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A Perturbed pK_a at the Binding Site of the Nicotinic Acetylcholine Receptor: Implications for Nicotine Binding

E. James Petersson, Anita Choi, David S. Dahan, Henry A. Lester, and Dennis A. Dougherty*

Division of Chemistry and Chemical Engineering, Division of Biology, California Institute of Technology, Pasadena, California 91125

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The nicotinic acetylcholine receptor (nAChR) is essential to synaptic transmission and is implicated in learning and memory, as well as a variety of neurological disorders including Alzheimer's disease, Parkinson's disease, and schizophrenia.[1] On binding acetylcholine (ACh) this integral membrane receptor undergoes a conformational change that permits cations to pass through a central pore, thereby converting a synaptic chemical signal to an electrical signal. The receptor comprises five subunits. The two ACh binding sites have been localized to the α/δ and α/γ interfaces, on the basis of extensive biochemical studies[1] and the crystal structure of ACh binding protein (AChBP), a soluble protein homologous to the extracellular domain of the nAChR.[2]

We have previously used unnatural amino acid mutagenesis (nonsense suppression) techniques to incorporate tyrosine derivatives with tethered quaternary ammonium groups (quats; e.g., TyrO3Q, Figure 1) at the agonist binding site.[3,4] These yielded constitutively active receptors (i.e. receptors that open in the absence of ACh), providing valuable geometrical information about the agonist binding site. Most agonists and antagonists of the nAChR (other than ACh), including nicotine, contain protonatable amines rather than quats, and it is generally assumed that the protonated, cationic species is the active form of the ligand. If so, one expects tethered amine analogues of TyrO3Q to produce pH-sensitive receptors, structures that are constitutively active only when the tethered group is protonated. We now report that the tethered amines TyrO3S and TyrO3T (Figure 1) indeed show a strong increase in constitutive activity at low pH. Titration of the amine side chain provides a probe of the local pK_a at the receptor binding site. We find that the phenomenological pK_a's of these tethered amines are far lower than their values in free solution.

The syntheses of TyrO3P and TyrO3S in appropriate forms for nonsense suppression were straightforward. Both the α-N of the amino acid and the side-chain amine were protected as nitroveratryoxycarbonyl groups. The tertiary amine of TyrO3T cannot be protected as an amide. Interestingly, the *N*-nitroveratryl (NV) side-chain protecting group, which has been used successfully in many similar contexts, was not viable for TryO3T because of inefficient photodeprotection of the quat group. Other studies with simpler model compounds confirm that nitrobenzyl-type photocleavage reactions are not efficient when converting a quaternary ammonium to a tertiary amine. We have found, however, that the dimethoxycoumarin (DMCm) group is an effective photocleavable protecting group for tertiary amines (see Supporting Information).

The tethered amine unnatural amino acids were incorporated into nAChR expressed in *Xenopus laevis* oocytes using now well-established protocols.[5] Channel activity was monitored with standard two-electrode, voltage-clamp electrophysiology. Along with the constitutive (standing) current, responses to added ACh, known channel-blockers such TMB-8 (8-(diethylamino)octyl trimethoxybenzoate) and QX-314 (lidocaine *N*-ethyl bromide), or agonist-free solutions of various pH were measured.

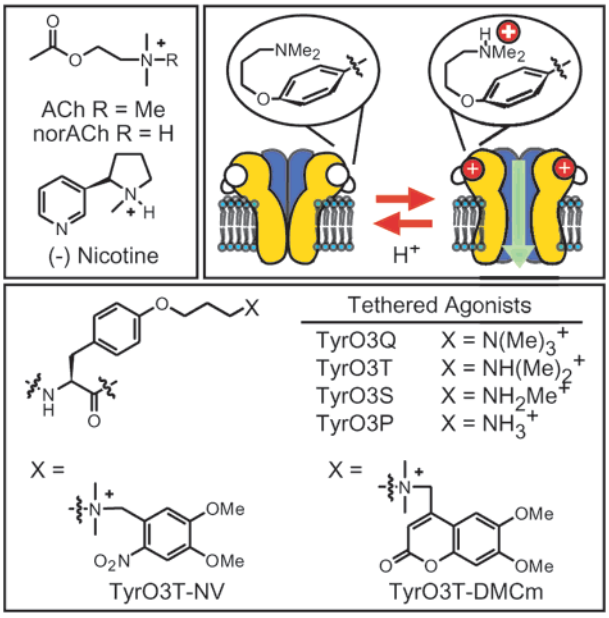

Figure 1. (Top left) ACh, norACh, and nicotine. (Top right) Incorporation of TyrO3T at α149 yields a receptor that is constitutively active at low pH. (Bottom) Tethered agonist unnatural amino acids.

