Molecular Pharmacology Fast Forward. Published on August 22, 2012 as doi:10.1124/mol.112.080853

MOL # 80853

Title page

α-Conotoxin PeIA[S9H,V10A,E14N] potently and selectively blocks α6β2β3 vs. α6β4 nicotinic acetylcholine receptors

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Molecular Pharmacology Fast Forward. Published on August 22, 2012 as DOI: 10.1124/mol.112.080853
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Running title page

Running title: PeIA[S9H,V10A,E14N] blocks α6β2β3 nicotinic receptors

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Number of text pages: 22

Number of tables: 4

Number of figures: 8

Number of references: 47

Number of words in the abstract: 231

Number of words in the introduction: 640

Number of words in the discussion: 1497

Abbreviations: ACh, acetylcholine; α-Ctx, α-conotoxin; nAChR, nicotinic acetylcholine receptor; *, denotes the possible presence of additional subunits.

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Abstract

Nicotinic acetylcholine receptors (nAChRs) containing α6 and β2 subunits modulate dopamine release in the basal ganglia and are therapeutically relevant targets for treatment of neurological and psychiatric disorders including Parkinson's disease and nicotine dependence. However, the expression profile of β2 and β4 subunits overlap in a variety of tissues including locus coeruleus, retina, hippocampus, dorsal root ganglia, and adrenal chromaffin cells. Ligands that bind α6β2 nAChRs also potently bind the closely related α6β4 subtype. To distinguish between these two subtypes, we synthesized novel analogs of a recently described α-conotoxin, PeIA. PeIA is a peptide antagonist that blocks several nAChR subtypes, including α6/α3β2β3 and α6/α3β4 nAChRs, with low nanomolar potency. We systematically mutated PeIA and evaluated the resulting analogs for enhanced potency and/or selectivity for α6/α3β2β3 nAChRs expressed in *Xenopus* oocytes (α6/α3 is a subunit chimera that contains the N-terminal ligand binding domain of the α6 subunit). Based on these results, second generation analogs were then synthesized. The final analog, PeIA[S9H,V10A,E14N], potently blocked acetylcholine-gated currents mediated by α6/α3β2β3 and α6/α3β4 nAChRs with IC_{50} values of 223 pM and 65 nM, respectively, yielding a >290-fold separation between the two subtypes. Kinetic studies of ligand binding to α6/α3β2β3 nAChRs yielded a k_{off} of 0.096 ± 0.001 min^{-1} and a k_{on} of 0.23 ± 0.019 min^{-1} M^{-9}. The synthesis of PeIA[S9H,V10A,E14N] demonstrates that ligands can be developed to discriminate between α6β2 and α6β4 nAChRs.

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Introduction

Mammalian neuronal nicotinic acetylcholine receptors (nAChRs) assemble in a pentameric stoichimetry from eight α and three β subunits to form various receptor subtypes. Selective ligands have played a critical role in identifying individual subtypes and defining their physiological functions. nAChR subtypes that contain the α6 subunit have a very restricted tissue distribution. In the central nervous system (CNS), α6-containing nAChRs are expressed in dopaminergic regions including the substantia nigra, ventral tegmental area, and the nucleus accumbens (Gotti et al., 2010; Yang et al., 2009). Dopaminergic neurons of the substantia nigra are gradually lost in Parkinson's disease which leads to disordered control over motor movement (Bordia et al., 2007). In reward centers of the brain, activation of α6-containing nAChRs enhances dopamine release and reinforces the addictive properties of nicotine (Brunzell et al., 2010; Exley et al., 2011; Jackson et al., 2009; Liu et al., 2012; Pons et al., 2008). In these midbrain areas, α6 subunits assemble with β2 subunits to form α6β2* nAChRs (asterisk denotes the possible presence of additional subunits); areas where expression of the β4 subunit is minimal or absent and therefore, few, if any, α6β4* nAChRs are likely to be present. Thus, ligands that bind α6* nAChRs can be used in these regions to selectively identify α6β2* nAChRs. However, in other areas such as the retina (Marritt et al., 2005; Moretti et al., 2004) and the locus coeruleus (Azam and McIntosh, 2006; Lena et al., 1999; Vincler and Eisenach, 2003) α6β4* nAChRs are present and may be co-expressed with the α6β2* subtype. Moreover, in the hippocampus of mouse, α6β4* nAChRs control the release of norepinephrine (Azam et al., 2010). Lastly, in the peripheral nervous system, α6β4* nAChRs are expressed by human adrenal chromaffin cells (Perez-Alvarez et al., 2012) and by rat dorsal root ganglion neurons (Hone et al., 2012), two cell populations that also have substantial β2 subunit expression. Thus, ligands

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that can discriminate between α6β2* and α6β4* nAChRs are needed to facilitate the study of α6β2* nAChRs in areas where multiple α6-containing nAChRs are potentially expressed.

Predatory cone snails (*Conus*) have evolved a rich, combinatorial-like library of neuropharmacologically active compounds. Among the principal components of the cone snail venom are the α-conotoxins (α-Ctxs), disulfide-rich peptide antagonists of nAChRs. Each species of cone snail produces several distinct α-Ctxs as part of its venom arsenal to immobilize prey. Subsets of these α-Ctxs are now widely employed by neuroscientists to block the function of mammalian nAChR subtypes (Armishaw, 2010; Azam and McIntosh, 2009). However, as the diversity of nAChR subtypes is progressively elucidated, the need for increasingly selective ligands correspondingly grows. Several existing α-Ctxs potently block α6 nAChRs. However, α-Ctxs that block α6/α3β2β3 nAChRs also potently block α6/α3β4 nAChRs (Dowell et al., 2003). Thus, further refinement of the specificity of these ligands is required.

α-Ctx PeIA, cloned from a cDNA library of *Conus pergrandis*, potently blocks several nAChRs subtypes including those that contain the α6 subunit (McIntosh et al., 2005). We used α-Ctx PeIA as a template to create ligands with novel specificity and tested them on *Xenopus* oocytes expressing cloned nAChRs. We used rat and mouse α6/3 subunit chimeras to model the α6β2 and α6β4 ligand binding domain because injection of non-chimeric α6 with β2 and β3 fails to reliably produce functional expression (Dash et al., 2011; Dowell et al., 2003; Kuryatov et al., 2000; Papke et al., 2005). However, comparison of data obtained from studies on heterologously expressed chimeric constructs of α6 with studies on native α6-containing nAChRs demonstrate that these chimeric constructs and native α6-containing nAChRs share a similar pharmacological profile (Bordia et al., 2007; Capelli et al., 2011; Perez-Alvarez et al., 2012). Using this strategy

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and through synthetic iteration of α-Ctx PeIA, we created a ligand that to our knowledge is the most selective α-Ctx to date for distinguishing between α6β2β3 and α6β4 nAChRs.

Materials and Methods

Rat α3, α4, and α7 nAChR subunit clones were provided by S. Heinemann (Salk Institute, San Diego, CA, USA), C. Luetje (University of Miami, Miami, FL, USA) provided the β2, β3, and β4 subunits in the high-expressing pGEMHE vector and α9 and α10 were provided by A. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). Mouse α6, β2, β3, and β4 subunit clones were provided by J. Stitzel (University of Colorado, Boulder, CO, USA). Construction of α6/α3 subunit chimeras has been previously described and consists of an α3 subunit where the first 237 amino acids of the ligand binding domain are replaced with the corresponding α6 amino acids (McIntosh et al., 2004). The rat α6/α4 chimera was provided by R. Papke (University of Florida, Gainesville, FL, USA)These chimeras were used because of poor expression of the non-chimeric forms of the α6 construct. The human β3-α6-β2-α4-β2 concatamer has been previously described (Kuryatov and Lindstrom, 2011). Acetylcholine chloride (ACh) and bovine serum albumin were obtained from Sigma Aldrich (St. Louis, MO, USA). N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) was purchased from Research Organics (Cleveland, OH, USA).

