alanine substitution of Tyr^{10} in PACAP27 resulted in an 1800-fold reduction of its binding affinity [18]. In VIP, replacement of Phe^6 or Tyr^{10} with Ala, gave rise to a more

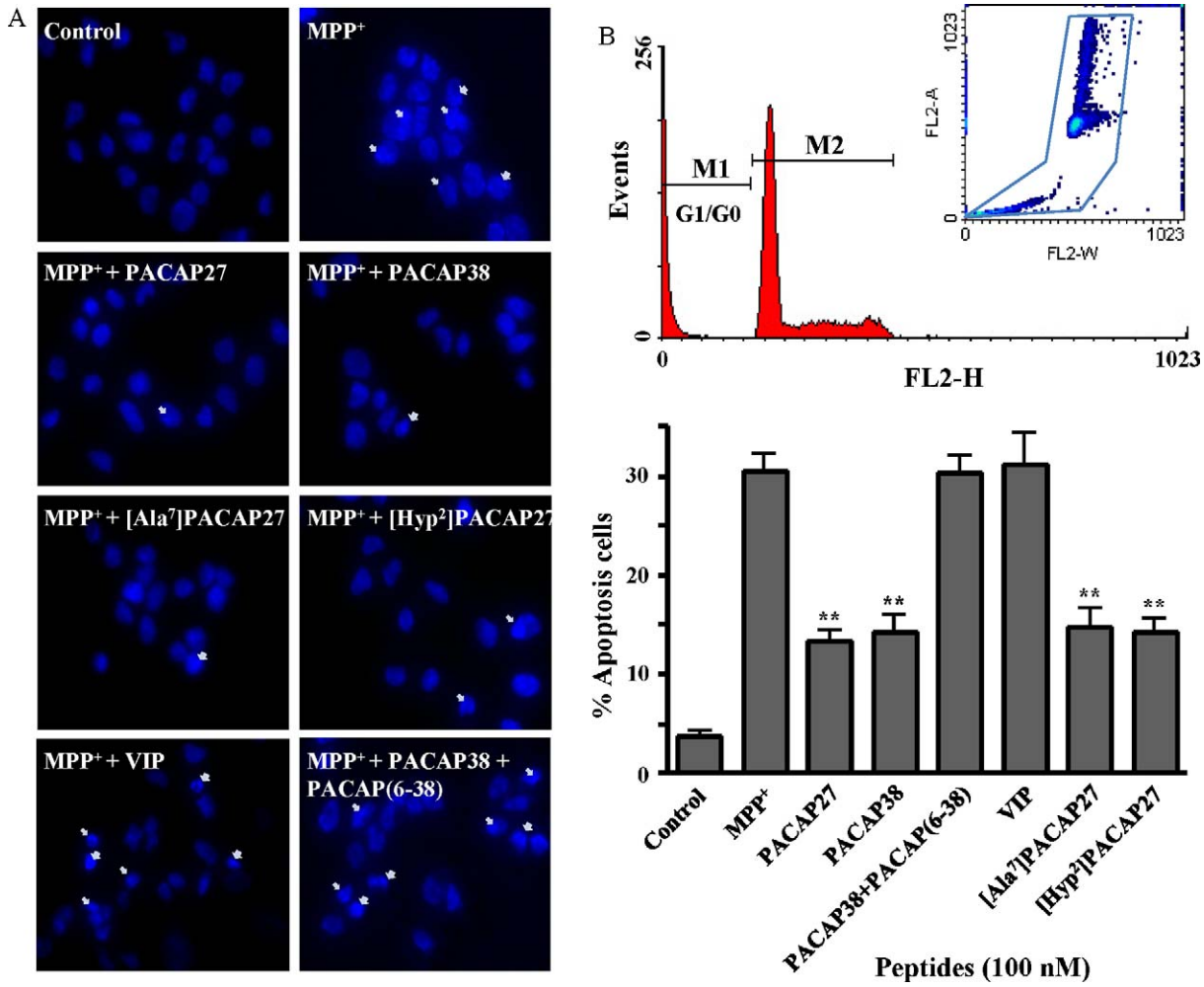

Fig. 4. Effect of PACAP and related analogs on SH-SY5Y cells apoptosis (DAPI staining and DNA content). In both cases, SH-SY5Y cells were pre-incubated 4 h with PACAP or its analogs (100 nM) before being treated with 0.5 mM MPP^+ in serum-free medium for 24 h. Chromatin condensation was visualized by fluorescence microscopy (A). DNA content was analyzed by flow cytometry using live gates to discriminate between doublets and cells exhibiting reduced DNA content corresponding to the sub G1/G0 diploid peak (B). Each value represents the mean ± S.E.M. of at least 4 independent assays. $^{**}P \leq 0.01$ versus MPP^+-treated cells.

