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ARTICLE TYPE

Molecular dynamics study of catestatin docked on nicotinic acetylcholine receptors to identify amino acids potentially involved in the binding of chromogranin A fragments

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Catestatin, a cationic and hydrophobic 21 amino acid fragment of chromogranin A, is known to be a non-competitive catecholamine release antagonist working through nicotinic acetylcholine receptors (nAChRs). Since this receptor is the target of several neuronal and non-neuronal disorder prophylaxes and treatments, this study aims at an elucidation of the binding of human catestatin to entire nAChR reconstructed in lipid bilayers by means of docking followed by full atomistic molecular dynamics simulations. The obtained results show that the minimum free energy for the binding of the peptide and the receptor reaches minimal values for locations at the pore site and in the outer beta subunit. This result is consistent with previous studies showing that catestatin occludes the pore opening. A new finding is an additional even stronger binding seat at the beta subunit and that membrane presence could be an important factor. Specific aminoacids involved in catestatin binding have been identified, indicating targets for point mutations studies. In addition to improving understanding of the interaction between peptide and muscle-type and even other nAChR subtypes, the results of this study provides directions for future peptidomimetic research.

Introduction

Chromogranin A (CgA), a 48 kDa protein, is a regulatory protein that can be found at a high concentration in neuroendocrine and neurosecretory granules of adrenal chromaffin cells and adrenergic neurons.[1, 2] CgA binds with Ca^{2+}, ATP, and catecholamines in the hormone storage vesicles. These bindings induce the accumulation of other secretory proteins in the dense core vesicles.[3] CgA also functions as a prohormone that can be cleaved into anti-insulin pancreastatin (hCgA250-301),[4] vasorelaxant vasostatin (hCgA1-76),[5] catecholamine release inhibitory peptide catestatin (hCgA352-372),[6] and pro-adrenergic serpinin.[7]

Catestatin is one of the CgA fragments. In humans, catestatin is cleaved in pheochromocytoma chromaffin granules at the dibasic sites,[8] and it is released by an exocytotic process.[9] Catestatin is a neuropeptide consisting of 21 amino acids (bovine $CgA_{344-364}$ and human $CgA_{352-372}$). The active core sequence for bovine catestatin is constituted by only 15 amino acids (bovine $CgA_{344-358}$), which is sufficient to inhibit nicotinic cholinergic stimulated catecholamine secretion, signal transduction and desensitization.[10] The peptide is a novel and potent inhibitor of catecholamine released from chromaffin cells and adrenergic neurons. For instance, Mahata S.K. *et al.* showed that the peptide acts as a non-competitive nicotinic cholinergic antagonist, which leads to the inhibition of nicotinic cation (Na^+ and Ca^{2+}) signal transduction.[11] It has been postulated that it occludes the nicotinic acetylcholine receptor (nAChR) pore and interacts with multiple subunits at the pore vestibule with a potency higher than that of substance P. In addition, Herrero *et al.* suggested that catestatin might regulate an autocrine process in neuroendocrine secretion through binding with different nAChR subtypes.[12] The blockage could be regulated by the intensity and duration of the presynaptic stimulus. In addition, catestatin is also a potent vasodilator in vivo. Its actions may involve histamine release and H1 receptor stimulation.[8] Finally, the studies of Sciamanna *et al.* show that nAChRs may influence the secretion of autocrine growth factors, thus being involved in the growth and metastasis of malignant neuroendocrine neoplasms through synergizing with the activity of voltage-sensitive Ca^{2+} channels.[13] In the light of these findings, it is important to discover the roles of molecular catestatin not only in the usually studied area of neuronal-type nAChRs, but also on muscle-type nAChRs,[14] especially given that these latter have been found to be expressed by neurons in the CNS and PNS.[15, 16] For instance, the SCC-37 cell line was the first neuroendocrine cell type found to express both types of nAChRs. Other examples of the co-existence of neuronal and muscle type receptors is the a-BTx receptor[17] from tissue of chick sympathetic ganglia, suggesting that normal cells of neural lineage are also able to co-express muscle and neuronal-type nAChRs.

