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Identification of a New Component of the Agonist Binding Site of the Nicotinic α7 Homooligomeric Receptor*

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Tryptophan 54 of the α7 neuronal nicotinic homooligomeric receptor is homologous to γ-Trp-55 and δ-Trp-57 of non-α subunits of *Torpedo* receptor labeled by *d*-tubocurarine. This residue was mutated on the α7-V201–5-hydroxytryptamine ($5HT)_3$ homooligomeric chimera, which displays α7 nicotinic pharmacology, and for which both equilibrium binding studies and electrophysiological recordings could be carried out in parallel. Replacement of Trp-54 by a Phe, Ala, or His causes a progressive decrease both in binding affinity and in responses (EC_{50} or IC_{50}) for acetylcholine, nicotine, and dihydro-β-erythroidine, without significant modification in α-Bgtx binding. Except for Gln-56, comparatively small effects are observed when the other residues of the 52–58 region are mutated into alanine. These data support the participation of Trp-54 to ligand binding, and provide evidence for a new "complementary component" of the α7 nicotinic binding site, distinct from its three-loop "principal component," and homologous to the "non-α component" present on γ and δ subunits.

Binding of ACh[1] to nicotinic acetylcholine receptors causes channel opening and receptor desensitization. Affinity labeling and expression experiments support the notion that the two ACh binding sites of $\alpha_2\beta\gamma\delta$ muscle-type *Torpedo* receptor result from the association of specific domains of the α subunits (referred to as *principal component* of the site) with regions of the non-α subunits (referred to as *complementary component* of the site) (1–6). This notion has been extended to neuronal nicotinic receptors, whose pharmacology varies with their α and β subunit composition (7–9).

The principal component of the nicotinic binding site of muscle and neuronal α subunits comprises three separate regions from the large N-terminal domain: loop A (Trp-86, Tyr-93), loop B (Trp-149), and loop C (Tyr-190, Cys-192, Cys-193, and Tyr-198) (numbering according to the *Torpedo* receptor) (10). Some evidence supports the participation of several residues of the γ and δ subunits of the *Torpedo* receptor to the complementary binding component (5, 11–13). In particular, sequencing experiments have shown that *d*-tubocurarine labeling of non-α *Torpedo* receptor subunits occurs on the homologous γ-Trp-55 and δ-Trp-57 residues (3, 11). Moreover, the γW55L mutation results in a 8-fold decrease in ACh apparent affinity and *d*-tubocurarine inhibitory potency from electrophysiological recordings in the *Xenopus* oocytes (14).

Neuronal nicotinic receptor subunits α7, α8, and α9 assemble as functional homooligomers in the *Xenopus* oocytes (15–18). Under these conditions, the nicotinic binding sites form from neighboring identical subunits, implying that each subunit potentially contains both the principal component (which includes loops A, B, and C as demonstrated for α7 receptor; Ref. 19) and the complementary component. In this work, we investigate this second aspect of α7 contribution and mutate Trp-54, which is homologous to the γ-Trp-55 and δ-Trp-57 of *Torpedo* receptor.

To combine biochemical and electrophysiological approaches, we used the α7-V201–$5HT_3$ chimera (referred to as wild-type) (20), which is efficiently transiently expressed in HEK 293 cells whereas α7 is not. While the chimera appears somewhat "artificial" receptor, it possesses the extracellular domain of α7. This confers to this construct a pharmacological profile of activation nearly identical to that of α7 in the *Xenopus* oocytes (20), supporting that the agonist binding site of both receptors are structurally homologous. Here, the pharmacological binding properties of the wild-type and mutant chimeras are characterized by electrophysiological recording in the *Xenopus* oocytes and parallel binding measurements in HEK 293 cells.

MATERIALS AND METHODS

Mutants were obtained as described previously (19) and subcloned in pMT_3 vector for expression (21). Electrophysiological recordings in the *Xenopus* oocytes were performed as described previously (22).

Chimeric cDNAs were transfected into HEK 293 cells by calcium phosphate precipitation (23). The level of expression was typically 5–20 fmol of ^{125}I-α-Bgtx (Amersham Corp.) binding sites/cm^2 of confluent cells. Receptor membranes were prepared as described previously (24).

