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Residues at the Subunit Interfaces of the Nicotinic Acetylcholine Receptor That Contribute to α-Conotoxin M1 Binding

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ABSTRACT

The two binding sites in the pentameric nicotinic acetylcholine receptor of subunit composition $\alpha_2\beta\gamma\delta$ are formed by nonequivalent α-γ and α-δ subunit interfaces, which produce site selectivity in the binding of agonists and antagonists. We show by sedimentation analysis that ^{125}I-α-conotoxin M1 binds with high affinity to the α-δ subunit dimers, but not to α-γ dimers, nor to α, γ, and δ monomers, a finding consistent with α-conotoxin M1 selectivity for the $\alpha\delta$ interface in the intact receptor measured by competition against α-bungarotoxin binding. We also extend previous identification of α-conotoxin M1 determinants in the γ and δ subunits to the α subunit interface by mutagenesis of conserved residues in the α subunit. Most mutations of the α subunit affect affinity similarly at the two sites, but Tyr93Phe, Val188Lys, Tyr190Thr, Tyr198Thr, and Asp152Asn affect affinity in a site-selective manner. Mutant cycle analysis reveals only weak or no interactions between mutant α and non-α subunits, indicating that side chains of the α subunit do not interact with those of the γ or δ subunits in stabilizing α-conotoxin M1. The overall findings suggest different binding configurations of α-conotoxin M1 at the α-δ and α-γ binding interfaces.

Nicotinic acetylcholine receptors are pentamers of homologous subunits with composition $\alpha_2\beta\gamma\delta$ that form a ring around a central channel (Galzi and Changeux, 1994; Karlin and Akabas, 1995). Two of its five subunit interfaces, $\alpha\gamma$ and $\alpha\delta$, form binding sites for the neurotransmitter acetylcholine. These two binding interfaces are not identical in their affinities for agonists and competitive antagonists (Damle and Karlin, 1978; Neubig and Cohen, 1979; Weiland and Taylor, 1979; Sine and Taylor, 1981). Because the α subunit is common to each binding interface, differences in affinity are attributed to the contributions of the γ and δ subunits (Blount and Merlie, 1989; Petersen and Cohen, 1990, Sine and Claudio, 1991).

Recent studies showed that certain α-conotoxins, 12–14-amino acid disulfide-linked peptides isolated from venom of cone snails (Myers *et al.*, 1991, 1993), bind with unusual selectivity to one of the two ligand binding sites on mouse and *Torpedo californica* receptors (Kreienkamp *et al.*, 1994; Hann *et al.*, 1994; Utkin *et al.*, 1994; Groebe *et al.*, 1995; Sine *et al.*, 1995a). α-Conotoxin M1 binds with high affinity to the $\alpha\delta$ site of the mouse receptor (K_D = 0.5 nM), whereas it binds five orders of magnitude less tightly to the $\alpha\gamma$ site (K_D = 20 mM) (Kreienkamp *et al.*, 1994; Sine *et al.*, 1995a). α-Conotoxin Ml is unique in that its degree of selectivity is greater than for any known ligand, and its site preference is opposite to that of curariform antagonists, which bind more tightly to the $\alpha\gamma$ site (Blount and Merlie, 1989; Petersen and Cohen, 1990; Sine and Claudio, 1991).

The high degree of sequence identity between the γ and δ subunits suggests that the polypeptide chains of the two subunits fold into similar basic scaffolds. Thus residues in equivalent positions of the linear sequence are predicted to occupy similar positions in three dimensional space. This idea is supported by the striking finding that three residues in equivalent positions of the γ and δ subunits confer virtually all of the selectivity for α-conotoxin Ml (Sine *et al.*, 1995a). Residues in the α subunit that stabilize α-conotoxin Ml have not been identified, but mutagenesis studies with agonists and antagonists revealed three regions of the α subunit that contribute to the binding interface (Sine *et al.*, 1994; Galzi *et al.*, 1991; Middleton and Cohen, 1991; Tomaselli *et al.*, 1991; O'Leary and White, 1994; O'Leary *et al.*,

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ABBREVIATIONS: HEK, human embryonic kidney.

1994; Sugiyama *et al.*, 1996). These three regions differ from binding site regions in the γ and δ subunits, because they are predicted to be located on the opposite face of the subunit. In this study we employ radioiodinated α-conotoxin M1 to show directly that its binding requires an intact subunit interface; high affinity binding is found only at the αδ interface. We then examine through residue replacement the relationships between amino acid determinants in the α subunit and those in the γ and δ subunits that govern α-conotoxin M1 binding.

Materials and Methods

Radioiodination of conotoxin M1. α-Conotoxin M1 (American Peptide Co.), 0.75 nmol (1.125 mg), was iodinated at its single tyrosine (Tyr-12) with 0.1 mCi of $Na^{125}I$ (Amersham) and 2.5 mg of lactoperoxidase (Sigma) in 100 ml of a 50 mM $NaPO_4$ buffer, pH 7.5. Free iodide was removed by selective adsorption on a Dowex 1X-8 (Bio-Rad) cationic resin (Marchot *et al.*, 1993). Labeled α-conotoxin M1 was stored as a 0.5 mM solution in a 1:1 methanol:50 mM $NaPO_4$ buffer, pH 7.5, at −20°. Dilute solutions (1 mM) were prepared in 1 mg/ml bovine serum albumin, 50 mM $NaPO_4$, pH 7.5, stored at 4°, and used within the next 3 weeks. Specific activities of 100 Ci/mmol were achieved, which corresponded to 0.05 atom of iodine incorporated per molecule of α-conotoxin M1. The ratio of labeled species was kept low to minimize formation of diiodo-α-conotoxin M1.