Very few studies aiming at an elucidation of the molecular level mechanisms of catestatin binding on the nAChR are

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available. The first simulation studies were conducted by Tsigelny *et al.*.[18] These docking studies only used the nAChR binding protein located on the extracellular binding domain of the Torpedo nAChR. It was shown that the peptide mainly interacts with the β and δ subunits, the hydrophobic region of the pore. In other studies, Taupenot *et al.* postulated that catestatin may interact with the δ, γ, and β subunits of nAChR at the receptor vestibule, which is responsible for the action of a non-competitive antagonist.[19] At the beginning of the 21st century, Unwin and co-workers provided high resolution electron microscopy (EM) data for nAChR proteins from the electric organ of Torpedo.[20-23] The most advanced molecular study of catestatin binding on the human $\alpha_3\beta_4$ nAChR was published by Sahu *et al.*.[24] The results presented in the molecular dynamics (MD) part of their study are, however, questionable due to (i) the lack of receptor stabilization by cholesterol molecules within its voids (cholesterol present only in membrane) as pointed out by Brannigan *et al.*,[25] and (ii) the lack of solvent molecules in the far extracellular part of the receptor (evident in Fig. 1 of Ref. [24]), where the authors found catestatin binding. Nevertheless, their results corroborate their experimental findings and they were able to formulate strong hypotheses based on those simulations.

The aim of the studies presented in this paper was to use the docking method followed by all-atom molecular dynamics simulations to thoroughly analyze the interactions of catestatin with the entire structure of the αβγδ nAChR,[23] which can be found in neuromuscular junctions. MD simulations were mainly conducted in order to elucidate potential binding sites for catestatin, along with their energetic profiles. We believe that these results provide guidelines and new molecular targets for the design of drugs for neuronal cardiac and hypertension disorders, and even for prevention of malignant neuroendocrine neoplasms.

Methods

Simulations of Catestatin

Catestatin exists in nature as a β-strand/loop/β-strand structure, which is stabilized by the hydrophobic nature of both β-strands. Since the molecule is very flexible, it is therefore hard to account for its native dynamic nature in a simplified docking study. However, the consideration of all available rigid configurations of catestatin in preliminary docking studies would be a satisfactory starting point for the subsequent molecular dynamics (MD) simulations we performed. Thus, in the first (docking) approach, we decided to use all 25 crystallized linear catestatin structures (human $CgA_{352-372}$, S_{352}SMKLSFRARAYGFRGPGPQL$_{372}$, PDB ID: 1LV4) in our docking analysis.[26] Such an approach is a reasonable and effective initial approximation of the catestatin dynamics. When all potential binding sites had been identified, the all-atom MD simulations, with 6 catestatin molecules placed near predetermined binding sites (one molecule per each of the 5 receptor subunits, and near the pore entry), were performed. MD simulations are suitable to provide a more realistic determination of the affinity of the peptide to available binding sites than is the case in docking, accounting for such factors as peptide dynamics, the presence of aqueous and hydrophobic environments and parameters such as pressure and temperature.

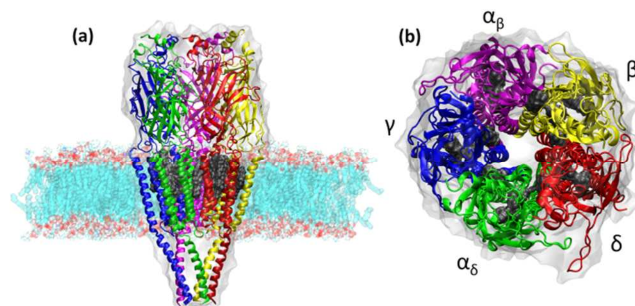

Fig. 1 *The whole structure of nAChR. Side (a) and top (b) views of nAChR embedded into the lipid membrane. The receptor consists of 5 subunits: 2αβγδ and grey spots in the transmembrane part of the protein indicate the embedded cholesterol molecules, α_β subunit is shown in violet, β in yellow, δ in red, α_δ in green, and γ in blue.*