The response of the wild-type nAChR to saturating concentrations of ACh shows a small inherent dependence on pH, with conductance being maximal at pH 7.5 and falling at lower and higher pH values (see Supporting Information for examples of primary electrophysiological data).[7] In sharp contrast, receptors that contain TyrO3T at position 149 of the α subunit, (α149) showed a systematic increase in constitutive current as the pH was lowered.[6] These currents can be blocked by TMB-8, showing that they arise from the opening of nAChRs. In an important control, we find that the pH dependence of the constitutive current of the system with the tethered quat, TyrO3Q, mirrors that of wild-type receptor. These observations establish that the increase in blockable current observed for TyrO3T at lower pH is due to the specific protonation of the side-chain amine. TyrO3S constitutive currents also increase with decreasing pH. However, receptors containing TyrO3P showed no constitutive activity, even at pH 5.5. Perhaps the protonated TyrO3P lacks the steric bulk to activate the receptor. Note that attempts to incorporate Lys at α149 led to no surface nAChR expression; conventional mutagenesis does not permit these studies (see Supporting Information).

* Corresponding author. E-mail: dad@igor.caltech.edu.

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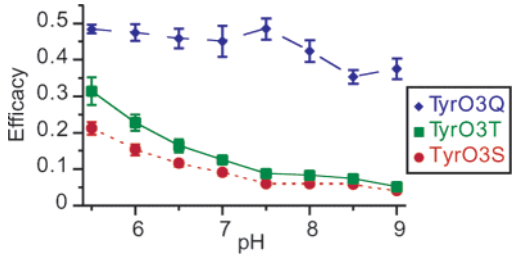

Figure 2. Tethered agonists at α149: tethered agonist efficacy as a function of solution pH. ACh efficacy = 1 at all pHs.

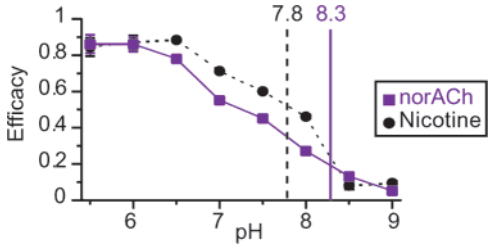

Figure 3. Tertiary agonists: norACh and nicotine efficacy as a function of solution pH. ACh efficacy = 1 at all pHs.

We define the *efficacy* of a tethered agonist as the ratio of the constitutive current that can be blocked by TMB-8 to the maximum current induced by saturating concentrations of ACh (corrected for basal conductance changes with pH). By considering only constitutive current that can be blocked, we eliminate any background (non-nAChR specific) leak current. The efficacies of TyrO3Q, TyrO3T, and TyrO3S at α149 are shown in Figure 2. TyrO3Q's efficacy, as expected, is insensitive to pH changes, when corrected for basal pH dependence. As described above, TyrO3T and TyrO3S exhibit strong titration behavior when incorporated at α149.

These adjusted measurements of receptor activation provide a straightforward way to assay the degree of protonation of a tethered amine at the binding site. Thus Figure 2 gives a phenomenological pK_a for the side chain. Since pH ≤ 5 compromises the oocyte membrane integrity,[8] we are unable to study the presumed plateau at low pH values. Nevertheless, it is clear that the side-chain pK_a of TyrO3T is ≤6 when incorporated at α149 of the nAChR, substantially shifted from its value in free solution (~9.3, measured for *N*,*N*-dimethylaminopropanol).[9] pK_a shifts of this magnitude are precedented. For example, Lys 115 in *Clostridium acetobutylicum* acetoacetate decarboxylase has an effective pK_a of 6.0, shifted by 4.5 units.[10] Our results suggest that the agonist binding site of the nAChR is relatively hydrophobic, consistent with the fact that the binding site is primarily formed by aromatic residues.

Nicotine (pK_a = 7.8)[12] is, of course, a noted tertiary amine agonist of the nAChR, and we have measured the pH-dependent nicotine efficacy in the present context (Figure 3). Efficacy is computed for nontethered agonists by normalizing the current from applications of saturating agonist concentrations to the maximal ACh-induced current. Interestingly, there is no pK_a shift for nicotine; the phenomenological pK_a is not measurably different from the solution pK_a of the drug. In contrast, norACh (pK_a = 8.3),[11] the closest possible protonatable analogue of ACh, shows a pK_a shift of ~1 unit, noticeable, although not as large as for the tethered amines.

These differences are interpreted as follows. We assume that only the cationic forms of agonists can activate the receptor. For the amine tethers and norACh, the protonatable amine can equilibrate with the medium when at the agonist binding site and the local microenvironment of the protein produces a pK_a shift. For nicotine, however, no pK_a shift is seen because the protonated amine does not equilibrate with the medium once it has bound to the receptor. The degree of receptor activation is then dependent only on the amount of protonated nicotine available to bind to the receptor, and the pH dependence of activation mirrors nicotine's normal pK_a.

This is the second recent line of evidence from our labs that indicates a difference between the binding modes of ACh and nicotine. We have recently shown that the potent cation−π interaction observed between ACh and αTrp149[3] is not evident for nicotine.[13] Taken together, these data strongly suggest that pharmacophore models for the muscle-type nAChR should be expanded to include two distinct agonist binding modes: an ACh-like mode and a nicotine-like mode.

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Supporting Information Available: Procedures for the syntheses of the tethered agonists and their ^{1}H NMR, ^{13}C NMR, and MS data; protocols for unnatural amino acid mutagenesis and oocyte electrophysiology have been published and are summarized; examples of primary electrophysiological data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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