Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 12088–12093, October 1998
Chemistry, Neurobiology

From *ab initio* quantum mechanics to molecular neurobiology: A cation–π binding site in the nicotinic receptor

(nicotinic acetylcholine receptor/cation–π interaction/unnatural amino acids)

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Communicated by John Abelson, California Institute of Technology, Pasadena, CA, August 6, 1998 (received for review June 3, 1998)

ABSTRACT **The nicotinic acetylcholine receptor is the prototype ligand-gated ion channel. A number of aromatic amino acids have been identified as contributing to the agonist binding site, suggesting that cation–π interactions may be involved in binding the quaternary ammonium group of the agonist, acetylcholine. Here we show a compelling correlation between: (*i*) *ab initio* quantum mechanical predictions of cation–π binding abilities and (*ii*) EC_{50} values for acetylcholine at the receptor for a series of tryptophan derivatives that were incorporated into the receptor by using the *in vivo* nonsense-suppression method for unnatural amino acid incorporation. Such a correlation is seen at one, and only one, of the aromatic residues—tryptophan-149 of the α subunit. This finding indicates that, on binding, the cationic, quaternary ammonium group of acetylcholine makes van der Waals contact with the indole side chain of α tryptophan-149, providing the most precise structural information to date on this receptor. Consistent with this model, a tethered quaternary ammonium group emanating from position α149 produces a constitutively active receptor.**

The nicotinic acetylcholine receptor (nAChR) is the longest known, best studied neuroreceptor (1, 2). It defines a superfamily that also includes receptors for serotonin, γ-aminobutyric acid, and glycine, as well as an invertebrate glutamate-gated channel. The nAChR functions at the vertebrate neuromuscular junction and in the central nervous system, and nicotine binding to the nAChR represents the initial chemical step in nicotine addiction. The receptor comprises five homologous subunits, arranged in a pentagonal array around a central pore that is presumed to be the ion channel (3). The embryonic muscle form of the receptor has a subunit stoichiometry of $\alpha_2\beta\gamma\delta$, and much work has established an important role for the α subunits in defining the agonist binding site. Pioneering photoaffinity labeling studies by Changeux identified a number of residues that are near the agonist binding site, and, surprisingly, all such residues are aromatic—Tyr or Trp (1). Subsequent studies confirmed important functional roles for many of these aromatics (4–6). An elegant crosslinking study by Karlin implicated an anionic residue on the γ/δ subunits that also influences agonist binding (7). These findings are summarized in Fig. 1.

The large number of aromatic amino acids at the agonist binding site of the nAChR led to the proposal that cation–π interactions may be important in binding acetylcholine (ACh) (8). The cation–π interaction is a general noncovalent binding force, in which the face of an aromatic ring provides a region of negative electrostatic potential that can bind cations with considerable strength (9, 10). In a biological context, it is the aromatic side chains of Phe, Tyr, and Trp that can be expected to be involved in cation–π interactions, and a large body of evidence indicates a prominent role for cation–π interactions in structural biology in general (9, 11). The physical underpinnings of the cation–π interaction are now well understood, so that one can use *ab initio* quantum mechanical calculations on small model systems to predict with some confidence how modifications of an aromatic system should affect its cation–π binding ability (12, 13). This knowledge, combined with extensions to site-directed mutagenesis allowing for *in vivo* unnatural amino acid incorporation (14–19) allows the systematic evaluation of potential cation–π binding sites. Previously, we have used unnatural amino acid mutagenesis to evaluate three conserved tyrosines at the agonist binding site (Fig. 1) (14, 20), with no results that suggested a cation–π interaction. However, theoretical studies of the cation–π interaction (12) and surveys of protein structures (10) clearly indicate that, of the natural amino acids, Trp presents the most potent cation–π binding site. We now report studies of four different tryptophans implicated in ACh binding (Fig. 1). At one, and only one, site—αTrp-149—EC_{50} correlates strongly

