Mimotopes of the Nicotinic Receptor Binding Site Selected by a Combinatorial Peptide Library†

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ABSTRACT: Peptide libraries allow selecting new molecules, defined as mimotopes, which are able to mimic the structural and functional features of a native protein. This technology can be applied for the development of new reagents, which can interfere with the action of specific ligands on their target receptors. In the present study we used a combinatorial library approach to produce synthetic peptides mimicking the snake neurotoxin binding site of nicotinic receptors. On the basis of amino acid sequence comparison of different α-bungarotoxin binding receptors, we designed a 14 amino acid combinatorial synthetic peptide library with five invariant, four partially variant, and five totally variant positions. Peptides were synthesized using SPOT synthesis on cellulose membranes, and binding sequences were selected using biotinylated α-bungarotoxin. Each variant position was systematically identified, and all possible combinations of the best reacting amino acids in each variant position were tested. The best reactive sequences were identified, produced in soluble form, and tested in BIACORE to compare their kinetic constants. We identified several different peptides that can inhibit the binding of α-bungarotoxin to both muscle and neuronal nicotinic receptors. Peptide mimotopes have a toxin-binding affinity that is considerably higher than peptides reproducing native receptor sequences.

Nicotinic acetylcholine receptors (nAchR)[1] belong to the superfamily of ligand-gated ion channels (*1*, *2*). They are integral membrane proteins resulting from the association of five homologous subunits. Muscle nicotinic acetylcholine receptor is the most extensively characterized member of the family. It is composed of four different subunits which assemble in the stoichiometry 2α, *β*, *γ* or *ϵ*, and *δ* to delineate a central ionic channel whose gating is induced by acetylcholine binding to specific sites located at the interfaces between α−*γ* and α−*δ* subunits (see refs *3* and *4* for reviews).

Members of the nicotinic receptor family are widely distributed in the central nervous system where they play a significant role in important functions such as learning and memory (*5*, *6*). Several different genes encoding α (α2 to α9) and *β* (*β*2 to *β*4) subunits of neuronal nicotinic receptors have been identified (reviewed in ref *7*). In the central nervous system, acetylcholine-binding α subunits can be assembled in a number of combinations with structural *β* subunits giving rise to nicotinic receptors with different functional and pharmacological properties (*8*).

Both muscle and neuronal nicotinic receptors have a number of specific ligands which compete with acetylcholine for binding and can modulate receptor functions acting as agonists or antagonists.

Chemical modification of specific receptor residues and site-directed mutagenesis have been used to localize receptor residues involved in binding of agonist and antagonist molecules (reviewed in ref *9*). From these studies, a sequence of the α subunit close to cysteine residues 192 and 193 has been determined as containing at least part of the nAchR ligand binding site.

Snake venom neurotoxins have been very useful tools for the localization of nAchR binding sites since they bind with extremely high affinity to nicotinic receptors and compete with acetylcholine and other nicotinic ligands (*10*, *11*). Among snake neurotoxins, α-bungarotoxin (α-bgt) not only binds with an affinity in the picomolar range to muscle nicotinic receptors but also selectively binds with a similar affinity to a subpopulation of central nervous system nicotinic receptors containing the α7 subunit (*5*−*7*). α-bgt binding neuronal receptors seem to be involved in the regulation of excitatory transmission in the brain (*12*−*14*) and to induce neuroprotective activity upon activation in different in vivo and in vitro experimental systems (*15*, *16*).

Previous studies have demonstrated that synthetic peptides reproducing the regions close to cysteines 192 and 193 of muscle and neuronal α7 nAchR retain receptor ability to bind α-bgt, even if with a much lower affinity (*9*, *17*−*21*).

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[1] Abbreviations: α-bgt, α-bungarotoxin; BCIP, bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DIC, diisopropylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fmoc, fluorenylmethoxycarbonyl; HBS, Hepes-buffered saline; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid cromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; OD, optical density; nAchR, nicotinic acetylcholine receptor; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; T-TBS, Tween−TBS.

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The goal of our study was to construct peptide mimics of the nAchR binding site which could be used as competitive inhibitors of ligand−receptor interaction. To obtain peptides with higher binding capacity than those reproducing native receptor sequences, we chose a synthetic peptide library approach.

Peptide libraries allow for selection of new molecules, defined as mimotopes, which are able to mimic the structural and functional features of a native protein (*22*, *23*). Both antigen−antibody and receptor−ligand recognition surfaces can be reproduced by mimotopes (*24*−*26*). This technology can thus be applied for the development of new reagents which could interfere with the action of specific ligands on their target receptors.

In previous studies, a nAchR binding site mimicking peptide that binds α-bgt and competes with the native receptor for binding was selected from a random phage-epitope library. The sequence of this randomly selected peptide is very similar to that of peptides reproducing the regions close to cysteines 192 and 193 of muscle and α7 neuronal nAchR (*27*).

In the present study we used a combinatorial synthetic peptide library to produce peptides mimicking the α-bgt binding site of nAchR. The advantage of this approach is to introduce a high number of amino acid combinations that could result in peptides with higher binding efficiency than those reproducing original sequences of receptor binding sites. Compared to the phage-epitope approach, combinatorial synthetic peptide libraries make it possible to drive and follow the progressive selection of the best reacting ligands, thus providing important information about critical positions and the best residue combinations.

We identified several different peptides which inhibit the binding of α-bgt to muscle and neuronal nAchR and bind α-bgt with an affinity that is considerably higher than peptides reproducing native receptor sequences.

EXPERIMENTAL PROCEDURES

Peptide Library Assembly. SPOT synthesis (*28*) was carried out on amino-PEG-cellulose membranes (300 nmol of NH_2/cm^2, ABIMED, Langerfeld, Germany) using a model ASP 222 automated SPOTsynthesizer (ABIMED) and diisopropylcarbodiimide (DIC)/*N*-hydroxybenzotriazole (HOBt) activation. In situ prepared 0.2 M HOBt esters of fluorenylmethoxycarbonyl- (Fmoc-) amino acid derivatives were used for the coupling reaction. Free amino functions on the spots were treated with a solution of bromophenol blue in dimethylformammide, which resulted in a blue staining that allowed for the visual monitoring of all synthesis steps.