Cell transfections. Human embryonic kidney 293 cells were transfected with cytomegalovirus-based expression vectors containing the respective cDNAs encoding the individual subunits by $Ca_3(PO_4)_2$ precipitation (Sine, 1993; Kreienkamp *et al.*, 1994). Typically plasmids containing cDNAs encoding α, β, γ, and δ subunits in the weight ratio 2:1:1:1, and α, β, and γ, or α, β, and δ in the ratio of 2:1:2 were transfected. The transfections of cDNAs encoding the above four and three subunits yielded pentameric receptors $\alpha_2\beta\gamma\delta$, $\alpha_2\beta\gamma_2$ or $\alpha_2\beta\delta_2$ expressed at the cell surface (Sine and Claudio, 1991), whereas transfection of a cDNA encoding α or δ subunit and cotransfection of two cDNAs encoding α and γ or α and δ required permeabilization of the cells to detect monomeric, dimeric, or tetrameric combinations of subunits within the cells (Green and Claudio, 1993; Kreienkamp *et al.*, 1995).

Association of ^{125}I-α-conotoxin M1 and ^{125}I-α-bungarotoxin with isolated and assembled subunits. Three days after transfection, cells were harvested by gentle agitation in phosphate-buffered saline, pH 7.4, containing 5 mM EDTA. After low speed sedimentation, cells were permeabilized with saponin-containing buffer (10 mM, EDTA, 0.1% bovine serum albumin, and 0.5% saponin in 10 mM $NaPO_4$, pH 7.4), and then incubated on ice with 5 nM ^{125}I-α-bungarotoxin (specific activity of 8–16 mCi/mg; DuPont-New England Nuclear, Boston, MA) or with ^{125}I-α-conotoxin M1 at the specified concentrations. Cells were then sedimented and washed free of excess unbound ligand with potassium Ringer's buffer. The pellets were solubilized on ice in 1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris·HCl, pH 7.5. After 4 hr, supernatants were layered over 3–30% sucrose gradients containing the same detergent buffer. Layered gradients were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 22 hr at 4°. Fractions were collected and assayed; the *S* values were determined as previously described (Kreienkamp *et al.*, 1995; Sugiyama *et al.*, 1996).

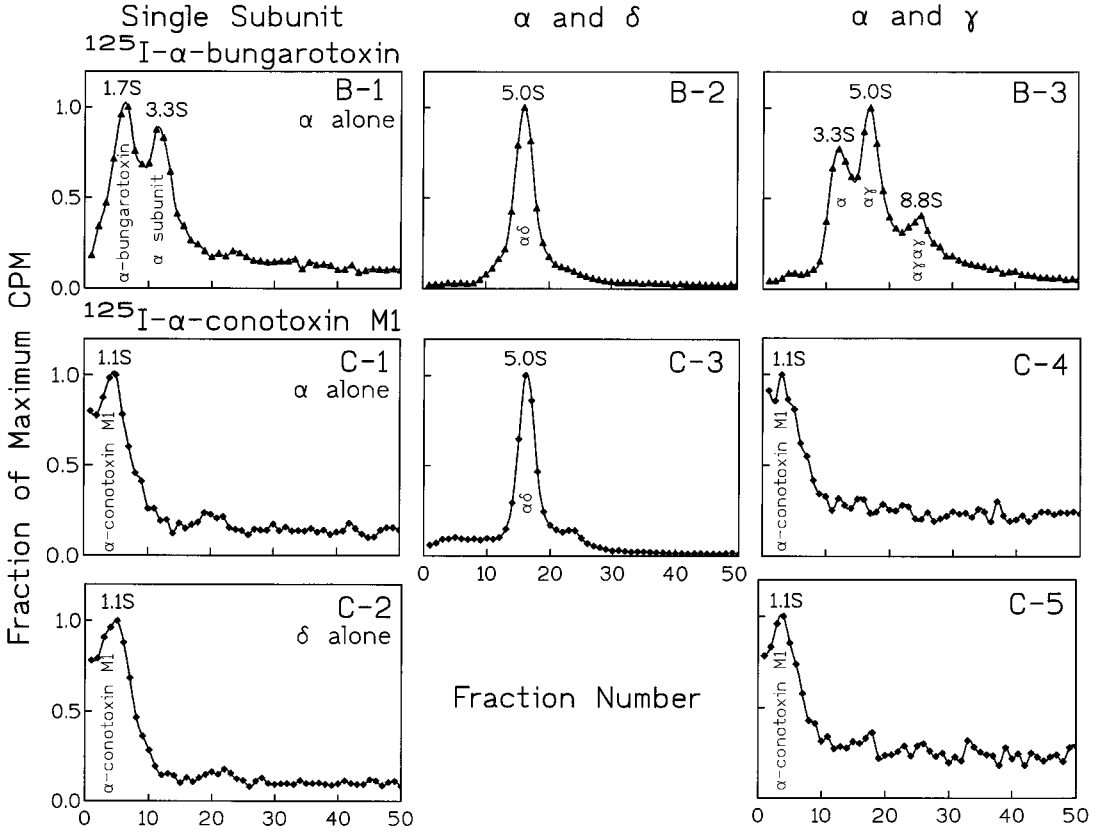

Fig. 1. Sucrose density gradient profiles of ^{125}I-α-Bungarotoxin (B1-B3) and ^{125}I-α-conotoxin M1 (C1–C5) association with nicotinic acetylcholine receptors subunits. HEK cells were transfected with cDNAs encoding the respective subunits, α (B1 and C1) and δ (C2) subunits, or two subunit combinations αδ (B2 and C3) or αγ (B3, C4, and C5). After 48 hr, cells were permeabilized with saponin. The respective radioiodinated toxins [~5 nM ^{125}I-α-bungarotoxin, 20 nM ^{125}I-α-conotoxin M1 (C1–C4), and 400 nM ^{125}I-α-conotoxin M1 (C5)] were allowed to bind. Excess toxin was removed by washing and the cells solubilized with Triton X-100. The solubilized material was layered on a 5–30% sucrose gradient, and fractions collected from the top of the tubes and counted. Comparisons between ^{125}I-α-conotoxin M1 and ^{125}I-α-bungarotoxin binding for particular subunits were made from the same platings of transfected cells.