Peptide Synthesis

Standard peptide chemistry was used to generate the α-Ctx peptides as previously described (Cartier et al., 1996) or synthesized on an AAPPTec Apex 396 automated peptide synthesizer (Louisville, KY, USA) applying standard solid-phase Fmoc (9-fluorenylmethyloxycarbonyl)

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methods. The peptides were initially constructed on a preloaded Fmoc-Rink Amide MBHA resin (substitution: 0.4 mmol/g^{-1}; Peptides International Inc, KY, USA). All standard amino acids were purchased from AAPPTec. Side-chain protection for the following amino acids was: His- and Asn-trityl (Trt), Ser-tert-butyl (*t*Bu). Cys residues were orthogonally protected by Trt for Cys^1 and Cys^3 and acetamidomethyl (Acm) for Cys^2 and Cys^4. The peptides were synthesized at 50 μmol scale. Coupling activation was achieved with 1 equivalent of 0.4 M benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2 equivalents of 2 M *N*,*N*-diisopropylethyl amine (DIPEA) in *N*-methyl-2-pyrrolidone (NMP) as the solvent. For each coupling reaction 10-fold excess of amino acid was used and the reaction was conducted for 60 min. The Fmoc deprotection reaction was carried out for 20 min with 20% (vol/vol) piperidine in dimethylformamide (DMF). The peptides were cleaved from the resin using Reagent K, trifuloroacetic acid/phenol/ethanedithiol/thioanisol/H_2O (9:0.75:0.25:0.5:0.5 by volume) and a two-step oxidation protocol was used to selectively fold the peptides in the correct disulfide configuration. Briefly, the first disulfide bridge was closed using 20 mM potassium ferricyanide and 0.1 M Tris, pH 7.5. The solution was allowed to react for 45 min and then the monocyclic peptide was purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Simultaneous removal of the Acm groups and closure of the second disulfide bridge was carried out by iodine oxidation. The monocyclic peptide and HPLC eluent was dripped into an equal volume of iodine (10 mM) in H_2O/trifluoroacetic acid/acetonitrile (78:3:25 by volume) and allowed to react for 10 min. The reaction was terminated by the addition of ascorbic acid diluted 20-fold with 0.1% (vol/vol) trifluoroacetic acid and the bicyclic product purified by RP-HPLC. The masses of the peptides were verified by matrix-assisted laser desorption ionization time-off-flight mass spectrometry at the Salk Institute for Biological Studies (San Diego, CA, USA) under

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the direction of Dr. J. Rivier. Mass spectrometry and HPLC data for the peptides are shown in Supplemental Table 1.

Two-Electrode Voltage-Clamp Electrophysiology of *Xenopus laevis* Oocytes.

Detailed methods for conducting electrophysiological experiments of nAChRs heterologously expressed in *Xenopus laevis* oocytes have been previously described in detail (Hone et al., 2009). Briefly, stage IV-V oocytes were injected with equal ratios of cRNA encoding cloned rat and mouse nAChR subunits α3, α4, α6/α3, α7, α9, α10, β2, β3, and β4 and used 1-4 days after injection. The oocyte membranes were clamped at a holding potential of -70 mV and continuously gravity perfused with standard ND96 solution and stimulated with 1-sec pulses of ACh once every min. The solution changes were controlled through a series of 3-way solenoid valves interfaced with a personal computer via a CoolDrive valve driver (Neptune Research & Development, West Caldwell, NJ, USA) and LabVIEW software (National Instruments, Austin, TX, USA). The ACh-gated currents (I_{ACh}) were acquired using an Oocyte OC-725 series voltage-clamp amplifier (Warner Instruments, Hamden, CT, USA), filtered through a 5 Hz low-pass Bessel style filter (model F1B1; Frequency Devices, Ottawa, IL, USA), and digitized at 50 Hz using a National Instruments USB-6009 digital to analog converter. The toxins were diluted in ND96 and perfusion applied up to 1 μM; concentrations ≥10 μM were bath applied in a static bath for 5 min. To determine the observed off-rates (k_{off}), the toxins were applied at 10 μM or 100 μM depending on the nAChR subtype and the potency of the toxin. PeIA, PeIA[V10], and PeIA[E14N] were applied at 100 μM for all subtypes. PeIA[S9H] was applied at 10 μM for all subtypes and PeIA[S9H,V10A,E14N] was applied at 10 μM for α6/α3β2β3 and at 100 μM for α6/α3β4 nAChRs.

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Data Analysis

The electrophysiology data were analyzed using GraphPad Prism software (La Jolla, CA, USA). Concentration-response curves for inhibition of I_{ACh} were generated by fitting the data to the Hill equation: % response = $100/\{1 + ([toxin]/IC_{50})^{nH}\}$. Data for observed on-rates (k_{obs}) were fit with a one-phase exponential equation and then analyzed by linear regression analysis to obtain an estimated value for k_{on}. Observed off-rate kinetics were assessed by fitting the I_{ACh} amplitudes with a one-phase exponential equation to obtain the plateau value of the I_{ACh} ; observed values were then normalized to the plateau value and displayed as a percentage of the response to ACh. Three-dimensional reconstructions of PeIA[S9H,V10A,E14N], MII, and PnIA were generated using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC). PeIA[S9H,V10A,E14N] was generated using the NMR structural coordinates of PeIA (Daly et al., 2011) as a template and the mutagenesis function of PyMOL. MII was also generated from NMR structural coordinates (Hill et al., 1998) and PnIA was generated from X-ray crystallography coordinates (Hu et al., 1996). Structures were generated without consideration of alternative rotameric positions of the side chains.

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Results

Single Amino Acid Substitutions of PeIA Confer Increased Potency and Selectivity for the α6/α3β2β3 Subtype. α-Ctx PeIA was originally isolated from the fish-eating species *Conus pergrandis* and at the time of its discovery was noted to be the first peptide ligand that potently blocked α9α10 but not α7 nAChRs (McIntosh et al., 2005). However, PeIA also potently blocks several other nAChR subtypes including those that contain the α6 subunit. PeIA shows a high degree of homology to other α-Ctxs that have 4 Cys residues that are connected by two disulfide bonds (Fig. 1A). Structure function studies of α-Ctx MII, indicated that residues of the second disulfide loop interact with residues of the α6 nAChR subunit (Azam et al., 2008; McIntosh et al., 2004; Pucci et al., 2011). His residues in the 9th and 12th positions, and Asn in the 14th position of MII are critical for conferring potency on α6/α3β2β3 nAChRs (McIntosh et al., 2004). For α-Ctx PnIA, Ala in the 10th position is critical for subtype selectivity and potency for β2-containing nAChRs (Hogg et al., 1999; Luo et al., 1999). Therefore, we reasoned that mutations in the homologous positions of PeIA might not only be permissive for retaining activity, but might also confer beneficial changes in selectivity. We synthesized analogs that incorporated these non-conserved amino acids into the second disulfide loop of PeIA (Fig 1A). These synthetic peptides were then evaluated for changes in potency and selectivity for the α6/α3β2β3 subtype using two-electrode voltage-clamp electrophysiology of *Xenopus* oocytes expressing cloned nAChRs. Substitution of Ser for His in the 9th position of PeIA increased the potency for inhibition of α6/α3β2β3 nAChRs by ~11-fold; increased potency was also observed on α3β2, α6/α3β4, and α3β4 nAChRs (Fig. 2A). In contrast, the S9H substitution resulted in an ~55-fold loss of potency on the α9α10 subtype (Fig. 2A). Next, we evaluated an Ala substitution for Val in the 10th position. Similar to the S9H analog, PeIA[V10A] showed increased activity on

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the α6/α3β2β3 nAChR, but in contrast showed a ~2-fold loss of activity on α6/α3β4 and α3β4 nAChRs (Fig. 2B). The net result of this substitution was an analog with ~126-fold selectivity for α6/α3β2β3 vs. the α6/α3β4 subtype. Finally, Asp was substituted for Glu14; although the activity was decreased for α6/α3β2β3 by ~2-fold, there was an ~3-fold loss of activity for α6/α3β4 and an ~15-fold loss for α3β2 nAChRs (Fig. 2C). Table 1 summarizes the changes in IC_{50} values for inhibition of the four closely related α3- and α6-containing nAChR subtypes and compares the selectivity of each peptide for α6/α3β2β3 relative to the α6/α3β4 subtype.

We next examined the kinetics of block and unblock by PeIA and its singly substituted analogs. To obtain k_{obs}, the oocytes were perfused with a concentration of peptide within 10-fold of the peptides' respective IC_{50} values; 1-sec pulses of ACh were applied once every min until a steady state block of the I_{ACh} was observed. To obtain k_{off}, a concentration of peptide that blocked >95% of the I_{ACh} (see *Materials and Methods*) was applied for 5 min in a static bath after which the peptide was washed out and I_{ACh} was monitored for recovery. As shown in Fig. 3, the kinetics of block and unblock by the parent peptide PeIA were similar for all four of the nAChR subtypes tested. Block and recovery from block was rapid and in most cases the $t_{1/2}$s were < 30 sec. In contrast to the relatively rapid kinetics of PeIA, those of the S9H analog were markedly slower (Fig. 3). Steady-state block by PeIA[S9H] of α6/α3β2β3 nAChRs using a 1 nM concentration required ~11 min ($t_{1/2}$ = 2.2 min). Recovery from block was also slow and full recovery required ~30 min ($t_{1/2}$ = 6.2 min) of washout. The kinetics of the V10A analog were also slower than the native peptide but faster than PeIA[S9H] (Fig. 3). Kinetic analysis of block and recovery of block by PeIA and PeIA[V10A] of α6/α3β2β3 and α6/α3β4 yielded $t_{1/2}$ times of <60 sec so an accurate comparison of the change in affinity between PeIA[V10A] and the native peptide could not be made. However, the ~2-fold increase in the IC_{50} value for PeIA[V10A] for

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inhibition of α6/α3β4 nAChRs implies that either k_{on} is slower or k_{off} is faster and contributes to the ~126-fold separation between the IC_{50} values for inhibition of α6/α3β2β3 vs. α6/α3β4 nAChRs. Similarly, the kinetics of PeIA[E14N] were too fast ($t_{1/2}$ times of <60 sec for all subtypes) to permit accurate measurement of k_{obs} and k_{off} under the conditions used in this study. Table 2 summarizes the kinetic data for block and recovery from block by PeIA and the three single substituted analogs.