Side chain protecting groups were as follows: *tert*-butyl ester for D and E, trityl for H, N, and Q, *tert*-butoxycarbonyl for K and W, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for R, and *tert*-butyl ether for S, T, and Y.

The introduction of randomized position X within the peptide sequence was achieved by applying 0.2 μL of a 40 mM equimolar mixture of the 19 L-amino acid HOBt esters (cysteine was omitted because of its tendency to oxidize). The Fmoc-amino acid mixture was activated in the same way as the other individual amino acid solutions, by HOBt/DIC addition. Spotting was repeated up to four times, after which the reaction was complete as judged by a color change of the spots from blue to yellow. After the final cycle, all of the peptides were N-terminally acetylated with 2% acetic anhydride. At the end of the synthesis, peptides were side chain deprotected using a mixture of trifluoroacetic acid/triisobutylsilane/water/dichloromethane (50/3/2/45).

Binding Assay. Cellulose-bound peptide libraries were soaked in ethanol to prevent hydrophobic interactions between the peptides. Nonspecific binding was blocked by incubating cellulose sheets overnight with 10 mL of 2% casein in Tris-buffered saline (50 mM Tris, 137 mM NaCl, 27 mM KCl, pH 7.0) containing 0.05% Tween 20 (T-TBS). The sheets were incubated overnight at 4 °C with 58 μg/10 mL biotinylated α-bungarotoxin (Molecular Probes) in T-TBS blocking buffer. Alkaline phosphatase-conjugated streptavidin (Bio-Rad) was then added at 1:3000 dilution in T-TBS blocking buffer for 1 h. Sheets were washed three times with T-TBS, and detection of α-bgt binding was achieved by incubating the sheets with bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) in substrate buffer (100 mM Tris, pH 8.9, 100 mM NaCl, 2 mM $MgCl_2$). Quantitative evaluation of the signal was obtained using a Umax Speedy II 2200 optical scanner.

Preparative Peptide Synthesis. Solid-phase synthesis was carried out on a MultiSynTech (Bochum, Germany) Syro automatic peptide synthesizer employing Fmoc chemistry with HOBt/DIC activation on a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamido-norleucyl-4-methylbenzhydrylamine resin (Rink amide MBHA resin). Side chain protections of the amino acids were as described for the SPOT synthesis. Peptides were cleaved from the resins and deprotected by treatment with trifluoroacetic acid containing ethanedithiol, water, triisobutylsilane, and anisole (93/2.5/2/1.5/1). After precipitation by ethylic ether, the crude peptides were purified by preparative HPLC using a Vydac C-18 column (25 cm × 1 cm, 10 μm) and characterized by amino acid analysis and mass spectrometry.

BIACORE. Biotinylated α-bgt diluted in Hepes-buffered saline (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% polysorbate 20, pH 7.4) (HBS) (Biacore AB, Uppsala, Sweden) was injected at 10 μg/mL over an SA sensor chip (Biacore AB) containing streptavidin covalently immobilized on the carboxymethylated dextran matrix. Peptides were diluted in HBS and injected over the α-bgt-SA matrix at a flow rate of 10 μL/min. For kinetic experiments, serial dilutions of each peptide (from 0.5 to 10 μg/mL) were injected in different cycles. Association and dissociation kinetic rate constants (k_{on} and k_{off}) and the equilibrium association constant K_A were calculated using the BIAevaluation 3.0 software (*29*).

For competition experiments, α-bgt at a concentration of 5 μg/mL, together with peptides ranging from 0.3 to 20 μg/mL, was injected over an immobilized biotinylated peptide.

Purification of nAchR from Torpedo Electric Organs and Neuroblastoma Cells. nAchR was extracted from electric organs of *Torpedo marmorata* by Triton X-100 and affinity-purified through a Sepharose−α-cobratoxin column as described by Lindstrom et al. (*30*).

Neuronal nicotinic receptors containing the α7 subunit were purified from the human neuroblastoma cell line IMR32. Cell membranes were extracted with 1% Thesit detergent, essentially following the procedure described by

Table 1: Design of the Peptide Combinatorial Library[a]

	187[b]	188	189	190	191	192	193	194	195	196	197	198	199	200
Torpedo marmorata α1	W	V	Y	Y	T	C	C	P	D	T	P	Y	L	D
human α1	S	V	T	Y	S	C	C	P	D	T	P	Y	L	D
mouse α1	W	V	F	Y	S	C	C	P	T	T	P	Y	L	D
rat α1	W	V	F	Y	S	C	C	P	N	T	P	Y	L	D
human α7	E	R	F	Y	E	C	C	K	E		P	Y	P	D
phage library peptide (27)	M	R	Y	Y	E	S	S	L	K	S		Y	P	D

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
combinatorial library	X	V R	Y F T	Y	E T S	S	S	X	X	X	X	Y	P L	D

[a] Sequence comparison of different α-bgt binding nAchR α subunits was used to design the combinatorial peptide library. The X indicates totally variant peptide positions where a mixture of 19 L-amino acids was introduced. [b] Receptor amino acid sequence numbering refers to the *Torpedo* nAchR α subunit.

Luther et al. (*31*). The detergent extract was then run through a Sepharose−α-cobratoxin column as for the *Torpedo* nAchR.

Competition ELISA. Microtiter plates (Falcon 3912, Becton Dickinson, Oxnan, CA) were coated overnight at 4 °C with purified muscle or neuronal nAchR (5 μg/mL in 0.05 M carbonate buffer, pH 9.6) and then blocked with 3% BSA in phosphate-buffered saline (PBS), pH 7.4, for 1 h at room temperature. α-bgt (1 μg/mL), together with peptides at concentrations ranging from 0.001 to 100 μg/mL, was then added and incubated for 1 h at room temperature. α-bgt binding to nAchR was revealed with peroxidase-conjugated streptavidin. Assays were performed in triplicate, and the half-maximal inhibition constant IC50 was calculated by nonlinear regression analysis of curves obtained by plotting OD values versus Log peptide concentration using the GraphPad Prism 3.02 software.