α-Conotoxin competition with ^{125}I-α-Bungarotoxin. Harvested cells were resuspended in potassium Ringer's buffer to measure ligand binding to receptors expressed on the cell surface. Specified concentrations of α-conotoxin M1 were added to each aliquot of cell suspension 60 min before measurement of the initial rate of ^{125}I-α-bungarotoxin binding. The fractional reduction in the initial rate corresponds to the fractional occupation of sites by α-conotoxin M1 (Sine and Taylor, 1981; Sine *et al.*, 1995a). K_D values for each mutant are given as averages from two separate cell transfections. Sufficient cells were transfected to generate an entire concentration profile with duplicate samples measured at each concentration. The $K_D$values typically varied by less than 20%.

Site-directed mutagenesis. Mutations of the individual subunits were generated by the method of Kunkel (1985); the entire mutagenic insert was sequenced to verify the mutation and rule out random polymerase errors.

Results

^{125}I-α-Conotoxin M1 binding to the receptor subunits. When cDNA encoding α subunit is cotransfected with cDNAs encoding either the γ or δ subunit, the resulting assembled subunits are retained within the cell. To detect association of labeled α-conotoxin, with monomeric subunits and the αγ and αδ assembled oligomers, the cells were permeabilized with saponin, incubated with labeled toxin, washed, solubilized, and centrifuged into sucrose density gradients. The gradients resolve free toxin as well as labeled toxin associated with monomeric, dimeric, and tetrameric subunit oligomers (Fig. 1).

Analysis of HEK cells transfected with equivalent amounts of cDNAs encoding only the α subunit, the α and γ pairs or the α and δ pairs resulted in ^{125}I-α-bungarotoxin association with α subunit, the assembled αγ and αδ subunit dimers, and the αγαγ tetramer (Fig. 1, B1–B3). In contrast, association of ^{125}I-α-conotoxin M1 is detected only with the αδ dimer, but neither with the αγ dimer nor with the αγαγ tetramer (Fig. 1, C1–C4). The absence of a peak or shoulder corresponding to α subunit monomer when cells were cotransfected with either α and γ or α and δ pairs of cDNAs indicates that free subunit monomer does not bind ^{125}I-α-conotoxin M1 with high affinity. To rule out the possibility that the δ subunit rather than the αδ dimer is responsible for α-conotoxin binding, we examined ^{125}I-α-conotoxin M1 association with the isolated δ subunit where expression of δ subunit after cDNA transfection has been confirmed with Western blot analysis using a δ subunit specific monoclonal antibody (mAb166) (Keller S and Taylor P, unpublished observations). As shown in Fig. 1, C2, only a free toxin peak is observed indicating that ^{125}I-α-conotoxin M1 does not associate with the δ subunit monomer.

Selectivity of 20 nM ^{125}I-α-conotoxin M1 for the αδ interface is revealed by the lack of dissociation of the complex as it migrates into the gradients. The results are consistent with previous studies of α-conotoxin M1 competition with ^{125}I-α-bungarotoxin binding to the intact receptor on the cell surface and cell surface receptors devoid of either the γ or δ subunit (Kreienkamp *et al.*, 1994; Sine *et al.*, 1995a). Even at 400 nM concentrations of ^{125}I-α-conotoxin M1, binding to the αγ subunit dimer could not be detected above background levels (Fig. 1, C5), yet parallel plates of cells expressing the αγ subunit combination showed ^{125}I-α-bungarotoxin association with αγ dimer and αγαγ tetramer (Fig. 1, B3).

Identification of α subunit determinants for α-conotoxin M1 binding. Site-directed labeling and mutagenesis studies have defined three linearly distinct regions in the α

Region A

Position (mouse α1)	88	89	90	91	92	93	94	95	96	97	98	99
Muscle												
Mouse α1	P	D	V	V	L	**Y**	N	N	A	D	G	D
Mouse β1	P	D	V	V	L	L	N	N	N	D	G	N
Mouse γ	P	D	I	V	L	E	N	N	V	D	G	V
Mouse δ	P	E	I	V	L	E	N	N	N	D	G	S
Mouse ε	P	E	I	V	L	E	N	N	I	D	G	Q
Neuronal												
Rat α2	P	D	I	V	L	Y	N	N	A	D	G	E
Rat α3	P	D	I	V	L	Y	N	N	A	D	G	D
Rat α4	P	D	I	V	L	Y	N	N	A	D	G	D
Rat α7	P	D	I	L	L	Y	N	S	A	D	E	R
Conserved in α	*	*			*	*	*		*	*		
α -Subunit Mutations	•	•	•	•	•	F	•	•	•	•	•	•
	•	•	•	•	•	S	•	•	•	•	•	•

Region B

Position (mouse α1)	144	145	146	147	148	149	150	151	152	153	154
Muscle											
Mouse α1	M	K	L	G	T	**W**	T	**Y**	**D**	G	**S**
Mouse β1	M	V	F	S	S	Y	S	Y	D	S	S
Mouse γ	L	I	F	Q	S	Q	T	Y	S	T	S
Mouse δ	L	K	F	S	S	L	K	Y	T	A	K
Mouse ε	L	I	F	R	S	Q	T	Y	N	A	E
Neuronal											
Rat α2	M	K	F	G	S	W	T	Y	D	K	A
Rat α3	M	K	F	G	S	W	S	Y	D	K	A
Rat α4	M	K	F	G	S	W	T	Y	D	K	A
Rat α7	L	K	F	G	S	W	S	Y	G	G	W
Conserved in α		*		*		*		*			
α -Subunit Mutations	•	•	•	•	•	F	•	F	N	•	•
	•	•	•	•	•	•	•	•	•	•	•