Combined Double and Triple Amino Acid Substitutions Confer Further Increases in Potency and Selectivity for α6/α3β2β3 nAChRs. All three of the single amino acid substitutions (Fig 2) produced improvements in either potency or selectivity for the α6/α3β2β3 subtype. Therefore, we combined the S9H substitution with either the V10A or the E14N substitution to produce second generation analogs. As shown in Fig. 4, PeIA[S9H,V10A] retained the high potency of the singly substituted S9H analog as well as the selectivity of the V10A analog. The IC_{50} value for block of α6/α3β2β3 was ~2-fold lower than that of PeIA[S9H] whereas a ~4-fold increase in the IC_{50} value was observed for α6/α3β4 nAChRs. Likewise, the selectivity ratio (~126-fold) of PeIA[V10A] for block of α6/α3β2β3 vs. α6/α3β4 was also retained. Interestingly, PeIA[S9H,E14N] retained high potency for the α6/α3β2β3 nAChR (IC_{50} = 753 pM) which is ~39-fold more potent than block of α6/α3β4 nAChRs. Finally, we combined all three amino acid substitutions to generate PeIA[S9H,V10A,E14N]. This triply substituted analog was ~290-fold more potent at blocking the α6/α3β2β3 subtype than the α6/α3β4 subtype (Fig 5A). As discussed above, oocytes injected with non-chimeric α6 in combination with β2 and β3 constructs fail to produce functional responses. However, injection of α6 with β4 does yield functional responses albeit relatively small in amplitudes. The α6β4-mediated currents evoked by 100 μM ACh were on average 10.2 ± 1.1 nA (n=5) and were blocked by PeIA[S9H,V10A,E14N]

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with an IC_{50} value of 66.0 (51.5-84.7) nM and similar to the 65.4 (43.4-98.6) nM value obtained using chimeric α6/α3β4 nAChRs (Fig. 5A). We also tested this triply substituted PeIA analog on cloned mouse and cloned human α6-containing nAChRs. The IC_{50} values obtained for mouse α6/α3β2β3 and α6/α3β4 were also similar to those obtained using the rat counterparts (Fig. 5A). Likewise, the activity was also similar when tested on concatameric human α6β2α4β2β3 (Fig. 5A) and chimeric rat α6/α4β2β3 nAChRs (Supplemental Figure 1). PeIA[S9H,V10A,E14N] also inhibited rat α3β2 nAChRs but was much less potent when tested on other rat subtypes including α3β4, α4β2, α4β4, α7, and α9α10 (Fig. 5B). Tables 3 and 4 summarize the activity of the doubly and triply substituted analogs on the nAChRs tested.

PeIA[S9H,V10A,E14N] Potently and Selectively Blocks the α6/α3β2β3 vs. the α6/α3β4 Subtype. To more fully examine the ability of PeIA[S9H,V10A,E14N] to discriminate between the α6/α3β2β3 and the α6/α3β4 subtypes, we conducted an in depth characterization of the pharmacological properties of the peptide. First, we determined k_{on} by perfusing oocytes expressing α6/α3β2β3 with five different concentrations of PeIA[S9H,V10A,E14N] from 100 pM to 5 nM until steady-state block was achieved (Fig 6A). The data were analyzed using a one-phase exponential to obtain the k_{obs} for each of the concentrations tested and the data plotted as a function of concentration. This analysis yielded a slope, or k_{on}, of 0.228 ± 0.019 min^{-1} M^{-9} (Fig. 6B). A k_{off} value of 0.096 ± 0.001 min^{-1} was obtained by applying a high concentration of peptide to the oocyte in a 5 min static bath (Fig. 6C). Since the dissociation constant is a ratio of k_{off} to k_{on}, an estimated K_i value of 423 pM was obtained. Applying this same methodology to oocytes expressing α6/α3β4 nAChRs over a concentration range of 10 nM to 500 nM yielded a k_{on} of 0.699 ± 0.023 min^{-1} M^{-7} (Fig. 6D,E) and an observed k_{off} value of 0.626 ± 0.02 min^{-1}. The estimated K_i for α6/α3β4 was ~90 nM, a value >200-fold higher than that determined for

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α6/α3β2β3 nAChRs. Thus, from a functional standpoint, a concentration of 5 nM would be expected to produce near complete inhibition of α6/α3β2β3 nAChRs but show little block of the α6/α3β4 subtype. Indeed, in the presence of 5 nM PeIA[S9H,V10A,E14N], the I_{ACh} mediated by α6/α3β2β3 nAChRs were only 3.0 ± 0.2% of control I_{ACh} whereas those mediated by α6/α3β4 nAChRs were on average 94.0 ± 4% (Fig. 7A-C).

Discussion

α-Ctx PeIA was originally described as the first ligand that could discriminate between α9α10 and α7 nAChRs (McIntosh et al., 2005). It is noteworthy that while PeIA shares a similar activity profile with other α-Ctxs that target α3β2 and α6/α3β2β3 nAChRs, specifically MII (Cartier et al., 1996; McIntosh et al., 2004) and PnIA (Hogg et al., 1999; Hone et al., 2012; Luo et al., 1999), it is only 42% similar to these peptides in non-cysteine amino acid homology (Fig. 1A). Despite their sequence differences, molecular modeling and solution NMR studies predict that PeIA, MII, and PnIA all share a similar 3-dimensional backbone structure and occupy the acetylcholine binding pocket in approximately the same orientation (Daly et al., 2011; Pucci et al., 2011; Rogers et al., 1999). These differences and similarities suggest that specific residues of the peptides might be critical for binding to α6β2-containing nAChRs. Thus, one aim of this study was to gain mechanistic insight into the binding of ligands with α6β2-containing nAChRs. To this end, we substituted specific amino acids of PeIA with those of MII and PnIA that are known to be critical for activity and evaluated the substituted analogs for changes in potency and selectivity for the α6/α3β2β3 subtype.

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Native PeIA shows a modest degree of separation (~13-fold) between the IC_{50} values for α6/α3β2β3 and the α6/α3β4 subtypes. Thus, coupled with the known activity on other nAChR subtypes, PeIA is a non-selective antagonist of the five nAChR subtypes listed in Fig. 1B. However, structure-function studies of α-Ctxs MII (McIntosh et al., 2004), PnIA (Hogg et al., 1999; Luo et al., 1999), and BuIA (Azam et al., 2010) suggested that PeIA would be a promising platform from which to develop α6β2-selective ligands. These studies demonstrated that specific amino acids of MII confer high potency for the α6/α3β2β3 subtype and that the 10th position in PnIA is critical for subtype selectivity and provided a rational strategy for engineering α6/α3β2β3 subtype-selective ligands using PeIA as a template.

In this report, we describe the synthesis and characterization of a potent antagonist of α6/α3β2β3 nAChRs achieved by substituting critical amino acids in the second loop of PeIA that increased potency and selectivity for α6/α3β2β3 vs. α6/α3β4 nAChRs. These amino acids included Ser9, Val10, and Glu14 and were substituted with His, Ala, and Asn, respectively. When tested on *Xenopus* oocytes that expressed rat α6/α3β2β3 and α6/α3β4 nAChRs, the S9H substitution had the largest impact on potency and shifted the IC_{50} value for inhibition of α6/α3β2β3 nAChRs by ~11-fold while the V10A substitution had the largest effect on selectivity and conferred a ~10-fold increase in separation between the two subtypes (Fig. 2A, B) over the native peptide. An additional ~5-fold increase in selectivity for α6/α3β2β3 vs. α6/α3β4 nAChRs over the native peptide was observed with the Glu for Asn substitution in the 14th position (Fig. 2C). The V10A and E14N substitutions were combined in pair-wise fashion with S9H with the expectation that double substitutions would retain the effects observed with each respective single substitution. Indeed, both PeIA[S9H,V10A] and PeIA[S9H,E14N] retained the selectivity of PeIA[V10A] and PeIA[E14N] and, interestingly, both doubly substituted analogs were even

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more potent than PeIA[S9H] (Fig 2A and 4A, B). Finally, PeIA[S9H,V10A,E14N] was synthesized combining all three single substitutions to generate a ligand that was ~50-fold more potent than native PeIA for inhibition of α6/α3β2β3 nAChRs (Fig. 1B and 5A) and produced an ~1800-fold improvement in selectivity for α6/α3β2β3 vs. α3β4 and an ~1700-fold improvement vs. α7 nAChRs (IC_{50} = 1.8 μM (McIntosh et al., 2005)) over native PeIA (5A,B). Most importantly, an ~280-fold improvement was observed for α6/α3β2β3 vs. the α6/α3β4 subtype (Fig. 5A).