RESULTS

Design of the Combinatorial Synthetic Peptide Library. Conserved amino acids in the 187−200 region of α subunits from muscle and neuronal nAChR that share the ability to bind α-bgt were taken as the basis for the design of the combinatorial synthetic peptide library (Table 1).

We introduced two invariant residues in peptide library positions 6 and 7, replacing the conserved cysteines 192 and 193 by two serines, to avoid uncontrolled postsynthetic thiol oxidation. We also introduced as invariant residues Y190, Y198, and D200 (peptide positions 4, 12, and 14, respectively), which are conserved in all α-bgt binding nAchR subunits. Furthermore, the peptide library was designed to represent all combinations of the most represented residues in positions 188, 189, 191, and 199 (peptide positions 2, 3, 5, and 13, respectively). The remaining five positions of the peptide library were composed of equally distributed mixtures of the 19 L-amino acids (cysteine was omitted) and are indicated with an X.

Screening of the Combinatorial Synthetic Peptide Library. The four partially variant positions of the library were defined in the first screening (Figure 1a). Results using biotinylated α-bgt on cellulose-bound peptides revealed that residues giving the highest binding at positions 2, 3, 5, and 13 were arginine, tyrosine, glutamic acid, and proline, respectively.

Position 8 was defined by systematically introducing in that position each of the 19 L-amino acids, while the remaining totally variant positions (1, 9, 10, and 11) were occupied by amino acid mixtures. The following peptide mixtures proved to give the strongest α-bgt binding: X-R-Y-Y-E-S-S-**L**-X-X-X-Y-P-D, X-R-Y-Y-E-S-S-**V**-X-X-X-Y-P-D, X-R-Y-Y-E-S-S-**T**-X-X-X-Y-P-D, X-R-Y-Y-E-S-S-**P**-X-X-X-Y-P-D, and X-R-Y-Y-E-S-S-**E**-X-X-X-Y-P-D. To identify the remaining variant positions 1, 9, 10, and 11, a sublibrary was constructed where each position was systematically defined, while the remaining three were composed of the amino acid mixtures. This was repeated for the five amino acids which gave the strongest binding in position 8 (L, V, T, P, and E) (Figure 1b). When this positional scanning sublibrary was tested for biotinylated α-bgt binding, as a general rule, peptide mixtures with leucine and threonine at position 8 showed the highest binding. All pools with the five different residues at position 8 accepted mainly histidine at position 1, while the presence of the negatively charged aspartic or glutamic acid in this position drastically reduced binding. Position 9 appeared to be less selective, in that either D, E, or K can be accepted. Proline and alanine were clearly selected for position 10, with a preference for proline. The hydrophobic amino acids alanine and tryptophan gave a particularly high response at position 11.

For each position, we chose amino acids giving results above the average response plus standard deviation; peptide sequences resulting from all possible combinations of the selected amino acids were synthesized and tested in the last sublibrary (Table 2).

On the basis of the obtained results, five peptides giving the highest α-bgt binding were resynthesized by standard solid-phase synthesis in order to be used as soluble products. Binding of soluble peptides to α-bgt was then analyzed using BIACORE.

α-bgt Binding of Soluble Peptides. Identical concentrations of different peptides were injected over a BIACORE flow cell where biotinylated α-bgt had been previously captured via streptavidin. Under this condition, a ranking of peptide−toxin binding can be directly visualized (Figure 2). Peptides p6 and p7 bound α-bgt more efficiently than other peptides selected from the synthetic library. In particular, peptides p8 and p10, which were still selected as efficiently binding α-bgt when immobilized on cellulose, proved to be very weak binders in solution. It is worth noting that both peptides have a lysine in position 1. The presence of a positive charge at the N-terminus, associated with the negatively charged

