

MOL # 80853

Title page

α -Conotoxin PeIA[S9H,V10A,E14N] potently and selectively blocks $\alpha6\beta2\beta3$ vs. $\alpha6\beta4$ nicotinic acetylcholine receptors

Arik J. Hone, Mick'l Scadden, Joanna Gajewiak, Sean Christensen, Jon Lindstrom, & J. Michael McIntosh

Interdepartmental Program in Neuroscience, University of Utah, Salt Lake City, Utah (J.M.M)

Department of Biology, University of Utah, Salt Lake City, Utah (A.J.H., M.S., J.G., S.C., J.M.M)

Department Psychiatry, University of Utah, Salt Lake City, Utah (J.M.M.)

Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, Pennsylvania (J.L.)

MOL # 80853

Running title page

Running title: PeIA[S9H,V10A,E14N] blocks $\alpha 6\beta 2\beta 3$ nicotinic receptors

Correspondence should be addressed to Dr. J. Michael McIntosh mcintosh.mike@gmail.com

Department of Psychiatry 50 North 1900 East Salt Lake City, UT, USA, 84132.

Telephone: 801-581-8370

Fax: 801-585-5010

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.

MOL # 80853

Abstract

Nicotinic acetylcholine receptors (nAChRs) containing $\alpha 6$ and $\beta 2$ 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 $\beta 2$ and $\beta 4$ subunits overlap in a variety of tissues including locus coeruleus, retina, hippocampus, dorsal root ganglia, and adrenal chromaffin cells. Ligands that bind $\alpha 6\beta 2$ nAChRs also potently bind the closely related $\alpha 6\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ nAChRs, with low nanomolar potency. We systematically mutated PeIA and evaluated the resulting analogs for enhanced potency and/or selectivity for $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs expressed in *Xenopus* oocytes ($\alpha 6/\alpha 3$ is a subunit chimera that contains the N-terminal ligand binding domain of the $\alpha 6$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs yielded a k_{off} of $0.096 \pm 0.001 \text{ min}^{-1}$ and a k_{on} of $0.23 \pm 0.019 \text{ min}^{-1} \text{ M}^{-1}$. The synthesis of PeIA[S9H,V10A,E14N] demonstrates that ligands can be developed to discriminate between $\alpha 6\beta 2$ and $\alpha 6\beta 4$ nAChRs.

MOL # 80853

Introduction

Mammalian neuronal nicotinic acetylcholine receptors (nAChRs) assemble in a pentameric stoichiometry 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 $\alpha 6$ subunit have a very restricted tissue distribution. In the central nervous system (CNS), $\alpha 6$ -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 $\alpha 6$ -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, $\alpha 6$ subunits assemble with $\beta 2$ subunits to form $\alpha 6\beta 2^*$ nAChRs (asterisk denotes the possible presence of additional subunits); areas where expression of the $\beta 4$ subunit is minimal or absent and therefore, few, if any, $\alpha 6\beta 4^*$ nAChRs are likely to be present. Thus, ligands that bind $\alpha 6^*$ nAChRs can be used in these regions to selectively identify $\alpha 6\beta 2^*$ nAChRs. However, in other areas such as the retina (Marriott et al., 2005; Moretti et al., 2004) and the locus coeruleus (Azam and McIntosh, 2006; Lena et al., 1999; Vincler and Eisenach, 2003) $\alpha 6\beta 4^*$ nAChRs are present and may be co-expressed with the $\alpha 6\beta 2^*$ subtype. Moreover, in the hippocampus of mouse, $\alpha 6\beta 4^*$ nAChRs control the release of norepinephrine (Azam et al., 2010). Lastly, in the peripheral nervous system, $\alpha 6\beta 4^*$ 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 $\beta 2$ subunit expression. Thus, ligands

MOL # 80853

that can discriminate between $\alpha 6\beta 2^*$ and $\alpha 6\beta 4^*$ nAChRs are needed to facilitate the study of $\alpha 6\beta 2^*$ nAChRs in areas where multiple $\alpha 6$ -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 $\alpha 6$ nAChRs. However, α -Ctxs that block $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs also potently block $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6$ 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 $\alpha 6/3$ subunit chimeras to model the $\alpha 6\beta 2$ and $\alpha 6\beta 4$ ligand binding domain because injection of non-chimeric $\alpha 6$ with $\beta 2$ and $\beta 3$ 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 $\alpha 6$ with studies on native $\alpha 6$ -containing nAChRs demonstrate that these chimeric constructs and native $\alpha 6$ -containing nAChRs share a similar pharmacological profile (Bordia et al., 2007; Capelli et al., 2011; Perez-Alvarez et al., 2012). Using this strategy

MOL # 80853

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 $\alpha 6\beta 2\beta 3$ and $\alpha 6\beta 4$ nAChRs.