^{125}I-α-Bgtx binding measurements were performed at 18 °C. For ^{125}I-α-Bgtx association experiments, receptor membranes (0.2–2 pmol of ^{125}I-α-Bgtx binding sites) were diluted in 250 μl of HEPES buffer (10 mM HEPES, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 82.5 mM NaCl, pH 7.2). ^{125}I-α-Bgtx was added, and, after a selected time, the sample was quickly diluted in 5 ml of phosphate-buffered saline buffer, filtered through GF-C filter (Whatman), rinsed with 5 ml of phosphate-buffered saline buffer, and counted. Nonspecific binding was determined in the presence of 1 mM L-nicotine. Association experiments in the presence of competitors were performed by preincubating the diluted membranes with the ligands for at least 10 min, and then measuring the initial rate of toxin binding after 5 min incubation with 2.5 nM ^{125}I-α-Bgtx. We verified that 3 μM nicotine equilibrated with the wild-type chimera under the present conditions in less than 1 min (data not shown). ACh was always used in combination with 0.1 mM eserine. For ^{125}I-α-Bgtx dissociation experiments, 2 ml (1–5 pmol) of diluted receptor membranes were preincubated with 5 nM ^{125}I-α-Bgtx for 30 min. 1 μM of

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[1] The abbreviations used are: ACh, acetylcholine; 5HT, 5-hydroxytryptamine; HEK 293 cells, human embryonic kidney 293 cells; α-Bgtx, α-bungarotoxin; DHβE, dihydro-β-erythroidine; GABA, γ-aminobutyric acid.

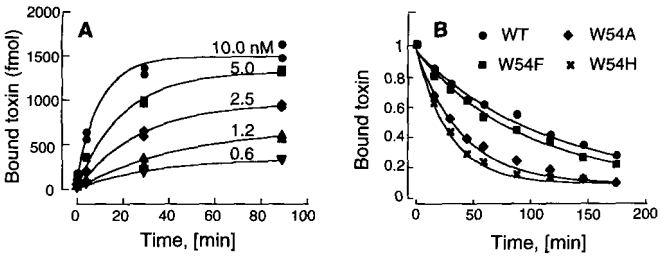

FIG. 1. *A*, time course of specific ^{125}I-α-Bgtx binding to the wild-type α7-V201–5HT$_3$ chimera; the *solid lines* are the best fits obtained with the theoretical exponential. *B*, time course of dissociation of ^{125}I-α-Bgtx with the wild-type and mutant α7-V201–5HT$_3$ chimeras. For each construct, data from three separate experiments were normalized to maximum values and then averaged. The *solid lines* are the best fits obtained with the theoretical single exponential.

unlabeled α-Bgtx was then added, and 200 μl of the mixture was filtered every 15 min to measure the decay of bound labeled ^{125}I-α-Bgtx. Nonspecific binding was measured by preincubating membranes with 1 μM unlabeled α-Bgtx.

RESULTS

Kinetics and Equilibrium Binding in Vitro of α-Bgtx and Nicotinic Ligands—Since the kinetics of α-Bgtx association and dissociation to the α7 receptor are slow, an accurate measurement of the dissociation constant of nicotinic ligands using ^{125}I-α-Bgtx as a labeled probe requires kinetic measurements. We thus used the methodology described by Weber and Changeux (25, 26) to analyze the binding of [^{3}H]α-Bgtx to membranes from *Torpedo* electric organs. The time courses of association and dissociation of ^{125}I-α-Bgtx with the wild-type chimera expressed in HEK 293 cells can be fitted by single exponentials (Fig. 1). This is consistent with a bimolecular mechanism of binding to an homogeneous class of sites, giving the rate constants and K_d values of ^{125}I-α-Bgtx (Table I). As shown in Fig. 2, ACh, nicotine, and DHβE decrease the initial rates of ^{125}I-α-Bgtx binding on membranes expressing the wild-type chimera. Fitting the competition data by the Hill equation yielded protection constants K_p (with which *Torpedo* ACh receptor-rich membranes were shown to represent actual dissociation constants; Ref. 26) and Hill coefficients. The wild-type chimera displays high affinity (1.1 nM) for ^{125}I-α-Bgtx and micromolar affinities for ACh (K_p = 83 μM), nicotine (K_p = 3.6 μM) and DHβE (K_p = 2.5 μM) (Tables I and II). These values are close to the K_i values measured in binding experiments on α7 homomeric receptors expressed in the *Xenopus* oocytes for α-Bgtx (1.6 nM), ACh (25 μM), and nicotine (0.5 μM) (27) confirming that the wild-type chimera exhibits a pharmacology closely related, if not identical, to that of α7.