Region C

Position (mouse α1)	180	181	182	183	184	185	186	-	187	188	189	190	191	192	193	194	195	196	197	198	199	200
Muscle																						
Mouse α1	E	A	**R**	G	**W**	**K**	H	(-)	W	V	**F**	**Y**	S	C	C	**P**	T	T	**P**	**Y**	L	D
Mouse β1	H	K	P	S	R	L	I	Q	L	P	G	D	Q	R	G	G	K	E	G	H	H	E
Mouse γ	H	R	P	A	K	M	L	(-)	L	D	S	V	A	P	A	E	E	A	G	H	Q	K
Mouse δ	H	R	A	A	K	L	N	(-)	V	D	P	S	V	P	M	D	S	T	N	H	Q	D
Mouse ε	Y	C	P	G	M	I	R	(-)	R	Y	E	G	G	S	T	E	G	P	G	E	T	D
Neuronal																						
Rat α2	N	A	T	G	T	Y	N	(-)	S	K	K	Y	D	C	C	(-)	A	E	I	Y	P	D
Rat α3	K	A	P	G	Y	K	H	(-)	E	I	K	Y	N	C	C	(-)	E	E	I	Y	Q	D
Rat α4	D	A	V	G	T	Y	N	(-)	T	R	K	Y	E	C	C	(-)	A	E	I	Y	P	D
Rat α7	G	I	P	G	K	R	N	(-)	E	K	F	Y	E	C	C	(-)	K	E	P	Y	P	D
Conserved in α				*								*		*	*					*		*
α -Subunit Mutations	•	•	V	•	Y	E	•	•	•	K	K	F	•	•	•	L	E	•	I	F	•	•
	•	•	•	•	•	•	•	•	•	D	•	T	•	•	•	(-)	•	•	•	T	•	•

Fig. 2. Amino acid sequences and studied residue mutations for the three domains in the α subunit which contribute to ligand binding. *Bold residues,* mutations.

subunit (regions A–C) that contribute to the ligand binding site (Dennis *et al.*, 1988; Galzi *et al.*, 1991; Middleton and Cohen, 1991; Tomaselli *et al.*, 1991; O'Leary and White, 1992; O'Leary *et al.*, 1994; Sine *et al.*, 1994; Fu and Sine, 1994; Keller *et al.*, 1995). Each region, presumably existing as a loop at the subunit interface, contains conserved aromatic residues, including Y93, W149, Y190, and Yl98 (Fig. 2). Here we mutate residues in these three regions and measure changes in α-conotoxin M1 affinity. Most mutations are substitutions for the aromatic residues, but also include substitutions of residues differing between muscle and neuronal α subunits. Binding of α-conotoxin M1 was measured by competition against the initial rate of ^{125}I-α-bungarotoxin binding, as described previously (Sine and Taylor, 1981).

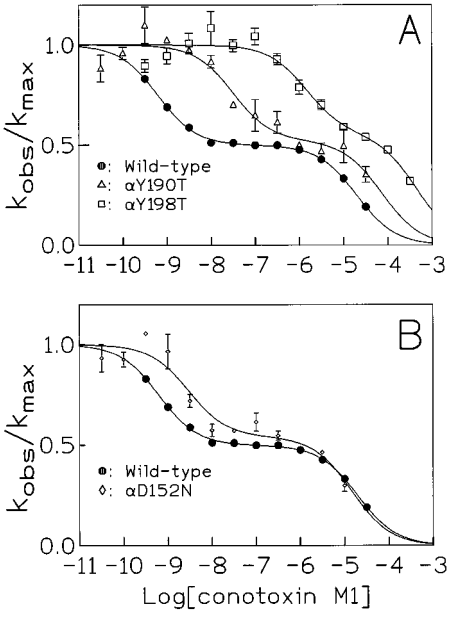

Fig. 3. α-Conotoxin M1 competition with the initial rate of α-bungarotoxin association at cell surface nicotinic receptors after transfection of HEK cells with cDNAs encoding the component subunits. Cells were transfected with wild-type or mutant α subunit cDNAs along with β, γ, and δ cDNAs. After 48 hr, the cells were incubated with α-conotoxin M1 at the specified concentrations, and initial rates of ^{125}I-α-bungarotoxin association were measured. A, ●, wild-type α subunit; △, Y190T; □, Y198T. B, ●, wild-type α subunit; ◇, D152N. A shows that the mutation in the α subunit affects the high affinity, αδ interface more than the low affinity, αγ interface. B shows a shift by the αD152N mutation only at the αδ interface. k_{obs} and k_{max} are the bimolecular rate constants for ^{125}I-α-bungarotoxin association in the presence and absence of α-conotoxin M1.

Mutation of conserved tyrosines in regions A, B, and C. Mutation of Y93, Y190, and Y198 to aliphatic hydroxyl side chains (S or T) reduces affinity for α-conotoxin M1 (Fig. 3 and Table 1). Furthermore, the Y190T and Y198T mutations result in a selective influence for the affinity of the αδ site is affected to much greater extent than the αγ site. Removal of the aromatic hydroxyl at Y151, Y190, and Y198 has no effect on α-conotoxin M1 affinity, whereas the Y93F mutation enhances affinity in a site-selective manner, increasing affinity for the αγ site without affecting the αδ site (Fig. 4 and Table 1). Thus, mutation of the four conserved tyrosines affects α-conotoxin affinity in a manner similar to other antagonists (O'Leary *et al.*, 1994; Sine *et al.*, 1994). Aliphatic hydroxyl substitutions dramatically reduce affinity for α-conotoxin as observed previously for agonists and antagonists, whereas the removal of the hydroxyl group by substitution of phenylalanine either has little influence or enhances affinity (Sine *et al.*, 1994, 1995b; Tomaselli *et al.*, 1991; O'Leary *et al.*, 1994). Interestingly, Y93F increases affinity of α-conotoxin (Fig. 4), whereas Y198F enhances *d*-tubocurarine affinity (Fu and Sine, 1994; Sine *et al.*, 1994).