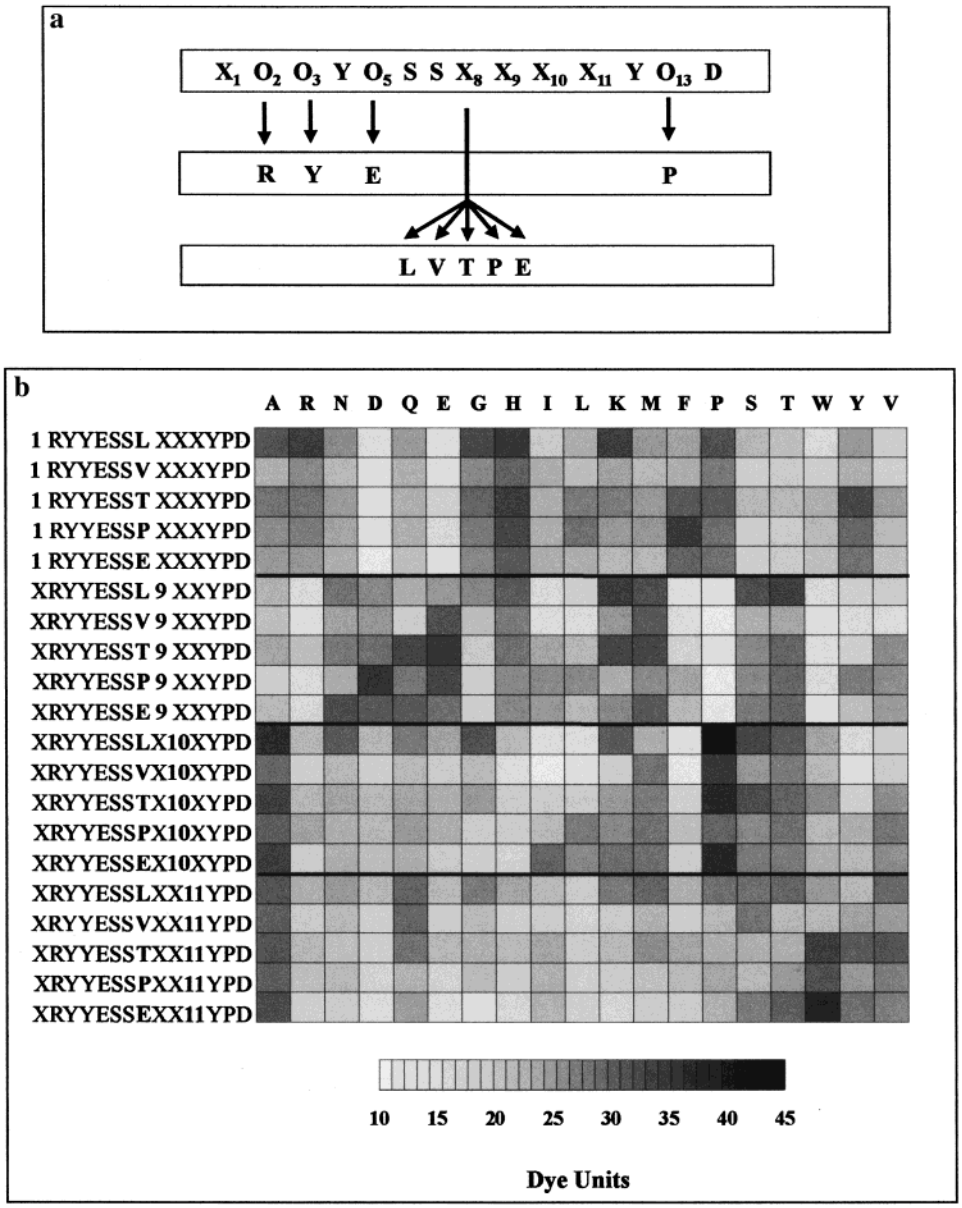

FIGURE 1: Definition of variant positions in the synthetic peptide combinatorial library. Partially variant positions 2, 3, 5, and 13 (indicated with O) were identified during the first screening of the combinatorial peptide library. Subsequent sublibraries were then constructed to define the totally variant positions X. The first X to be identified was position 8 (a). (b) Totally variant positions 1, 9, 10, and 11 were then systematically defined using each of the 19 natural L-amino acids (cysteine was excluded), while the remaining three were composed of the amino acid mixtures. This was repeated in association with each of the five amino acids which gave the strongest α-bgt binding in position 8 (L, V, T, P, and E). Dye units are directly proportional to the amount of dye precipitated on the spots as determined through calibration of the scanning device.

invariant aspartic acid at the C-terminus, might induce a different peptide conformation in solution compared with that obtained when the C-terminus is bound to the cellulose matrix. Peptides reproducing the 187−200 native sequence of different α subunits were tested in the same assay. All of the peptides reproducing receptor native sequences gave lower binding in respect to peptides selected from the library, with the human α7 187−200 and human muscle 187−200 giving the weakest binding.

Since results obtained by testing peptides in solution indicated p6 and p7 as the best α-bgt binding peptides, a number of amino acid substitutions were introduced in the peptide p6 sequence using a standard solid-phase technique. Peptides p6 and p7 differ only for the presence of a glutamic acid in the place of lysine in position 9, and p6 is better than p7 for α-bgt binding in solution. Therefore, glutamic acid in position 9 was also synthesized in association with leucine in position 8. This combination had not been synthesized in the last sublibrary (see Table 2) since it had not been selected among the best α-bgt binders. Moreover, since peptides with valine in position 11 proved to be weaker binders in solution compared with peptides having a tyrosine in the same position, we introduced the aromatic residue tryptophan in position 11 in association with leucine in position 8. Tryptophan had been in fact selected from the library in position 11 only when position 8 was occupied by T, P, or E. In conclusion, the following substitutions were introduced: T8/L (peptide p6.6), T8/L and Y11/W (peptide p6.7), T8/L and E9/K (peptide p6.8), T8/L, E9/K, and Y11/W (peptide p6.9), and Y11/W (peptide p6.3). Modified peptides were then tested in BIACORE as described above (Figure 3). All but peptide p6.3 proved to bind α-bgt more efficiently

Table 2: Construction and Results of the Final Peptide Sublibrary[a]

peptide mixture	selected amino acids in variant positions				no. of different peptides synthesized
	1	**9**	**10**	**11**	
1 R Y Y E S S ***L*** **9 10 11** Y P D	H	K	P	A	72
	R	T	A	V	
	K	M		T	
				Q	
1 R Y Y E S S ***V*** **9 10 11** Y P D	H	E	P	A	24
	R	M	A	Q	
	G				
1 R Y Y E S S ***T*** **9 10 11** Y P D	H	E	P	W	192
	F	Q	A	A	
	P	K	S	V	
	G	M		Y	
1 R Y Y E S S ***P*** **9 10 11** Y P D	F	D	A	W	24
	H	E	P	A	
		T			
1 R Y Y E S S ***E*** **9 10 11** Y P D	H	N	P	W	72
	F	Q	A	A	
		D	I	T	
		M			

[a] All combinations of the best reacting amino acids selected from the definition of the totally variant positions 1, 9, 10, and 11 for the five preselected amino acids in position 8 were synthesized in the last peptide sublibrary.

than peptide p6.

Peptide kinetic rate constants were calculated by running different concentrations of each peptide on biotinylated α-bgt in BIACORE; results are reported in Table 3. The modified peptides p6.6, p6.7, and p6.9 have a K_A one Log higher than p6, p7, and p9 and than the previously described phage library derived peptide (*27)* (Table 3). The K_A of peptide p6.7 is higher than that of all peptides reproducing receptor native sequences and is about 15 times higher than that of *Torpedo* 187−200 which, among peptides reproducing native receptor sequences, resulted in having the highest affinity for α-bgt.

