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Analysis of Binding Centers in Nicotinic Receptors with the Aid of Synthetic Peptides

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Abstract—We studies the receptor-binding specificity of the synthetic peptide HAP (High Affinity Peptide) and its analogues, which are regarded as a model of the orthosteric site nicotinic acetylcholine receptors (nAChR). Using radioligand analysis, electrophysiology tests, and calcium imaging, we assessed the ability of HAP to interact with nAChR antagonists: long α-neurotoxins and α-conotoxins. A high affinity of HAP for α-bungarotoxin and the absence of its interaction with α-cobratoxin and α-conotoxins was found. The synthesized analogues of HAP in general retained the properties of the original peptide. Thus, HAP cannot be a model of a ligand-binding site.

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Earlier, neurotoxins were used to isolate receptor from natural sources. Today, they remain important pharmacological tools in studies of the mechanisms of interaction of ligands with receptors. α-Neurotoxins (α-NT) from snake venom helped to isolate the nicotinic acetylcholine receptor (nAChR) from the electric organ of the ray and currently serve as highly sensitive markers of different receptor subtypes [1, 2]. The interest to the synthetic fragments of α-NT and nAChR is determined by the fact that the fragment of the central loop C of α-subunit, comprising residues Cys192–Cys193, is a part of the ligand-binding sites in all nAChRs. Synthetic peptides derived from this region bind to radioiodine-labeled α-bungarotoxin (α-Bt), though with a lower affinity than in the case of the whole nAChR [3–5]. The binding of the synthetic fragment of nAChR to the whole α-NT is considered as a simplified model of toxin interaction with the native receptor, which gave practical results: marker nucleotide sequences were inserted into the genes encoding different receptors (e.g., γ-aminobutyric acid ionotropic receptors), which made it possible to detect these receptors in cells using fluorescent toxins [6].

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α-NT Have a three-finger fold [2]. Their central loop is very important for the binding to nAChR; on the structural basis of this loop, its synthetic fragments exhibiting high affinity in binding to nAChR were obtained [7]. Currently, much attention is given to the elatively short neurotoxic peptides rather than to proteins. This primarily refers to the α-conotoxins from the venoms of marine mollusks *Conus*. They are used not only as tools for identifying nAChR subtypes but also as potential drugs [8, 9].

Among the synthetic fragments of toxins or receptors, the record-holder was the 13-mer peptide WRYYESSLLPYPD, which was derived from the combinatorial peptide library and is highly homologous to a fragment of loop *C* (two vicinal serine residues occupy the position Cys192–Cys193). This peptide bound α-Bt with the same high affinity that nAChR (2 nM) and was called HAP (High Affinity Peptide) [10]. In the crystalline structure of the complex with α-Bt [11], HAP has a conformation generally similar to loop *C* in the crystalline structure of the acetylcholine-binding protein (AChP) [12], which is a standard structural model of the ligand-binding domain of nAChR. Thus, HAP can be considered as a possible model of the orthosteric site of nAChR, in which agonists (acetylcholine) and competitive antagonists such as α-Bt or α-conotoxins are bound. Since the authors of [10, 11] used only α-Bt, we decided to test whether HAP can also interact with other antagonists. Testing this hypothesis was the subject of this study.

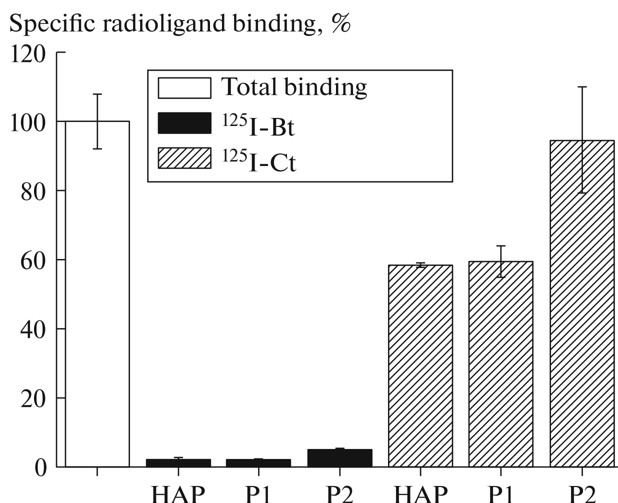

Fig. 1. Inhibition of the specific binding of ^{125}I-Bt and ^{125}I-Ct to *T. californica* nAChR by HAP and peptides P1 and P2. The total binding of each radioligand to nAChR in the absence of HAP was taken as 100%. Here and in Figs. 3, 4, data are represented as $M \pm m$, number of measurements $n = 4$.

The high-affinity peptide, its analogues, and α-conotoxins were obtained by solid-phase peptide synthesis. Their purity and structure were confirmed by HPLC and mass spectrometry.