Mutation of charged, aromatic, and proline residues in region C. α-Conotoxin M1 is amidated on its carboxyl-terminus, lacks negatively charged side chains, but contains three or four positively charged side chains depending on pH. We therefore focused on charged residues in loop C, which differ between muscle and neuronal α subunits (Fig. 2). Re-

TABLE 1
Dissociation constants for α-conotoxin M1 and nicotinic acetylcholine receptor complexes

Receptor mutation[a]	K_D, (αδ)	$K_D$mt(αδ)/$K_D$wt, (αδ)	K_D, (αγ)	$K_D$mt(αγ)/$K_D$wt, (αγ)	Subunit selectivity of the mutation
	nM		*μM*		
Wild type (4)	0.55 ± 0.06	1.00	18.3 ± 0.93	1.00	
Region A					
Y93F (2)	0.46	0.85	1.59	0.086	γ
Y93S (2)	0.88	1.63	113	6.14	γ
Region B					
W149F (2)	0.62	1.15	14.9	0.81	None
Y151F (2)	0.83	1.54	21.3	1.16	None
D152N (2)	3.20	5.93	12.4	0.67	δ
Region C					
R182V (2)	0.64	1.19	11.2	0.61	None
W184Y (2)	0.65	1.20	13.6	0.74	None
K185E (2)	0.50	0.93	18.3	0.99	None
V188K (2)	0.98	1.81	120	6.52	γ
V188D (2)	0.68	1.26	14.8	0.80	None
F189K (2)	1.06	1.96	29.8	1.62	None
Y190F (2)	0.75	1.39	26.3	1.43	None
Y190T (2)	17.1	31.7	48.8	2.65	δ
P194L (2)	0.60	1.11	36.4	1.98	None
P194deletion (2)	0.51	0.94	17.5	0.95	None
T195E (2)	1.54	2.80	15.6	0.85	δ
P197I (2)	1.95	3.61	38.9	2.13	None
Y198F (2)	0.77	1.43	11.3	0.61	None
Y198T (2)	1340	2480	297	16.1	δ

[a] Data represent the average of K_D values calculated from two separate transfections, except for the wild-type (wt) α subunit where is a mean of four values. K_D values are determined from curves of α-conotoxin M1 competition with the initial rate of α-bungarotoxin binding as shown in Figs. 3 and 4. mt = mutant.

placement of positively charged residues with R182V or K185E, or insertion of a positive charge with F189K, fails to affect α-conotoxin M1 affinity (Table 1). By contrast, inserting a positive charge with V188K selectively decreases α-conotoxin Ml affinity for the αγ site without affecting affinity for the αδ site (Fig. 4 and Table 1). Thus, electrostatic interactions with α-conotoxin M1 seem localized to position 188 and specifically affect the αγ site.

We looked for additional sources of stabilization in region C by studying the mutations W184Y and V188D. However, these mutations, one of which adds a negative charge, do not affect α-conotoxin M1 affinity (Table 1).

A small loop bounded by prolines at the 194 and 197 positions is a characteristic feature of the α1 subunit, not found in neuronal α subunits (Fig. 2). Deletion of P194 or the P194L substitution has little effect on α-conotoxin M1 binding. The P197I mutation reduces α-conotoxin M1 affinity 2–4-fold at both sites, whereas the T195E mutation also reduces α-conotoxin M1 affinity, primarily at the αδ site.

Mutations in loop B. Mutations of the conserved W149 and Y151 to phenylalanine do not significantly affect α-conotoxin Ml binding (Table 1). The lack of effect of W149F on α-conotoxin M1 affinity contrasts with the large reduction in agonist affinity associated with this mutation (Sine *et al.*, 1994). Previous studies showed that D152N introduces a glycosylation consensus site that becomes glycosylated, and further that agonist and antagonist affinities are markedly reduced (Sugiyama *et al.*, 1996). For α-conotoxin M1, D152N reduces affinity slightly for the αδ site without affecting the αγ site. Thus, the aromatic residues examined in region B make no contribution to α-conotoxin M1 affinity, whereas the negative charge at position 152 enhances its affinity.

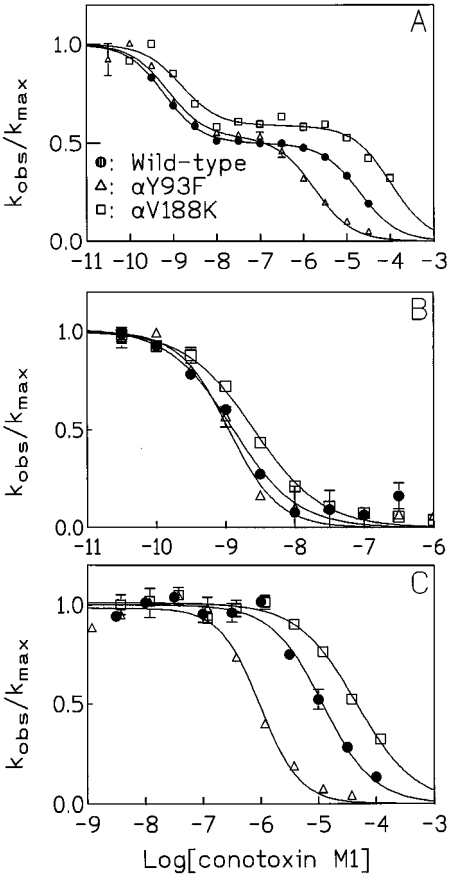

Fig. 4. α-Conotoxin M1 competition with the initial rate of α-bungarotoxin association at cell surface nicotinic receptors after cotransfection of the cDNAs encoding the individual subunits. cDNAs encoding wild-type (●) or mutant V188K (□), Y93F (△) α subunits along with specified combinations of other subunits were transfected into HEK cells and α-conotoxin M1 binding was measured on the cell surface as described in Methods. A, Wild-type α subunits, V188K or Y93F mutant α subunit with β, γ, and δ subunits. B, Wild-type α subunits, V188K or Y93F mutant α subunits with β and δ subunits. C, Wild-type α subunits or V188K or Y93F mutant subunits with β and γ subunits. Dissociation constants were calculated assuming high and low affinity sites of equal population and are listed in Table 1. The assay is identical to that shown in Fig. 3.