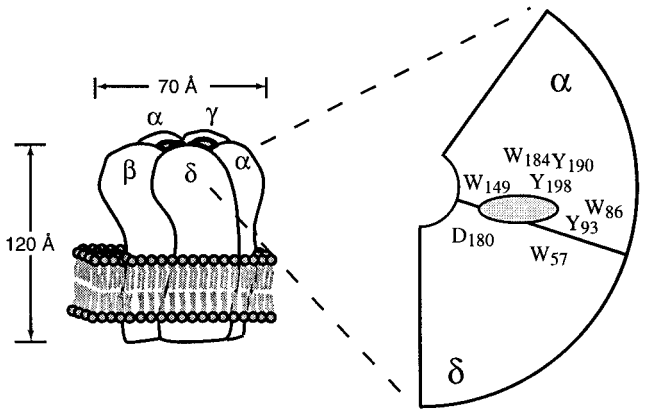

FIG. 1. Structural features of the nAChR. (*Left*) Global layout, adapted from data by Unwin (3), but with alternative ordering of subunits around the central axis (2, 20). (*Right*) Schematic of the agonist binding site (gray oval) viewed from the synapse, showing the large number of aromatic residues from three noncontiguous regions of the α subunit and key residues from the δ subunit also thought to contribute to binding. A comparable arrangement exists at the α/γ interface. Adapted from drawings previously presented by Galzi and Changeux (1) and Karlin and Akabas (2). Amino acid codes: W, tryptophan, Y, tyrosine, D, aspartate.

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Abbreviations: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; dTC, *d*-tubocurarine.

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with the cation–π binding ability of the aromatic side chain. We take this correlation as clear evidence that the α149 side chain is in van der Waals contact with the quaternary ammonium group of ACh in the bound state of the receptor. Consistent with this model, a tethered quaternary ammonium group emanating from position α149 produces a constitutively active receptor.

METHODS

General Procedures. Protocols for mutagenesis, mRNA preparation, aminoacyl-tRNA synthesis, oocyte injection, and electrophysiology are as described in several previous publications (14, 15, 20). The patch pipette and bath solutions for single channel recording were 115 mM NaCl/10 mM Hepes—NaOH/1 mM $CaCl_2$/2 mM KCl, pH 7.4. Patch recordings were conducted at room temperature 36 h after mRNA injection. Single-channel currents were filtered at 5 kHz and stored on a digital data recorder (48-kHz sampling rate, Neuro-corder DR-384, Neuro Data Instruments, New York). The data were played back, redigitized at 20 kHz, and filtered at 3 kHz.

Synthesis of Unnatural Amino Acids. Unnatural amino acids were either commercially available or were prepared by using a method developed by Roberts *et al.* (21). This method simplifies the synthesis of Trp analogs to the synthesis of the corresponding indole side chains. In most cases, indole synthesis followed standard procedures. Tetrafluoroindole was prepared by the method of Rajh (22). 5,7-Difluoroindole and 5,6,7-trifluoroindole were prepared by the reaction of CuI/dimethylformamide with the analogous 6-trimethylsilylacetylenylaniline [the latter prepared by reaction of the fluorinated aniline with trimethylsilylacetylene catalyzed by bis(triphenylphosphine)palladium dichloride and CuI (23)]. Nvoc-Tyr-O3Q (Nvoc, 6-nitroveratryloxycarbonyl) cyanomethyl ester was prepared by reaction of Nvoc-4-*O*-(3-iodopropyl)tyrosine cyanomethyl ester with trimethylamine. Further synthetic details are available in ref. 24. Structures of all the pdCpA-amino acid conjugates were confirmed by fast atom bombardment or electrospray ionization–MS analysis.

RESULTS AND DISCUSSION

Using nonsense codon suppression procedures and oocyte expression, we incorporated and evaluated electrophysiologically (14, 15) a series of Trp derivatives at four sites of the mouse muscle nAChR—α86, α149, α184, and γ55/δ57—all of which are naturally Trp and all of which have been implicated in defining the agonist binding site (Fig. 1). Fig. 2 shows Trp derivatives that have been incorporated by the suppression method at one or more of these sites. At all sites and for all derivatives, responses display hallmarks of functional nAChR molecules: desensitization with prolonged exposure to agonist, and dose-response relations with Hill coefficients between 1 and 2 (Fig. 3).

Strongly electron-withdrawing groups such as cyano (CN) and fluoro substantially weaken the cation–π interaction. Fluorine is generally considered to be a substituent that provides a negligible steric perturbation, and so direct comparison of fluorinated tryptophans with the wild type is justified. In considering the CN group, modeling shows that 5-Br-Trp and 5-CN-Trp are quite similar in size, but they differ considerably in cation–π binding ability, making this also a useful comparison. Such a comparison is greatly preferable to, for example, 5-CN-Trp vs. Trp, where both steric and electronic structure are changing.