Competition among Different Peptides for α-bgt Binding. Competition experiments were run in BIACORE in order to test whether different peptides were mutually competing for α-bgt binding. Biotinylated peptide p6.7 was immobilized on a SA sensor chip, and α-bgt was injected in solution in the presence of different peptides (Figure 4). We found that all synthetic peptides selected from the library competed with peptide p6.7, which confirms that they are all directed to the same toxin region. Synthetic peptides reproducing the homologous sequences of human muscle and human α7 receptors very weakly inhibited the binding of α-bgt to peptide p6.7, which is not surprising considering the difference in their α-bgt binding affinity. Inhibition of p6.7 binding to α-bgt by *Torpedo* 187−200 is very similar to that obtained with peptide p6, which is in agreement with their very similar K_A and IC50 (Table 3).

Competition between Peptides and Nicotinic Receptors for α-bgt Binding. The ability of receptor binding site mimicking synthetic peptides to inhibit the binding of α-bgt to nicotinic receptors was tested in ELISA. nAchR affinity purified from *Torpedo* electric organs and IMR32 neuroblastoma cells, respectively, was used in this assay. The polypeptide composition of affinity-purified receptors was analyzed by SDS−PAGE. Four polypeptide bands were detected in the receptor preparation from *Torpedo* electric organs whose molecular weight is compatible with that of the α, *β*, *γ*, and *δ* receptor subunits. In the case of IMR32 neuroblastoma cells, receptor affinity purification produces two bands of 58 and 54 kDa, respectively, which is in accordance to what is reported in a previous α7 subunit purification and characterization from IMR32 cells (*32*).

All tested peptides were observed to inhibit the binding of α-bgt to both *Torpedo* and α7 neuronal nAchR (Figure 5 and Table 3). The IC50 of each peptide was calculated in inhibition experiments using either the *Torpedo* or α7 neuronal nAchR. Peptides p6.6, p6.7, and p6.9 were more effective in inhibiting α-bgt binding than peptides p6 and p7, with a decrease in IC50 of about one Log, which corresponds to the difference in their K_A (Table 3). The IC50 of peptides p6.6, p6.7, and p6.9 is lower than that of peptides reproducing native α subunit sequences. The IC50 of peptide p6.7 is 30 times lower than that of the previously described phage library derived peptide (*27*) and 50 times lower than the IC50 of *Torpedo* 187−200.

DISCUSSION

We designed and constructed a combinatorial synthetic peptide library with the aim of selecting mimotopes of the α-bgt binding site of nicotinic receptors. Peptide mimotopes have already been reported as promising new drugs that can be able to interfere with either antigen−antibody or ligand−receptor interactions (*24*−*26*). A common technical approach to obtain mimotopes is the selection from phage epitope libraries. In this case, biologically active peptides can be obtained even with no information about the molecular basis of native interactions, and peptides that have no correlation with the primary structure of the mimicked protein can be theoretically selected (*33*).

The phage epitope approach has been already used to select peptide mimotopes of the nicotinic receptor binding site (*27*). Interestingly, in that case the selected active peptide had some sequence correlation with the region around critical cysteines 192 and 193 of α-bgt binding nicotinic receptor subunits and competed with peptides reproducing receptor native sequences. Nonetheless, selection from phage libraries has some possible disadvantages. First, no information can be obtained about amino acid combinations different from the finally selected sequences, while this could be useful to reconstruct the molecular basis of the interaction. Second, high-affinity peptides could be lost, since phage viability is not compatible with drastic elution conditions.

With the development of peptide synthesis techniques, combinatorial synthetic peptide libraries are becoming a feasible alternative approach to phage display of amino acid sequences. In particular, SPOT synthesis on cellulose membranes (*28*, *34*) has the advantage of allowing for the synthesis of hundreds of different peptides on the same sheet and for the selection of peptides on the same synthesis support. This overcomes problems connected to peptide solubility and to differences in adhesion of peptides to supports that might be used for the screening.

The combinatorial peptide library that we constructed was designed on the basis of sequence comparison among different α-bgt binding nicotinic receptor subunits and had five invariant, four partially variant, and five totally variant positions. We chose to limit the number of invariant positions with the aim of increasing possible amino acid combinations,

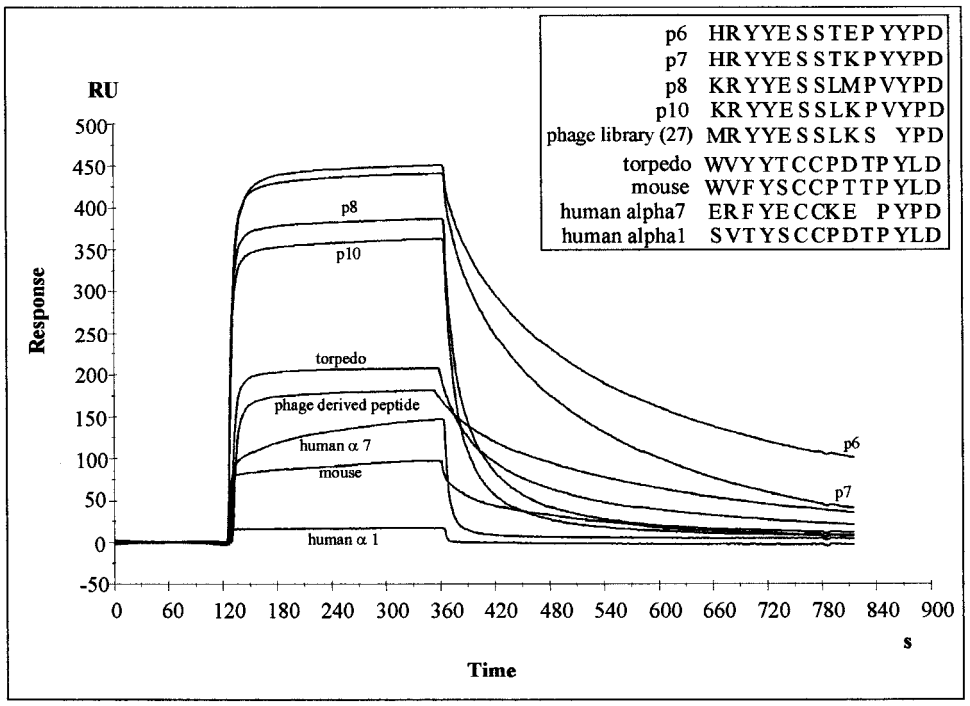

FIGURE 2: Binding to α-bgt of synthetic peptides. Peptides (10 μg/mL) were injected over a BIACORE SA sensor chip where biotinylated α-bgt had been previously captured via streptavidin. The flow rate was 10 μL/min. Regeneration of the matrix was achieved by injecting 25 mM NaOH at the end of each cycle. Peptide sequences are indicated in the inset.