Simulations of nAChR channel

Atomic coordinates for the nAChR have been acquired from the whole structure of Torpedo marmorata nAChR at 4 Å resolution (PDB ID: 2BG9).[23] All subunits (labeled α_β, β, γ, α_δ and δ) consist of extracellular agonist binding domains composed mostly of β-sheets, a transmembrane domain composed of a four-helix bundle, and an intracellular vestibule domain. A previous docking study showed that voids exist between transmembrane helices that accommodate cholesterol molecules.[25] This is supported by observations that the EM density maps revealed that the void density is similar to that of the surrounding lipids, thus indicating cholesterol presence. Furthermore, the molecular dynamics simulations conducted without cholesterol in receptor membrane pockets have shown an abnormal and extremely unstable complex that differs significantly from the experimentally determined structure.[25] In contrast, MD conducted on nAChR in a system containing cholesterol embedded in these voids has resulted in a conformation consistent with the experimentally determined structure. For all these reasons, we used the mandatory nAChR/cholesterol set in the current studies (see Figure 1).

Following Brannigan *et al.*,[25] there are several missing loops in the structure. The β8-β9 loop in the agonist-binding domain of the β-, δ-, and γ-subunit is missing from the 2BG9 entry. This loop was modeled by the authors using MODELLER.[27] The missing M3-MA loop was resolved by placing restraints on both ends of the MA helix in the vestibule domain rather than attempting to model this loop with 100 residues. This simplification of missing M3-MA loops should not affect the outcome of the simulation, since the binding sites are located in the extracellular part of the protein.

Automated Docking of catestatin

AutoDock 4.2 was used in combination with the Lamarckian genetic algorithm (LGA) for the docking studies to search for a globally optimized conformation.[28] The LGA was used to model the interaction/binding between the ligand and nAChR and to describe the relationship between the ligand and the macromolecule by the translation and orientation of 25 experimental conformations of the catestatin. First, the equilibrated structure of nAChR containing all hydrogen atoms together with cholesterol molecules and the lipid bilayer was extracted from MD simulations. Simultaneously, the Gasteiger

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Table 1 Energy of the catestatin binding to the active sites of nAChR

Site	Panel of Figure 2	occurence	VdW [kcal/mol]	El. [kcal/mol]	dG(estim) [kcal/mol]
pore	**(a)**	31%	-5.14±0.67	-6.48±0.73	**-11.62**±0.70
δ domain	**(b)**	16%	-4.74±0.70	-6.15±0.77	**-10.89**±0.63
α_δ domain	**(c)**	4%	-4.18±0.54	-6.16±0.89	**-10.34**±0.68
α_β domain	**(d)**	20%	-3.75±0.76	-6.71±0.90	**-10.46**±0.55
β domain	**(e)**	7%	-4.63±0.65	-5.80±1.00	**-10.43**±0.90
γ domain		none			

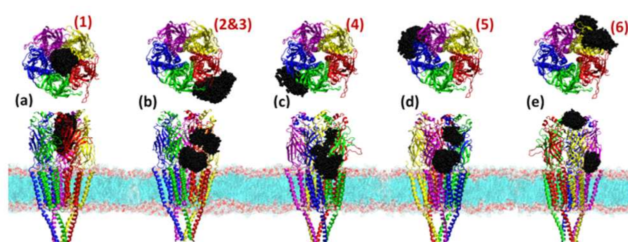

Fig. 2 *Binding sites of catestatin on aChR: inside the pore (a), on the extracellular parts of the δ (b), α_δ (c), α_β (d) and β (e) domains. Each red number corresponds to a binding site investigated further in molecular dynamic simulations.*

charges were computed and added to atoms of the ligand. Then, the 3D grid was created using the genetic algorithm to form the grid parameter file. The grid spacing was 0.5 Å in all dimensions, and the grid map consisted of 240×240×320 points. During each of the 25 docking experiments, 50 LGA runs were carried out. Modes of binding were extracted with the 0.5 Å RMSD cut-off method. At the end of each multiple-run docking experiment, a cluster analysis was performed. The best binding modes were selected using the binding energy score.

