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Cysteine accessibility analysis of the human alpha7 nicotinic acetylcholine receptor ligand-binding domain identifies L119 as a gatekeeper

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ABSTRACT

A large number of structurally diverse ligands have been produced to selectively target α7 nicotinic acetylcholine receptors (nAChRs). We applied the method of scanning cysteine accessibility mutations (SCAM) to the ligand-binding domain of the α7 nAChR to identify subdomains of particular importance to the binding and subsequent activation by select agonists. We evaluated the activity of four structurally distinct α7 agonists on wild-type human α7 and 44 targeted mutants expressed in *Xenopus* oocytes. Responses were measured prior and subsequent to the application of the sulfhydryl reagent methanethiosulfonate ethylammonium (MTSEA). One mutant (C116S) served as a Cys-null control, and the additional mutants were made in the C116S background. In many cases, the insertion of free cysteines into the agonist-binding site had a negative effect on function, with 12 of 44 mutants showing no detectable responses to ACh, and with only 19 of the 44 mutants showing sufficiently large responses to permit further study. Several of the cysteine mutations, including W55C, showed selectively reduced responses to the largest agonist tested, 2-methoxy,4-hydroxy-benzylidene anabaseine. Interestingly, although homology models suggest that most of the introduced cysteine mutations should have had good solvent accessibility, application of MTSEA had no effect or produced only modest changes in the agonist response profile of most mutants. Consistent with previous studies implicating W55 to play important roles in agonist activation, MTSEA treatment further decreased the functional responses of W55C to all the test agonists. While the cysteine mutation at L119 itself had relatively little effect on receptor function, treatment of L119C receptors with MTSEA or alternative cationic sulfhydryl reagents profoundly decreased activation by all agonists tested, suggesting a general block of gating. The homologous mutation in heteromeric nAChRs produced similar results, provided that the mutation was placed in the beta subunit complementary surface of the ligand-binding domain. Structural models locate the L119 residue directly across the subunit interface from the C-loop of the primary face of the binding domain. Our data suggest that a covalent modification of L119C by MTSEA or other cationic reagents might block the binding of even small agonists such as TMA through electrostatic interactions. Reaction of L119C with small non-polar reagents increases activation by small agonists but can block the access of large ligands such as benzylidene anabaseines to the ligand-binding domain.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of a large superfamily of ligand-gated ion channels that are all characterized by several key structural similarities, including a signature disulfide-constrained loop that is thought to mediate the intramolecular conformational changes linking ligand binding and ion channel activation (Millar and Gotti, 2009). This "Cys-loop" superfamily also includes GABA and glycine receptors, which mediate inhibitory neurotransmission in the central nervous system (CNS). Although nAChRs are associated more with neuromodulation and presynaptic functions than with synaptic transmission, important roles in behavior and cognition can be ascribed to nAChRs in the brain (Gotti et al., 2006).

There are two main classes of nAChRs in the brain and peripheral nervous system. One class consists of homomeric pentamers of the α7 subunit, while the other class consists of heteromeric pentamers containing both alpha-type and non-alpha (beta)

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subunits. Homomeric α7 receptors are also found in many non-neuronal cells (Gahring and Rogers, 2005; Wessler and Kirkpatrick, 2008), where they have been shown to mediate multiple kinds of signal transduction (Arredondo et al., 2006; de Jonge et al., 2005; Marrero and Bencherif, 2009; Parrish et al., 2008).

The ligand-binding domain (LBD) of nAChRs for acetylcholine (ACh) is at the interface between subunits, a surface containing primary elements contributed by one subunit and complementary elements contributed by the adjacent subunit. In heteromeric neuronal nAChRs, α subunits (α2, α4, or α6) have evolved to contain the special subdomains of the primary face of the LBD, associated with three structural elements referred to as the A-, B-, and C-loops (Sine, 2002). Certain non-alpha subunits (β2 and β4 among the neuronal nAChR subunits, and δ, γ, and ϵ among the muscle nAChR subunits) have lost the specialized features of the primary LBD surface, but contain specializations associated with the complementary LBD surface, associated with three structural elements referred to as the D, E, and F loops (Sine, 2002). Alpha7 receptors are considered a primordial nAChR form of the Cys-loop receptors (Le Novere et al., 2002) and retain structural elements of both the primary and complementary surfaces of the LBD. Consequently, while heteromeric nAChR are limited to two LBDs in each pentamer at the interface between dissimilar subunits, data suggest that α7 receptors contain five potential LBDs, one at each α7–α7 interface.

Since numerous studies have supported a role for α7 receptors in cognitive and neuroprotective processes in the CNS and also as regulators of peripheral inflammation, the development of α7-selective agonists has been of interest to many scientists and pharmaceutical groups. There is a large amount of structural diversity in the compounds identified as α7-selective agonists and partial agonists due to the fact that at least three distinct structural motifs can be associated with the selective activation of α7 nAChR (Horenstein et al., 2008). The smallest molecule which can activate α7 (and other neuronal nAChR) is the tetramethyl ammonium ion (TMA, Fig. 1A), yet the α7 LBD can also evidently accommodate vastly larger molecules such as the selective partial agonist 2-methoxy-4-hydroxy-benzylidene anabaseine (2MeO4OHBA, also 4OH-GTS-21). Although numerous homology models of the α7 LBD have been published, they have been largely based on the distantly related snail ACh binding protein (AChBP) and so provide only limited insight into the binding of diverse ligands to the wild-type receptor. Therefore, we have applied the method of scanning cysteine accessibility mutations (SCAM) to the LDB of α7 nAChR.

SCAM has been used to successfully identify portions of the *Torpedo* nAChR subunits which contribute to the ion conduction pathway (Akabas and Karlin, 1995; Akabas et al., 1994, 1992; Zhang and Karlin, 1996), residues associated with the binding of agonists and competitive antagonists (Gay et al., 2008; McLaughlin et al., 1995; Spura et al., 1999; Sullivan et al., 2002), and domain changes associated with positive allosteric modulation (Barron et al., 2009). The method involves systematically substituting cysteines, one at a time, for each of the residues in the domains of interest. The accessibility of the cysteine residues can be probed with a small, positively charged, sulfhydryl reagent such as methanethiosulfonate ethylammonium (MTSEA), which has a diameter of ≈6 Å, smaller than nicotine or anabaseine, and much smaller than the benzylidene anabaseines. Alternatively, sulfhydryl reagents which are larger or with specific functional groups, varying in charge or H-bonding properties, can be used. With this approach we have identified important functional subdomains of the α7 agonist-binding site and associated portions of the receptor, the accessibilities of which regulate agonist binding and receptor activation.

2. Materials and methods

2.1. nAChR clones and mutants

The wild-type human nAChR clones were provided by Dr. Jon Lindstrom (Univ. Pennsylvania, Philadelphia, PA). Mutations to cDNA clones were introduced using the QuikChange kit from Stratagene according to the manufacturer's instructions. The mutations were confirmed with automated fluorescent sequencing.

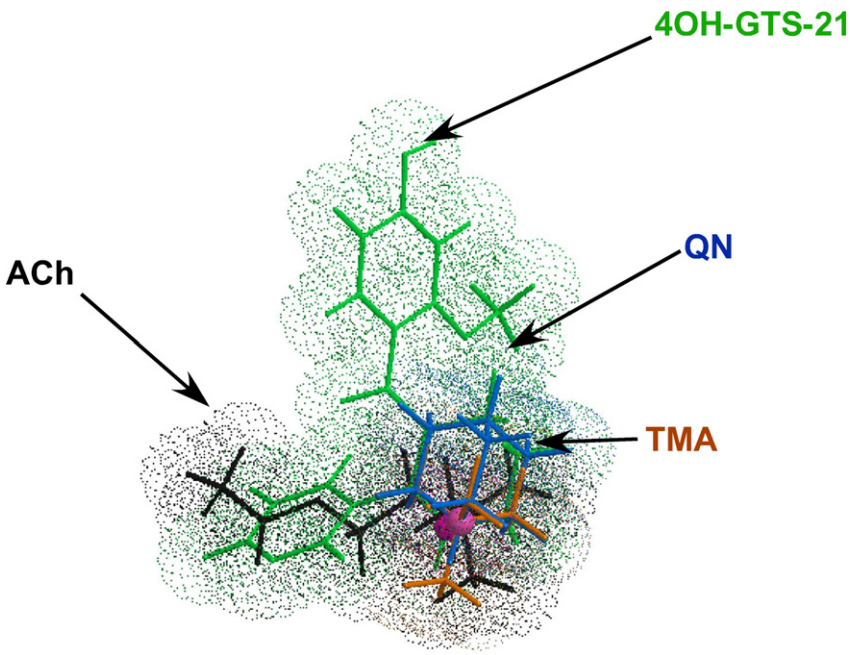

Fig. 1. Agonist and receptor structures. The structures of the four molecules were optimized using Chem 3D Ultra v. 6.0 (CambridgeSoft, Cambridge, MA): ACh, QN, TMA, and 2MeO4OHBA's energies were first minimized by semi-empirical computation MOPAC (AM1) to minimum RMS gradient of 0.001 while charge equals 1 separately. The ACh structure was then overlaid with 2MeO4OHBA (both contain H-bonding acceptor), and QN was overlaid with TMA. The overlaid two sets of structures were superimposed manually. The core ammonium cation of each is shown in purple. The color scheme for the agonist is: Black, ACh; Green, 2MeO4OHBA; Blue, QN; Orange, TMA.

