

Construction of a Mini-library and Structure-Functional Studies of Maxadilan, a Non-mammalian Potent Vasodilatory Peptide, which Interacts with the PAC-1 Receptor

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Introduction

Maxadilan (Maxa, Fig. 1) isolated from salivary gland lysates of the blood feeding sand fly, *Lutzomyia longipalpis*, consists of 61 amino acids with 2 disulfides. It is a potent vasodilator that acts selectively as an agonist of the PAC-1 receptor although it has no similarity in the primary structure to PACAP [1]. PACAP has been implicated in several physiological processes for which a number of different receptors are known. Hence synthetic Maxa and its related peptides should provide a useful tool for dissecting the physiological actions of the PACAP receptor systems. Maxa has an endothelium-independent vasodilator action with the accumulation of intracellular cAMP and subsequent vasodilatation. Maxa is recognized only by PAC-1 receptors, to which potent agonists and antagonists may have important clinical applications.

The total synthesis of Maxa has been successfully performed by Nokihara *et al.* [2,3], although difficult sequences had been envisaged after incorporation of residue 34. The present paper describes further improvements in the synthesis of Maxa and summarizes the structural requirements for PAC-1 receptor recognition.

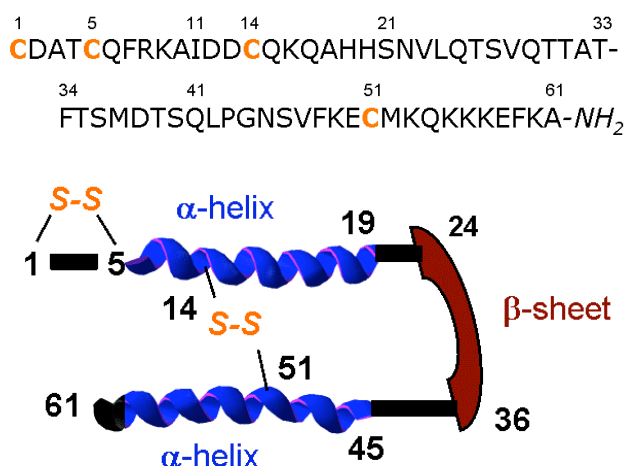


Fig. 1. Primary and predicted secondary structure of Maxa.

Results and Discussion

Maxa-mini libraries designed in the present study are shown in Fig. 2. Additional improvements in Maxa synthesis, focusing on higher efficiency, were accomplished in the present study as follows:

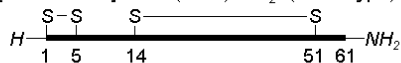
Chem-Matrix resin [4] was used as polymer in SPPS, coupling was carried out using HATU (1eq) / HOAt (1eq) / TMP (1.5eq) in the super solvent system [3] at 45 °C, Fmoc-removal was performed with 0.1 M HOBt in 25% piperidine [5] in the same solvent system, and pseudoproline [6] was incorporated at the positions 3-4 and 32-33. The cleavage cocktail used was TFA/TIS/Water/EDT=85/5/5/5% (for 90 min) and Bu₄NBr was added for the final 5 min to suppress Met-oxidation [7]. As shown in Fig. 3 the elution profile of the crude Maxa is dramatically improved. Hence, we have developed a novel reverse-phase HPLC column based on wide pore silica. HiPep-Intrada, that is suitable for structured peptides or proteins in addition to the previous ODS-column for peptide separation (HiPep-Cadenza) [8]. Low purity peptides show better resolution on the first column while higher quality peptides can also be analyzed on the ODS-column. Disulfide(s) formation has been performed as previously reported [3] and the desired peptides were finally purified by HPLC using preparative HiPep-Intrada or HiPep-Cadenza columns.

Maxa does not contain a Tyr-residue, which is a useful residue for modification in a number of biochemical studies. Hence two Tyr-containing Maxa analogs, Nα-Tyr-Maxa and Tyr⁷-Maxa, have been prepared. Since both showed very similar properties to wild type Maxa, they can be easily used as radio-ligands.

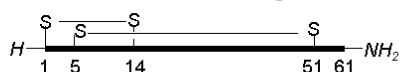
The structural requirements for the function of Maxa were elucidated using a rat brain membrane binding assay [9] and by the melanophore assay [10]. The binding of ¹²⁵I PACAP-27 to the rat cortical membranes was saturated within 2 hours at 4°C and displaced with non radiolabelled PACAP-38 with an IC₅₀ of approximately 4.90 nM. PACAP-27 has a lower affinity (20.5 nM) than PACAP-38 and VIP did not displace the binding even at concentrations over 10000 nM. Such binding data suggest that the PAC-1 receptor is present in the system used. Recently clones for numerous G-protein coupled receptors (GPCRs) have been available for drug discovery and the melanophore assay has potential for high throughput screening of interactions between ligand and GPCR. Maxa-related peptides prepared in the present paper were assayed for functional activity by measuring pigment dispersion in melanophores stably expressing the rat PAC-1 receptor. The results of binding and melanophore assays are summarized in Table 1.