Effect of Mutations at Position 54 on the Pharmacological Response to Nicotinic Effectors and on Ligand Binding in Vitro—To investigate the role of the Trp side chain at position 54, we constructed mutants W54F, W54A and W54H. The time courses of the currents evoked by ACh and nicotine on oocytes expressing each mutants were similar to those observed for the wild-type chimera (Fig. 3) (20). However, although both W54F and W54A mutations yielded a 4–6-fold increase in ACh EC$_{50}$, introduction of a histidine at this position caused a 190-fold increase (Fig. 2, *D–F*). In contrast, W54F mutation had no significant effect on nicotine elicited currents, while the W54A and W54H mutations resulted, respectively, in 10- and 50-fold increases in nicotine EC$_{50}$. All mutations caused a comparable 10–20-fold increase in the IC$_{50}$ of the competitive antagonist DHβE. Fig. 3 also shows that the currents elicited by saturating concentrations of ACh and nicotine were identical with W54A and W54F, whereas in W54H ACh-evoked currents were only about 30% of the nicotine-evoked currents.

TABLE I
Kinetic constants and calculated dissociation constants of ^{125}Iα-Bgtx for α7-V201–5HT$_3$ wild-type and mutant chimeras
All values are shown as the mean and standard deviation from triplicate experiments.

	K_{on}	K_{off}	K_d
	$min^{-1}nM^{-1}$	min^{-1}	nM
Wild-type	$(8.2 \pm 3.0)10^{-3}$	$(7.5 \pm 1.6)10^{-3}$	1.1 ± 0.6
W54F	$(10.3 \pm 3.0)10^{-3}$	$(10.0 \pm 2.7)10^{-3}$	1.0 ± 0.5
W54A	$(14.3 \pm 3.5)10^{-3}$	$(20.0 \pm 7.0)10^{-3}$	1.5 ± 0.7
W54H	$(11.0 \pm 1.0)10^{-3}$	$(26.0 \pm 7.2)10^{-3}$	2.4 ± 0.9

All constructs yielded ^{125}I-α-Bgtx binding sites when expressed in HEK 293 cells. Mutations of Trp-54 did not change the k_{on} of ^{125}I-α-Bgtx, and only slightly increased the k_{off} of ^{125}I-α-Bgtx in the case of the W54A and W54F (2- and 3-fold increase, respectively) (Table I). The mutations caused increases in the K_p of ACh, nicotine, and DHβE, with no significant changes of Hill coefficients (Fig. 2, *A–C*). The increases in K_p values for the three ligands were close to the increases in apparent dissociation constants observed in electrophysiological recordings (Table II). For nicotine, the 1-, 10-, and 60-fold shifts in K_p are almost identical to the 1.5-, 10-, and 50-fold shifts in EC$_{50}$, for mutants W54F, W54A, and W54H, respectively. For ACh, the shifts in K_p (3.5-, 10-, and 44-fold) correlate reasonably well with the shifts in EC$_{50}$ (4-, 6-, and 190-fold). All mutations caused similar increases in DHβE K_p (3–10-fold) and IC$_{50}$ (10–20-fold). W54H mutation also resulted in a 10-fold increase in *d*-tubocurarine K_p (data not shown).

Involvement of Residues at Positions 52–59 in the Agonist Binding Site—We explored the possible contribution of amino acids neighboring Trp-54 by mutating those present from position 52 to 59 into alanines (Fig. 4). These mutants yielded ^{125}I-α-Bgtx binding sites, except in the case of the mutation of the canonical residue Trp-59, with which no toxin binding nor electrophysiological response were observed. This feature can result from either a poor expression of the receptor or a disruption of the nicotinic binding site. The N52A, I53A, and L55A mutants appeared identical to the wild-type chimera when tested for ACh and nicotine inhibition of ^{125}I-α-Bgtx binding (data not shown). The M57A and Y58A mutations caused small although significant effects on receptor pharmacology, and the Q56A mutation caused a 3- and 20-fold increase in K_p and a 8- and 7- fold increase in EC$_{50}$, for ACh and nicotine, respectively (Table II).