2.2. Preparation of RNA

After linearization and purification of cloned cDNAs, RNA transcripts were prepared *in vitro* using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

2.3. Expression in Xenopus oocytes

Mature (>9 cm) female *Xenopus laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as the source of oocytes. Prior to surgery, frogs were anesthetized by placing the animal in a 1.5 g/L solution of MS222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis MO) for 30 min. Oocytes were removed from an abdominal incision.

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 15 mM HEPES (pH 7.6), 12 g/l tetracycline). Subsequently, stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 2–7 days after injection. Although the absolute magnitude of the evoked current responses increased over time, the normalized values of the experimental responses did not vary significantly over time.

2.4. Chemicals

Sulfhydryl reagents were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO). Fresh ACh and sulfhydryl reagent stock solutions were made daily in Ringer's solution and diluted.

2.5. Electrophysiology

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City CA) (Stokes et al., 2004). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Cells were automatically perfused with bath solution, and agonist solutions were delivered from a 96-well plate. Both the voltage and current electrodes were filled with 3 M KCl. The agonist solutions were applied via disposable tips, which eliminated any possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications. Flow rates were set at 2 ml/min for experiments with α7 receptors and 4 ml/min for other subtypes. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Unless otherwise indicated, drug applications were 12 s in duration followed by 181 s washout periods.

2.6. Experimental protocols and data analysis

Each oocyte received two initial control applications of ACh (300 μM unless otherwise indicated), an experimental drug application, and then a follow-up control application of ACh. The peak amplitude and the net charge (Papke and Papke, 2002) of experimental responses were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. After each experimental measurement cells were rechallenged with ACh at the control concentration. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration unless otherwise noted. For experiments involving potential covalent modification of cysteine residues with sulfhydryl reagents, data both before and after the application of the sulfhydryl reagent were normalized to the average of the two initial ACh controls.

2.7. Molecular modeling

A homology model for the α7 nAChR was constructed using the *Aplysia californica* AChBP structure (PDB ID 2PGZ) (Hansen et al., 2005). A ClustalW alignment of the AChBP and human α7 sequence was generated and submitted to the Swiss Model structure server. The resulting monomeric model was superimposed twice on the A and B chains of the AChBP pentameric crystal structure in order to generate a dimer model. The model was then examined for clashes which where resolved by variation of side chain rotomers, or in combination with constrained minimization using the GROMOS force field resident in the Swiss-Pdb Viewer 4.0 software package, followed by Amber 10 molecular mechanics refinement with the bound 2PGZ ligand (cocaine hydrochloride) included to prevent collapse of the LBD during structural optimization.

3. Results and discussion

3.1. Construction and characterization of the cysteine-null C116S α7 pseudo-wild-type

There are four conserved cysteine residues in the extracellular domain of every nicotinic alpha subunit which are required for function, two of which form a disulfide bond and stabilize this eponymous element of every protein in the Cys-loop ligand-gated ion channel superfamily. The other pair of conserved cysteines is a vicinal pair in the C-loop of the primary face of the agonist-binding site, and this is a defining feature of the nicotinic alpha subunits. The vicinal cysteines are also disulfide coupled, and so none of the four conserved cysteines are free to react readily with applied sulfhydryl reagents. However, the human α7 contains an additional free cysteine in the extracellular domain at residue 116. In order to apply the SCAM approach, it was first necessary to mutate the C116 residue and thus prevent spurious modifications at that site, as well as to eliminate the possibility of disulfide formations between that residue and the additional cysteines introduced by mutation. An α7C116S mutant was therefore constructed to serve as our pseudo-wild-type background for subsequent mutants. The α7C116S mutant readily formed homomeric receptors when expressed alone in *Xenopus* oocytes. Responses of the α7C116S mutant showed concentration-dependent desensitization indistinguishable from wild-type α7, and the EC_{50} of the ACh-evoked responses of the α7C116S receptors was 29.4 ± 0.7 μM, not significantly different from the C116 wild-type (Fig. 2). Likewise, the relative responses of α7C116S receptors to tetramethyl ammonium (TMA), quinuclidine (QN), and 2MeO4OHBA, compared to ACh, were not significantly different from those of the C116 wild-type (Table 1) in regard to both potency and efficacy as determined with concentration–response studies relative to ACh controls (not shown).

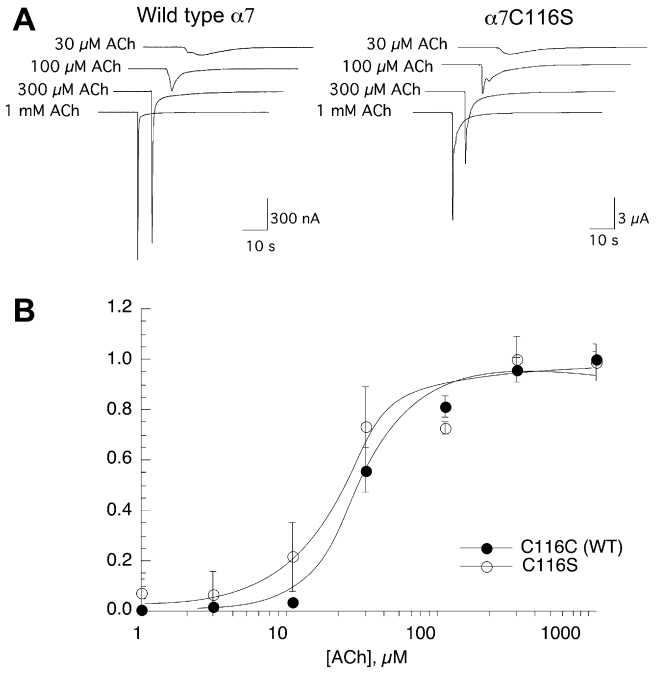

Fig. 2. Characterization of C116S Cys-null α7. A) Comparison of raw data obtained from wild-type (C116C α7) and cells expressing α7C116S over a range of ACh concentrations. B) ACh concentration–response studies for α7 C116C (wild-type) and α7C116S. Points plotted are the average net charge responses of at least four oocytes (±SEM), normalized to 300 μM ACh control responses.

Table 1
Characterization of the wild-type, Cys-null, and α7C116S,L119C mutant responses to ACh and test agonists.

	EC_{50} values, μM		
	α7C116C	α7C116S	α7C116S,L119C
ACh	27 ± 3[a]	29.4 ± 0.7	30 ± 4
TMA	30 ± 3[a]	25 ± 1	144 ± 38
QN	7.2 ± 0.5[a]	5.6 ± 0.6	13.4 ± 2.2
2MeO4OHBA	4.0 ± 1 4[b]	6 ± 1 4	1.6 ± 0.4
(I_{max})	(0.44 ± 0.03)	(0.54 ± 0.05)	(1.2 ± 0.6)

For all three receptors the efficacies of TMA and QN were equivalent to that of ACh. 2MeO4OHBA was a partial agonist for α7C116C and α7C116S but appeared to be a full agonist for α7C116S,L119C.
[a] Wild-type α7 data from Horenstein et al. (2008)
[b] Wild-type α7 data from Papke and Papke (2002)

3.2. Initial characterization of cysteine mutants in the cysteine-null C116S α7 pseudo-wild-type

A homology model of the α7 LBD domain was used to select the residues for cysteine mutations. These residues were 92% conserved among the seven known vertebrate α7 sequences and only 30% identical to the homologous residues in other human nAChR subunits. A total of 43 double mutants of human α7 were studied (Table 2), with single residues mutated to cysteine generated in the C116S Cys-null pseudo control. Data from these double mutants were compared to the Cys-null control and to wild-type human α7. However, since wild-type α7 subunits naturally contain a free cysteine at residue 116, for the purposes of our SCAM analysis we can consider the wild-type as α7C116C.