Materials and Methods

Rat $\alpha 3$, $\alpha 4$, and $\alpha 7$ nAChR subunit clones were provided by S. Heinemann (Salk Institute, San Diego, CA, USA), C. Luetje (University of Miami, Miami, FL, USA) provided the $\beta 2$, $\beta 3$, and $\beta 4$ subunits in the high-expressing pGEMHE vector and $\alpha 9$ and $\alpha 10$ were provided by A. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). Mouse $\alpha 6$, $\beta 2$, $\beta 3$, and $\beta 4$ subunit clones were provided by J. Stitzel (University of Colorado, Boulder, CO, USA). Construction of $\alpha 6/\alpha 3$ subunit chimeras has been previously described and consists of an $\alpha 3$ subunit where the first 237 amino acids of the ligand binding domain are replaced with the corresponding $\alpha 6$ amino acids (McIntosh et al., 2004). The rat $\alpha 6/\alpha 4$ 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 $\alpha 6$ construct. The human $\beta 3$ - $\alpha 6$ - $\beta 2$ - $\alpha 4$ - $\beta 2$ 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)

MOL # 80853

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 Cys¹ and Cys³ and acetamidomethyl (Acm) for Cys² and Cys⁴. 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, trifluoroacetic acid/phenol/ethanedithiol/thioanisole/H₂O (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 H₂O/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-of-flight mass spectrometry at the Salk Institute for Biological Studies (San Diego, CA, USA) under

MOL # 80853

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 $\alpha 3$, $\alpha 4$, $\alpha 6/\alpha 3$, $\alpha 7$, $\alpha 9$, $\alpha 10$, $\beta 2$, $\beta 3$, and $\beta 4$ 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 $\geq 10 \mu\text{M}$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ and at 100 μM for $\alpha 6/\alpha 3\beta 4$ nAChRs.

MOL # 80853

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 = $100 / \{1 + ([\text{toxin}] / IC_{50})^{n_H}\}$. 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.

MOL # 80853

Results

Single Amino Acid Substitutions of PeIA Confer Increased Potency and Selectivity for the $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 9\alpha 10$ but not $\alpha 7$ nAChRs (McIntosh et al., 2005). However, PeIA also potently blocks several other nAChR subtypes including those that contain the $\alpha 6$ 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 $\alpha 6$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs (McIntosh et al., 2004). For α -Ctx PnIA, Ala in the 10th position is critical for subtype selectivity and potency for $\beta 2$ -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 $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs by ~11-fold; increased potency was also observed on $\alpha 3\beta 2$, $\alpha 6/\alpha 3\beta 4$, and $\alpha 3\beta 4$ nAChRs (Fig. 2A). In contrast, the S9H substitution resulted in an ~55-fold loss of potency on the $\alpha 9\alpha 10$ 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

MOL # 80853

the $\alpha 6/\alpha 3\beta 2\beta 3$ nAChR, but in contrast showed a ~ 2 -fold loss of activity on $\alpha 6/\alpha 3\beta 4$ and $\alpha 3\beta 4$ nAChRs (Fig. 2B). The net result of this substitution was an analog with ~ 126 -fold selectivity for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. the $\alpha 6/\alpha 3\beta 4$ subtype. Finally, Asp was substituted for Glu14; although the activity was decreased for $\alpha 6/\alpha 3\beta 2\beta 3$ by ~ 2 -fold, there was an ~ 3 -fold loss of activity for $\alpha 6/\alpha 3\beta 4$ and an ~ 15 -fold loss for $\alpha 3\beta 2$ nAChRs (Fig. 2C). Table 1 summarizes the changes in IC_{50} values for inhibition of the four closely related $\alpha 3$ - and $\alpha 6$ -containing nAChR subtypes and compares the selectivity of each peptide for $\alpha 6/\alpha 3\beta 2\beta 3$ relative to the $\alpha 6/\alpha 3\beta 4$ 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 $t_{1/2s}$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ 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