Molecular Dynamics

The model of the nAChR channel with protein voids filled with 15 cholesterol molecules was taken directly from Brannigan *et al.*[25] This was then inserted into a fully hydrated lipid bilayer patch POPC (1-palmitoyl-2-oleoyl-sn-glycerol-phosphatidyl-choline) equilibrated over 200 ns 240 Å × 320 Å. The protein was positioned in such a way that Trp residues of each subunit were located at the lipid/water interface and then overlapping lipids were extracted. Two slabs of water molecules were added from both sides to re-solvate the hydrophilic parts of the protein and the whole system was electrostatically neutralized using a physiological concentration of NaCl.

Initially, the energy of the system was minimized for 400 steps. Next, a 7 ns relaxation of the remaining environment of the elements was performed with a fixed position for all atoms of the protein, in order to get a clung lipid bilayer. A harmonic potential with a force constant of 1 kcal/mol/Å^2 was applied at the initial positions in the 2BG9 structure. This was followed by a further 15 ns relaxation of all atoms, except the Cα of transmembrane helices. Thus, the resultant protein RMSD deviates from the experimental structure by about 5 Å, and reaches a plateau after 10 ns of relaxation. Next, simulation run was performed for a further 60 ns.

All MD simulations were carried out on an NPT ensemble using NAMD 2.6.[29] Short- and long-range forces were calculated every 1 and 2 time-steps, respectively, with an integration time of 2.0 fs. The Langevin dynamics algorithm and the Langevin piston Nosé-Hoover method were used to maintain a 300 K temperature and a 1 bar pressure in the system.[30] Long-range electrostatic forces were taken into account using the particle mesh Ewald (PME) approach.[31] The force field parameters for lipids were taken from CHARMM27 with the united atoms extension,[32] while for cholesterol molecules we followed the description from Ref. [33]. The intra- and intermolecular potentials for water were modeled within the TIP3P approach.[34]

Results

Using the docking pre-analysis, we identified many available sites for each configuration of catestatin, resulting within and onto the nAChR receptor. As already mentioned, the catestatin sites are quite large. The large spots in Figure 2 illustrate this feature.

The extracellular domain is the high scoring binding site

Most high-scoring sites were found on the extracellular binding domain. 85% of the determined configurations occur on the extracellular side (see Figure 2), while only 15% of configurations are located at the vestibule on the intracellular side of the membrane (data not shown). The Autodock scoring function, an empirical estimate of the free energy of binding, indicates that these sites have the same energies. As shown in Table 1, scores for the energy of minimized catestatin/nAChR complex were found to be in the range between -11.6 kcal/mol and -10.3 kcal/mol. The determined energy dispersion was small, indicating that docking sites had a similar physical complementarity. Using steric constraints for the transmembrane protein part and performing docking on proteins with lipid environments, we found that the extracellular domain is a more probable target for catestatin than the intracellular one.

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Table 2 Amino acids involved in long-term interactions with catestatin peptide

Site	amino acid	subunit	% of time bound	Site location
β site (6)	Asp162	β	74.23	**1.0 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
	Pro491	β	68.76	
	Phe161	β	28.04	
	Glu191	β	26.11	
	Arg218	β	26.10	
	Asn490	β	17.68	
α_β site (5)	Cys216	α_β	80.79	**1.0 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
	Pro218	α_β	71.27	
	Cys217	α_β	46.69	
	Tyr214	α_β	35.40	
	Thr215	α_β	34.25	
	Asp219	α_β	22.61	
γ/α_δ site (4)	Asp42	γ	99.51	**5.7 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
	Ile99	α_δ	48.45	
	Tyr96	α_δ	43.39	
	Asp95	α_δ	36.54	
	Pro105	α_δ	26.75	
	Ala94	α_δ	23.49	
	Leu104	α_δ	21.04	