For the evaluation of data in a study such as this, the first consideration should be the impact of each mutation on receptor function and/or assembly. A valuable perspective on this comes from determining the relative conservation of the original residues with the nAChR gene family. Three levels of comparison are presented in Table 2. The first column is a calculation of the frequency at which the residue found in human α7 is also found in other human nAChR subunits, including the other neuronal subunits (α2, α3, α4, α5, α6, β2, β3, β4) and the muscle receptor subunits (α1, β1, γ, δ, ϵ), as well as α9 and α10, subunits with expression largely limited to the inner ear and with pharmacological profiles that are atypical for nAChR. The second column is a calculation of the frequency at which the residues believed to be in the portion of α7 contributing to the primary face of the agonist-binding site are also found in other human α-type subunits: α1, α2, α3, α4, and α6. (Note that α5 was excluded from this comparison, since it is not believed to assemble into an agonist-binding site.) The third column is a calculation of the frequency at which the residues believed to be in the portion of α7 contributing to the complementary face of the agonist-binding site are also found in other human non-α-type subunits: γ, δ, ϵ, β2, and β4 (note that β1 and β3 were excluded from this comparison, since they are not believed to assemble into agonist-binding sites). The fourth column provides a comparison between human α7 and seven other vertebrate α7 sequences. It is interesting to note that while some residues (G114 and W60) are conserved in all human nAChR subunits and all α7 subunits, regardless of species, other residues are only found in α7 subunits, most notably S113 and C116.

Conservation through evolution is only one factor that is relevant to the effects of single point mutations. The physiochemical properties of the specific native residues are also important, relative to how they will be altered by the mutation. While mutations like serine to threonine or aspartic acid to glutamic acid may be judged as rather conservative, affecting mainly the size of the residue, mutation of specific residues to cysteine may alter multiple

Table 2
Comparisons of human α7 (C116C) and cysteine mutants of α7C116S.

Loop	Mutant	[a]Human nAChR (15)	Sequence identity					
			[b]LBD 1° (5)	[c]LBD 2° (5)	[d]Vert. α7 (7)	a. a. score	ΔHP	ACh response
D	Cys-null	0		0	1	0.62	54	+++++
	W55C	0.87		1	1	1.55	−48	++++
	L56C	0.60		0.4	1	1.77	−48	+
	Q57C	0		0	1	1.81	59	++
	M58C	0.07		0.2	0.86	2.13	−25	+
	S59C	0		0	0.57	0.62	54	++
	W60C	1		1	1	1.55	−48	−
E	V110C	0.47		1	1	1.43	−27	+
	N111C	0		0	1	1.22	77	+
	S112C	0.27		0.2	0.71	0.62	54	++
	S113C	0		0	1	0.62	54	−
	G114C	1		1	1	0.52	49	−
	H115C	0.07		0	0.86	1.34	41	+
	C116C	*0*		*0*	*1*	*0*	*0*	+++++
	Q117C	0.07		0	1	1.81	59	+++
	Y118C	0		0	1	1.44	−14	−
	L119C	0.33		1	1	1.77	−48	+++++
	P120C	0.87		1	1	0.78	95	−
B	W149C	0.73	1		1	1.55	−48	−
	S150C	0.13	0.2		0.71	0.62	54	++
	Y151C	0.93	1		1	1.44	−14	+
	G152C	0.07	0		1	0.52	49	+
	G153C	0.33	0.2		1	0.52	49	+
	W154C	0	0		1	1.55	−48	+
	S155C	0	0		1	0.62	54	−
	L156C	0.07	0		1	1.77	−48	++
F	I165C	0		0	1	1.92	−50	++++
	S166C	0.13		0	0.86	0.62	54	−
	G167C	0.07		0.2	0.71	0.52	49	+++
	Y168C	0.2		0.2	1	1.44	−14	−
	I169C	0.13		0	1	1.92	−50	+
C	K182C	0.07	0		0.86	1.04	72	++
	R183C	0.2	0		1	1.41	63	+
	S184C	0	0		0.43	0.62	54	++
	E185C	0.07	0		1	1.42	80	+
	R186C	0.2	0.2		0.29	1.41	63	+++
	F187C	0	0		1	2.46	−51	++
	Y188C	0.47	1		1	1.44	−14	+
	E189C	0.07	0.2		0.86	1.42	80	++
	K192C	0	0		1	1.04	72	+
	E193C	0.47	0.8		1	1.42	80	−
	P194C	0.2	0		1	0.78	95	−
	Y195C	0.73	1		1	1.44	−14	+
	P196C	0.47	0.6		1	0.78	95	+++
	D197C	0.73	1		1	1.01	104	−

Mutants were made in the C116S α7 background. The mutations were in the various LBD subdomains as indicated, with loops A, B, and C being in the primary face and loops D, E, and F in the complementary face. The wild-type, which retains a Cys at 116, is outlined. The "a. a. score" is an estimate of the relative differences among multiple physiochemical properties between cysteine and the original amino acid (adapted from Taylor WR. 1986 (Taylor, 1986). ΔHP is the relative difference in the hydrophobicity index (Monera et al., 1995) between the original amino acid and cysteine. Positive values indicate that the cysteine substitution increases the hydrophobicity at the site. "ACh response" refers to a qualitative assessment of function based on comparing the net charge responses to 300 μM ACh of the mutants to that of the Cys-null (C116S) and wild-type controls. The sequence identity measurements compare the original amino acid in human α7 to: a) all human nAChR subunits other than α7, to all subunits forming heteromeric receptors and contributing to either b) the primary (1°) alpha subunit face of putative ligand-binding domains (α1, α2, α3, α4, α6) or c) the complementary (2°) non-alpha subunit face (γ, δ, ϵ, β2, β4), and d) to α7 subunits from seven other vertebrate species (*Macaca mulatta* (Rhesus Macaque), *Rattus norvegicus* (rat), *Mus musculus* (mouse), *Bos taurus*, *Danio rerio* (zebrafish), *Pan troglodytes* (chimpanzee), *Gallus gallus* (chick)). The numerical values for sequence identity in the table are calculated as the number of subunits in the comparison group with the same residue at that location as human α7 divided by the total number of sequences in the specific comparison group. A score of 1.0 means that all subunits shared the same residue, and a score of 0 means that the residue is unique to human α7. Mutants with two or more +'s for ACh response were tested with multiple agonists and for the effects of MTSEA.

physiochemical properties. In order to generate systematic, albeit somewhat arbitrary, measures of physiochemical distance, we used a multifactorial Venn diagram plot of amino acid properties (Taylor, 1986) and calculated linear distances between the original residues and cysteine. This two-dimensional space factored in size, polarity, aromaticity, hydrophobicity, and charge. These values are given as the "a. a. score" in Table 2. This scale ranked amino acids like glycine and serine close to cysteine (smallest numbers) and the large hydrophobic aromatic amino acid phenylalanine farthest (largest numbers).

The LBD of nAChR is often characterized as a large hydrophobic pocket (Sine, 2002), and so changes in hydrophobicity arising from mutation of specific residues to cysteine may also have large impact on the basic function of the receptors. Using a previously published amino acid hydrophobicity index (Monera et al., 1995), we calculated the predicted changes in hydrophobicity that would arise with each of the cysteine mutations (ΔHP in Table 2). Positive values indicate that the mutation of the original residue to cysteine would increase the hydrophobicity at the site (*e.g.* S to C), while negative values indicate predicted decreases (*e.g.* W to C).

All of the mutants were initially screened for their responsiveness to applications of 300 μM ACh. The majority of the cysteine mutants tested responded relatively poorly to ACh or showed no detectable responses. Of the 43 mutants made in the C116S background, 12 failed to show any detectable responses to ACh, even when tested repeatedly with different injection sets and several days of incubation post RNA microinjection. Of the remaining 21 mutants, only the C116S,L119C double mutant gave responses that were typically as large as the wild-type C116C α7 or the Cys-null C116S α7 (marked "+++++" in Table 2). Some mutants had responses that were on the average 30–60% that of the C116S controls (marked "++++" or "+++" in Table 2). Mutants marked "++" had typical responses between 10% and 30% that of the C116S controls. Mutants marked with a single plus sign in Table 2 showed ACh responses that were above our level of detection but <10% that of typical control responses. The observed variation in the responsiveness of the cysteine mutants to ACh may be due to many factors, and neither the genetic conservation scores nor the physiochemical scores in Table 2 showed any clear correlations to the level of ACh response. The limited degree to which loss of function can be ascribed to either evolutionary or physiochemical distance sheds light on the holistic character of the receptor's chemical transduction, such that receptor function may be perturbed by any number of small alterations in the reactive domains of the receptor.

The distributions of functional and non-functional mutation sites are shown in Fig. 3A. Cysteine substitutions were best tolerated through a band of sequence on the complementary face of the LBD and on the back edge of the primary face. Interestingly, one site at which α7 subunits differ from all other human nAChR subunits is C116, and this site fits into the band of sites seen in Fig. 3A. Somewhat curiously, there are no free cysteines in any LBD of any of the other human neuronal nAChR subunits, and the C116 residue is conserved in α7 in species as distantly related as zebrafish to man. Nonetheless, the mutation of the residue to serine had no readily detectable effect on the function of the human receptors expressed in oocytes (Fig. 2, Table 1). Likewise, it has previously been reported that mutation of this residue in α7 expressed in SH-EP1 cells did not interfere with receptor expression or function (Dunckley et al., 2003).