MOL # 80853

inhibition of $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs. All three of the single amino acid substitutions (Fig 2) produced improvements in either potency or selectivity for the $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ was ~2-fold lower than that of PeIA[S9H] whereas a ~4-fold increase in the IC_{50} value was observed for $\alpha 6/\alpha 3\beta 4$ nAChRs. Likewise, the selectivity ratio (~126-fold) of PeIA[V10A] for block of $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ was also retained. Interestingly, PeIA[S9H,E14N] retained high potency for the $\alpha 6/\alpha 3\beta 2\beta 3$ nAChR (IC_{50} = 753 pM) which is ~39-fold more potent than block of $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ subtype than the $\alpha 6/\alpha 3\beta 4$ subtype (Fig 5A). As discussed above, oocytes injected with non-chimeric $\alpha 6$ in combination with $\beta 2$ and $\beta 3$ constructs fail to produce functional responses. However, injection of $\alpha 6$ with $\beta 4$ does yield functional responses albeit relatively small in amplitudes. The $\alpha 6\beta 4$ -mediated currents evoked by 100 μ M ACh were on average 10.2 ± 1.1 nA (n=5) and were blocked by PeIA[S9H,V10A,E14N]

MOL # 80853

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 $\alpha 6/\alpha 3\beta 4$ nAChRs (Fig. 5A). We also tested this triply substituted PeIA analog on cloned mouse and cloned human $\alpha 6$ -containing nAChRs. The IC_{50} values obtained for mouse $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ were also similar to those obtained using the rat counterparts (Fig. 5A). Likewise, the activity was also similar when tested on concatameric human $\alpha 6\beta 2\alpha 4\beta 2\beta 3$ (Fig. 5A) and chimeric rat $\alpha 6/\alpha 4\beta 2\beta 3$ nAChRs (Supplemental Figure 1). PeIA[S9H,V10A,E14N] also inhibited rat $\alpha 3\beta 2$ nAChRs but was much less potent when tested on other rat subtypes including $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 7$, and $\alpha 9\alpha 10$ (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 $\alpha 6/\alpha 3\beta 2\beta 3$ vs. the $\alpha 6/\alpha 3\beta 4$ Subtype. To more fully examine the ability of PeIA[S9H,V10A,E14N] to discriminate between the $\alpha 6/\alpha 3\beta 2\beta 3$ and the $\alpha 6/\alpha 3\beta 4$ subtypes, we conducted an in depth characterization of the pharmacological properties of the peptide. First, we determined k_{on} by perfusing oocytes expressing $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $0.228 \pm 0.019 \text{ min}^{-1} \text{ M}^{-9}$ (Fig. 6B). A k_{off} value of $0.096 \pm 0.001 \text{ min}^{-1}$ 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 $\alpha 6/\alpha 3\beta 4$ nAChRs over a concentration range of 10 nM to 500 nM yielded a k_{on} of $0.699 \pm 0.023 \text{ min}^{-1} \text{ M}^{-7}$ (Fig. 6D,E) and an observed k_{off} value of $0.626 \pm 0.02 \text{ min}^{-1}$. The estimated K_i for $\alpha 6/\alpha 3\beta 4$ was ~90 nM, a value >200-fold higher than that determined for

MOL # 80853

$\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs. Thus, from a functional standpoint, a concentration of 5 nM would be expected to produce near complete inhibition of $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs but show little block of the $\alpha 6/\alpha 3\beta 4$ subtype. Indeed, in the presence of 5 nM PeIA[S9H,V10A,E14N], the I_{ACh} mediated by $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs were only $3.0 \pm 0.2\%$ of control I_{ACh} whereas those mediated by $\alpha 6/\alpha 3\beta 4$ nAChRs were on average $94.0 \pm 4\%$ (Fig. 7A-C).

Discussion

α -Ctx PeIA was originally described as the first ligand that could discriminate between $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs (McIntosh et al., 2005). It is noteworthy that while PeIA shares a similar activity profile with other α -Ctxs that target $\alpha 3\beta 2$ and $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 6\beta 2$ -containing nAChRs. Thus, one aim of this study was to gain mechanistic insight into the binding of ligands with $\alpha 6\beta 2$ -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 $\alpha 6/\alpha 3\beta 2\beta 3$ subtype.