DISCUSSION

Mutation of Trp-54 causes parallel increases in binding affinity (K_p) and in apparent affinity for channel activation (EC$_{50}$ or IC$_{50}$) for ACh, nicotine, and DHβE. This is compatible with the simple notion that mutations of Trp-54 specifically alter the nicotinic binding site. In this respect, the different efficacies observed for ACh and nicotine for the W54H mutant can be interpreted either by a channel block of ACh, since the recordings were performed in the presence of a high concentration of agonist, or by the fact that the mutation affects to a different extent the affinity for one of these agonists in the resting and active states of the receptor. In contrast, the kinetics and equilibrium dissociation constants of ^{125}I-α-Bgtx were not significantly altered, showing that modifying the side chain of Trp-54 did not disrupt the α-Bgtx binding site and thus the general tertiary and quaternary structure of the related part of the pentamer. Altogether, these mutagenesis results are consistent with a local alteration of the ACh binding domain. Moreover, the labeling of *Torpedo* γ-Trp-55 and δ-Trp-57 by *d*-tubocurarine supports the notion that Trp-54 contributes directly to ligand binding. Consistent with this view, we find

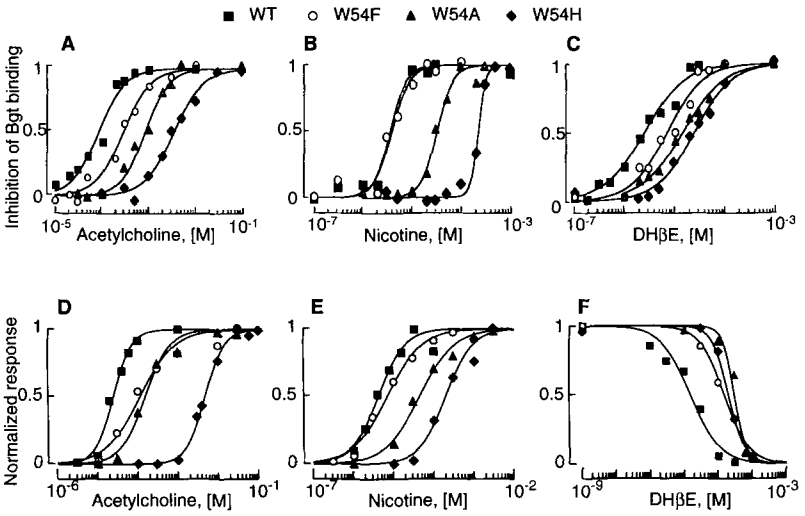

FIG. 2. *A–C*, effect of ACh, nicotine, and DHβE on the initial rate of ^{125}I-α-Bgtx binding to the α7-V201–5HT$_3$ wild-type and mutant chimeras. Effect of these ligands are expressed as their capacity to slow down the initial rate of specific ^{125}I-α-Bgtx binding normalized to the maximum values. Each point represents the mean value of three separate experiments. *D–F*, ACh and nicotine dose-response relationship, and DHβE inhibition of ACh-evoked responses, of α7-V201–5HT$_3$ wild-type and mutant chimeras. Responses evoked by 3-s application of increasing concentrations of ACh and nicotine were measured in 3–8 cells from several batches held at −100 mV. In DHβE experiments, several (3–7) oocytes were perfused with half-maximally effective dose of ACh and increasing concentrations of DHβE. Peak currents are normalized to the maximum values and averaged. In all cases, lines are the best fits obtained with the empirical Hill equation.

TABLE II

Affinity constants of α7-V201-5HT$_3$ wild-type and mutant chimeras

The normalized currents and binding data are determined as described in Fig. 2. K_p, EC_{50}, and IC_{50} are presented as the mean and standard deviation. ND, not determined.

	ACh				Nicotine				DHβE			
	K_p	n_H	EC_{50}	n_H	K_p	n_H	EC_{50}	n_H	K_p	n_H	IC_{50}	n_H
	μM		μM		μM		μM		μM		μM	
WT	83 ± 12	1.6	25 ± 6.7	2	3.6 ± 0.3	2.0	4.3 ± 0.7	1.3	2.5 ± 0.3	1.1	1.7 ± 1.0	1
W54F	280 ± 25	1.5	110 ± 18	1	3.8 ± 0.4	1.9	7.7 ± 1.9	1	7.0 ± 1.2	1.2	15 ± 5.4	1.2
W54A	840 ± 85	1.7	160 ± 35	1.5	32 ± 3.3	1.9	45 ± 10	1	14 ± 1.2	1.0	34 ± 0.5	2.5
W54H	3500 ± 350	1.4	4700 ± 470	1.8	220 ± 64	2.1	200 ± 11	1.4	24 ± 1.6	1.2	21 ± 2.5	2
Q56A	230 ± 42	3.4	200 ± 10	1.4	72 ± 3.5	1.8	30 ± 0.6	1.4	ND	ND	ND	ND
M57A	27 ± 7.2	2.4	6.4 ± 0.3	2	4.5 ± 0.8	1.8	2.1 ± 0.3	1.5	ND	ND	ND	ND
Y58A	180 ± 18	1.6	25 ± 0.5	2	15 ± 0.7	1.9	8.3 ± 0.7	1.5	ND	ND	ND	ND