The ACh concentration–response data for five of the best-functioning mutants are shown in Fig. 3B. Although some mutants showed reduced Hill slopes, most had ACh EC_{50} values similar to wild-type α7 and the Cys-null C116S α7 (Table 1). One notable exception was the W55C mutant, which showed a 5–6-fold higher EC_{50} for ACh. The tryptophan 55 residue has been previously shown to be pivotal in determining the gating of both α7 and α4β2 nAChR by a range of structurally diverse agonists (Williams et al., 2009).

The failure of the numerous mutants to function and for many of the others to respond less well than wild-type could be due to several possible factors, including failure to express. Hypothetically, inserted cysteines might cause spurious disulfide bonds to form during protein maturation, leading to incorrectly folded proteins. It is also possible that the presence of inserted cysteines hindered assembly due to mismatches at the protein–protein interfaces. Alternatively, the inserted cysteines may have impeded agonist binding, disrupted the gating process, or otherwise inactivated the LBD. Of course, it is likely that the various mutants were non-functional for different reasons, some for failure to form complexes, some from failure to bind agonist, and some from failure to activate. While ligand-binding studies are sometimes used to discriminate failure to express from failure to function, binding studies might easily be misleading with mutations directly in the LBD. Additionally, the processes of gating and binding are inseparably convoluted in the detection and measurement of macroscopic current responses (Colquhoun, 1998) due to the allosteric character of the receptors and the interdependence of binding and activation.

We tested the responses of the functional cysteine mutants to the set of four structurally diverse agonists (Fig. 1): ACh (300 μM), TMA (1 mM), QN (100 μM), and 2MeO4OHBA (30 μM). The results are shown in Fig. 4. In panel A, the data for TMA, QN, and 2MeO4OHBA are expressed relative to the control ACh responses of that particular mutant. To illustrate the effect of the cysteine mutations on the relative responses of the specific agonists compared to the Cys-null control, the ratio of the mutant responses to the C116S responses are shown in Fig. 4B. Significant changes in the agonist response profiles were seen for W55C (relative to ACh, responses to TMA, QN, and 2MeO4OHBA were all reduced, $p < 0.001$), Q57C (relative to ACh, responses to QN ($p < 0.001$) and 2MeO4OHBA ($p < 0.05$) were reduced), and S150C (relative to ACh, responses to 2MeO4OHBA were reduced, while responses to TMA and QN were increased, $p < 0.01$). For I165C and G167C, responses to 2MeO4OHBA (relative to ACh) were reduced ($p < 0.05$), without significant effects on the relative responses to TMA or QN. Note that, although the responses of the L119C mutant to 2MeO4OHBA were not significantly different from the C116S Cys-null control at the probe concentration of 30 μM, full concentration–response studies of this mutant to all three probe agonists (Table 1) indicated that 2MeO4OHBA was, in fact, fully as efficacious as ACh for this mutant.

As noted above, the well-conserved W55 residue can accept mutations that produce major alterations in pharmacological profile without necessarily large overall decreases in function (Williams et al., 2009). The mutation at S150, which resulted in increased hydrophobicity, decreased 2MeO4OHBA activity relative to ACh. However, caution is needed in the interpretation of activity of an agonist relative to ACh (Colquhoun, 1998). In the case of S150C, it is possible that the mutation increased activation by ACh while leaving activation by 2MeO4OHBA relatively unaffected. Consistent with this hypothesis, analysis of a homology model with a docked BA suggests no direct contact in the region of 150–152 (Williams et al., 2009).

Mutations of I165 or G167 to cysteine decreased the relative efficacy of 2MeO4OHBA. The I165C mutation represents a decrease in hydrophobicity and steric bulk, while the mutation at G167 results in an increase in hydrophobicity and steric bulk. These mutations may therefore give a clue regarding the interactions of 2MeO4OHBA in the LBD for binding and/or activation. I165 is in Van der Waals contact with I179 as part of a loop structure that helps frame the (−) face of the LBD. According to docking studies of BA compounds, this loop, which includes I165 and G167, is in contact

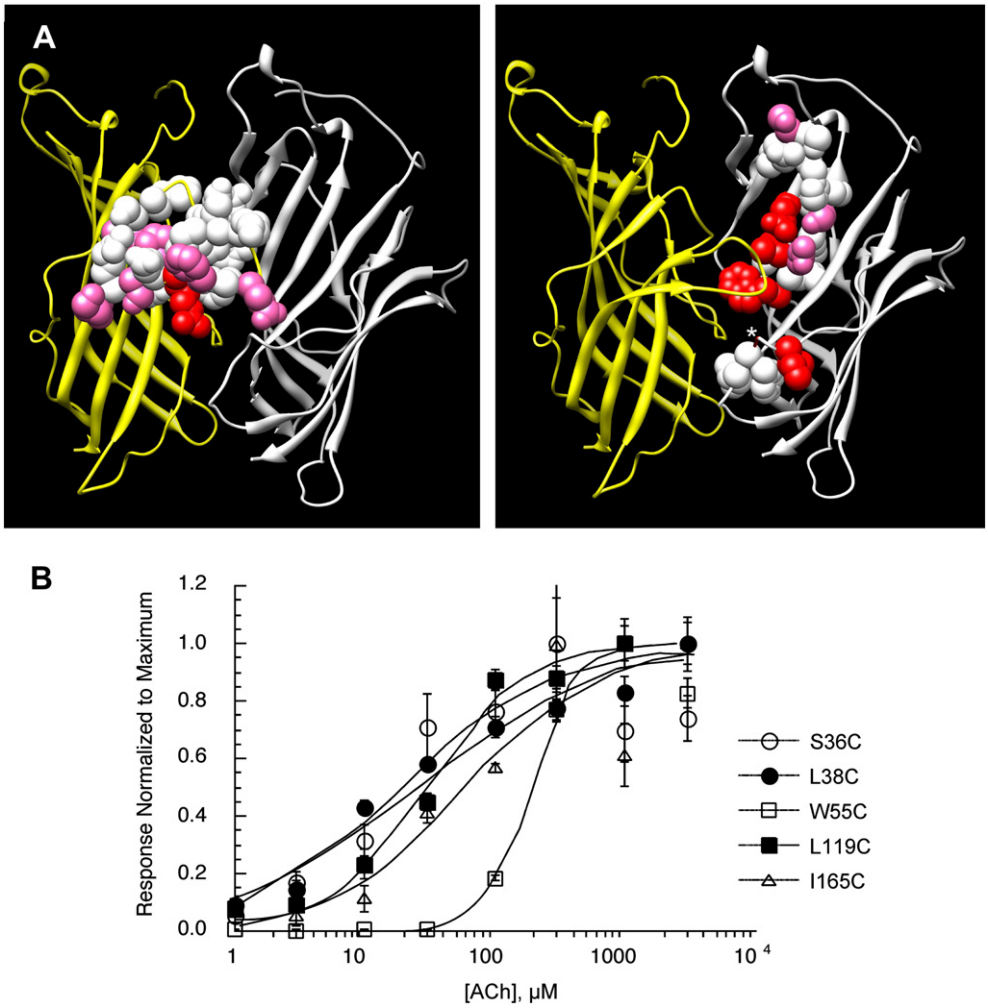

Fig. 3. A) The image on the right highlights residues on the complementary face of the LBD, while the image on the left is the subunit with the LBD primary face. Residues that could be mutated to cysteine and yield receptors that had good ACh responses (scores of 3–5 in Table 2) are in red. Residues that when mutated gave small measurable responses are in pink, and residues that when mutated gave non-functional receptors are in white. The position of C116S is indicated on the right structure(*), where the backbone is colored in red. B) ACh concentration–response data for five of the most functional mutants. Points plotted are the average net charge responses of at least four oocytes (±SEM), normalized to each mutant's empirically determined maximal ACh-evoked response. The ACh EC_{50} values for S36C, L38C, W55C, L119C, and I165C were 20 ± 9, 24 ± 5, 180 ± 20, 30 ± 4, and 60 ± 26, respectively.

with bound BA compounds. This close interaction may be weakened in I165C, such that contacts with bound BA compounds become compromised. The added bulk of the G167C mutation may interfere with binding of BA compounds directly.