MOL # 80853

Native PeIA shows a modest degree of separation (~13-fold) between the IC₅₀ values for $\alpha 6/\alpha 3\beta 2\beta 3$ and the $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6\beta 2$ -selective ligands. These studies demonstrated that specific amino acids of MII confer high potency for the $\alpha 6/\alpha 3\beta 2\beta 3$ subtype and that the 10th position in PnIA is critical for subtype selectivity and provided a rational strategy for engineering $\alpha 6/\alpha 3\beta 2\beta 3$ subtype-selective ligands using PeIA as a template.

In this report, we describe the synthesis and characterization of a potent antagonist of $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs achieved by substituting critical amino acids in the second loop of PeIA that increased potency and selectivity for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ nAChRs, the S9H substitution had the largest impact on potency and shifted the IC₅₀ value for inhibition of $\alpha 6/\alpha 3\beta 2\beta 3$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ 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

MOL # 80853

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 $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs (Fig. 1B and 5A) and produced an ~1800-fold improvement in selectivity for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 3\beta 4$ and an ~1700-fold improvement vs. $\alpha 7$ nAChRs ($IC_{50} = 1.8 \mu M$ (McIntosh et al., 2005)) over native PeIA (5A,B). Most importantly, an ~280-fold improvement was observed for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. the $\alpha 6/\alpha 3\beta 4$ subtype (Fig. 5A).

The kinetics of block and recovery from block were assessed on $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs and compared to those obtained for the $\alpha 6/\alpha 3\beta 4$ subtype (Fig. 6A-F). These studies yielded a K_i value for $\alpha 6/\alpha 3\beta 2\beta 3$ (423 pM) that was >200-fold lower than that obtained for $\alpha 6/\alpha 3\beta 4$ (~90 nM) nAChRs (Fig. 6A-F). Thus, at reasonable concentrations (5-10x the K_i) for inhibition of $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs little inhibition of $\alpha 6/\alpha 3\beta 4$ nAChRs would be expected (Fig. 7). PeIA[S9H,V10A,E14N] was also tested on six other non- $\alpha 6$ -containing nAChR subtypes and although inhibition of the two major CNS subtypes, $\alpha 4\beta 2$ and $\alpha 7$, was observed, the IC_{50} values were ~930- and ~1800-fold higher, respectively, than the IC_{50} value for $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs (Fig. 5A,B). The peptide does, however, retain high potency for the $\alpha 3\beta 2$ subtype and cannot be used to discriminate between this subtype and $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs.

An analog of α -Ctx MII, MII[E11A], also shows some selectivity (40-fold) for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ nAChRs nAChRs (McIntosh et al., 2004). PnIA also preferentially targets $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs but with ~50-fold lower potency (Hone et al., 2012) than PeIA[S9H,V10A,E14N] and with significantly overlapping activity on the $\alpha 7$ 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

MOL # 80853

10th, 11th, and 13th positions yet the PeIA analog is ~7-fold more selective for $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6$ 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 $\alpha 6$ 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 $\alpha 6\beta 2$ -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 $\alpha 6\beta 2$ binding site. It would therefore be highly informative to conduct a positional scanning study on PeIA coupled with site-directed mutagenesis of the $\alpha 6$ subunit to gain further mechanistic insights into the interaction between ligands and the $\alpha 6\beta 2$ -containing nAChR.

The $\alpha 6\beta 2^*$ 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

MOL # 80853

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 $\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ (Grady et al., 2007; Perez et al., 2008) and in the ventral tegmental area where the predominant subtypes are $\alpha 6\beta 2^*$, $\alpha 4\beta 2^*$, $\alpha 7$, and a currently uncharacterized $\beta 4$ -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 $\alpha 6$, $\beta 2$, and $\beta 4$ subunit mRNAs have been found (Quik et al., 2000). Furthermore, $\alpha 6\beta 4^*$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$, but not $\alpha 3\beta 2$ nAChRs, and therefore the presence of $\alpha 6\beta 2^*$ nAChRs cannot be ruled out. The $\alpha 6\beta 4^*$ subtype has also been recently identified in a population of rat dorsal root ganglion neurons that also express $\beta 2$ -containing nAChRs of the $\alpha 6\beta 2^*$ and/or the $\alpha 3\beta 2^*$ 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 $\beta 2$ -containing nAChRs in these neurons. The fact that PeIA[S9H,V10A,E14N] blocks rat, mouse, and human $\alpha 6\beta 2$ -containing nAChRs with similar potencies while maintaining selectivity for $\alpha 6\beta 2$ - vs $\alpha 6\beta 4$ -containing nAChRs will allow the toxin to be used across species and thus should prove particularly useful for distinguishing $\alpha 6\beta 2^*$ from $\alpha 6\beta 4^*$ 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 $\alpha 3\beta 2$ than $\alpha 3\beta 4$ nAChRs. This exquisite selectivity for $\alpha 3\beta 2$ nAChRs may be useful for distinguishing between these two subtypes in neurons such as those found in intracardiac ganglia (Bibevski et al., 2000)