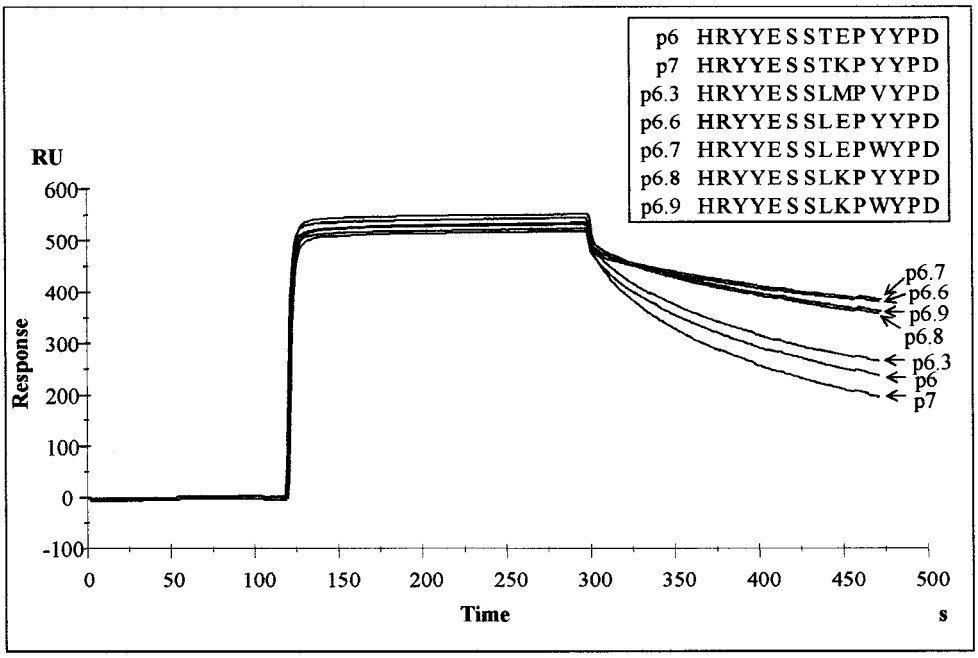

FIGURE 3: Binding to α-bgt of modified synthetic peptides. Amino acid substitutions were inserted in the peptide p6 sequence. Binding of substituted peptide to α-bgt was analyzed in BIACORE and compared to that of unmodified sequences. The flow rate was 10 μL/min. Regeneration of the matrix was achieved by injecting 25 mM NaOH at the end of each cycle. Peptide sequences are indicated in the inset.

thus increasing the possibility to select mimotopes that could better reproduce the receptor recognition surface. In this view, we also chose to concentrate most of the totally variant positions between residues 193 and 199. NMR solution structure of a synthetic peptide reproducing the sequence 185−196 of *Torpedo* nAchR in its complex with α-bgt indicated, in fact, that binding to α-bgt was mainly due to peptide N-terminal residues (*35*). This opens the possibility to construct an efficient mimotope by changing residues which seem to be less involved in the binding of the native peptide to α-bgt. This choice was also supported by data from chimeric analysis of α-bgt binding and nonbinding subunits which indicate that the single point mutation of K189 in the α-bgt insensitive α3 subunit with the correspondent Y of Torpedo α1 subunit is enough to induce significant sensitivity to α-bgt (*36*). Moreover, we inserted totally variant positions in place of T196 and P197 of the *Torpedo* receptor sequence. T196 is conserved in all muscle subunits but is lacking in α7 neuronal subunits, which still bind α-bgt. P197 is instead invariant in all muscle and

Table 3: α-bgt Binding Affinity and Competition with Muscle and Neuronal nAchR of nAchR Binding Site Mimicking Peptides[a]

peptide	sequence	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_A (M^{-1})	IC50 *Torpedo* AchR (M)	IC50 α7 AchR (M)
p6	HRYYESSTEPYYPD	1.13×10^4	2.68×10^{-3}	4.22×10^6	6.4×10^{-6}	8.95×10^{-7}
p7	HRYYESSTKPYYPD	8.79×10^3	4.64×10^{-3}	1.9×10^6	5.4×10^{-6}	1.07×10^{-6}
p9	HRYYESSTEPVYPD	6.94×10^3	6.81×10^{-3}	1.02×10^6	7.4×10^{-5}	nd
p6.6	HRYYESSLEPYYPD	3.59×10^4	7.59×10^{-4}	4.73×10^7	6.1×10^{-7}	4.6×10^{-7}
p6.7	HRYYESSLEPWYPD	4.31×10^4	7.33×10^{-4}	5.88×10^7	1.22×10^{-7}	1.99×10^{-7}
p6.9	HRYYESSLKPWYPD	5.3×10^4	8.93×10^{-4}	5.93×10^7	8.5×10^{-7}	2.94×10^{-7}
Torpedo	WVYYTCCPDTPYLD	1×10^4	2×10^{-3}	4×10^6	6.18×10^{-6}	nd
mouse	WVFYSCCPTTPYLD	4×10^3	5×10^{-3}	8.1×10^5	1.45×10^{-5}	nd
human α1	SVTYSCCPDTPYLD	0.019×10^3	6.52×10^{-3}	2.91×10^3	1.2×10^{-3}	nd
human α7	ERFYECCKE-PYPD	0.287×10^3	6.37×10^{-3}	4.5×10^4	2×10^{-4}	nd
phage library peptide (27)	MRYYESSLKS-YPD	5.69×10^3	1.92×10^{-3}	2.96×10^6	3.86×10^{-6}	nd

[a] Kinetic rates and K_A were calculated in BIACORE using the BIAevaluation 3.0 software. IC50 was calculated from ELISA competition experiments using affinity-purified nAchR from *Torpedo* electric organs or IMR32 neuroblastoma cells.