The kinetics of block and recovery from block were assessed on α6/α3β2β3 nAChRs and compared to those obtained for the α6/α3β4 subtype (Fig. 6A-F). These studies yielded a K_i value for α6/α3β2β3 (423 pM) that was >200-fold lower than that obtained for α6/α3β4 (~90 nM) nAChRs (Fig. 6A-F). Thus, at reasonable concentrations (5-10x the K_i) for inhibition of α6/α3β2β3 nAChRs little inhibition of α6/α3β4 nAChRs would be expected (Fig. 7). PeIA[S9H,V10A,E14N] was also tested on six other non-α6-containing nAChR subtypes and although inhibition of the two major CNS subtypes, α4β2 and α7, was observed, the IC_{50} values were ~930- and ~1800-fold higher, respectively, than the IC_{50} value for α6/α3β2β3 nAChRs (Fig. 5A,B). The peptide does, however, retain high potency for the α3β2 subtype and cannot be used to discriminate between this subtype and α6/α3β2β3 nAChRs.

An analog of α-Ctx MII, MII[E11A], also shows some selectivity (40-fold) for α6/α3β2β3 vs. α6/α3β4 nAChRs nAChRs (McIntosh et al., 2004). PnIA also preferentially targets α6/α3β2β3 nAChRs but with ~50-fold lower potency (Hone et al., 2012) than PeIA[S9H,V10A,E14N] and with significantly overlapping activity on the α7 subtype (Luo et al., 1999). We note that the sequence of PeIA[S9H,V10A,E14N] is 75% identical to that of MII[E11A] in terms of non-cysteine amino acids, differing in the second loops by residues in the

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10th, 11th, and 13th positions yet the PeIA analog is ~7-fold more selective for α6/α3β2β3 vs. α6/α3β4 nAChRs than MII[E11A]. Interestingly, when we substituted Ala for Asn in the 11th position to make PeIA more MII[E11A]-like, a ~5-fold loss of potency was observed (data not shown). Additionally, substitution of Asn with that of native MII (Glu) in the 11th position of PeIA[S9H,V10A] also resulted in a loss in potency of ~12-fold (data not shown). The α6 ligand binding pocket contains 3 Glu and 1 Asp residues that are thought to interact with residue 11 of MII and its analogs (Azam et al., 2008; Pucci et al., 2011). Thus, the introduction of the negatively charged amino acid Glu may produce a repulsive electrostatic interaction between Glu and the highly negatively charged α6 subunit interface. This would be consistent with the results of others who found that replacement of Glu with a positively charged Arg in the 11th position increased the potency of MII (Pucci et al., 2011). However, this mechanism would not account for the increased potency observed with MII[E11A] since Ala is uncharged. Thus, although the 3-dimensional backbone structures of PeIA[S9H,V10A,E14N], MII, and PnIA are predicted to be similar (Fig. 8), differences in amino acid side chains are critical for activity on the α6β2-containing nAChR. Specific amino acid side chains may directly interact with residues of the receptor or influence different conformations of neighboring amino acid side chains. These possibilities together with our results with PeIA suggest a more complex interaction between α-Ctxs and the α6β2 binding site. It would therefore be highly informative to conduct a positional scanning study on PeIA coupled with site-directed mutagenesis of the α6 subunit to gain further mechanistic insights into the interaction between ligands and the α6β2-containing nAChR.

The α6β2* subtype has received considerable attention recently and consequently significant efforts have been undertaken to develop highly specific ligands in order to study this receptor subtype in areas where multiple nAChR subtypes are potentially expressed (Letchworth

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and Whiteaker, 2011; Pivavarchyk et al., 2011; Pucci et al., 2011). Significant heterogeneity of nAChR expression typifies many neuronal populations in the nervous system and such is the case in striatum where the predominant subtypes are α6β2* and α4β2* (Grady et al., 2007; Perez et al., 2008) and in the ventral tegmental area where the predominant subtypes are α6β2*, α4β2*, α7, and a currently uncharacterized β4-containing nAChR (Jones and Wonnacott, 2004; Wooltorton et al., 2003; Wu et al., 2004; Yang et al., 2009). In certain regions of the primate brain such as the cerebellum, substantial overlap of α6, β2, and β4 subunit mRNAs have been found (Quik et al., 2000). Furthermore, α6β4* nAChRs have recently been identified in human adrenal chromaffin cells and characterized using analogs of MII (Perez-Alvarez et al., 2012). However, the analogs used in this study also potently block α6/α3β2β3, but not α3β2 nAChRs, and therefore the presence of α6β2* nAChRs cannot be ruled out. The α6β4* subtype has also been recently identified in a population of rat dorsal root ganglion neurons that also express β2-containing nAChRs of the α6β2* and/or the α3β2* subtypes (Hone et al., 2012). While PeIA[S9H,V10A,E14N] does not distinguish between these two subtypes, MII[H9A,L15] does (McIntosh et al., 2004). Thus an approach utilizing the selectivity profiles of both toxins may be useful for identifying the β2-containing nAChRs in these neurons. The fact that PeIA[S9H,V10A,E14N] blocks rat, mouse, and human α6β2-containing nAChRs with similar potencies while maintaining selectivity for α6β2- vs α6β4-containing nAChRs will allow the toxin to be used across species and thus should prove particularly useful for distinguishing α6β2* from α6β4* nAChRs in tissues where these receptors are potentially co-expressed. Finally, it is noteworthy that PeIA[S9H,V10A,E14N] is 1300-fold more potent at blocking α3β2 than α3β4 nAChRs. This exquisite selectivity for α3β2 nAChRs may be useful for distinguishing between these two subtypes in neurons such as those found in intracardiac ganglia (Bibevski et al., 2000)

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and superior cervical ganglia (David et al., 2010; Mao et al., 2006) that predominantly express a mixed population of α3-containing nAChRs.

Molecular Pharmacology Fast Forward. Published on August 22, 2012 as DOI: 10.1124/mol.112.080853
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Acknowledgements

We thank Bob Shackman at the DNA/Peptide Synthesis core facility at University of Utah for assistance with peptide synthesis, Miguel Ruiz, Phuong Tran, and Melissa McIntyre for assistance with peptide folding, and William Lowe at the Salk Institute for performing the MALDI mass spectrometry.

Molecular Pharmacology Fast Forward. Published on August 22, 2012 as DOI: 10.1124/mol.112.080853
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Conducted experiments: Hone, Scadden, Gajewiak, Christensen

Contributed new reagents or analytic tools: Lindstrom

Performed data analysis: Hone and Scadden

Contributed to the writing of the manuscript: Hone, Gajewiak, McIntosh

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Molecular Pharmacology Fast Forward. Published on August 22, 2012 as DOI: 10.1124/mol.112.080853
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Footnotes

This work was supported by grants from the U.S. National Institutes of Health [MH-53631 and GM-48677] and by the National Institute of Neurological Disorder and Stroke [NS-11323].

The authors declare there are no conflicts of interest.

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Figure Legends

Figure 1. Amino acid sequence comparison between α-Ctxs PeIA, MII, and PnIA. A) Conserved cysteines are shown in bold; residues of PeIA that were selected for substitution with those of either MII or PnIA are shown underscored. B) Concentration-response analysis of the activity of PeIA on *Xenopus* oocyte expressed rat nAChRs. PeIA blocked I_{ACh} mediated by α3β2, α3β4, α6/α3β2β3, α6/α3β4, and α9α10 nAChRs with IC_{50} values of 9.73 (8.11-11.7) nM, 1.5 (1.3-1.7) μM, 11.1 (8.17-15.0) nM, 148 (124-176) nM, and 33.0 (28.0-33.9) nM, respectively. Error bars denote the SEM of the data from 3-5 oocytes for each determination. The 95% CIs for the IC_{50} values are given in parentheses.

Figure 2. Concentration-response analysis of the activity of singly substituted PeIA analogs on *Xenopus* oocyte expressed rat α3β2, α3β4, α6/α3β2β3, α6/α3β4, and α9α10 nAChRs. The error bars for the data denote the SEM from 4-8 oocytes for each determination. IC_{50} values and confidence intervals are shown in TABLE 1.

Figure 3. Kinetic analysis of the activity of PeIA and single substituted analogs on *Xenopus* oocyte expressed rat α3β2, α3β4, α6/α3β2β3, and α6/α3β4 nAChRs. The toxins were applied as described in *Materials and Methods* and the data fit to a single exponential equation. The error bars denote the SEM of the data from 3-9 oocytes for each determination. Note the different time scale used for PeIA[S9H]. See TABLE 2 for a summary of the values obtained.