MOL # 80853

and superior cervical ganglia (David et al., 2010; Mao et al., 2006) that predominantly express a mixed population of α 3-containing nAChRs.

MOL # 80853

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.

MOL # 80853

Author contributions

Participated in Research Design: Hone and McIntosh

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

MOL # 80853

References

- Armishaw CJ (2010) Synthetic alpha-Conotoxin Mutants as Probes for Studying Nicotinic Acetylcholine Receptors and in the Development of Novel Drug Leads. *Toxins (Basel)* **2**(6):1471-1499.
- Azam L, Maskos U, Changeux JP, Dowell CD, Christensen S, De Biasi M and McIntosh JM (2010) alpha-Conotoxin BuIA[T5A;P6O]: a novel ligand that discriminates between alpha6beta4 and alpha6beta2 nicotinic acetylcholine receptors and blocks nicotine-stimulated norepinephrine release. *FASEB J* **24**(12):5113-5123.
- Azam L and McIntosh JM (2006) Characterization of nicotinic acetylcholine receptors that modulate nicotine-evoked [3H]norepinephrine release from mouse hippocampal synaptosomes. *Mol Pharmacol* **70**(3):967-976.
- Azam L and McIntosh JM (2009) Alpha-conotoxins as pharmacological probes of nicotinic acetylcholine receptors. *Acta Pharmacol Sin* **30**(6):771-783.
- Azam L, Yoshikami D and McIntosh JM (2008) Amino acid residues that confer high selectivity of the alpha6 nicotinic acetylcholine receptor subunit to alpha-conotoxin MII[S4A,E11A,L15A]. *J Biol Chem* **283**(17):11625-11632.
- Bibeovski S, Zhou Y, McIntosh JM, Zigmond RE and Dunlap ME (2000) Functional nicotinic acetylcholine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. *J Neurosci* **20**(13):5076-5082.
- Bordia T, Grady SR, McIntosh JM and Quik M (2007) Nigrostriatal damage preferentially decreases a subpopulation of alpha6beta2* nAChRs in mouse, monkey, and Parkinson's disease striatum. *Mol Pharmacol* **72**(1):52-61.
- Brunzell DH, Boschen KE, Hendrick ES, Beardsley PM and McIntosh JM (2010) Alpha-conotoxin MII-sensitive nicotinic acetylcholine receptors in the nucleus accumbens shell regulate progressive ratio responding maintained by nicotine. *Neuropsychopharmacology* **35**(3):665-673.
- Capelli AM, Castelletti L, Chen YH, Van der Keyl H, Pucci L, Oliosi B, Salvagno C, Bertani B, Gotti C, Powell A and Mugnaini M (2011) Stable expression and functional characterization of a human nicotinic acetylcholine receptor with alpha6beta2 properties: discovery of selective antagonists. *Br J Pharmacol* **163**(2):313-329.
- Cartier GE, Yoshikami D, Gray WR, Luo S, Olivera BM and McIntosh JM (1996) A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors. *J Biol Chem* **271**(13):7522-7528.
- Daly NL, Callaghan B, Clark RJ, Nevin ST, Adams DJ and Craik DJ (2011) Structure and activity of alpha-conotoxin PeIA at nicotinic acetylcholine receptor subtypes and GABA(B) receptor-coupled N-type calcium channels. *J Biol Chem* **286**(12):10233-10237.
- Dash B, Bhakta M, Chang Y and Lukas RJ (2011) Identification of N-terminal extracellular domain determinants in nicotinic acetylcholine receptor (nAChR) alpha6 subunits that influence effects of wild-type or mutant beta3 subunits on function of alpha6beta2*- or alpha6beta4*-nAChR. *J Biol Chem* **286**(44):37976-37989.
- David R, Ciuraszkiewicz A, Simeone X, Orr-Urtreger A, Papke RL, McIntosh JM, Huck S and Scholze P (2010) Biochemical and functional properties of distinct nicotinic acetylcholine receptors in the superior cervical ganglion of mice with targeted deletions of nAChR subunit genes. *Eur J Neurosci* **31**(6):978-993.
- Dowell C, Olivera BM, Garrett JE, Staheli ST, Watkins M, Kuryatov A, Yoshikami D, Lindstrom JM and McIntosh JM (2003) Alpha-conotoxin PIA is selective for alpha6 subunit-containing nicotinic acetylcholine receptors. *J Neurosci* **23**(24):8445-8452.
- Exley R, Maubourguet N, David V, Eddine R, Evrard A, Pons S, Marti F, Threlfell S, Cazala P, McIntosh JM, Changeux JP, Maskos U, Cragg SJ and Faure P (2011) Distinct contributions of nicotinic