At three sites—α86, α184, and γ55/δ57—4,5,6,7-F_4-Trp, 5-CN-Trp, and 5-Br-Trp gave EC_{50} values for ACh that were not largely different (<2-fold) from wild type (Table 1), ruling out a strong cation–π interaction at these sites. The results for 4,5,6,7-F_4-Trp support the notion that F does not provide a strong steric perturbation.

At αTrp-149, however, a very different pattern is seen (Table 2). 5-CN-Trp gives an EC_{50} that is very much greater than wild type and, importantly, much greater than 5-Br-Trp. To further establish that this is not a steric effect, we determined that 5-Me-Trp, which is sterically similar to 5-CN- and 5-Br-Trp but resembles wild-type Trp in a cation–π interaction, shows wild-type behavior (Table 2). Strongly electron-withdrawing groups such as CN or F could influence the hydrogen-bonding ability of the indole NH. However, any prominent role for such a hydrogen bond is ruled out by several mutants of Table 2 [N-Me-Trp, 1-Np-Ala, 2-Np-Ala, and benzothienylalanine (Np, naphthylalanine)]. Note that the EC_{50} for 5-CN-Trp is too large to be accurately measured directly, because at the ACh concentrations required to cover the dose–response relation, ACh itself becomes an effective

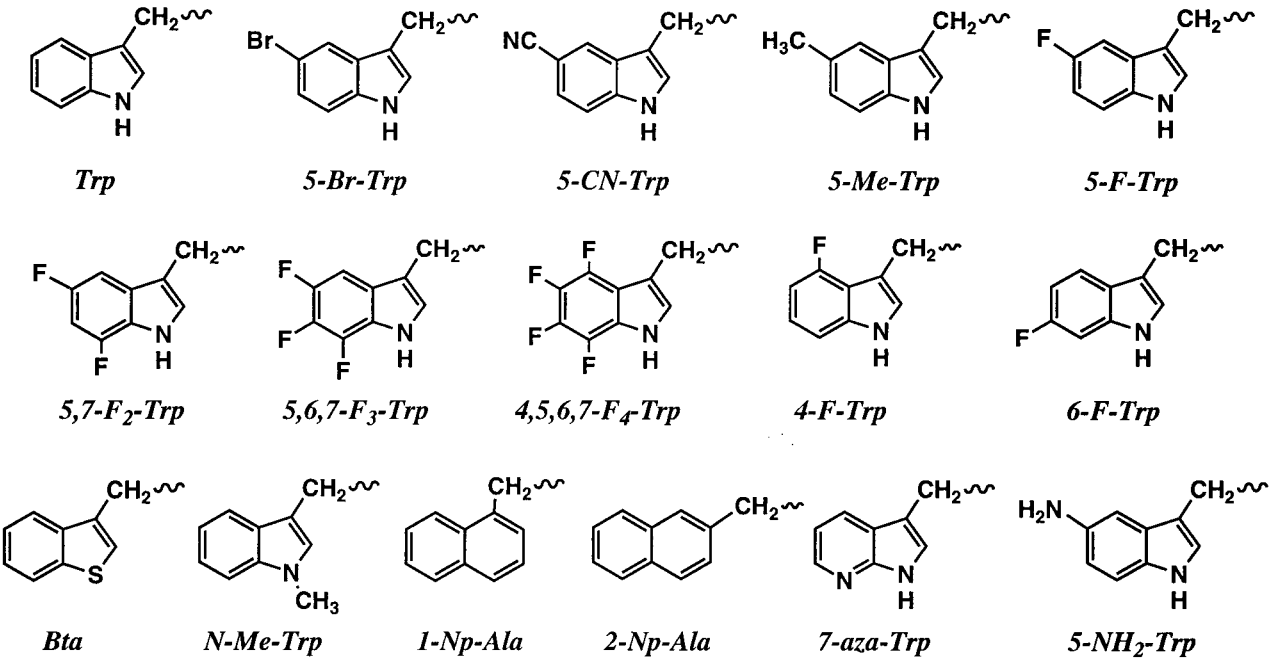

FIG. 2. Structures of Trp derivatives incorporated by the nonsense-suppression method. Bta, benzothienylalanine; Np-Ala, naphthylalanine.