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Figure 4. Concentration-response analysis of the activity of doubly substituted PeIA analogs on *Xenopus* oocyte expressed rat α3β2, α3β4, α6/α3β2β3, and α6/α3β4 nAChRs. The error bars for the data denote the SEM from 3-5 oocytes for each determination. For a summary of the IC_{50} values and confidence intervals see TABLE 3.

Figure 5. Concentration-response analysis of the activity of PeIA[S9H,V10A,E14N] on *Xenopus* oocyte expressed nAChR subtypes. The error bars for the data denote the SEM from 4-5 oocytes for each determination; r, rat; m, mouse; h, human. For a summary of the IC_{50} values and confidence intervals see TABLE 4.

Figure 6. Comparison of PeIA[S9H,V10A,E14N] kinetics on rat α6/α3β2β3 vs. α6/α3β4 nAChRs expressed in *Xenopus* oocytes. A) The k_{obs} was determined for 5 concentrations of PeIA[S9H,V10A,E14N] from 100 pM to 5 nM by perfusing the oocytes with the toxin until a steady-state level of block was achieved. B) The data were fit to an exponential equation and the observed rates plotted as a function of PeIA[S9H,V10A,E14N] concentration to obtain k_{on}. C) To obtain the observed off-rate kinetics (k_{off}), a high concentration of toxin was applied in a static bath for 5 min after which the perfusion was resumed and the I_{ACh} monitored for recovery (see Materials and Methods for the concentrations used). The same analysis was performed for α6/α3β4 nAChRs (D-F). The error bars in A-C denote the SEM from 4 individual determinations and from 5 in D-F.

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Figure 7. Block of α6/α3β2β3 and α6/α3β4 nAChRs by PeIA[S9H,V10A,E14N]. Oocytes expressing α6/α3β2β3 (A) and α6/α3β4 (B) nAChRs were continuously perfused with 5 nM PeIA[S9H,V10A,E14N] until a steady-state level of block was achieved; % response was then quantified for comparison (C). The error bars in C denote the SEM from 4 oocytes expressing the α6/α3β2β3 subtype and from 6 expressing the α6/α3β4 subtype. Significance was determined by a *t*-test and compared to a theoretical response mean of 100%; ***, $p<0.001$.

Figure 8. Comparison of the 3-dimensional structures of PeIA[S9H,V10A,E14N], MII, and PnIA. (A) α-Ctx PeIA[S9H,V10A,E14N] with residues substituted from MII shown in blue (His9) and (Asn14), and from PnIA shown in red (Ala10). Residues shown in white are non-homologous with MII. (B) α-Ctx MII with His9 and Asn14 shown in blue. (C) α-Ctx PnIA with Ala10 shown in red. Images were generated using PyMOL as described in *Material and Methods.*

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Tables

TABLE 1
IC_{50} values for inhibition of rat nAChRs expressed in *Xenopus* oocytes

	α3β2	α6/α3β2β3	α3β4	α6/α3β4	α6/α3β4 to α6/α3β2β3 ratio
PeIA	9.7 (8.1-11.7) nM	11.1 (8.2-15.0) nM	1.5 (1.3-1.7) μM	147 (124-176) nM	13
PeIA[S9H]	713 (480-1060) pM	991 (932-1054) pM	66 (52-84) nM	14.5 (11.3-18.6) nM	15
PeIA[V10A]	2.2 (2.0-2.5) nM	2.4 (2.1-2.7) nM	2.7 (2.2-3.3) μM	302 (278-330) nM	126
PeIA[E14N]	149 (122-182) nM	22.0 (19.6-24.6) nM	1.9 (1.5-2.4) μM	406 (385-427) nM	18

( ), 95% CI.

Table 2
Kinetic analysis of block and recovery from block by PeIA and its analogs

	α3β2				α6/α3β2β3			
	k_{off} min^{-1}	k_{obs} min^{-1}	k_{on} min^{-1} M^{-1}	K_i (M^{-9})	k_{off} min^{-1}	k_{obs} min^{-1}	k_{on} min^{-1} M^{-1}	K_i (M^{-9})
PeIA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PeIA[S9H]	0.062 ± 0.001 (0.059-0.064)	0.18 ± 0.01 (0.161-0.204)	0.118 ± 0.01 x 10^{9} [(a)]	0.525 [(b)]	0.11 ± 0.01 (0.10-0.12)	0.31 ± 0.01 (0.29-0.34)	0.200 ± 0.01 x 10^{9} [(a)]	0.550[(b)]
PeIA[V10A]	0.456 ± 0.008 (0.440-0.472)	0.55 ± 0.04 (0.46-0.62)	0.94 ± 0.04 x 10^{8} [(a)]	4.85 [(b)]	N.D.	N.D.	N.D.	N.D.
PeIA[E14N]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

	α3β4				α6/α3β4			
	k_{off} min^{-1}	k_{obs} min^{-1}	k_{on} min^{-1} M^{-1}	K_i (M^{-9})	k_{off} min^{-1}	k_{obs} min^{-1}	k_{on} min^{-1} M^{-1}	K_i (M^{-9})
PeIA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PeIA[S9H]	0.032 ± 0.001 (0.030-0.034)	0.088 ± 0.002 (0.084-0.093)	0.56 ± 0.002 x 10^{6} [(a)]	57[(b)]	0.078 ± 0.001 (0.075-0.081)	0.21 ± 0.01 (0.184-0.240)	0.132 ± 0.01 x 10^{8} [(a)]	5.9 [(a)]
PeIA[V10A]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PeIA[E14N]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[(a)], calculated from $k_{obs} = k_{on}[\text{Toxin}] + k_{off}$.; ; [(b)], calculated from $K_{i,=} k_{off}/ k_{on}$; N.D., not determined; ( ), 95% CI;

±, SEM. from 3-9 oocytes.

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TABLE 3
IC_{50} values for inhibition of rat nAChRs by doubly substituted analogs of PeIA

	α3β2	α6/α3β2β3	α3β4	α6/α3β4	α6/α3β4 to α6/α3β2β3 ratio
PeIA[S9H,V10A]	793 (675-932) pM	506 (455-564) pM	307 (240-394) nM	65.1 (50.0-84.8) nM	129
PeIA[S9H,E14N]	1.15 (1.02-1.09) nM	753 (656-864) pM	128 (109-151) nM	29.5 (24.4-34.6) nM	39

( ), 95% CI.

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TABLE 4
Activity of PeIA[S9H,V10A,E14N] on nAChRs expressed in *Xenopus* oocytes.

nAChR Subtype	IC_{50}	95% Confidence Interval	IC_{50} ratios (compared to rat α6/α3β2β3)
rα3β2	335 pM	264-423	1.5
rα3β4	441 nM	370-527	1,970
rα4β2	209 nM	169-258	937
rα4β4	37.6 μM	32.9-43.0	>10,000
rα6/α3β2β3	223 pM	186-266	1
mα6/α3β2β3	470 pM	393-652	2.0
hα6β2α4β2β3	759 pM	685-842	3.4
rα6β4	66.0 nM	51.4-84.7	296
rα6/α3β4	65.4 nM	43.4-98.6	293
mα6/α3β4	75.6 nM	61.1-96.3	339
rα7	421 nM	356-498	1,880
rα9α10	27.8 μM	15.5-50.0	>10,000

R, rat; m, mouse; h, human concatamer.