Disulfide isomer and linker of full length

M1 [S-S^{1-5,14-51}] Maxa(1-61)-NH₂ (Wild Type)



M4 [S-S^{1-14, 5-51}] Maxa(1-61)-NH₂



M2 [Cys(Acm)^{1,5}, S-S¹⁴⁻⁵¹] Maxa(1-61)-NH₂

M3 [Ala^{1,5}, S-S¹⁴⁻⁵¹] Maxa(1-61)-NH₂

M5 [Cys(Acm)^{1,14}, S-S⁵⁻⁵¹] Maxa(1-61)-NH₂

M6 [Cys(Acm)^{1,51}, S-S⁵⁻¹⁴] Maxa(1-61)-NH₂

M7 [Cys(Acm)^{1,5,14,51}] Maxa(1-61)-NH₂

M8 [Ala^{1,5,14}, Leu⁵¹] Maxa(1-61)-NH₂

N-terminal fragment (C-terminal truncated fragment) with disulfide

M9 [Ala^{1,5}, S-S¹⁴⁻⁵¹] Maxa(1-53)-NH₂

M10 [Cys(Acm)^{1,5}, S-S¹⁴⁻⁵¹] Maxa(1-53)-NH₂

Middle fragment (N- and C-terminal truncated fragment) with disulfide

M11 [S-S¹⁴⁻⁵¹] Maxa(14-53)-NH₂

M12 [S-S¹⁴⁻⁵¹] Maxa(14-51)-NH₂

C-terminal fragment (N-terminal truncated fragment) with or without disulfide

M13 [S-S¹⁴⁻⁵¹] Maxa(14-61)-NH₂

M14 [Cys(Acm)^{14,51}] Maxa(14-61)-NH₂

M15 [Leu⁵¹] Maxa(20-61)-NH₂

M16 [Leu⁵¹] Maxa(27-61)-NH₂

M17 [Leu⁵¹] Maxa(34-61)-NH₂

Without middle fragment with a disulfide

M18 [S-S¹⁻⁵] Maxa(1-5+53-61)-NH₂

M181 [S-S¹⁻⁵] Maxa(1-13+53-61)-NH₂

M19 [Cys(Acm)^{1,5}, S-S¹⁴⁻⁵¹] Maxa(1-23+43-61)-NH₂

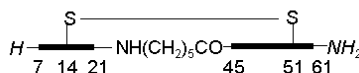
Without N-terminal and middle fragment

(connected *via* amino hexanoic acid) with a disulfide

M20 [S-S¹⁴⁻⁵¹] Maxa[7-21-NH(CH₂)₅CO-50-61]-NH₂

M21 [S-S¹⁴⁻⁵¹] Maxa[7-20-NH(CH₂)₅CO-45-61]-NH₂

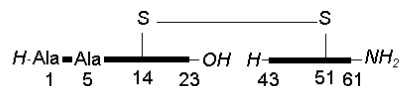
M22 [S-S¹⁴⁻⁵¹] Maxa[7-21-NH(CH₂)₅CO-45-61]-NH₂



N- and C-terminal fragment connected with a disulfide bond between 14-51

M23 [Cys(Acm)^{1,5}] Maxa(1-18) / S-S¹⁴⁻⁵¹ / Maxa(45-61)-NH₂

M24 [Ala^{1,5}] Maxa(1-23) / S-S¹⁴⁻⁵¹ / Maxa(43-61)-NH₂



M25 [Cys(Acm)^{1,5}] Maxa(1-18) / S-S¹⁴⁻⁵¹ / Maxa(38-61)-NH₂

Tyr containing Maxa derivatives with a disulfide as in wild type

M28 NαTyr-[Ala^{1,5,14}, S-S¹⁴⁻⁵¹] Maxa(1-61)-NH₂

M29 [Ala^{1,5,14}, Tyr⁷, S-S¹⁴⁻⁵¹] Maxa(1-61)-NH₂

Fig. 2. Designed Maxadilan Mini-Libraries.