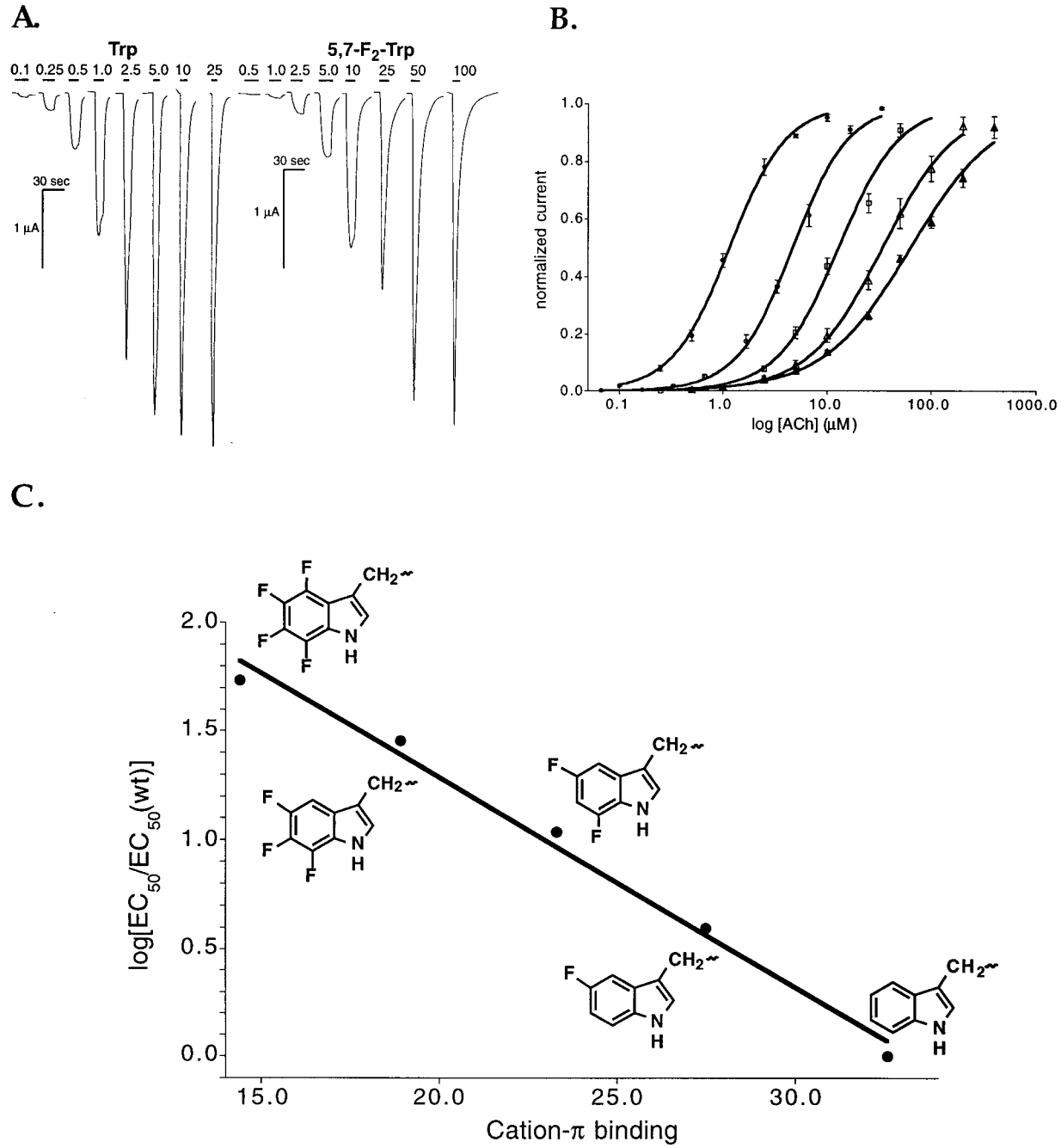

FIG. 3. (*A*) Representative voltage-clamp current traces from oocytes expressing Trp (wild type, *Left*) and 5,7-F_2-Trp (*Right*) at α149. Bars represent application of ACh (μM). (*B*) Dose–response relations and fits to the Hill equation for (left to right): Trp; 5-F-Trp; 5,7-F_2-Trp; 5,6,7-F_3-Trp; and 4,5,6,7-F_4-Trp. EC_{50} values are given in Table 2. (*C*) Plot of $\log[EC_{50}/EC_{50}(\text{wild type})]$ vs. quantitative cation–π binding ability at α149 for the same residues as in *B*. Data are from Table 2. The data fit the line $y = 3.2 - 0.096x$, with a correlation coefficient $r = 0.99$. Error bars are approximately the size of the markers.