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Figure 1

A

PeIA GCCSHPACSVNHPELC
MII GCCSNPVCHLEHSNLC
PnIA GCCSLPPCAANNPDYC

B

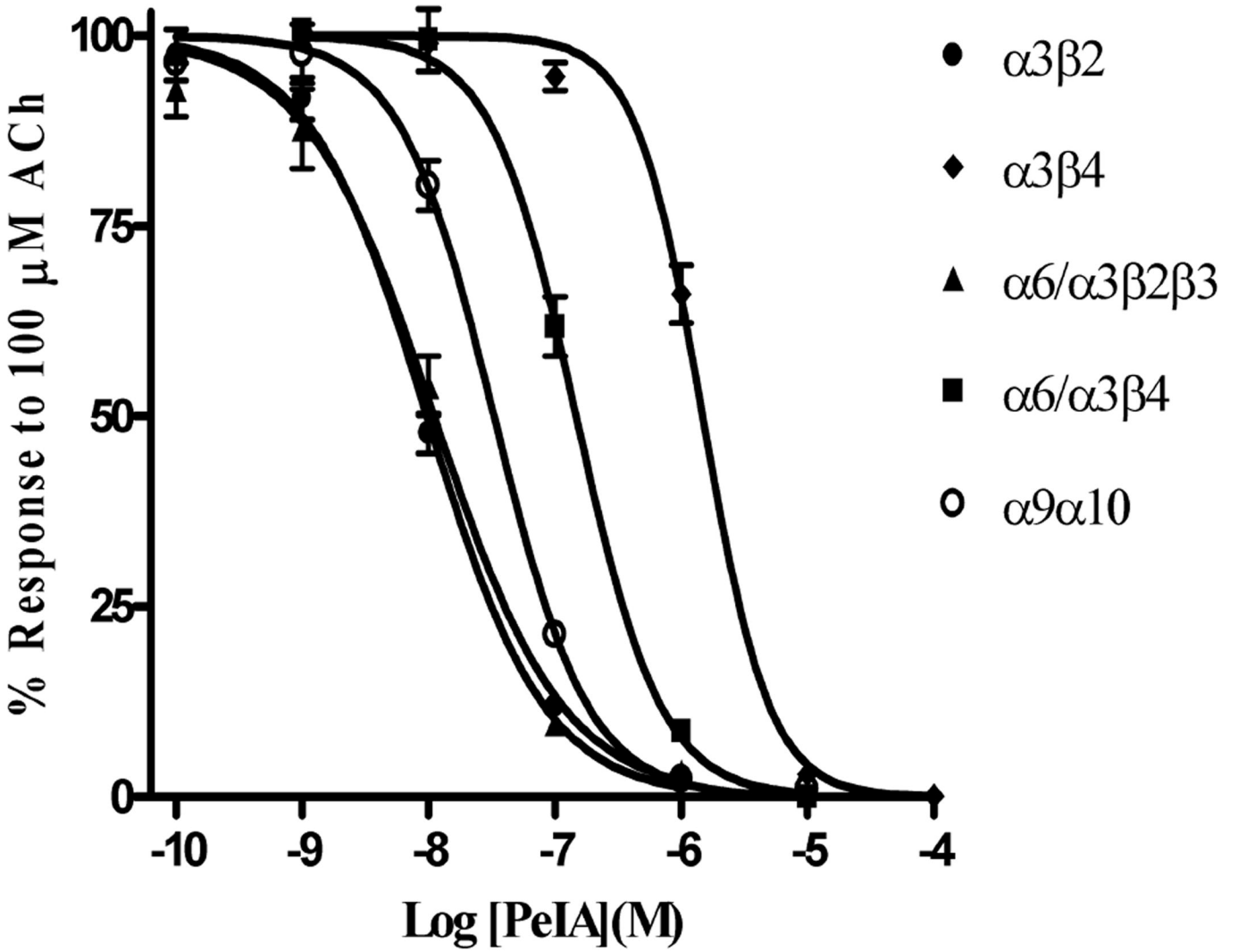
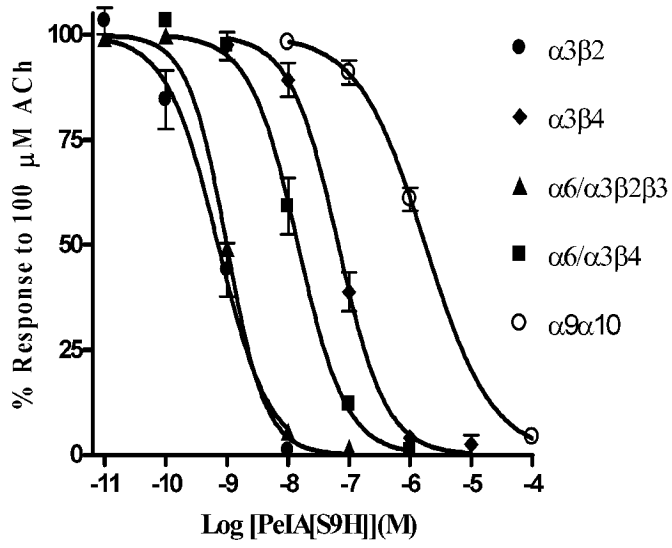


Figure 2

A



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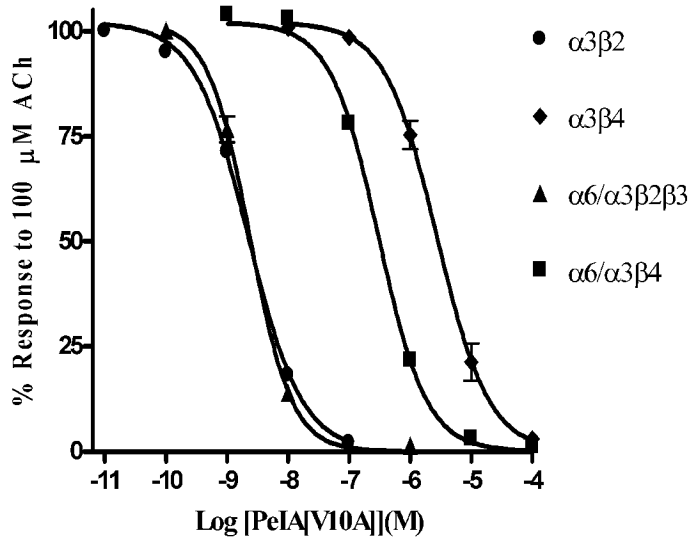