3.3. *Sulfhydryl modification of cysteine mutants in the cysteine-null C116S α7 pseudo-wild-type*

We were interested to test the hypothesis that MTSEA modification of functional cysteine mutants would selectively regulate the activation by larger agonists. After obtaining an initial agonist response profile, the mutants were then treated with the sulfhydryl reagent MTSEA (2 mM for 60 s) and tested again for their responses to ACh and the three experimental agonists (Fig. 5). For the most part, the sulfhydryl reactions had either no effect or affected activation by the entire panel of agonists. As evaluated by pairwise *t*-tests between each single cell's response prior to and following MSTEA, the MTSEA treatment produced significant decreases in the responses of W55C (responses to TMA, QN, and 2MeO4OHBA were all reduced, $p < 0.001$), L119C (responses to all agonists were reduced >95%, $p < 0.0001$), S150C (responses to ACh, TMA, and 2MeO4OHBA were reduced, $p < 0.05$), and G167C (responses to 2MeO4OHBA were reduced, $p < 0.05$). Interestingly, with P196C, responses to ACh, TMA and 2MeO4OHBA were significantly increased following MTSEA treatment ($p < 0.05$). There was a selective effect on 2MeO4OHBA activation at G167C both before and after MTSEA treatment, suggesting potential importance of sequence in this region for activation of α7 by large hydrophobic agonists such as 2MeO4OHBA.

The largely neutral impact of MTSEA treatment for most mutants may reflect the fact that mutations were inaccessible to the reagent or in noncritical regions of the LBD, so that covalent modifications did not impede agonist binding or gating. The likelihood of the later hypothesis may be increased by the fact that mutants used for the MTSEA experiments were those already demonstrated to be tolerant to the cysteine mutation itself. Moreover, based on an analysis of solvent accessibility using the CASTp server (Dundas et al., 2006) and visual inspection of homology models, it is likely that most of the mutants presented in Fig. 5 were accessible to sulfhydryl reagent. The two noteworthy exceptions were C116 and L156, which, based on inspection of the homology model, appeared to be protected from bulk solvent by neighboring residues.

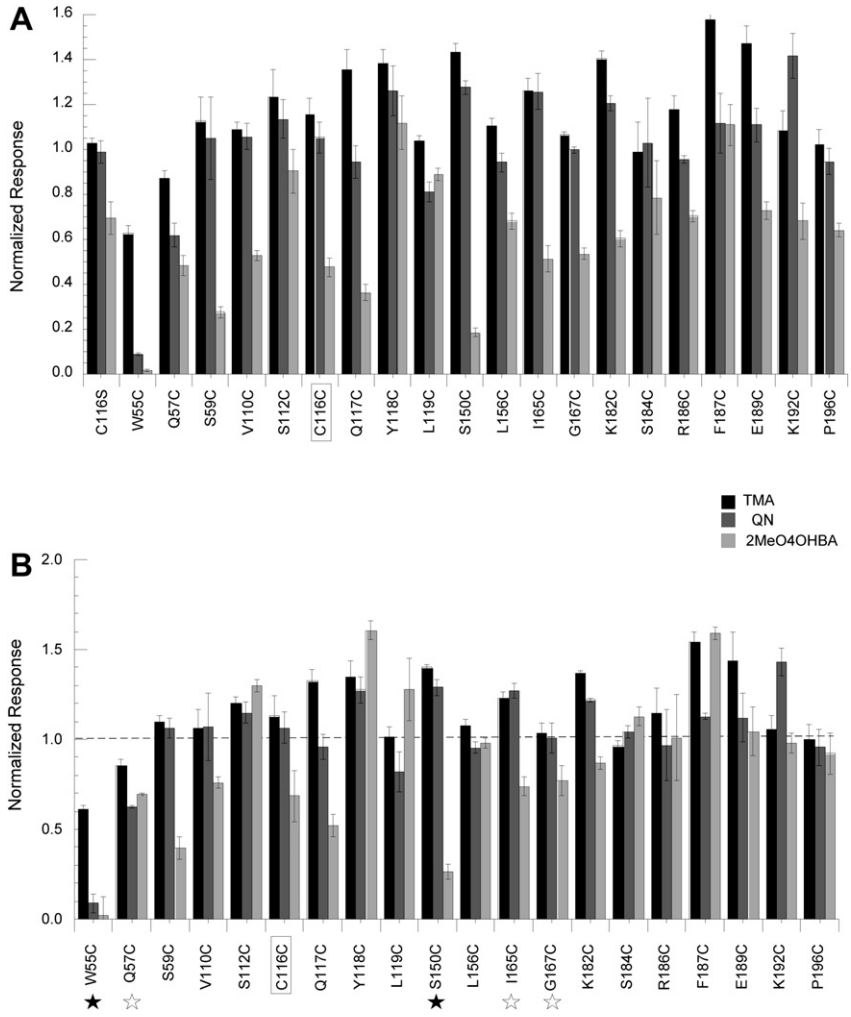

Fig. 4. The responses of human α7 wild-type (boxed) and mutants to ACh and experimental agonists. Cells were given two applications 300 μM ACh followed by applications of 1 mM TMA, 100 μM QN, and 30 μM 2MeO4OHBA, with additional applications of 300 μM ACh between the TMA, QN, and 2MeO4OHBA applications to confirm the stability of the ACh responses. A) Data from the Cys-null mutant, wild-type, and cysteine double mutants normalized to the control ACh responses of the specific cell types. B) The ratios of the experimental agonist responses relative to ACh compared to the response profile of the Cys-null pseudo-wild-type. Agonist applications were 12 s followed by 5 min washes. Statistical determinations are based on unpaired *T*-tests between the responses of the Cys-null pseudo-wild-type responses to each experimental agonist, normalized to the Cys-null ACh responses, and the mutant responses to each experimental agonist, normalized to the mutant's ACh responses. Mutants which had response profiles significantly different from the C116S Cys-null controls are marked with stars. Open stars indicate $p < 0.05$ and filled stars indicate $p < 0.01$; see text for details.

The most robust effect of MTSEA treatment was obtained with the C116S,L119C α7 double mutant. Responses of this mutant prior to MTSEA were indistinguishable from either the wild-type or Cys-null controls both in amplitude (Table 2) and in the response profile for the four agonists (Fig. 6) at the probe concentrations, although 2MeO4OHBA showed increased efficacy compared to ACh in full concentration–response studies (Table 1).

Several agents were tested to see if they could protect α7C116S,L119C mutants from MTSEA inactivation. Partial protection was obtained when the agonists ACh (1 mM), TMA (1 mM), or 2MeO4OHBA (1 mM) were co-applied with the MTSEA, with ACh giving the greatest protection (35%) and 2MeO4OHBA giving no more than 10% protection. TMA provided approximately 20% protection (data not shown). Co-application of 1 mM mecamylamine with MTSEA provided no protection. However, when the α7–selective competitive antagonist methyllycaconitine (100 μM) was co-applied with MTSEA, there was a 20% protection of ACh responses. This level of protection was equivalent to the residual antagonism measured after the application of methyllycaconitine alone (data not shown).

To test whether α7C116S,L119C mutants would be equally affected by other sulfhydryl reagents as by MTSEA, three alternative compounds were used. These were: [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET), [4-(trimethylammonium)benzyl] methanethiosulfonate (TMB-SH), and 2-(tributylammonium)ethyl methanethiosulfonate (TBAE-SH). As shown in Fig. 7, all of these reagents were equally effective at reacting with L119C and eliminating the responsiveness to all four of the test agonists.

Structural modeling of the α7 subunits (Brejc et al., 2001) places the L119 residue in the E-loop of the complementary face of the LBD. In heteromeric receptors this would be part of the specialized domains of the non-alpha subunits (Fig. 6B). While the placement of this modifiable residue in α7 impacts all of five potential binding sites, the effect of the homologous mutation in subunits of heteromeric receptors would depend on the specific subunit(s) in which the mutation was placed. In the case of α4β2 receptors, placement of the corresponding mutation in β2 would, as in the case of α7, presumably impact the function of all of the agonist-binding sites, and, although limited to 2 rather than 5 sites, the impact on function should be equally profound (assuming equal