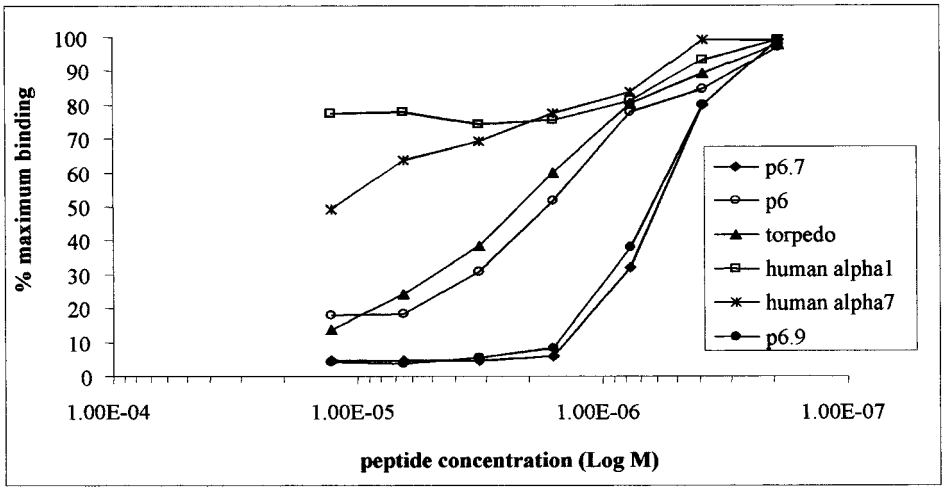

FIGURE 4: Competition among different synthetic peptides for α-bgt binding. Biotinylated peptide p6.7 was immobilized on an SA sensor chip. α-bgt at a concentration of 5 μg/mL, together with peptides ranging from 0.3 to 20 μg/mL, was then injected at a flow rate of 10 μL/min. Results are reported as percentage of the maximum binding obtained by injecting α-bgt in the absence of competing peptides.

neuronal α-bgt binding subunits. Nonetheless, the choice to introduce a totally variant position in place of P197 was justified by the attempt to avoid rigidity in peptide structure, which might derive from two close prolines in the sequence. Cysteines 192 and 193 were substituted with two invariant serines. This choice was dictated by the necessity to avoid postsynthetic oxidation. The importance of the disulfide bridge between C192 and C193 in α-bgt binding synthetic peptides is debated since contrasting results have been described (*19*, *37*−*40*). Nonetheless, results obtained with peptides selected from a random phage library (*27*) indicate that peptides with two serines in positions 192 and 193 can efficiently bind α-bgt.

We first defined the amino acids giving the best results in partially variant positions and then proceeded by defining each totally variant position. At the end of the positional scanning, we could identify amino acids giving the best results in terms of peptide−α-bgt binding for each variant position. All possible combinations of the best reacting amino acids were then synthesized and tested again in the last sublibrary. Five selected peptides from this sublibrary were synthesized again in a soluble form, and their α-bgt binding affinities were compared by BIACORE. We found that results from screening of cellulose-bound peptides did not completely correspond to those obtained with soluble peptides. In particular, the presence of two opposite charges at the peptide N- and C-terminus seemed to interfere with soluble peptide activity. As a consequence, a number of amino acid substitutions were introduced in the sequence of the highest affinity peptide, and modified peptides were synthesized and tested again in a soluble form. Some amino acids, which had been excluded during the last steps of SPOT synthesis since they were not included among those giving the highest α-bgt binding, were reintroduced in the sequence. All but one of the modified peptides showed an affinity constant at least one Log higher than peptides which had been directly selected from SPOT synthesis.

All selected peptides were able to inhibit the binding of α-bgt to nicotinic receptors, both muscular and neuronal, with an IC50 correlating with their affinity constants.

Our peptide mimotopes have some significant differences in their sequences compared to native receptors. In particular, amino acids in positions 1, 10, and 11 cannot be found in any corresponding sequence from either muscle or neuronal α-bgt binding receptors. Nonetheless, all selected mimotopes proved to be more efficient in α-bgt binding than peptides reproducing native receptor sequences. Peptides reproducing the 187−200 sequence from different α-bgt binding nicotinic receptor subunits were different in their α-bgt binding affinity and IC50. Torpedo 187−200 proved to be the most efficient with a K_A of 4×10^6 M and an IC50 of 6 μM, which are very similar to those reported for the *Torpedo* 181−200 (*19*)