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pore site (1)

amino acid	subunit	% of time bound	Site location
Asp1135	β	98.81	**3.3 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
Asp105	γ	97.27	
Phe116	γ	83.99	
Ala119	γ	82.14	
Leu103	γ	73.37	
Glu117	γ	71.10	
His128	α_β	68.87	
Asp123	α_β	66.44	
Asp121	α_δ	64.24	
Tyr127	δ	62.72	
Met129	α_δ	62.52	
Asp123	α_δ	59.21	
Met129	α_β	52.30	
Tyr121	γ	50.69	
Ala125	α_δ	46.67	
Phe124	α_δ	40.82	
Tyr167	γ	39.52	
Ile126	α_δ	37.94	
Val127	α_δ	35.83	
Tyr120	γ	34.78	
Trp134	γ	34.51	
Ser123	β	24.98	
Ile126	β	24.71	
His128	α_δ	24.59	
Gln111	β	23.50	
Val118	γ	21.66	

δ site (2)

amino acid	subunit	% of time boun	Site location
Tyr193	δ	68.60	**0.7 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
Ile198	δ	55.33	
Ile199	δ	48.39	
Trp162	δ	47.84	
Ile241	δ	46.05	
Asp225	δ	34.15	
Ile214	δ	28.21	
Asp161	δ	25.08	
Glu196	δ	22.55	
His215	δ	18.50	
Ile195	δ	17.20	

δ/α_δ site (3)

amino acid	subunit	% of time bound	Site location
Tyr214	α_δ	78.92	**1.3 nm** above membrane surface *(center of mass of NH_3 groups in lipid bilayer)*
Glu153	α_δ	67.07	
Lys209	α_δ	64.30	
Glu207	δ	63.54	
Gln164	α_δ	45.06	
Phe205	δ	44.08	
Tyr213	α_δ	43.29	
Ala204	δ	38.11	
Cys166	α_δ	33.08	
Tyr214	α_δ	25.01	
Leu170	α_δ	21.53	
Glu196	δ	19.96	

Catestatin binds non-specifically to nAChR at the receptor pore

We found that catestatin binds willingly inside the pore in the extracellular domain. 31% of occurrences for all configurations were found to be in this location (see Figure 2a). No specific interaction with one peculiar domain was found at the docking stage; however, further MD simulations show a slight catestatin preference toward the *γ* subunit (see Table 2 for contact amino acids). Our results are in agreement with previous docking studies presented by Tsigelny et al. [18] and Taupenot *et al.*.[19]

There is a single potential binding site in each domain

Docking data show that the catestatin interacts with α_β (20%), β (7%), and α_δ (4%) subunits of nAChR at the outer side of the receptor vestibule. Moreover, a non-negligible score was obtained for the δ domain (16 % with -10.9 kcal/mol on average), while no direct occurrence was observed at the γ domain. There is no evidence for symmetric catestatin binding. This result is

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unexpected, since Autodock should reflect the fivefold pseudo-symmetry of the nAChR receptor. Our further MD simulations support this observation, showing that amino acids involved in external direct contact with catestatin are not present in the γ subunit, except for a single Asp42 amino acid (see Table 2). Binding sites were found according to docking using MD at δ, δ/α_δ, γ/α_δ, α_β, and β subunits.

The analysis of docking locations presented in Figure 2b-e reveals that each binding subunit (α_β, β, α_δ, and δ) has two energetically equivalent sub-sites present at each extracellular domain. One of these is located at the lipid bilayer interface, whereas the other is found above in the bulk region. The only difference was observed for the β subunit (Figure 2e), where the second sub-site is located far away at the entry of the vestibule. No overlap between the two sub-sites has been found. However, the MD simulations show that only one of these two sub-sites is dynamically relevant. This is due to the dynamic nature of the peptide. The peptide, when approaching the membrane, changes its conformation before binding to the receptor. Exceptionally, this scenario does not happen when catestatin approaches the interface of γ/α_δ subunits (see Table 2). Catestatin tries to bind at this location in its unfolded state, since the binding seat is positioned almost 6 nm above the membrane surface (calculated as the mean position of NH_3 groups of lipid headgroups). However, this binding location was not found in docking studies and, accordingly, in MD simulations exhibits a repulsive electrostatic component (see Table 3) suggesting a highly unstable or unspecific binding site. This specific γ/α_δ location resembles that of the GABA active pocket in homology with $GABA_A$ receptors.[35] All other outer binding sites found in MD are located only 1-3 nm above the membrane surface. Taking into account the simulation time required for binding (at least ns of simulation) and the positive (repulsive) total binding energy of +6.8 kcal/mol for γ/α_δ location (see Table 3), we were able to speculate that membrane presence is a crucial factor for catestatin (or chromogranin A) binding or binding preparation to nAChR. Since catestatin is an amino acid chain, this statement could be as important as it is for all proteins folded at the water/membrane interface using an anisotropic environment-driven force.