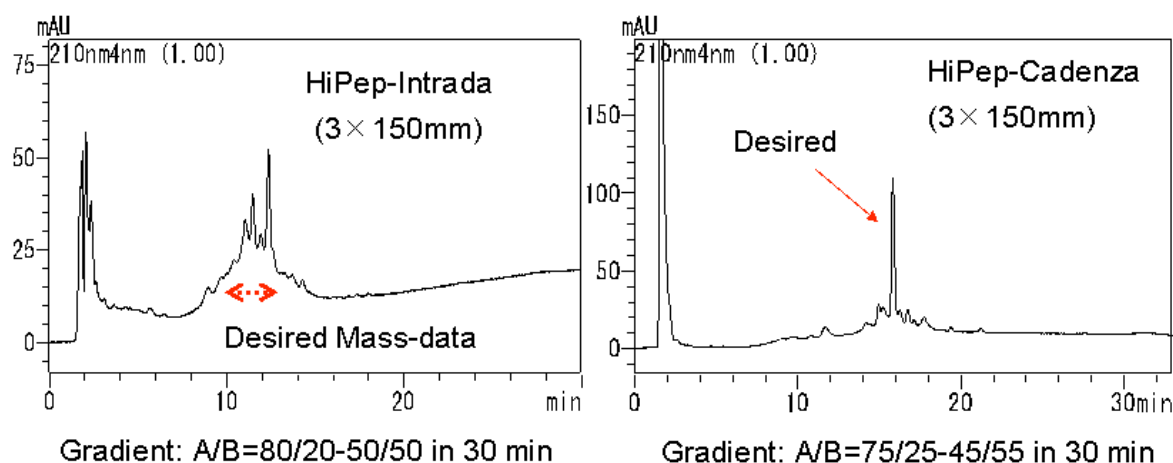


Fig. 3. HPLC-Profiles of crude [Cys(Acm)^{1,5}(SH)^{14,51}] Maxa(1-61) after cleavage.

An ion-trap LCMS system was routinely used to detect the desired peptides. Upper: Eluate 10.5-12.5 min contained materials with the calc. mass of 7010.0 of Cys^{1,5}S(Acm) 61amino acids, found 2337.9 [M+3H]³⁺. Major peak of lower panel: found 2338.0 [M+3H]³⁺. Flow rate: 0.5 mL/min, monitored at 210 nm, linear gradient: A = 0.1%TFA, B = 0.1%TFA, 90% acetonitrile.

Table 1. Binding of [¹²⁵I]PACAP-27 (IC₅₀) and Melanophore Assay (EC₅₀).

Peptides	IC ₅₀ (nM)	EC ₅₀ (nM)	Peptides	IC ₅₀ (nM)	EC ₅₀ (nM)
PACAP-27	20.51	Not Tested (NT)	M18	>1000	inactive
PACAP-38	4.90		M181	>1000	NT
Human VIP	>10000		M19	3.43	inactive
M1 (Wild-type)	3.89	0.01	M20	>1000	inactive
M2	13.06	0.20	M21	228	0.5–5μM
M3	8.27	0.15	M22	174	inactive
M4	8.95	0.02	M23	6.12	inactive
M5	7.33	0.04	M24	2.0	antagonist
M6	>1000	1.10	M25	34.5	inactive
M7	47.14	NT	M26	>1000	NT
M8	>1000	100	M27	>1000	NT
M9	>1000	20	M28	2.34	NT
M10	>1000	NT	M29	3.49	NT
M11, 12	>1000	NT			
M13	>1000	10			
M14,15,16,17	>1000	inactive			

Of the three possible disulfide isomers 1-51/5-14 could not be synthesized. Since the disulfide isomer of Maxa (M4), having two disulfide bonds at positions 1-14 and 5-51, also has a relatively high affinity, the three dimensional structures of both peptides must be similar. Deletion of the first disulfide bond of Maxa by substitution with Cys(Acm) (M2) or Ala residues (M3) also resulted in only small changes in the high affinity values. A considerably lower affinity was found in M7, the full length molecule of 61 amino acid residue without disulfide bonds (instead tetra Acm derivative). Subsequent replacement of all the Cys residues by Ala and Leu caused a total loss in binding affinity (M8). These data suggest the first disulfide was not essential, but the second disulfide is required for high affinity binding to the PAC-1 receptor. The Maxa derivative with the central section deleted between residues 24 to 42 (M19) maintains a high affinity, suggesting the first disulfide bond and middle region of the peptide from Leu at 24 through Leu 42 are not required for binding. In contrast Maxa analogs without C- and/or N-terminal fragments (M9-M17) did not show any inhibitory effects on the specific binding of ¹²⁵I-PACAP-27. These data suggest that a particular conformation is required for recognition of PAC-1, the middle region of Maxa is not required for binding, while N- and C-terminal fragments alone, as well as various central fragments, show no binding even when disulfide position 14-51 exists.