Mutagenesis of residues in both the α subunits and non-α subunits. Previous studies identified three residues in equivalent positions of the γ and δ subunits that confer higher affinity of α-conotoxin M1 for the αδ over the αγ interface (Sine *et al.*, 1995a). To account for the selective effect of αY93F and αV188K for the αγ interface, we reasoned that either α-conotoxin M1 affinity is enhanced by interaction between determinants in the α and γ subunits or α-conotoxin M1 orients differently at the αγ and αδ interfaces. Thus we investigated these two possibilities by examining α-conotoxin Ml binding to receptors containing mutations in both the α and γ subunits.

We first examined the site selective mutations αY93F and αV188K incorporated into pentameric receptors lacking the δ subunit. The resulting cell surface receptors have the composition $\alpha_2\beta\gamma_2$, which should contain two equivalent αγ binding sites (Sine and Claudio, 1991). Incorporating αY93F into these pentamers increases α-conotoxin M1 affinity, whereas incorporating αV188K decreases affinity for both αγ sites (Fig. 5A and Table 2), as observed for one of the two sites in $\alpha_2\beta\gamma\delta$ pentamers (Fig. 4 and Table 1). Moreover, when coexpressed with the series of mutant γ subunits, the αY93F and αV188K mutations affect α-conotoxin M1 affinity to the same extent as observed when coexpressed with the wild-type γ subunit (Fig. 5A). Thus, αY93F and αV188K do not interact

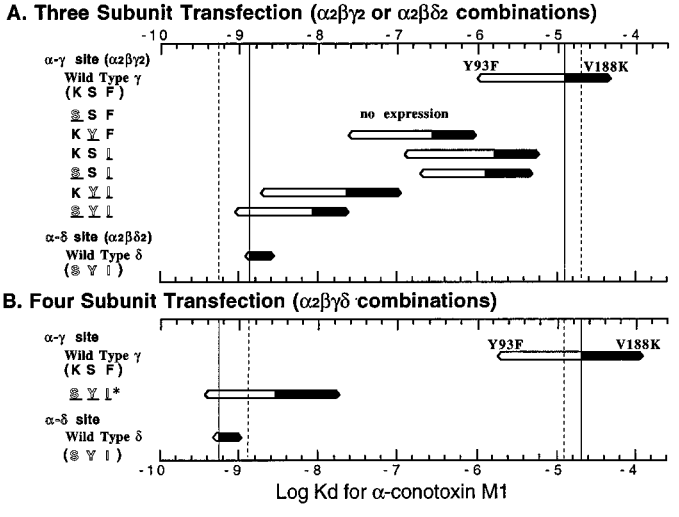

Fig. 5. Interrelationships in α-conotoxin M1 dissociation constants for α subunit mutant receptors containing mutations in the γ and δ subunits. Data are determined from concentration profiles similar to those shown in Figs. 3 and 4. A, Cotransfection of three subunits: α (wild-type or mutant Y93F or V188K), wild-type β, either wild-type or mutant γ, or wild-type δ were cotransfected. B, Cotransfection of four subunits: α (wild-type or mutant Y93F or V188K), wild-type β, wild-type or mutant γ, and wild-type δ were cotransfected. The dissociation constants determined are shown on a logarithmic plot relative to wild-type α subunits.

with residues in the γ subunit that confer selective binding of α-conotoxin M1.

Curiously, neither αY93F nor αV188K significantly affect affinity when paired with the wild-type δ subunit and expressed as $\alpha_2\beta\delta_2$. However, they affect affinity strongly when paired with the triple mutant of the γ subunit (γSYI) that increases affinity to approach that conferred by the δ subunit (Fig. 5). These findings suggest that other residues unique to the δ subunit nullify the effects of αY93F and αV188K, perhaps by conferring a different orientation of α-conotoxin M1 at the αγ and αδ interfaces. Alternatively, constraints imposed by the δ subunit may preclude Y93F in the α subunit from achieving a higher affinity than in the wild-type receptor α-δ interface.

We also examined the influence of the a Y93F and V188K mutations on α-conotoxin M1 affinity when the receptor is assembled from four distinct subunits as $\alpha_2\beta\gamma\delta$, rather than as $\alpha_2\beta\gamma_2$ or $\alpha_2\beta\delta_2$. α-Conotoxin M1 affinity for the αδ site is slightly higher, whereas the affinity for the αγ site is slightly lower in the $\alpha_2\beta\gamma\delta$ pentamer, compared with the pentameric combinations of the three respective subunits (Table 2 and Fig. 5B). Hence, the difference in $K_D$values for the two sites is larger in the receptor assembled from four distinct subunits ($\alpha_2\beta\gamma\delta$) than three ($\alpha_2\beta\gamma_2$ or $\alpha_2\beta\delta_2$). This may reflect longer range interactions between binding sites. Nevertheless, the influence of the α subunit mutations is the same in the pentameric assemblies of four and three distinct subunits (Fig. 5B).