marked loss of affinity toward the VPAC2 receptor than the VPAC1 [25]. Interestingly, the ability of [Ala^7]PACAP27 to bind and activate efficiently PAC1 and VPAC1 with almost no activation of VPAC2 exposes the presence of a putative hydrogen bond involving the hydroxyl moiety of Thr^7. Noteworthy, it has already been pointed out that Thr^7 in VIP represented a specific key pharmacophore for VPAC2 selectivity [31,32]. Altogether, these results support the presence of an N-capping motif for PACAP that could be different to that found in VIP. For instance, based on this study and others [25,30], it appears that a type IA N-capping motif might be necessary for a proper interaction with VPAC2. Whether or not another type of N-capping, that would be present upon binding to the receptor as part of the recognition process, is a key element for the stabilization of the bioactive conformation and plays a role in the PAC1/VPAC1 receptor selectivity will need further investigation.

4.4. Evaluation of the neuroprotective potency of PAC1/VPAC1 selective analogs

Due to the advantages presented by a PAC1/VPAC1 analog in terms of possible treatment of neurodegenerative diseases, we investigated the putative neuroprotective properties of two analogs ([Hyp^2]PACAP27 and [Ala^7]PACAP27) in a common *in vitro* model of Parkinson's disease [22,33]. Pathologically, PD is characterized by the loss of mesencephalic dopaminergic neurons. The SH-SY5Y cell line possesses many characteristics of dopaminergic neurons that make it suitable as an *in vitro* model for PD research. For instance, SH-SY5Y cells possess the ability to synthesize dopamine (DA), and express dopamine transporter (DAT), a protein expressed only in dopaminergic neurons within the central nervous system. Because DAT is a prerequisite for MPP^+ incorporation into neurons where it selectively and potently inhibits the mitochondrial complex I electron transport chain [34], SH-SY5Y has been widely utilized to study mechanisms of MPP^+-induced neurotoxicity and the pathogenesis underlying MPP^+-induced PD-like symptoms.

Following treatment with 0.5 mM MPP^+, SH-SY5Y cells underwent an early stage of apoptosis, as shown by chromatin condensation and DNA content measurement (Fig. 4A and B). It has to be mentioned that the serum reduction content may induce cellular apoptosis and cell death by autophagy [35]. However, it was recently demonstrated that PACAP is able to abolish autophagy through the activation of adenylyl cyclase [36]. Similar results were also described with Neuro-2a neuroblastoma cells [22] and with sympathetic neurons [37]. Characterization and time course of MPP^+-induced apoptosis in human SH-SY5Y neuroblastoma cells demonstrated that a high concentration of MPP^+ (5 mM)