Table 3 Free energy of binding as calculated based on molecular dynamics simulations using the Linear Interaction Energy method

site	VdW [kcal/mol]	El. [kcal/mol]	dG [kcal/mol]
pore	-5.7	-57.9	**-63.6**±49.2
δ outerside	-3.6	-13.0	**-16.6**±65.7
δ/α_δ outerside	-1.9	-49.9	**-51.8**±38.3
γ/α_δ outerside	-0.3	+7.1	**+6.8**±53.2
α_β outerside	-8.6	-17.6	**-26.2**±48.3
β outerside	-3.5	-73.1	**-76.6**±34.9

The interaction energy between catestatin and receptors is affected by the flexibility of the former

The large size of the binding sites and the very small dispersion of the docking scoring functions (standard deviation of about 0.6 kcal/mol, see Table 1) may indicate that the 25 catestatin models used do not reflect the dynamic nature of the peptide. This may prevent the performance of a meaningful docking analysis. In order to account for any biases resulting from limited statistical sampling of catestatin structures, we performed a binding energy analysis using MD results for comparison. In Table 3 calculated van der Waals and electrostatic energies of interaction between peptides and receptors in each of discovered binding sites are presented. The strongest interactions were observed in the β site (-76.6 kcal/mol) and within the pore (-63.6 kcal/mol). A slightly weaker affinity is noted in the δ/α_δ site with a value of -51.8 kcal/mol, while other sites have much lower affinity (-26.2 kcal/mol for the α_β site and -16.6 kcal/mol for the δ site). The GABA-like γ/α_δ site has an interaction energy equal to +6.8 kcal/mol. The linear interaction energy method[36, 37] employed for analysis is very sensitive to the reference energy calculated for the unbound state; therefore, calculated values cannot be directly compared to those determined in experiments. This method, however, can correctly identify favorable and unfavorable binding states. High values of uncertainty (sometimes larger than the calculated values of interaction) indicate high ligand dynamics, as in all the cases of catestatin to nAChR binding.

Conclusions

Molecular simulations regarding binding of ligands in general, and catestatin in particular, with nAChRs receptors can help to identify seats of interactions. Such studies are of particular importance for systems which are inherently dynamic or when there is no complete information on the receptor 3D structure. In the case of catestatin, its interaction with receptors was the subject of intense studies using docking methodologies even before the whole nAChR structure had been determined.[23, 38] The studies presented here are intended to elucidate the interaction of catestatin with the whole structure of nAChR, as proposed by Unwin.[23] In the course of computer simulations, it has been shown that catestatin docks at the proximal vestibule of the receptor pore, thus confirming previous findings that catestatin can regulate nicotinic signal transduction by occluding the receptor pore. The other outcome of these studies is the identification of a new high affinity binding site on the β subunit near the membrane surface. These findings indicate that there are potentially different modes of action for the peptide than those proposed previously, and that the presence of a lipid bilayer may be an important factor in peptide folding before alternative binding. The characterization of association kinetics and identification of specific amino acids involved in the interaction are of particular value for the planning of experimental strategies aimed at the uncovering of the molecular mechanisms of chromogranin A/nAChR interactions. Alternatively, they may assist in the design of catestatin mimetic syntheses strategies to control several disorders related to nAChR receptors.

Notes and references

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