The αY190T and αY198T mutations show a predominant, but not completely selective, influence on reducing the affinity of the high affinity, αδ interface (Fig. 3A and Table 1). When these mutations are coexpressed as pentamers with the γ subunit triple mutant (γSYI), a reduction in α-conotoxin M1 affinity similar to that found for the δ subunit is seen; the absolute affinities are enhanced over coexpression with wild-type γ because of the γSYI mutation (Fig. 6). Examination for the opposite situation, the substitution of γ subunit containing side chains into the δ template, yielded diminished receptor expression with the mutant α subunits and precluded a precise quantitation of affinity. Nevertheless, the influence of αY190T and αY198T mutations seems to depend on the binding affinity of α-conotoxin M1 and, here again, may reflect a slightly different binding position for α-conotoxin M1 between the high and low affinity binding sites.

TABLE 2

α-Conotoxin MI dissociation constants (K_D) for wild-type and mutant nicotinic receptors expressed from three and four subunit combinations

K_D values are averages from two separate transfections. In each transfection, a complete concentration profile is generated using duplicate sampling at each concentration (compare Figs. 3, 4, and 6). Transfection of α/β/γ/δ (2:1:1:1), α/β/γ (2:1:2), and α/β/δ (2:1:2) in the specified cDNA ratios yields the above stoichiometry of subunits (Sine and Claudio, 1991; Sine 1993).

Receptor subunit composition	K_D, (αδ) (nM)	$K_D$mt (αδ)/ $K_D$wt, (αδ)	K_D, (αγ) (μM)	$K_D$mt(αγ)/ $K_D$wt, (αγ)
Wild-type				
$\alpha_2\beta\gamma\delta$	0.54	1.00	18.4	1.00
$\alpha_2\beta\delta_2$	1.34	1.00		
$\alpha_2\beta\gamma_2$			11.7	1.00
αY93F				
$\alpha_2\beta\gamma\delta$	0.46	0.85	1.59	0.086
$\alpha_2\beta\delta_2$	1.01	0.77		
$\alpha_2\beta\gamma_2$			0.96	0.082
αV188K				
$\alpha_2\beta\gamma\delta$	0.98	1.81	120	6.52
$\alpha_2\beta\delta_2$	2.74	2.09		
$\alpha_2\beta\gamma_2$			44.7	3.82

Discussion

Subunit contributions to α-conotoxin M1 association. Radioiodination to form ^{125}I-α-conotoxin M1 enables one to monitor directly the α-conotoxin association with various subunit combinations. High affinity binding where bound ^{125}I-α-conotoxin M1 is retained by the receptor subunits after sedimentation is evident only for the αδ dimer. The same procedure shows α-bungarotoxin association with α subunit monomers, αδ dimers, αγ dimers, and αγαγ tetramers. These findings not only document directly the site selectivity of α-conotoxin M1, but also reveal that α-conotoxin M1 requires an intact αδ subunit interface for high affinity binding.

Residues conferring α-conotoxin M1 selectivity. Previous studies have defined three residues in the δ subunit (S36, Y113, and I178), which confer high affinity binding of α-conotoxin M1 to the αδ interface (Sine *et al.*, 1995a). When substituted with the corresponding residues in the γ subunit (K34, S111, and F172), this triad of mutations in the δ template confers an α-conotoxin M1 affinity approaching that of the γ subunit (Sine *et al.*, 1995a). To account for αδ and αγ interfaces showing nearly a 10,000-fold difference in α-conotoxin M1 affinity, either the γ subunit diminishes the influence of determinants on the α subunit to α-conotoxin binding or determinants on the δ subunit are major contributors to the high affinity of α-conotoxin M1. In turn, the δ subunit could enhance affinity either directly through its own interactions with conotoxin or by altering the conformation of the α subunit. Because the differences in α-conotoxin M1 affinity between αδ and αγ interfaces are diminished and actually inverted for the *T. californica* receptor (Hann *et al.*, 1994; Utkin *et al.*, 1994; Groebe *et al.*, 1995; Sine *et al.*, 1995a) and affinity of the αε subunit interface is intermediate to the αδ and αγ interfaces in mouse (Sine S, unpublished observations), the γ, δ, and ε subunits likely contribute to stabilization of the α-conotoxin complex both directly and by affecting the conformations of the neighboring α subunit.

To pursue the question of subunit contributions further, we

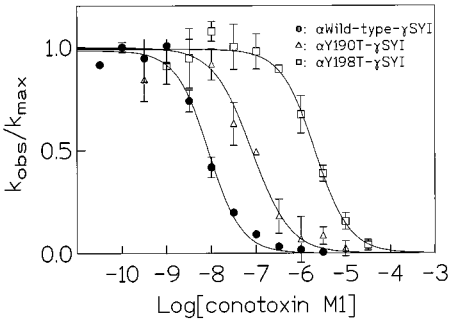

Fig. 6. Influence of α subunit mutations of Y190T and Y198T on the binding of α-conotoxin M1 to receptors of $\alpha_2\beta\gamma_2$ composition containing the three γ subunit mutations required to increase affinity. The γ subunit mutations (K34S, S111Y, and F172I) substitute three residues found at homologous positions in the δ subunit into the γ subunit template. By substitution of three residues of the δ subunit sequence into a γ subunit, high affinity for α-conotoxin M1 is conferred as well as the sensitivity to tyrosine substitutions in the α subunit resulting in a loss of affinity.

examined individual residues in three regions in the α subunit known to influence agonist and alkaloid antagonist binding to the receptor. Because several of the α-conotoxins such as M1 are relatively selective for the muscle subtype of receptor (i.e., α1 subunit-containing receptors), we also relied on differences in the sequence between α1 and the neuronal α subunits (α2, α3, α4, and α7) of receptor. In the α1 subunit, the region between residues 180 and 200 has been particularly well studied. Two tyrosines, Y190 and Y198, are conserved in all α subunits, and their modification affects the binding of both agonists and antagonists (Tomaselli *et al.*, 1991; O'Leary and White, 1992; Fu and Sine, 1994; O'Leary *et al.*, 1994; Sine *et al.*, 1994). Moreover, a diterpene antagonist, lophotoxin, actually conjugates with Y190 (Abramson *et al.*, 1989). The Y190F and Y198F mutations have a marked influence on agonist and *d*-tubocurarine binding, but show little influence on α-conotoxin M1 association. However, Y93F enhances α-conotoxin affinity. Removal of aromaticity by substituting T for Y has a substantial influence on both α-conotoxin M1 (Table 1 and Figs. 3 and 6), and the nonpeptide antagonists (Sine *et al.*, 1994), particularly at the high affinity αδ interface. Thus, aromaticity may be required to stabilize the quaternary amine moiety as well as the cationic peptide.