C

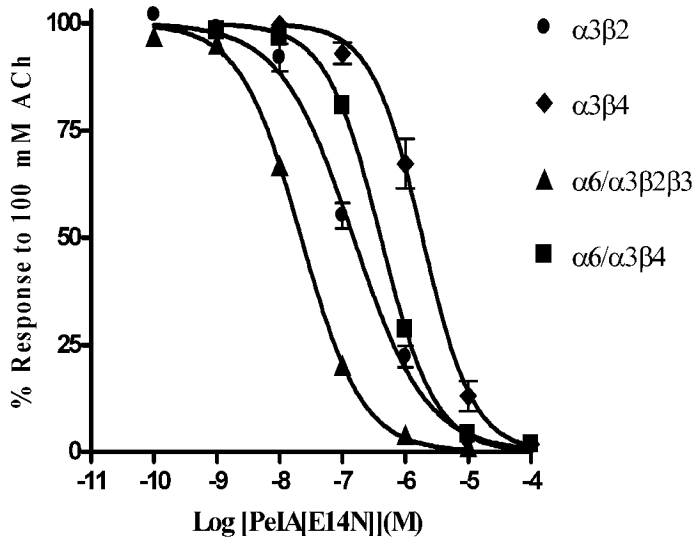

Figure 3

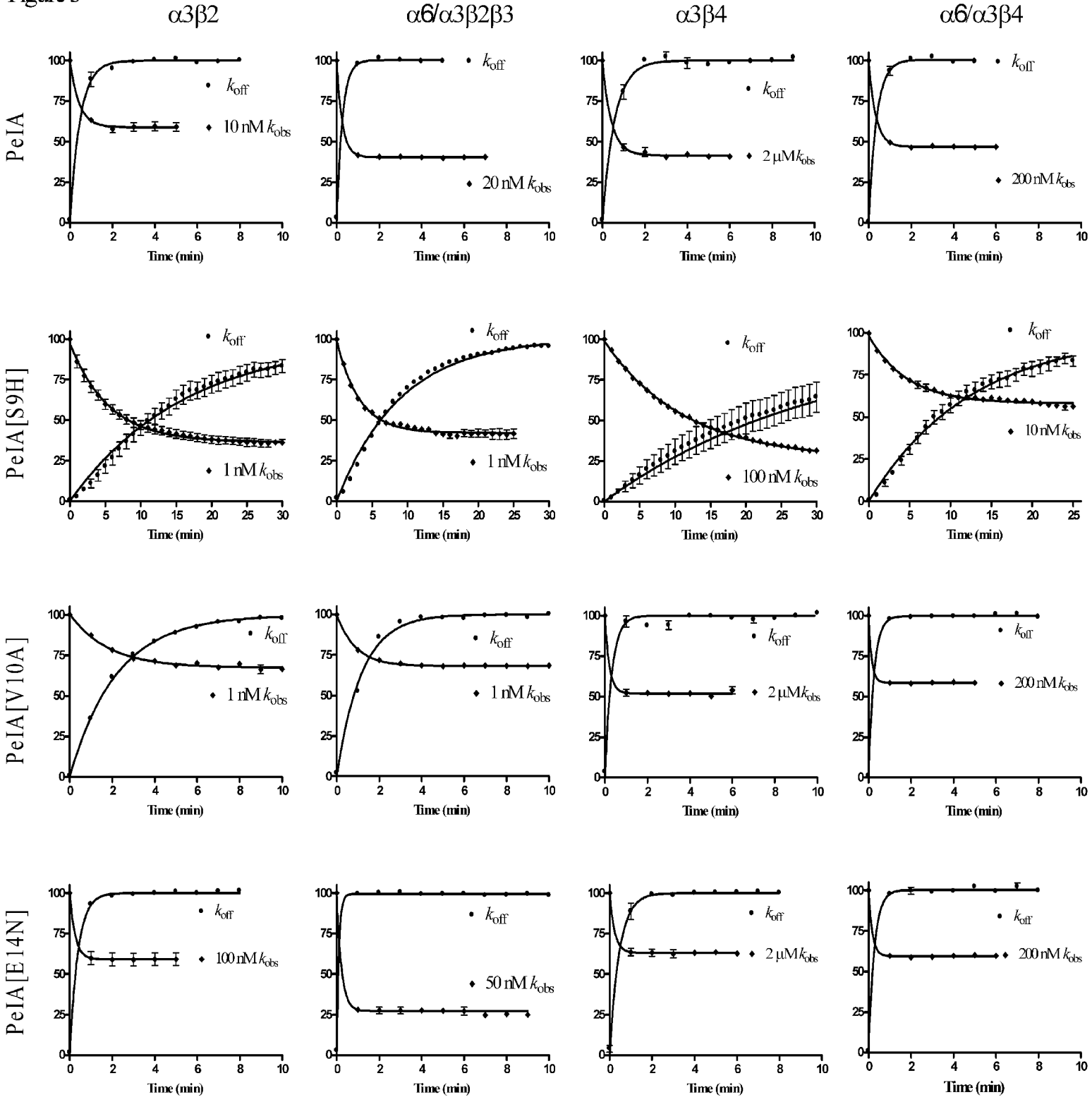

Figure 4

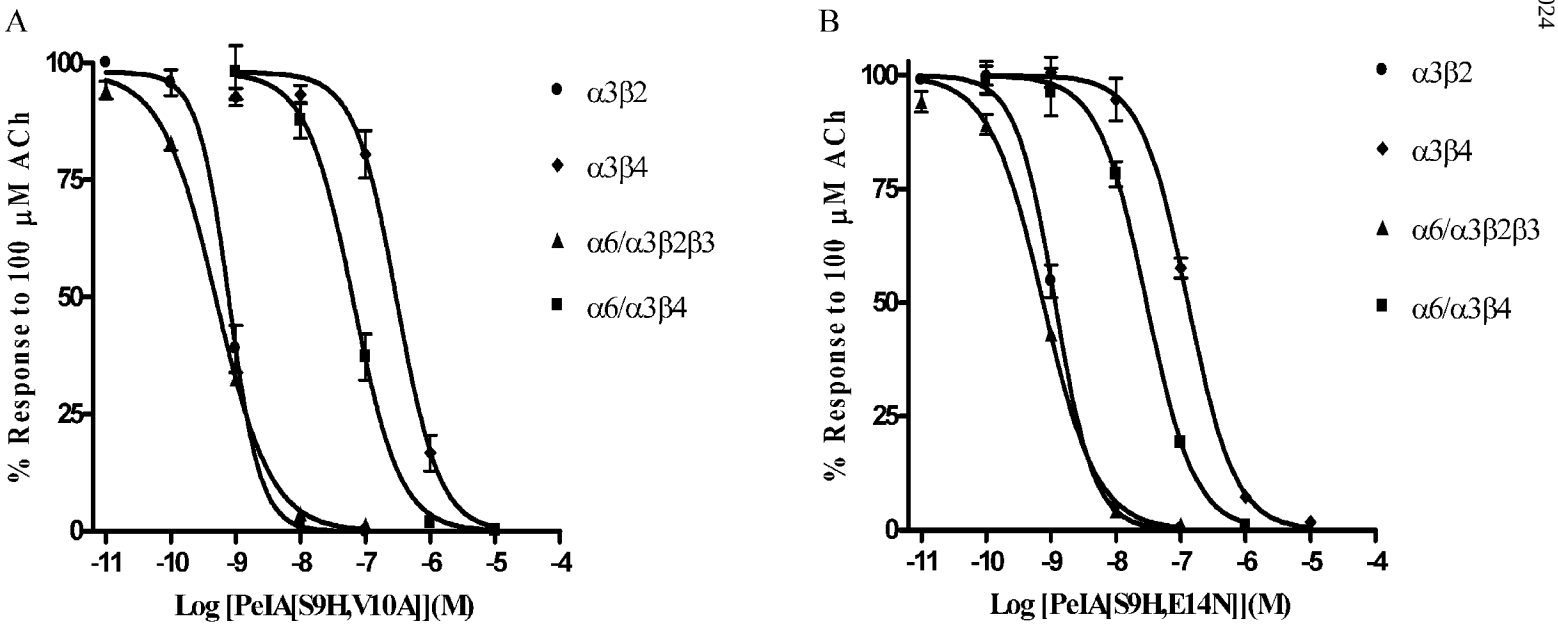
A
% Response to 100 µM ACh
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
-4
Log [PeIA[S9H,V10A]](M)
α3β2
α3β4
α6/α3β2β3
α6/α3β4
B
% Response to 100 µM ACh
Log [PeIA[S9H,E14N]](M)
α3β2
α3β4
α6/α3β2β3
α6/α3β4

Figure 5

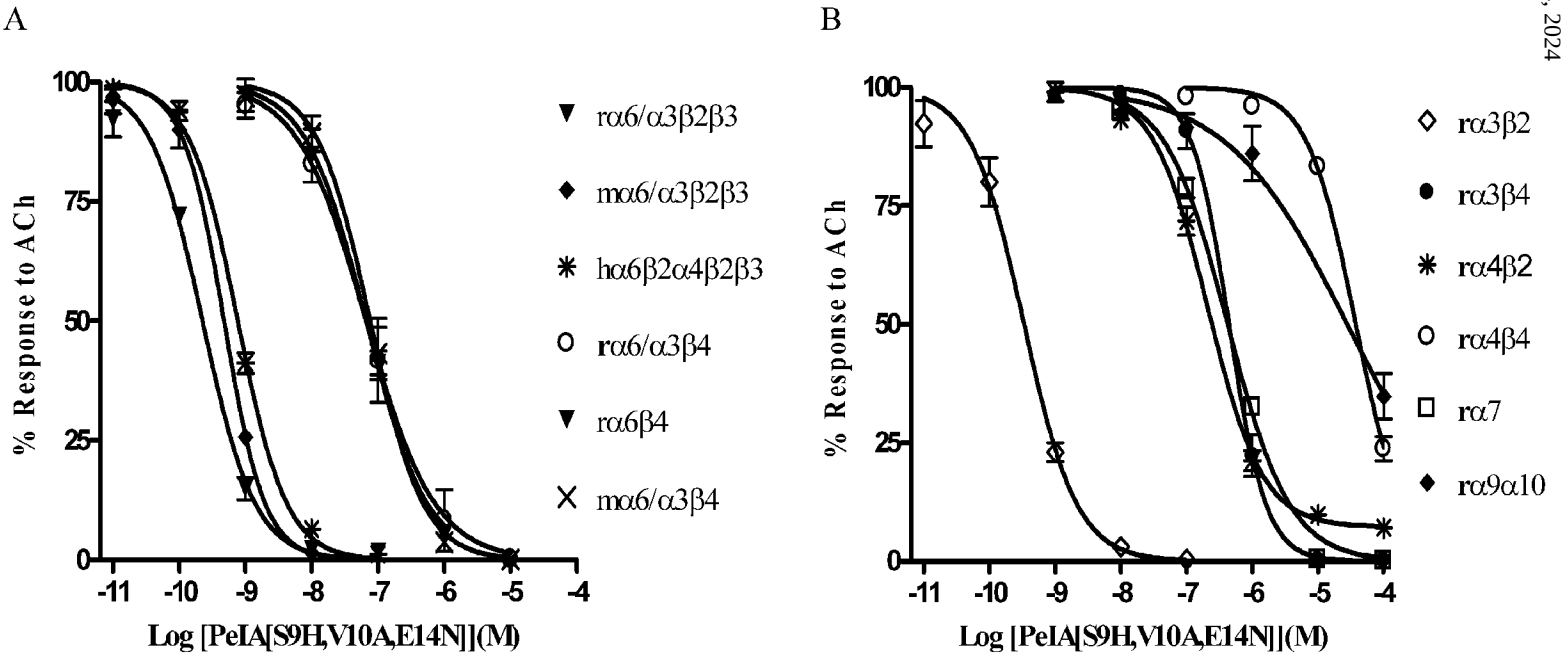
A
B
% Response to ACh
Log [PeIA[S9H,V10A,E14N]](M)
rα6/α3β2β3
mα6/α3β2β3
hα6β2α4β2β3
rα6/α3β4
rα6β4
mα6/α3β4
rα3β2
rα3β4
rα4β2
rα4β4
rα7
rα9α10