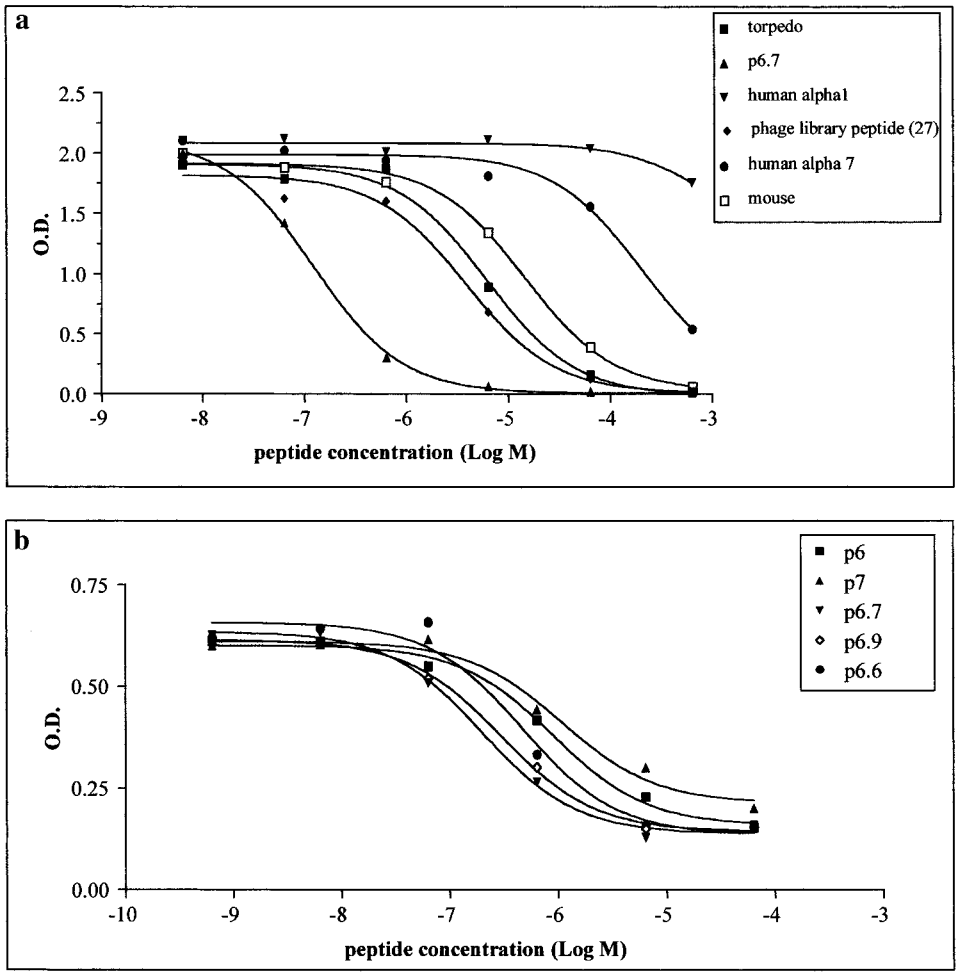

FIGURE 5: Inhibition of α-bgt binding to *Torpedo* and neuronal α7 nicotinic receptors by synthetic peptides. EIA plates were coated with 5 μg/mL affinity-purified nAchR from *Torpedo* electric organs (a) or from IMR32 human neuroblastoma cells (b). Biotinylated α-bgt (1 μg/mL), together with peptides at concentrations ranging from 0.001 to 100 μg/mL, was then added to the wells. Binding was revealed by peroxidase-conjugated streptavidin. Assays were performed in triplicate, and the half-maximal inhibition constant IC50 was calculated by nonlinear regression analysis of curves obtained by plotting OD values versus Log peptide concentration using the GraphPad Prism 3.02 software.

and 181−198 (*41*) synthetic peptides and close to the K_A and IC50 of the phage library derived peptide described in ref *27*. Conversely, the human muscle α187−200 synthetic peptide resulted as very weakly binding α-bgt, and the K_A of this peptide is at the lower limit of the BIACORE detection sensitivity. This is in agreement with results already reported for synthetic peptides reproducing the binding site sequence of human muscle α subunit (*17*, *42*).

Peptide mimotopes p6.6, p6.7, and p6.9 have an affinity constant and an IC50 at least 10 times better than the *Torpedo* 187−200 peptide. Some possible explanations for the poor affinity of peptides reproducing the nicotinic receptor 187−200 sequence from different α subunits can be derived from the results of our combinatorial synthetic peptide library. Peptides reproducing *Torpedo* and mouse muscle α subunits have a W at their N-terminal position, while human muscle and human α7 receptor sequences have respectively an S and an E. The library screening revealed that W, S, and E give extremely low α-bgt binding when inserted in library position 1.

In conclusion, we constructed peptide mimotopes of the α-bgt binding site of nicotinic receptors which can efficiently inhibit the binding of the snake neurotoxin to both muscle and neuronal receptors. The selected peptide mimotopes are more efficient than native receptor sequences in α-bgt binding. This result was achieved by combining a selection from a cellulose-bound synthetic peptide library with BIACORE analysis, which allowed the optimization of peptide activity in solution.

The fact that peptide mimotopes can be more efficient binders than the corresponding native sequence is not too surprising considering that amino acid substitutions can result in a better mimicry of the conformational structure of a binding site, which could not be reproduced by the native sequence when this is cut "out" of the protein three-dimensional structure. Some data indicating that modified peptides reproducing the nicotinic receptor binding site sequence could result in higher α-bgt binding have already been reported in the case of the human muscle α 185−200 sequence, where substitution of S191 with A produced a peptide which was more efficient than the corresponding native sequence in inhibiting α-bgt binding to the receptor in solution (*40*).

Our results indicate that mimotopes of receptor binding sites, able to reproduce the recognition surface better than native sequences, can be selected from peptide libraries. Large peptide libraries can thus be a source of receptor-competing molecules which can be obtained even in the lack

of information on the three-dimensional structure of the receptor.

It should be noted that our combinatorial peptide library could be tested with different nicotinic receptor agonists and antagonists in order to select potential specific competitors of the receptor binding site. Since receptor binding sites are often targets of different ligands which can produce opposite effects on receptor activity, the possibility to produce "artificial" binding sites with selected specificity for a predetermined receptor ligand can have important therapeutic applications. At least in the case of nAchR, binding of different molecules does not always involve the same receptor residues inside the acetylcholine binding site (*20*, *21*). This opens the possibility to select high-affinity mimotopes of the receptor binding site which could be specific for only one predetermined receptor ligand.

During the revision of the present paper, the production of a peptide mimotope that binds α-bgt with high affinity has been described (*43*). The IC50 of the *Torpedo* 187−200 synthetic peptide reported in ref *43* is 2.6×10^{-8} M, which is about 200 times lower than the value we find for the same peptide. It should be noted that, although the ELISA inhibition test used in ref *43* is very similar to ours, in their case all α-bgt binding peptides are incubated with α-bgt prior to addition to receptor-coated wells. This preincubation can easily determine a lower value of peptide IC50.

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