Several further modifications of residues in this region showed that creating a positive charge at residue 188 markedly decreased binding affinity, whereas a negative charge at this position slightly enhanced affinity; these changes were evident only at the αγ interface. Modification of residues between the two prolines at position 197 produced complex behavior: substitutions or deletions at position 194 were largely without influence, whereas substitutions at position 197 slightly lowered α-conotoxin M1 affinity. The T195E mutation reduced α-conotoxin M1 affinity selectively at the αδ interface. Unfortunately, we were unable to obtain reproducible binding with the T196E mutation. The cumulative influence of residues 194–197, when modified to residues found in neuronal receptors (Fig. 2), could account for part of the selectivity of α-conotoxin M1 for the muscle type of receptor.

The Y93F mutation enhances α-conotoxin M1 binding affinity at the low affinity, αγ site, whereas substitution of serine decreases the affinity at this site (Fig. 4). Modifications of aromatic residues at positions 149 and 151 are without influence, whereas the D152N mutation selectively reduces the affinity at the αδ interface (Fig. 3). Hence, the three regions of linear sequence known to affect the affinity of agonists and nonpeptidic antagonists also influence α-conotoxin binding. However, the similarities of residue contributions to α-conotoxin M1 and other nonpeptidic antagonists apply to regional segments of sequence but not necessarily to the individual amino acids.

Relationship between α subunit mutations and γ/δ subunit mutations. We examined whether linkage relationships exist between mutations in the α subunit and those in γ and δ subunits or whether the determinants on each subunit act independently of each other as would be expected if they bound to different portions of the α-conotoxin molecule. A strong linkage relationship is apparent between S36 and I178 on the δ subunit, where the individual substitutions, S36K or I178F, has a small or no influence on α-conotoxin K_D values, yet when both substitutions are made, a marked loss in affinity or increase in K_D evident (Sine *et al.*, 1995a). Using a mutant cycle analysis (Carter *et al.*, 1984) for mutations at corresponding positions 36 and 178 in the δ subunit template, we may set the following cycle:

$$\begin{array}{ccc} & \overset{S_1}{\rightharpoonup} & \\ \delta_{S36,I178} & & \delta_{K36,I178} \\ I_2 \downarrow & & \downarrow I_1 \\ & \underset{S_2}{\rightharpoonup} & \\ \delta_{S36,F178} & & \delta_{K36,F178} \end{array}$$

Where

$$\Omega = \frac{S_1}{S_2} = \frac{I_2}{I_1} = \frac{K_{SI}K_{KF}}{K_{SF}K_{KI}} = 142$$

Then

$$\Omega^{\ddagger} = RT \ln \Omega = 2.9 \text{ kcal/mol}$$

In this formulation, the dissociation constants K, for α-conotoxin and the receptor site are subscripted by the residues at positions 36 and 178. S_1, S_2, I_1, and I_2 designate the ratio of dissociation constants for the species at the base of the arrow relative to the tip of the arrow. The values are represented as absolute values without reference to sign.

Hence, a comparatively large linkage or coupling energy is seen between positions 36 and 178 in the δ subunit for the binding of α-conotoxin M1. When similar mutant cycles were constructed to relate mutations in the α subunit with those in γ and δ subunits, linkage relationships between the three γ/δ positions at residues 34/36, 111/113, and 172/178 with residues 93 and 188 in the α subunit were not evident ($\Omega^{\ddagger} \leq 0.35$ kcal/mol); rather, the energetic contributions to α-conotoxin M1 binding of residues in the α subunit seem independent of those in the γ and δ subunits (compare Fig. 5). Hence, the coupling energy contributing to α-conotoxin binding occurs within rather than between subunits. The independence of mutations between subunits indicate that distinct surfaces of the α-conotoxin molecule interact with the α and γ/δ subunit faces.

Our findings that the αγ and αδ interfaces possess disparate affinities for α-conotoxin M1 and that certain residues in the α subunit selectively affect binding at the γ or δ interface, suggest that the amino acid contributions from the common α subunit as well as the distinct γ and δ subunits differ in the stabilization of α-conotoxin M1. Hence, the orientations of the bound α-conotoxin molecules at the two sites are likely to be distinct.

Recent x-ray crystallographic (Guddat *et al.*, 1996; Hu *et al.*, 1996) and NMR studies (Han *et al.*, 1997) of α-conotoxin-G1, P1VA, and Pn1A show a triangular, wedgelike structure stabilized by the disulfide bonds between Cys3 and Cys8 and between Cys4 and Cys14. Fitting the sequence of α-conotoxin M1 into the structural template shows that the amino terminus (either Gly1 or the side chain of Arg2), Pro6, and Arg10 are found at the vertices of the triangle. The arginines at positions 2 and 10 are some 15 Å apart, suggesting that different receptor subunits could harbor the anionic sites that stabilize the cationic loci. Because, in addition to the amino terminus, only one cationic charge at position 10 is conserved as Arg or Lys among the α-conotoxins (Myers *et al.*,

1991, 1993), structure modification of α-conotoxins and analysis of their binding to mutant receptors should yield further details on the orientation of the bound peptide.

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