channel blocker. As in previous work (20), we have rendered these relatively insensitive receptors more responsive by introducing a single Leu-9′ → Ser mutation in the M2 region of the β subunit (residue 262) (25, 26). This mutation lowers EC_{50} by a consistent factor of ≈40, but by itself does not activate receptors. The M2 9′ residue is thought to lie some 50 Å from the binding site, and for the muscle receptor, the increased sensitivity probably arises because the channel is open longer and opens more frequently (C. Labarca, G. N. Filatov, H. Zhang, J. Li, and H.A.L., unpublished work), rather than because a desensitized state becomes conducting, as occurs for the analogous mutation in the homomultimeric α7 receptor (27). It remains formally possible that there are subtle changes in the structure of the binding site because of incorporation of the 9′ mutation (H. Zhang, P. Deshpande, C. Labarca, H.A.L., and J. Li, unpublished work) (28). The validity of extending the measurable range of ACh sensitivities by using the βLeu-9′ → Ser mutation is supported by three entries in Table 2 (Trp, 5-Br-Trp, and 5-F-Trp), for which the ratios of EC_{50} values with and without the 9′ mutation are the same.

While the results with 5-CN-Trp were suggestive, the most intriguing data come from studies of the fluorinated Trp derivatives. Beginning with 5-F-Trp and moving on to di-, tri-, and tetrafluoro derivatives produces a series of closely related compounds with substantial electronic but minimal steric changes. Within such a series, the ever-present concerns that

Table 1. EC_{50} for selected Trp mutants at α86, α184, and $\gamma55/\delta57$, showing that all three sites are substantially insensitive to the electronic properties of the Trp side chain

	EC_{50},† μM		
Side chain*	α86	α184	$\gamma55/\delta57$‡
Trp	50	50	50
5-Br-Trp	53	48	23
5-CN-Trp	61	46	25
4,5,6,7-F_4-Trp	45	62	92

*See Fig. 2 for side-chain structures.
†Hill coefficients are all in the range 1.5–2.0.
‡For all studies of this Trp, mutations were introduced into both the γ55 and δ57 sites, and both mutant subunits were expressed, so both agonist binding sites have the mutant Trp.

site-directed mutagenesis has led to major structural reorganizations are minimized. This level of control over structural perturbations represents a substantial advantage of the unnatural amino acid method.

As shown in Table 2, progressive fluorination at position α149 leads to a systematic increase in EC_{50}. We have developed previously a quantitative measure of cation–π binding ability for simple ring systems. Briefly, the method (12, 13) involves *ab initio* quantum mechanical calculation of the binding energy for a Na^+ ion to the ring of interest. Fig. 3*C* shows that fluorination at position α149 produces a compelling correlation between EC_{50} and cation–π binding ability. Over a range of nearly two orders of magnitude in EC_{50}, a linear relationship is seen between $\log(EC_{50})$ and the quantitative cation–π binding ability of the side chain.

Table 2. EC_{50} and cation-π binding ability for various Trp mutants at position α149

Side chain*	Cation–π binding,† kcal/mol	EC_{50},‡§ μM	Corrected EC_{50},§¶ μM
Trp	32.6	1.2	50
5-F-Trp	27.5	4.7	200
5,7-F_2-Trp	23.3	13	(550)‖
5,6,7-F_3-Trp	18.9	34	(1400)‖
4,5,6,7-F_4-Trp	14.4	65	(2700)‖
5-Br-Trp	27.8	2.0	88
5-CN-Trp	21.5	114	(4750)‖
5-Me-Trp	33.4	—	49
4-F-Trp	27.9	—	56
6-F-Trp	27.4	—	48
1-Np-Ala	28.9	—	51
2-Np-Ala	28.9	—	82
7-aza-Trp	26.0	—	130
Bta	26.9	—	174
N-Me-Trp	33.7	—	95
5-NH_2-Trp	36.4		280