Despite the poor similarity of the sequences of Maxa and PACAPs, sequence alignment suggests positions 34-35 of Maxa aligned with positions 6-7 of PACAP and PACAP(6-27) which are known to be responsible for antagonistic properties. This may then suggest that M13-17 might show antagonistic properties. In fact, neither antagonistic nor agonistic effects were found. The middle

region deleted analogs (M18, M181 and M19) showed no binding. The secondary structure of Maxa is predicted to be a β-sheet sandwich between two α-helices from Ala¹⁰-Asn²² and Val⁴⁷-Ala⁶¹ (Fig. 1). As both M18 and M181 did not show binding to PAC-1 receptors, it appears the central region, that incorporates the β sheet and ends of the two helices, is critical for recognition of the PAC-1 receptor binding sites. When the central segment was replaced by a short hexanoic moiety with a disulfide (M20, M21 and M22) only the C-terminal segment analogs with the longer fragment 45-61 have weak binding. This indicates that in addition to the presence of a disulfide bond helical elongation plays a key roll. Indeed the shorter middle region deleted analogs (M23, M24 and M25) showed significant binding, in particular M24 binds with high affinity to PAC-1 receptors. This implies the segment between 19-23 is important and binding sites for PAC-1 receptors are located in both the Cys¹-Val²³ and Pro⁴³-Ala⁶¹ regions of the molecule.

The agonistic/antagonistic properties of peptides M19 – M27 on PAC-1 receptors were then studied by measuring the accumulation of cAMP in PC12 cells either alone at a concentration of 100 nM peptide or in competition experiments with peptides M19–M27 to accumulated cAMP induced by addition of 1 nM PACAP-27 as PACAP-27 caused the accumulation of cAMP in PC12 cells. Clearly M24 showed the highest affinity among the non-full length Maxa analogs and is an antagonistic of the PAC-1 receptor in terms of c-AMP accumulation. Most of the peptides, apart from M24 and M25, behave as agonists in the PC12 cell system. M24 alone (100 nM) does not cause a significant increase in cAMP production in PC12 cells, with a low value of 11.1 % compared to the control and M24 at 100nM is likely to suppress cAMP accumulation stimulated with 1 nM PACAP-27 by 353 %.

The dimer of N-terminal fragment (M25) also showed weak antagonistic effects. In terms of cAMP accumulation, M1 was less potent than the other derivatives even though it showed a higher affinity for the PAC-1 receptor. The possibility that M1 itself behaves as an antagonist of the PAC-1 receptor could not be excluded from our results. Recently, Reddy et al. have reported that a recombinant Maxa showed ~ 5 pM EC₅₀ in the melanophore assay, which suggested wild Maxa could also bind to a PAC-1 receptor as an agonist [11]. The precise mechanism responsible for cAMP accumulation must await further studies.

In conclusion, we have efficiently prepared wild type Maxa and constructed Maxa-mini libraries. Antagonistic effects were observed in a two-chain peptide 1-23 and 43-61 linked with a disulfide bridge between 14-51. The middle region (position 24-42) was not required for binding, although this region showed antagonistic effects on PAC-1 receptors. Clearly synthetic Maxa and its related peptides should provide useful tools for further physiological/biological studies focusing on the PACAP receptor system and should provide new insights into the pathophysiology. The tyrosinated analogs of Maxa (M28 and M29), showed remarkably high binding affinities, suggesting their use as excellent radio ligands for histochemical studies.

Acknowledgments

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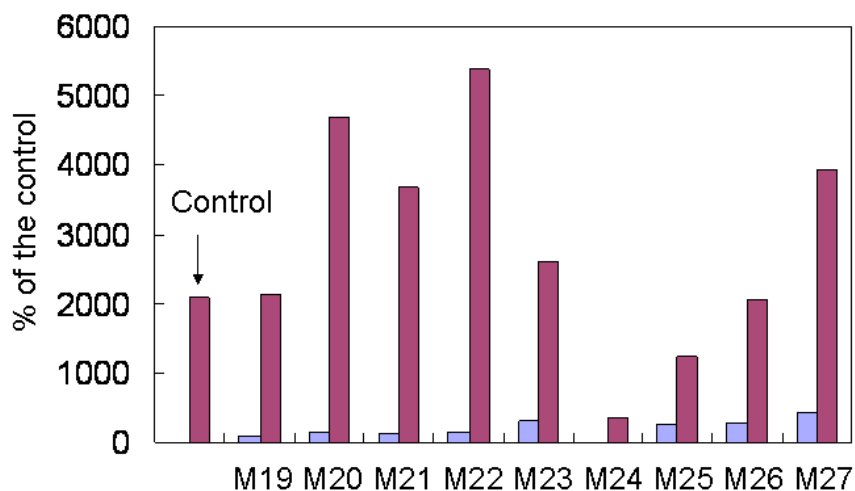


Fig. 4. Effects of Maxadilan analogues 10^{-7} M on PACAP-27 induced cAMP accumulation in PC12 cells.