The binding of HAP to long α-neurotoxins or α-conotoxin was detected by the radioligand assay. *T. californica* membranes or GH4C1 cells (1.25 or 1 nM of toxin-binding sites, respectively) were incubated with 10 or 30 μM peptides when using ^{125}I-Bt or ^{125}I-α-cobratoxin (Ct) or with 120 μM peptides when using α-conotoxin ^{125}I-PnIA [L5R, A10L, D14R] at 20°C for 3 h in 20 mM Tris-HCl buffer (pH 7.9) containing 1 mg/mL bovine serum albumin, after which 0.2–0.4 nM appropriate radioligand was added, and the mixture was incubated for another 5 min. The binding was terminated by filtration and washing through GF/C filters. The level of nonspecific binding was determined in the presence of 200-fold excess of α-Ct or PnIA [L5R, A10L, D14R] (PnIA[RLR]).

Electrophysiological experiments with two-electrode voltage-clump procedure were performed with the oocytes that were surgically obtained from adult female *Xenopus laevis*. Injections of plasmid DNA with inserted genes of muscular nAChR subunits and subsequent measurements of currents were performed as described in [13]. The solutions containing HAP and α-Bt were mixed 10–15 min before application on oocyte.

Calcium imaging tests were performed on Neuro2a mouse neuroblastoma cells expressing human muscular nAChR and genetically encoded calcium sensor Case12. Cells were incubated with 2 μM α-conotoxin GI or MI in the presence or absence of 20 μM HAP. The changes in the intracellular calcium content in response to application of 10 μM acetylcholine were evaluated by the Case12 emission in the green region of the spectrum (516 nm).

Previously, the binding of HAP to α-Bt was detected by its ability to inhibit the binding of biotinylated α-Bt to muscular nAChR subtype of *T. californica* [10]. In our study, we have confirmed this effect by competitive radioligand assay. Preincubation of 10 μM HAP with ^{125}I-α-Bt completely inhibited its binding to *T. californica* nAChR (Fig. 1).

Since the competitive radioligand analysis does not allow excluding another type of interaction of HAP with α-Bt, which does not prevent the binding of the latter to nAChR, we performed electrophysiological experiments in *Xenopus laevis* oocytes expressing mouse muscular nAChR. The preincubation of oocytes with 20 μM HAP did not affect the ion current induced by 10 μM acetylcholine (ACh) (Fig. 2). After the incubation of oocytes with ^{125}I-Bt, the value of ion currents decreased twice; however, the preincubation of α-Bt with HAP abolished the inhibition of nAChR response to ACh (Fig. 2). Thus, we have shown that HAP itself does not modify the effect of the agonist ACh and confirmed the binding of HAP to α-Bt.

We tested the ability of HAP to interact with α-conotoxin using a radioligand assay. High-affinity protein (120 μM) was preincubated with ^{125}I-α-conotoxin-PnIA[RLR], which interacts with the α7 subunit of human nAChR, expressed in GH4C1 cells [14]. In the presence of HAP, the binding of ^{125}I-PnIA[RLR] to nAChR α7 did not decrease; conversely, the radioactivity adsorbed on the filters slight increased (Fig. 3).

Due to the fact that the radioligand assay showed a high nonspecific binding, we performed calcium imaging tests using α-conotoxins GI and MI, specific muscular nAChR antagonists. Both α-conotoxins completely blocked the responses of cells expressing the muscular nAChR to the application of 10 μM ACh. The presence of 20 μM HAP in the reaction medium did not prevent this inhibition of in response to the ACh application (Fig. 4); i.e., HAP did not bind to α-conotoxins.

Since HAP bound only to α-Bt among all compounds studied, we tested its interaction with α-cobratoxin, which blocks α7 and muscular AChR with nearly the same affinity as α-Bt. Figure 1 shows that, in contrast to ^{125}I-Bt, when preincubation with 10 μM HAP completely inhibited the binding to nAChR, the preincubation of ^{125}I-Ct with 30 μM HAP led to the inhibition of the binding of this toxin to nAChR by at most 40%.

The observed significant difference in the binding of HAP to ^{125}I-Bt and ^{125}I-Ct seemed unusual due to the close affinity of these α-NTs to α7 and muscular

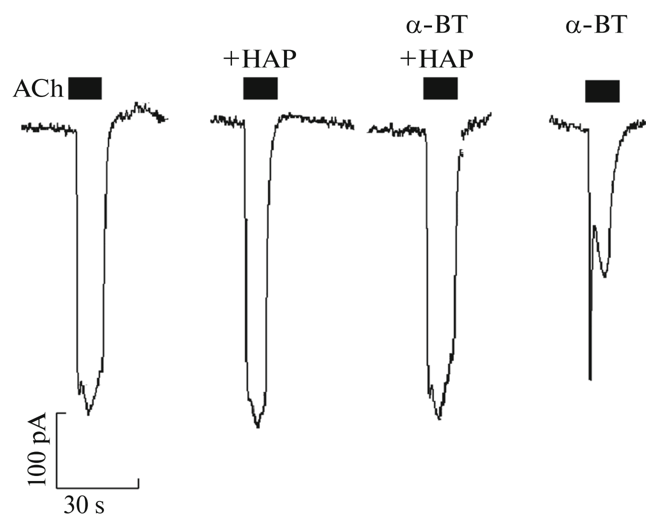

Fig. 2. Responses of oocytes expressing muscular nAChR. Ionic currents in response to application of 10 μM ACh, ACh after preincubation with 20 μM HAP, ACh after preincubation with a mixture of 20 μM HAP and 2 μM α-Bt, and after 5-min incubation with 2 μM α-Bt.