*See Fig. 2 for side-chain structures.
†The cation-π binding is defined as the negative of the binding energy (kcal/mol) of a generic probe cation (Na^+) to the appropriate aromatic ring using *ab initio* HF 6-31G** calculations with full geometry optimization, as described previously (12). Since the 6-31G** basis set is not available for Br, the 6-311G** basis set was used for 5-Br-Trp, and then the value was corrected based on a linear correlation ($r = 0.997$) between binding energies for a collection of ten aromatics determined with the two different basis sets. Calculations were performed with GAUSSIAN 94 (34) or SPARTAN (35).
‡For receptors with a βLeu-9′ → Ser mutation, see text.
§Hill coefficients are generally in the range 1.3–1.8, but for the relatively insensitive structures (5-CN-Trp and 4,5,6,7-F_4-Trp with βLeu-9′ → Ser mutations), values as low as 1.0 are observed.
¶For receptors that are otherwise wild type.
‖Obtained by multiplying the entries of the adjacent column by a constant factor of 42, to compensate for the βLeu-9′ → Ser mutation; see text.

Of course, EC_{50} is not a binding constant—it is a composite number reflecting both agonist binding and gating. By making the plot of Fig. 3*C*, we are not assuming that gating is unimportant; we are only assuming that variations in EC_{50} across this series of closely related structures occur primarily because of variations in agonist binding. This is a reasonable assumption, given the location of the residue. Similarly, the cation–π calculations ignore solvation issues and entropy effects and model ACh as a much simpler ion. The plot of Fig. 3*C* does not assume these effects are negligible, only that they are relatively constant across the series. On going from one point to the next in Fig. 3*C*, all that is changing is one H to F in a molecular weight 290,000 protein—it is hard to imagine, for example, that dramatic changes in ΔS would result from such a change. The high quality of the correlation suggests that we have isolated an important interaction in the receptor—a cation–π interaction at αTrp-149.

To the extent that EC_{50} can be considered as proportional to a binding constant in this particular situation, $\log(EC_{50})$ is the appropriate quantity to compare with the cation–π binding energy. The EC_{50} change on going from Trp to 4,5,6,7-F_4-Trp corresponds to $\geq$2 kcal/mol of binding energy. This is a substantial quantity for disruption (but not deletion) of a single noncovalent contact, and it requires that the ACh and the Trp side chain are interacting directly.

Table 2 presents EC_{50} values for all the derivatives of Fig. 2, and inclusion of these data produces a similar plot (correlation coefficient $r = 0.90$). Although the range spanned by the additional entries is not large, for most of the residues a decrease in cation–π binding implies an increase in EC_{50}. There are a few outliers, as expected for a much broader range of structural variation. The EC_{50} value for 5-NH_2-Trp is much higher than expected, and the differences among the various monofluoro tryptophans are not easily rationalized. These differences could indicate specific interactions in the receptor, and the 5-NH_2-Trp result in particular is under further investigation in relation to the possibility that the amine is protonated in the receptor.

We consider the data of Table 2 and Fig. 3*C* to provide convincing evidence that the quaternary ammonium group of ACh makes close contact with the side chain of αTrp-149 in the nAChR. Note that the maximum in cation–π binding occurs over the six-membered ring of Trp (9, 13), and the calculated cation–π binding energies of Table 2 and Fig. 3*C* involve such a geometry, so the quaternary ammonium of ACh must be positioned over the 6-ring of αTrp-149 (Fig. 4). Since the aromatic rings of Phe and Tyr align with the 5-ring of Trp — poorly positioned for a binding interaction — one might expect them to perform poorly at α149. Indeed, Changeux has found that an analogous Trp-to-Phe mutation in the α7 neuronal nAChR markedly increased EC_{50} (29), and we find that the same mutation at α149 in the muscle-type receptor increases EC_{50} roughly 100-fold.

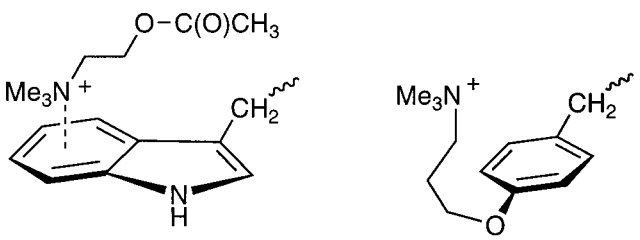

FIG. 4. (*Left*) Schematic of the quaternary ammonium of ACh positioned over the 6-ring of αTrp-149. (*Right*) Structure of Tyr-O3Q, showing that the tethered quaternary ammonium can be positioned in roughly the same location as the quaternary ammonium of ACh.