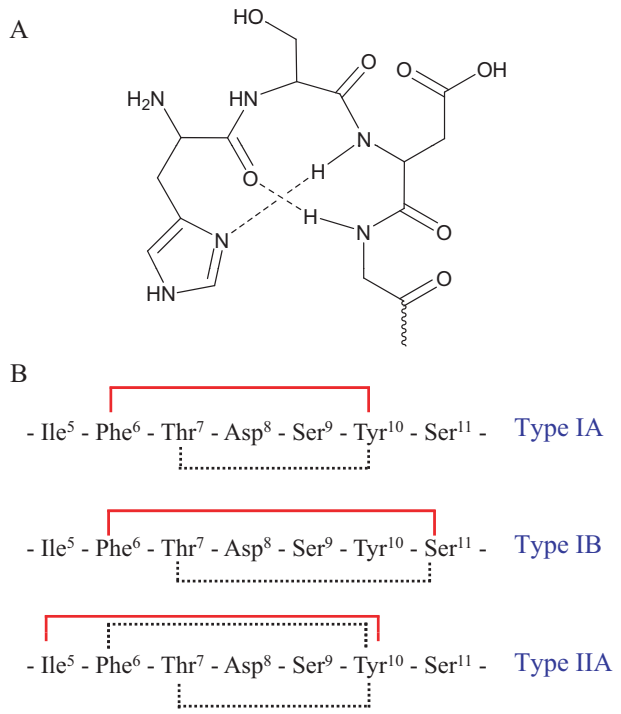

Fig. 5. Representation of the putative Asx-turn like structure present in the N-terminal domain of PACAP (A). Schematic representation of N-capping motifs (IA, IB, IIA) potentially present in PACAP (B). These particular motifs are characterized by hydrophobic interactions between side-chain groups (red line), and by hydrogen bonds between side-chain and backbone amide protons (black broken line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

was unable to produce necrosis [38]. Altogether, these observations strongly suggest that, in our experimental model, the neuroblastic cells are mostly in an apoptotic state following MPP^+ treatment. The neuroprotective effect of PACAP on cerebellar granule neurons, cortical neurons, dopaminergic neurons as well as pheochromocytoma cells has been extensively studied demonstrating that the inhibition of caspase-3 is directly linked downstream to the activation of PAC1 [5,39–41]. The adverse effect of MPP^+ on SH-SY5Y, constitutively expressing PAC1 and VPAC2 receptors [19], was partially reversed by the pre- or co-treatment of the cells with PACAP isoforms. Interestingly, this effect was not observed with VIP and the PAC1 antagonist, *i.e.* PACAP(6-38), abolished the PACAP-induced protection (Fig. 4B). These results confirmed that both PACAP isoforms protect dopaminergic neurons from apoptosis and/or autophagy through PAC1 activation. Pre-treatment experiments in *in vitro* models of neurodegenerative diseases are often chosen over co- and post-treatment because it avoids the competition between the progression of the insult and the neuroprotective effect of the tested substance. However, from a therapeutic point of view, co- and post-treatment investigations might give the promise of the therapeutic potential of a drug since they are more closely related to the pathological condition. Noteworthy, PD's first symptoms generally appear when about 80% of the dopaminergic neurons are dead. It is at that particular moment, when the first diagnostic is made, that a drug therapy is warranted. Our results showed that [Hyp^2]PACAP27 and [Ala^7]PACAP27 were able to protect dopaminergic cells against the toxicity of MPP^+ with the same efficacy as PACAP27 and PACAP38 in both pre- and co-treatment experiments (Fig. 3B and C). Hence, with the aim of a therapeutic use of PACAP-based derivatives, it is extremely appealing to observe the highly significant efficiency of our first-generation of compounds in co-treatment experiments, because it demonstrates that such PACAP-derived compounds might be able to stop the progression of the disease. Surely, to be effective *in vivo* these compounds need to cross the BBB to reach their biological target. Both PACAP isoforms possess this ability although there is a saturable transport for PACAP38 whereas PACAP27 rather uses passive diffusion to enter the CNS. Interestingly, when injected into blood, a larger proportion of PACAP27 enters the brain [42]. Moreover, PACAP27 has a higher plasma half-life than PACAP38 [43]. Thus, the protective effect of the PAC1/VPAC1 selective PACAP27 analogs herein described, *i.e.* [Hyp^2]PACAP27 and [Ala^7]PACAP27 not only supports their potential as promising drug candidates for the treatment of PD but also corroborates the involvement of the PAC1 receptor in cell survival.

In summary, we report the first step of a strategy aiming at developing PACAP-based drug candidates for the treatment of Parkinson's disease, as well as other neurodegenerative diseases. Our results highlighted the importance of His^1, Asp^3 and Phe^6 of PACAP for binding affinity and biological activity toward PAC1 and VPAC receptors and demonstrated the critical role of the hydroxyl group of Thr^7 for the recognition and activation of VPAC2 since [Ala^7]PACAP27 proved to be a PAC1/VPAC1 preferential analog. Finally, we demonstrated the potential benefits of PAC1/VPAC1 analogs as therapeutic compounds for the treatment of Parkinson's disease. Further experiments will be needed to assess the full potency of such compounds in a more complex system integrating the interaction of neurons with activated microglial cells.

Acknowledgments

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