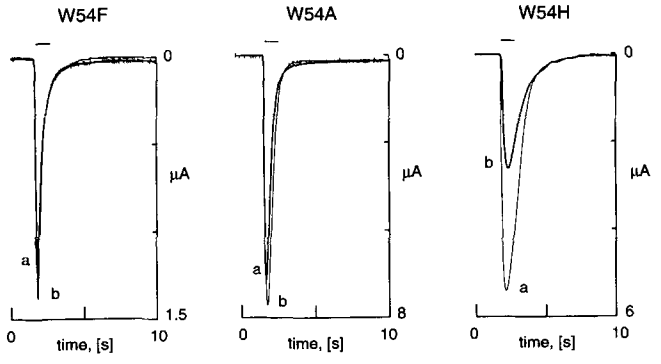

FIG. 3. **Current evoked by saturating concentrations of nicotine (3 mM, *trace a*) and ACh (30 mM, *trace b*) in the *Xenopus* oocytes expressing α7-V201–5HT$_3$ W54F, W54A, and W54H chimeras.**

		50		54	56		61
α7	chicken	T	T	N	I	W	L	Q	M	Y	W	T	D
α1	Torpedo	E	T	N	V	R	L	R	Q	Q	W	I	D
β1	Torpedo	T	T	N	V	F	L	N	L	A	W	T	D
γ1	Torpedo	T	T	N	V	W	I	E	I	Q	W	N	D
δ1	Torpedo	T	S	N	V	W	M	D	H	A	W	Y	D
α2	rat	T	T	N	V	W	L	K	Q	E	W	N	D
α3	rat	E	T	N	L	W	L	K	Q	I	W	N	D
α4	rat	T	T	N	V	W	V	K	Q	E	W	H	D
β2	rat	T	T	N	V	W	L	T	Q	E	W	E	D
β4	rat	T	T	S	I	W	L	K	Q	E	W	T	D
α1GABA$_A$	rat	T	I	D	V	F	F	R	Q	S	W	K	D
5HT3	rat	T	T	Y	I	W	Y	R	Q	Y	W	T	D
α1Glycine	rat	R	V	N	I	F	L	R	Q	Q	W	N	D

FIG. 4. **Comparison of the amino acid sequences of some nicotinic, GABA$_A$, 5HT$_3$, and glycine receptors (reviewed in Ref. 31) containing Trp-54 of chicken α7 nicotinic receptor (15).**

that the presence of electronegative aromatic residues (Trp and Phe as compared to Ala) favors agonist binding, while the introduction of a positive charge with a histidine causes a dramatic decrease in agonist affinity, suggesting a direct involvement of Trp-54 in the quaternary ammonium binding pocket of nicotinic ligands.

It is noteworthy that this residue is highly conserved in all subunits expected to contribute a complementary binding component: in muscle γ and δ subunits and neuronal β2, β4, α8, and α9 subunits. However, it is absent in the corresponding regions of muscle α and β subunits (Fig. 4). A tryptophan, however, is present at a homologous position in the neuronal α2–4 subunits, which are not expected to contribute a complementary component. A similar feature is seen for residues composing loops A and B, which, while implicated in the principal binding component of muscle and neuronal receptors, are also conserved in β2 and β4 subunits. This observation may point to a particular function of these neuronal subunits.

Mutations of residue Gln-56, and to a smaller extent that of residues Met-57 and Tyr-58, affect the apparent affinities of ACh and nicotine. These positions are occupied by different residues in *Torpedo* γ and δ subunits, and in β neuronal subunits (Fig. 4). This suggests that amino acids located at these positions could be involved in the non-equivalence of the two *Torpedo* receptor binding sites (13, 28) and/or in the pharmacological diversity of neuronal receptors distinct from the non-α subunits of muscle receptor.

Labeling and mutagenesis experiments have shown that residue Phe-65 of the α1 subunit of GABA$_A$ receptors contributes to the binding site of GABA (29, 30). This residue is homologous

to α7Trp-54, and α subunits of $GABA_A$ receptors appear to be functionally homologous to nicotinic non-α subunits (10). The notion that residues homologous to α7Trp-54 contribute to a complementary component of the agonist binding site in addition to its principal one may thus be extended to other members of the superfamily of ligand-gated ion channels.

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