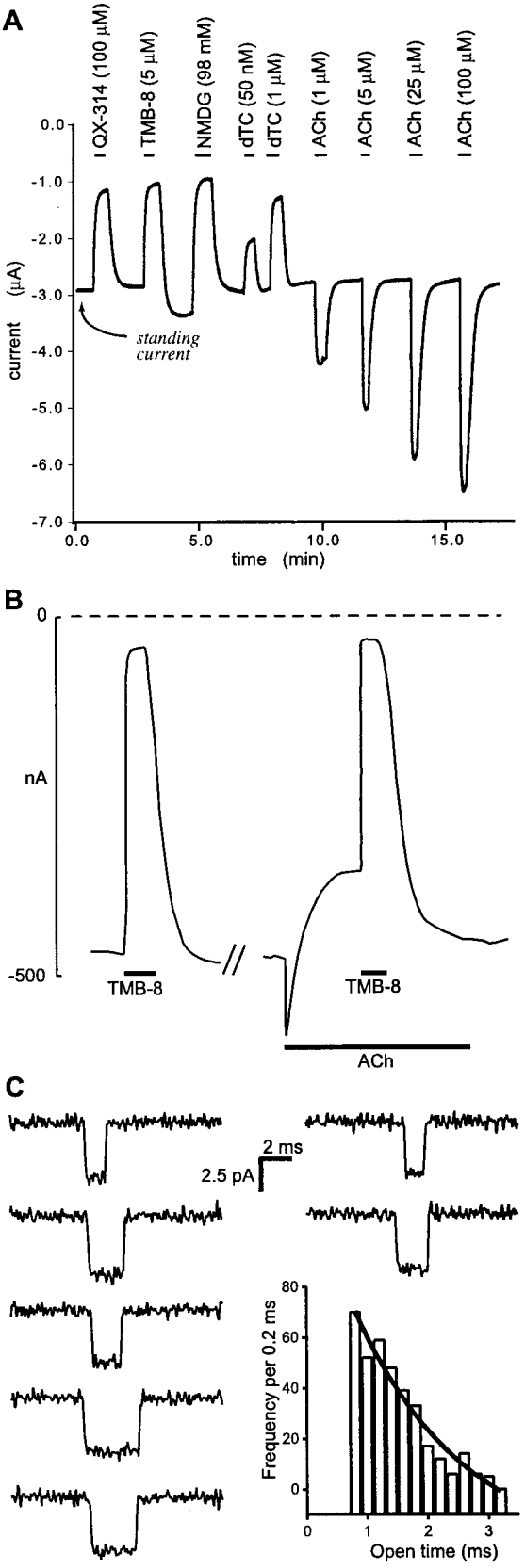

FIG. 5. (*A*) Standing inward currents and ACh responses in oocytes expressing nAChRs with Tyr-O3Q incorporated at α149 and a Leu-9′ → Ser mutation in M2 of the β subunit. The initial current of almost −3 μA, the standing current as a result of the mutant channels, is substantially eliminated by channel blockers such as QX-314 and TMB-8 or the cholinergic antagonist dTC. Application of ACh leads to increased currents. (*B*) ACh-induced desensitization affects the standing current. During the first application of TMB-8 (5 μM), the standing outward current was reduced reversibly to nearly zero. ACh (25 μM) was then added, inducing an inward current that desensitized. During the desensitization phase, the standing current was reduced. TMB-8 was added again during the desensitization phase. The TMB-8 deflection reached the former plateau level rather than producing a net outward current. These interactions show that ACh desensitizes the conductance mechanism that produces the standing current; therefore, the standing current is produced by functional nAChR. (*C*) Single-channel records and open-time distribution from receptors containing Tyr-O3Q at position α149. The traces show selected openings from cell-attached patches recorded at −80 mV. The open-time distribution from a typical patch is fitted (least-squares) to a single exponential decay with a time constant of 1.4 ms. Openings less than 0.6 ms were excluded from the analysis to eliminate contributions from the spontaneous openings of βLeu-9′ → Ser receptors, which have a time constant of 0.3 ms (H. Zhang *et al.*, personal communication).