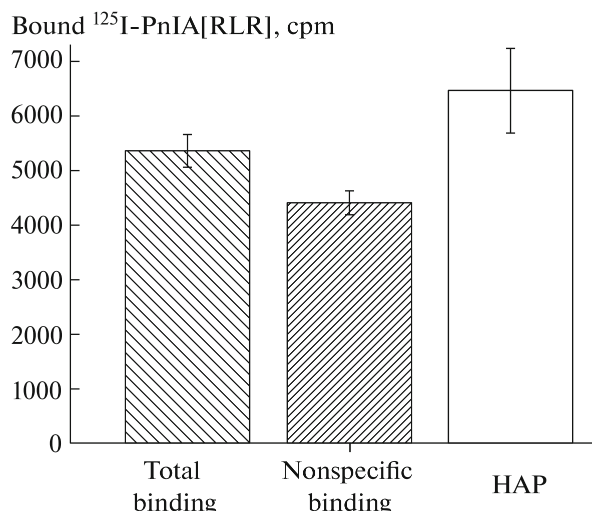

Fig. 3. Effect of HAP on the specific binding of ^{125}I-PnIA[RLR] to α7 nAChR of GH4C1 human cells. The nonspecific binding was determined in the presence of 200-fold excess of PnIA[RLR].

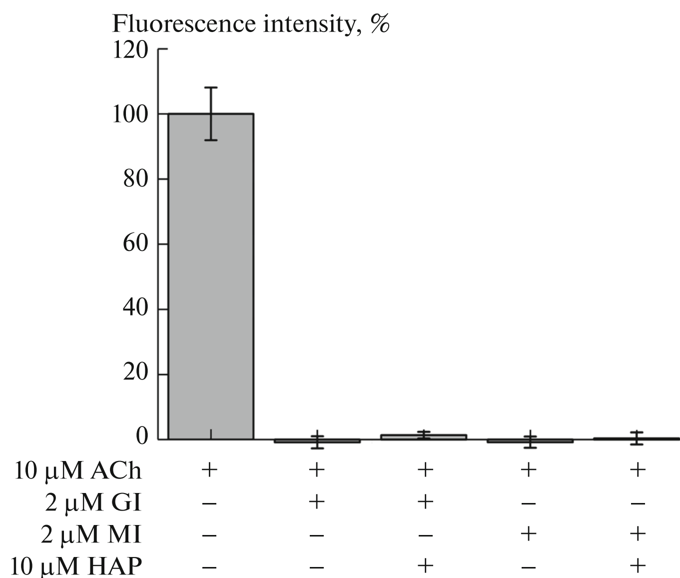

Fig. 4. Calcium imaging on Neuro2a cells. The fluorescence intensity of Case 12 in response to the addition of ACh, ACh with GI, ACh with GI and HAP, ACh with MI, and ACh with MI and HAP to the incubation medium. The fluorescence intensity level in response to 10 μM ACh was taken as 100%.

nAChR and the similarity of the crystalline structures of their complexes [2, 15]. Basing on the crystalline structure of the complex of HAP with α-Bt [11], we docked α-Ct to HAP and analyzed the molecular dynamics. The energy of this complex was substantially lower than that of the complex of HAP with α-Bt. The analysis pointed out the possible amino acid substitutions in HAP that might increase the affinity for α-Ct: R2V2 and E5S5 (synthesized peptides P1 and P2). Peptides P1 and P2 almost did not differ from HAP in the interaction with α-Bt (Fig. 1). In terms of binding to ^{125}I-Ct, analogue P1 was also almost identical to HAP, whereas analogue P2 was inactive. In other words, we have not yet been able to explain such significant differences in the interaction of these related α-neurotoxins with HAP.

Thus, α-Bt and α-Ct with a very similar affinity bind to the muscular and neuronal α7 AChR but vary

greatly in their interaction with a short peptide HAP, which is homologous to a fragment of the active site of AChR. The use of the α-Bt-HAP crystalline structure for computer simulation did not allow us to explain the marked differences in the effectiveness of their binding. New computer methods are required to pass from X-ray diffraction analysis of models (e.g., α-Bt-HAP) to the creation of ligands with high affinity and selectivity. For example, the recently proposed protein topography method allowed constructing several new analogues for α-conotoxin PnIA, interacting with AHR α7 with a high affinity [14].

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