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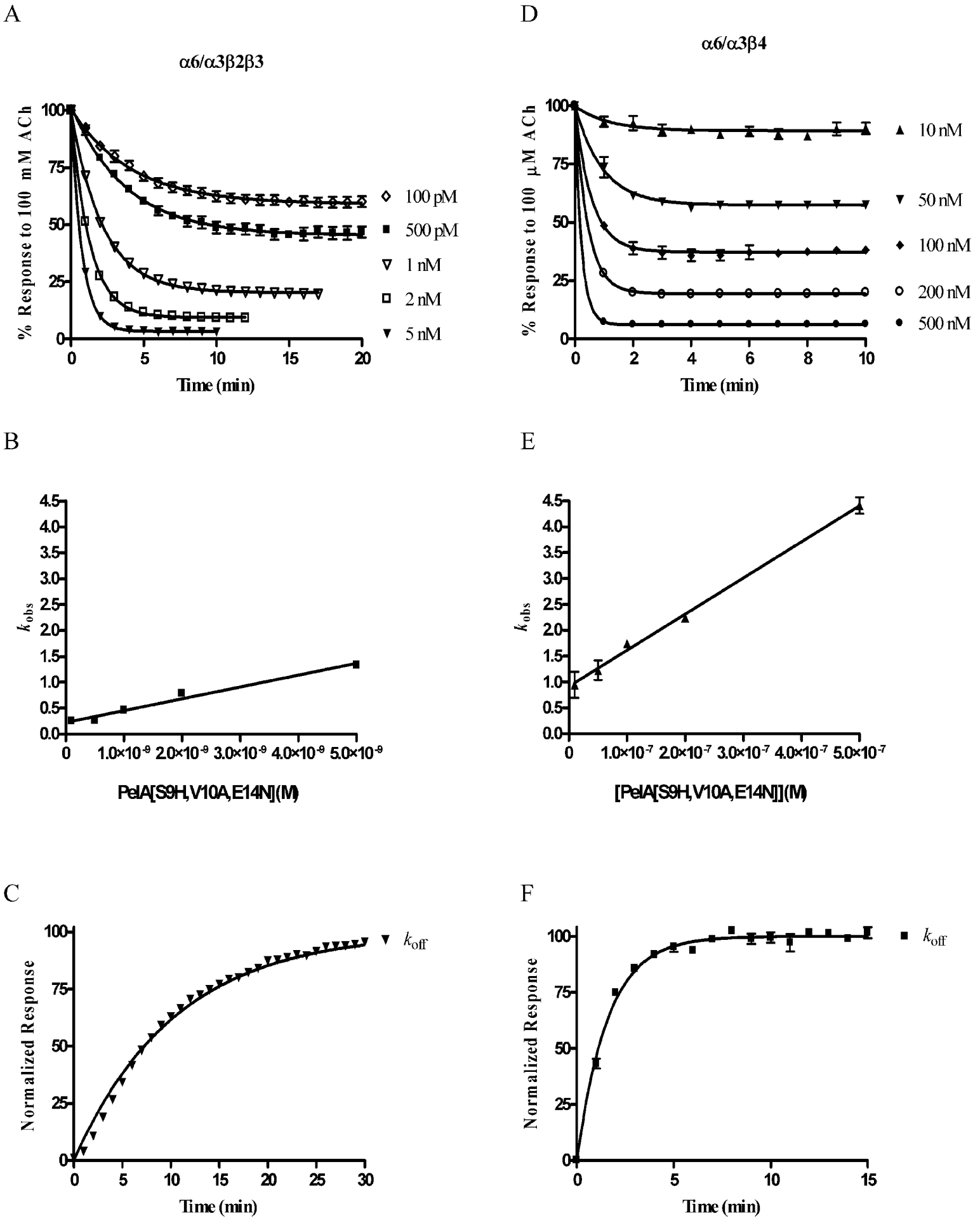
Figure 6



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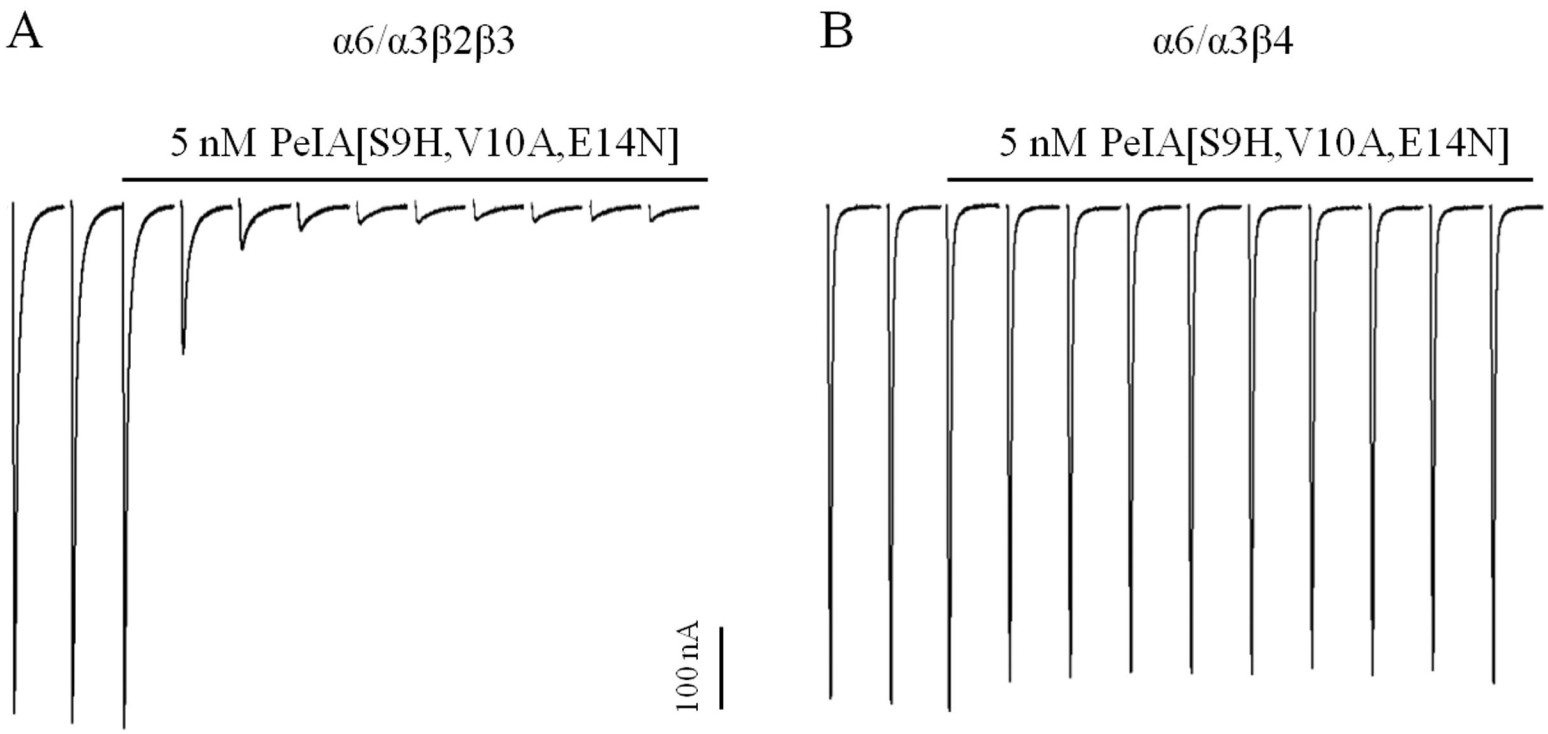

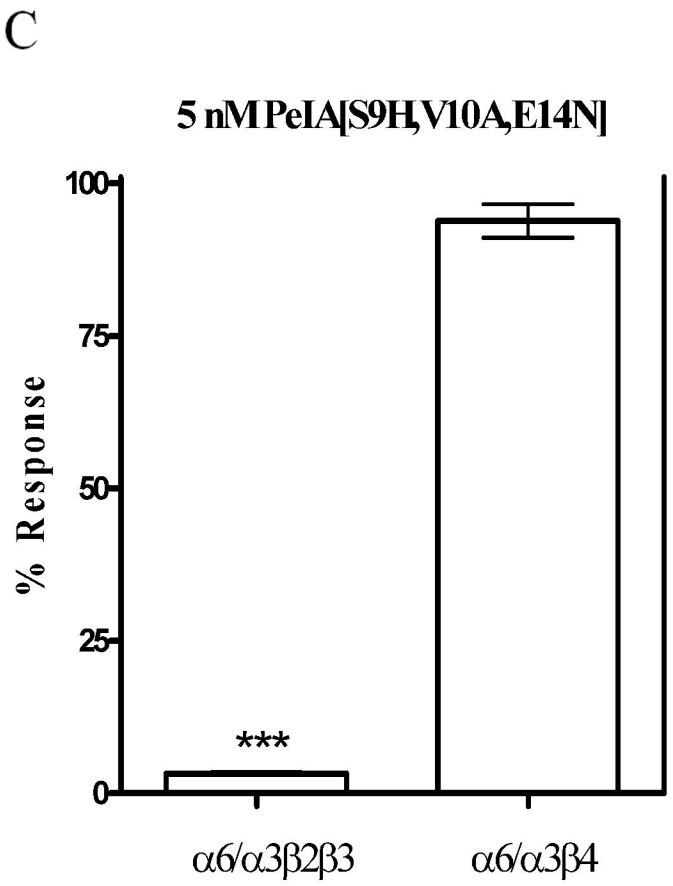

Figure 8