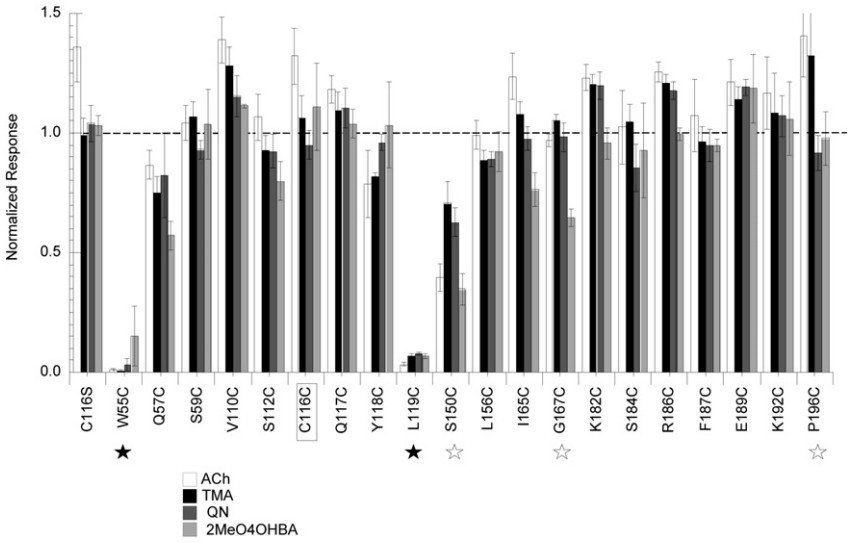

Fig. 5. MTSEA effects on human α7 wild-type (boxed) and mutants to ACh and experimental agonists. Following the acquisition of the data shown in Fig. 4, cells were treated with 2 mM MTSEA for 60 s and then given additional applications of 300 μM ACh, 1 mM TMA, 100 μM QN, and 30 μM 2MeO4OHBA. The ratios of the average responses to each agonist after MTSEA to the average responses before MTSEA are plotted. Statistical determinations are based on paired *T*-tests on the responses of the individual cells before and after MTSEA. Mutants which showed significant MTSEA effects are marked with stars. Open stars indicate $p < 0.05$ and filled stars indicate $p < 0.01$; see text for details.

efficiency of the MTSEA reaction). In contrast, since the α4 subunits are hypothesized to not form agonist-binding sites in their domains which correspond to the complementary face of binding site, homologous mutations to the α7L119C should have relatively little effect on function. The effects of mutations corresponding to α7L119C in α4β2 receptors are shown in Fig. 8. The mutations produced no apparent differences in ACh responses compared to wild-type prior to MTSEA treatment. The responses of α4β2 receptors with the homologous mutation (T124C) placed in the α4 subunit showed a small but statistically significant reduction in responses to 30 μM ACh after MTSEA, while the wild-type α4β2 did not. However, in contrast with the small effect obtained with the mutation in α4, when the mutation (L121C) was placed in β2, there was a profound reduction in the ACh-evoked response when co-expressed with either the α4T124C mutant or the wild-type α4. It was also the case that when α3 was co-expressed with the β2L121C mutant, that MTSEA reduced responses to both high (1 mM) and low (30 μM) concentrations of ACh by greater than 95%, while MTSEA had no significant effect on the responses of cells expressing α3 and wild-type β2 (data not shown).

We also used an L119C homolog to test whether β3 subunits are likely to contribute to agonist-binding sites when they are present in heteromeric nAChR. It has been shown that β3 is often co-assembled with α6 and b2 subunits, particularly in dopaminergic neurons. However, although functional effects of β3 expression have been identified (Papke et al., 2007), based on sequence analysis it has been proposed that the β3 subunits do not contribute to the agonist-binding sites (Gotti et al., 2006) but rather serve as structural subunits, like β1 in muscle-type nAChR (Kuryatov et al., 2008). In order to test this hypothesis, we evaluated the relative MTSEA sensitivity of receptors containing β2L121C and β3T126C, when these subunits were co-expressed with either α3 or the chimeric α6/3 subunit (Dowell et al., 2003). The expression of the β3T126C subunit with either α3 and β2 or α6/3 and β2 produced no sensitivity to MTSEA in the ACh responses (data not shown). However, the homologous mutation (L121C) in the β2 subunit decreased the response to ACh following MTSEA treatment by at least 97% any time it was present. These results strongly support the hpothesis that the β3 subunit does not locate in the complementary face of an agonist-binding site, even when the α6 extracellular domain is present in the primary face, and that the role of β3 within a receptor is structural.

The LBD of α7 has been the subject of numerous site-directed mutagenesis studies (Galzi et al., 1991; Gay et al., 2008; Grutter et al., 2003; Horenstein et al., 2007; Williams et al., 2009). While some mutations produce profound alterations in function or expression (Galzi et al., 1991), other mutations, even at highly conserved residues, produce only modest effects (Williams et al., 2009). Although SCAM approaches have been highly successful at delimiting ion conduction pathways (Akabas and Karlin, 1995; Akabas et al., 1994, 1992; Zhang and Karlin, 1996), our experiments suggest that the stringent requirements for structure and function, and the relatively nonconservative nature of cysteine substitutions, may limit the usefulness for the direct application of SCAM approaches in the study of ligand binding and channel gating. However, the covalent attachment of a SCAM label to a suitable cysteine residue provides a fixed marker and experimental geometric constraint with which homology models may be verified (Stewart et al., 2006). In particular, our data confirm the placement of the α7 L119 residue (and the homologous residues in other nAChR) in the complementary face of the LBD, in crucial proximity to the C-loop of the primary face.

While the relatively nonconservative L119C mutation itself had no detectable effect on receptor function, the covalent extension of that residue with any sulfhydryl reagent was sufficient to prevent agonist activation. Fig. 9 shows an overlay of TMA docked in the α7 wild-type LBD with a homology model of the of the L119C mutant following covalent modification by the sulfhydryl reagent MTSET, a somewhat larger molecule than MTSEA (for structural comparisons see Fig. 7). The close positioning of the L119 residue to the C-loop suggests that sulfhydryl reactions with any of a variety of reagents and L119C would produce modifications that might prevent the C-loop from moving deeper into the LBD, a process believed to be a necessary event for the initiation of gating (Hansen et al., 2005; Mukhtasimova et al., 2005), effectively putting a wedge in the gate and preventing the necessary conformational changes required for ion channel activation. However, the model cannot exclude the alternative hypothesis that labeling of L119C with a sulfhydryl reagent results in sufficient occupancy of the LBD to preclude agonist binding, especially for larger agonists and bulkier