The model proposed here provides the most precise information to date on the location of ACh when bound to the nAChR. This model suggests that any means of positioning a quaternary ammonium in close proximity to αTrp-149 might produce an active receptor. In classic experiments, tethered agonists were obtained by attaching structures containing quaternary ammonium groups to the cysteines produced by reducing the highly conserved Cys-192-Cys-193 disulfide of the α subunit, leading to the conclusion that the quaternary ammonium group interacts with a moiety within 9 Å of the disulfide (30, 31). We applied a similar strategy at position α149, incorporating the unnatural amino acid Tyr-O3Q (Tyr-*O*-$(CH_2)_3$-$N(CH_3)_3^+$, see Fig. 4). Modeling clearly shows that the quaternary ammonium of Tyr-O3Q can achieve the same position as that proposed in the binding of ACh.

As shown in Fig. 5, incorporation of Tyr-O3Q at position α149 produces a constitutively active receptor. In the absence of added ACh, standing inward currents (500–3000 nA, Fig. 5*A*) and substantially reduced resting membrane potentials are consistently seen in *Xenopus* oocytes expressing the mutant channel. The standing currents are presumably partially desensitized due to the prolonged activation. The standing currents are inhibited by α-bungarotoxin, a specific, high-affinity antagonist for nicotinic receptors (data not shown). Exposure of the oocytes to channel blockers such as QX-314 and TMB-8 or the competitive antagonist *d*-tubocurarine (dTC) immediately and reversibly reduces the standing inward current (Fig. 5*A*) and leads to more typical resting membrane potentials. We note that the tethered quaternary ammonium appears to be a fairly weak agonist, as standing currents are observed only when the previously mentioned Leu-9′ → Ser mutation is incorporated into the M2 region of the β subunit. Application of ACh produces additional inward currents (Fig. 5 *A* and *B*), in agreement with the idea that the tethered quaternary ammonium group is a partial agonist; apparently, additional features of the ACh molecule are required for full activation. Blockage by dTC appears to be roughly half-maximal at a value (50 nM, Fig. 5*A*) near its K_i for wild-type receptor (14). This result suggest that the dTC binding site does not overlap with the cation–π site or that the tethered group can be displaced by dTC. Prolonged exposure (>3 min) to high ACh concentrations led to desensitization of both the standing currents (Fig. 5*B*) and the responses to further ACh applications (data not shown), further evidence that the response arises directly from nAChR. Single-channel recordings showed that the standing current was due to openings of 43-pS conductance (Fig. 5*C*), quite comparable to the wild-type nAChR (42 pS). The combined data of Fig. 5 clearly establish that positioning a quaternary ammonium group very near the region normally occupied by αTrp-149 can be enough to activate the receptor.

The present study illustrates the power of combining theoretical chemistry with modern molecular biology and electrophysiology to produce high-precision insights into the workings of a complex biological system. We conclude that αTrp-149 is the primary cation–π binding site in the nAChR. When ACh binds, the quaternary ammonium group makes van der Waals contact with the 6-ring of the indole side chain. The aligned position is highly conserved as a Trp in all muscle and neuronal α subunits. In non-α subunits, however, the analogous residue is not Trp, but is generally Tyr, Gln, and Leu in β, γ, and δ, respectively. The residue is therefore not a "structural, canonical residue," as has been suggested for Trp-86, Tyr-151, and Tyr-198 (1). The aligned position in 5-HT_3 (serotonin) receptors is also Trp, while it is Tyr in γ-aminobutyric acid and glycine receptors. It will be interesting to see whether these aligned residues in other ligand-gated ion channels are also cation–π binding sites. It will also be interesting to probe the role of α149 in the complex pharmacology of both muscle and neuronal nAChR. The only high-resolution structure of a natural ACh binding site is that of acetylcholinesterase (32, 33). The quaternary ammonium group of ACh makes van der Waals contact with Trp-84, as in the model proposed here. Further away, but probably contributing to binding, is the carboxylate of Glu 199. The primary carboxylate implicated in the nAChR is δAsp-180/γAsp-174, on the basis of work by Karlin (7) (Fig. 1). If the binding arrangement in the nAChR is similar to that of the esterase, then αTrp-149 lies very close to δAsp-180/γAsp-174 and therefore must be at the interface between two subunits. This notion can be tested by using the suppression methodology.

We thank M. W. Nowak, A. P. West, Jr., C. Labarca, H. Dang, Y. Tong, and Y. Li for helpful discussions. This work was supported by the National Institutes of Health (NS-34407 and NS-11756) and the California Tobacco-Related Disease Research Program. J.P.G. is grateful to the California Institute of Technology and to Eastman Kodak for fellowship support.

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