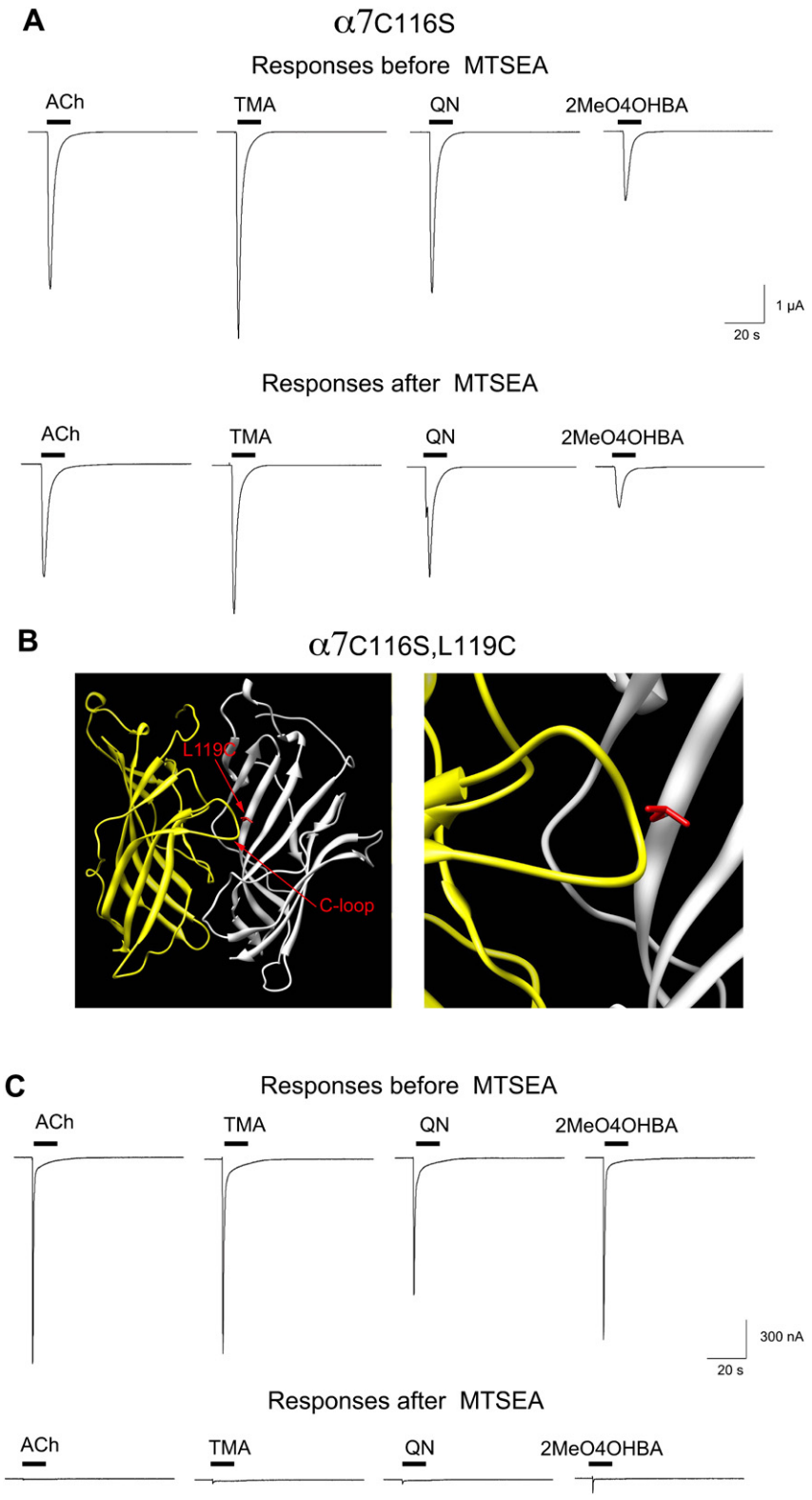

Fig. 6. MTSEA inactivation of the α7L119C mutant. A) Raw data traces showing the lack of MTSEA effect on the agonist-evoked responses of cells expressing α7C116S. B) Location of the L119 residue in a homology model of α7 (Celie et al., 2004). The overview at left shows an α7–α7 homo dimer and the location of the L119 residue in relation to the C-Loop in the primary face of the agonist-binding site. The image on the right shows the proximity at increased magnification. Images were created in Deep View, Swiss-Pdb Viewer (Guex and Peitsch, 1997), from the crystal structure model of AChBP (PDB 1I9B) (Brejc et al., 2001). C) Raw data traces showing the effect of MTSEA on the agonist-evoked responses of cells expressing the α7C116S,L119C double mutant.

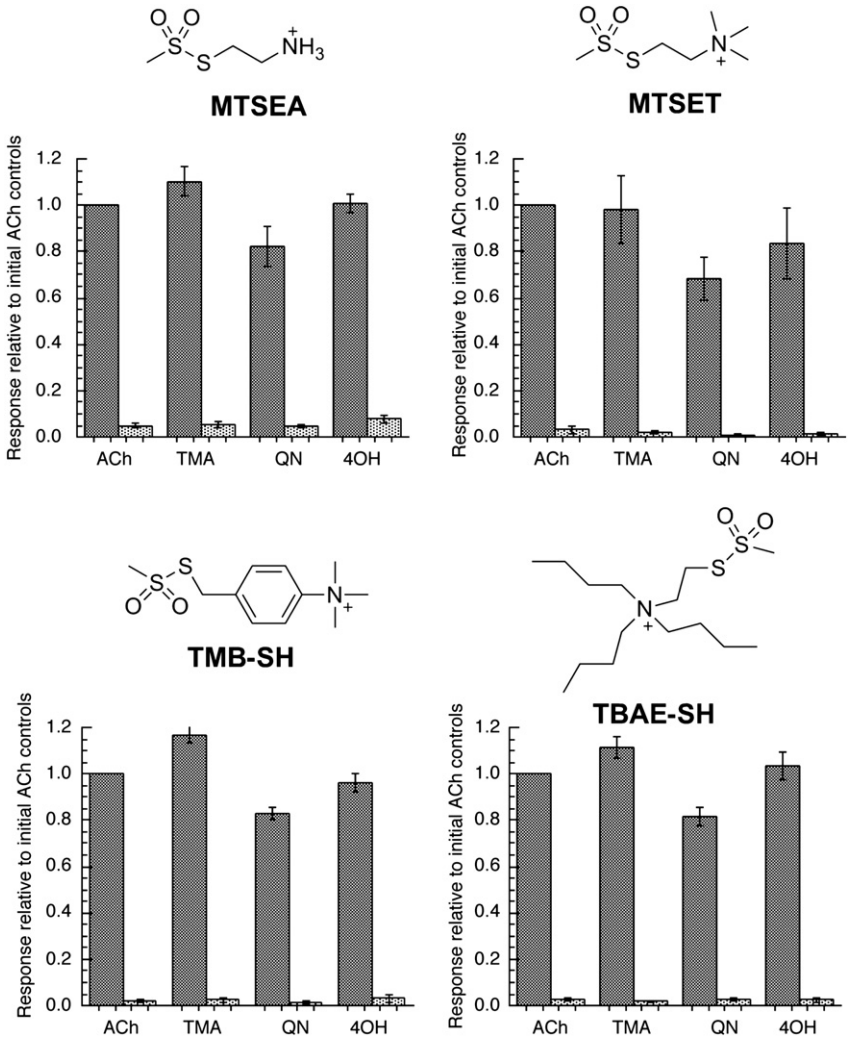

Fig. 7. Effects of MSTEA and alternative sulfhydryl reagents on the human α7C116S,L119C double mutant to ACh and experimental agonists. Control responses were obtained as described for Fig. 2, and then cells were tested with one of the sulfhydryl reagents shown (2 mM for 60 s). All of the agents tested were equally effective at suppressing the agonist-evoked responses.

sulfhydryl reagents. Interestingly though, the model also suggests a third hypothesis, that positively charged agonists may be excluded from the LBD due to electrostatic repulsion from the positively charged sulfhydryl reagents. This explanation could even be applied to the failure of the smallest agonist TMA to activate the receptor following reaction with the relatively small sulfhydryl reagent MTSEA, since the charge separation of those two molecules would be similar to that shown for TMA and MTSET (approximately 3 Å).

To test the hypothesis that the profound block of α7C116S,L119C receptors by MTSEA and the other cationic sulfhydryl reagents shown in Fig. 7 was due, at least in part, to the insertion of the positive charge itself into LBD, we tested two additional pairs of alternative reagents. One pair, carboxymethyl methanethiosulfonate (MTSCM) and 2-carboxyethyl methanethiosulfonate (MTSCE), were carboxylic acids, and so potentially could place negative charges in the LBD. The other pair had uncharged end groups, a methyl in the case of methyl methanethiosulfonate (MMTS) and an ethyl in the case of ethyl methanethiosulfonate (EMTS). The results, shown in Fig. 10, suggest that MTSCM did react with the cysteine at 119, since there was an effective block of the large experimental agonist 4OH-GTS-21. It seems likely that this selective block may have been due to steric occlusion of 4OH-GTS-21, the largest of our probe molecules, by this relatively small covalent modification. However, we cannot rule out other potential direct interactions between the benzylidene anabaseine 4OH-GTS-21 and the modified residue.

There were no significant effects of the larger anionic sulfhydryl reagent MTSCE, suggesting that it may have failed to react with the cysteine at 119. As is shown in Fig. 7, the cysteine at 119 was able to react readily with far larger cationic reagents, suggesting that MTSCE had steric accessibility. The lack of apparent labeling is likely to be multifactorial and was not pursued further.

Interestingly, reaction with the small non-polar reagent MMTS produced significant potentiation of the net charge responses to ACh, TMA, and QN ($p < 0.05$) and a trend toward larger 4OH-GTS-21 responses ($p < 0.1$). The basis for this effect is unclear, but it may have been due to a general increase in P-open or a reduction in the stability of desensitization, since the application of the reagent itself stimulated a significant amount of current (not shown), which was atypical of agonist-induced responses. Moreover, the application of MMTS had similar potentiating effects on cells expressing wild-type α7 receptors and so the potentiating effects were not due to a covalent modification of 119C. The effects of EMTS, in general,

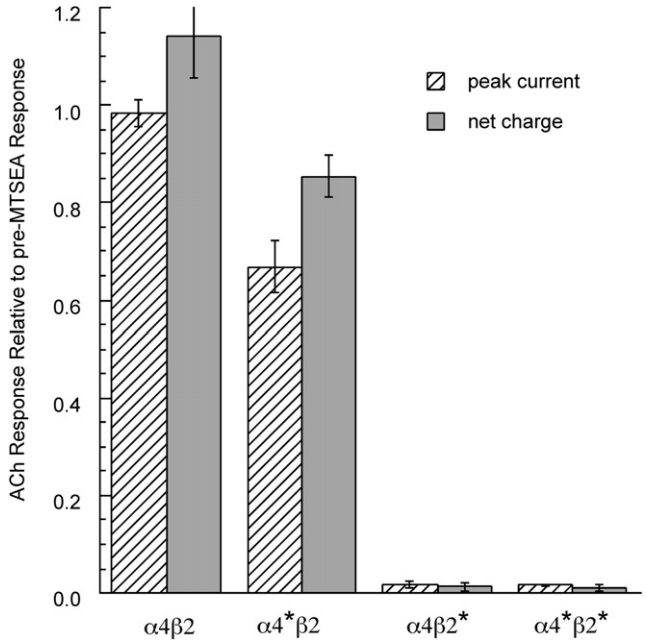

Fig. 8. The effect of MTSEA treatment on the responses of wild-type and mutant α4β2 receptors. The peak current and net charge responses to control applications of ACh following MSTEA treatments are shown, normalized to ACh controls from the same cells prior to MTSEA treatment. While cells expressing wild-type α4 and β2 showed no effect of MTSEA treatment, cells expressing α4T119C (the homologous mutation to α7L119C) and the wild-type β2 (α4*β2 in the figure) showed a small decrease in ACh response ($p < 0.05$). However the decrease in the α4*β2 responses (34 ± 5% and 15 ± 4% for peak current and net charge, respectively) was much less than what was obtained when the homologous mutation (L121C) was in β2. For receptors containing β2L121C, MTSEA produced a greater than 98% inhibition of both peak current and net charge, regardless of whether β2L121C was expressed with wild-type α4 (α4β2*) or with α4T119C (α4*β2*).

were similar to those of MMTS, and EMTS also produced some activation when applied alone (not shown) both to mutant and wild-type receptors. However in the L119C mutant, EMTS only appeared to potentiate the responses evoked by the smaller agonists, and the effects were not statistically significant. Nonetheless, modification of L119C by EMTS did produce a statistically significant selective reduction in the 4OH-GTS-21-evoked responses. Since the size of EMTS approaches that of MTSCM, this might also have been due to selective reduction of the access of 4OH-GTS-21 to the LBD.

Taken together, these results suggest there is a critical sized modification of the cysteine at 119 that can selectively occlude the large agonist 4OH-GTS-21 from the LBD and that this is most likely due to steric rather than electrostatic effects. These results are also most consistent with the potential importance of the cationic character of MTSEA for blocking activation by even the small agonist TMA.

In conclusion, our data indicate that many of the amino acids in the LBD of the α7 nAChR play critical and unique roles in channel activation, limiting the utility of scanning cysteine accessibility mutations, since many mutations led to non-functional receptors. For the most part, residues which allow the receptors to retain function after mutation to cysteine were either tolerant to, or inaccessible for, sulfhydryl modification. Our data also highlight the important position of the L119 residue in the complementary face of the LBD, such that when modified to cysteine, it can serve as a veritable gatekeeper to receptor activation. It seems likely that the leucine residue in native receptors may likewise be important for the coordination of intersubunit dynamics during normal gating. Additionally, our work suggests that, due to their ability to react with sulfhydryl reagents, the W55C and L119C mutants will serve useful as tools for further functional investigations. For example, these and other cysteines placed in the LDB may serve as tethering points for agonist or antagonist analogs (Wang et al., in press) or permit conditional modulation of single binding sites in heteromeric receptors (Williams et al., in press).

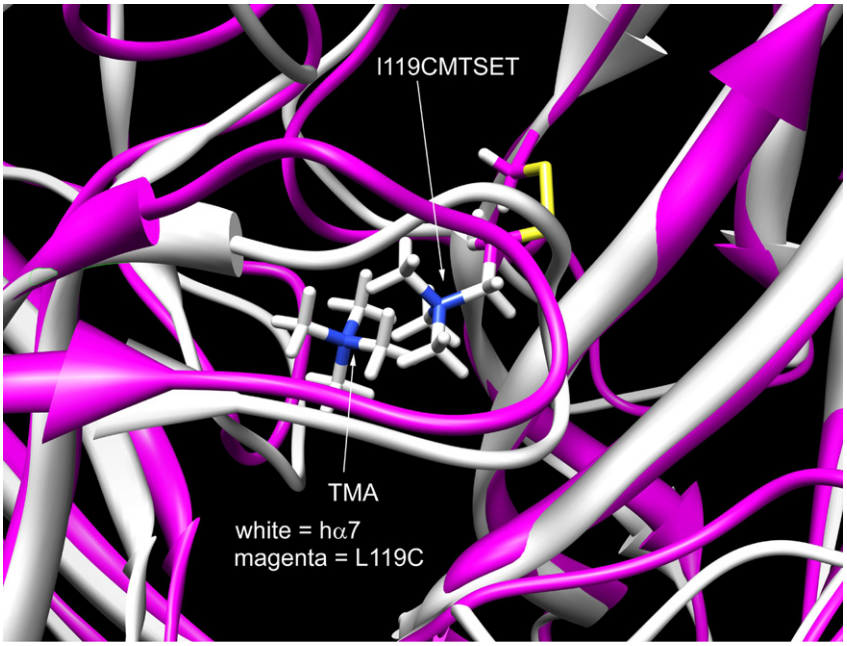

Fig. 9. Overlay of the human α7 wild-type nAChR with TMA docked in the LBD (white) with the α7L119C mutant covalently modified with the sulfhydryl reagent MTSET (magenta). The structure with MTSEA is predicted to be similar, except for the substitution of hydrogens for the methyl groups on the quaternary nitrogen of the sulfhydryl reagent (see Fig. 7). The overlay is consistent with steric interference in movement of the C-loop in the modified mutant. Although it suggests that a small agonist like TMA might bind deep enough in the pocket to avoid steric interaction with a labeled L119C, it also suggests that such binding might be disfavored by the required proximity of two positive charges.

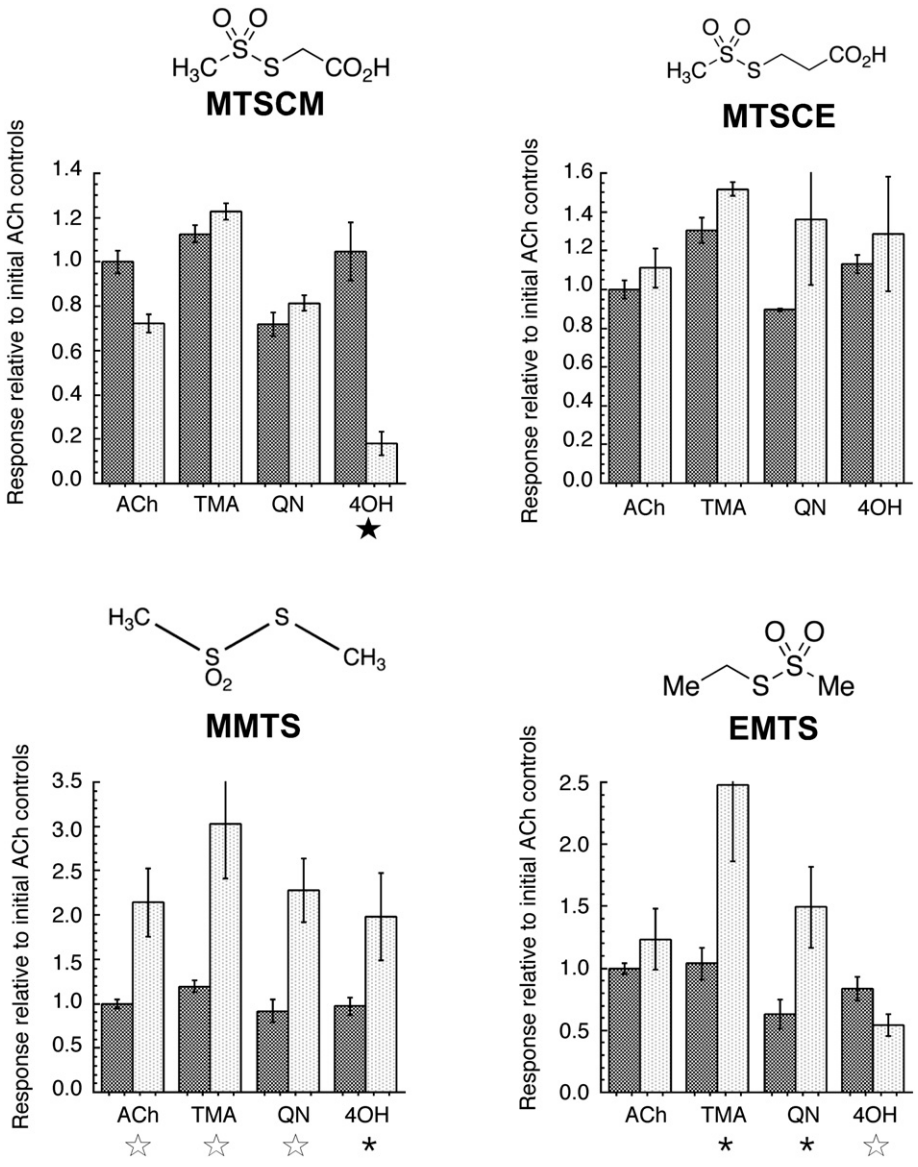

Fig. 10. Effects of non-cationic sulfhydryl reagents on the human α7C116S,L119C double mutant to ACh and experimental agonists. Control responses were obtained as described for Fig. 2, and then cells were tested with each of the sulfhydryl reagents shown (2 mM for 60 s). Solid stars represent statistically significant effects of the sulfhydryl application at $p < 0.01$. Open stars represent statistically significant effects of the sulfhydryl application at $p < 0.05$, and asterisks mark cases where p was <0.1 and >0.05.

Acknowledgements

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