MOL # 80853

- acetylcholine receptor subunit alpha4 and subunit alpha6 to the reinforcing effects of nicotine. *Proc Natl Acad Sci U S A* **108**(18):7577-7582.
- Gotti C, Guiducci S, Tedesco V, Corbioli S, Zanetti L, Moretti M, Zanardi A, Rimondini R, Mugnaini M, Clementi F, Chiamulera C and Zoli M (2010) Nicotinic acetylcholine receptors in the mesolimbic pathway: primary role of ventral tegmental area alpha6beta2* receptors in mediating systemic nicotine effects on dopamine release, locomotion, and reinforcement. *J Neurosci* **30**(15):5311-5325.
- Grady SR, Salminen O, Lavery DC, Whiteaker P, McIntosh JM, Collins AC and Marks MJ (2007) The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochem Pharmacol* **74**(8):1235-1246.
- Hill JM, Oomen CJ, Miranda LP, Bingham JP, Alewood PF and Craik DJ (1998) Three-dimensional solution structure of alpha-conotoxin MII by NMR spectroscopy: effects of solution environment on helicity. *Biochemistry* **37**(45):15621-15630.
- Hogg RC, Miranda LP, Craik DJ, Lewis RJ, Alewood PF and Adams DJ (1999) Single amino acid substitutions in alpha-conotoxin PnIA shift selectivity for subtypes of the mammalian neuronal nicotinic acetylcholine receptor. *J Biol Chem* **274**(51):36559-36564.
- Hone AJ, Meyer EL, McIntyre M and McIntosh JM (2012) Nicotinic acetylcholine receptors in dorsal root ganglion neurons include the alpha6beta4* subtype. *FASEB J* **26**(2):917-926.
- Hone AJ, Whiteaker P, Christensen S, Xiao Y, Meyer EL and McIntosh JM (2009) A novel fluorescent alpha-conotoxin for the study of alpha7 nicotinic acetylcholine receptors. *J Neurochem* **111**(1):80-89.
- Hu SH, Gehrmann J, Guddat LW, Alewood PF, Craik DJ and Martin JL (1996) The 1.1 A crystal structure of the neuronal acetylcholine receptor antagonist, alpha-conotoxin PnIA from *Conus pennaceus*. *Structure* **4**(4):417-423.
- Jackson KJ, McIntosh JM, Brunzell DH, Sanjakdar SS and Damaj MI (2009) The role of alpha6-containing nicotinic acetylcholine receptors in nicotine reward and withdrawal. *J Pharmacol Exp Ther* **331**(2):547-554.
- Jones IW and Wonnacott S (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J Neurosci* **24**(50):11244-11252.
- Kuryatov A and Lindstrom J (2011) Expression of functional human alpha6beta2beta3* acetylcholine receptors in *Xenopus laevis* oocytes achieved through subunit chimeras and concatamers. *Mol Pharmacol* **79**(1):126-140.
- Kuryatov A, Olale F, Cooper J, Choi C and Lindstrom J (2000) Human alpha6 AChR subtypes: subunit composition, assembly, and pharmacological responses. *Neuropharmacology* **39**(13):2570-2590.
- Lena C, de Kerchove D'Exaerde A, Cordero-Erausquin M, Le Novere N, del Mar Arroyo-Jimenez M and Changeux JP (1999) Diversity and distribution of nicotinic acetylcholine receptors in the locus ceruleus neurons. *Proc Natl Acad Sci U S A* **96**(21):12126-12131.
- Letchworth SR and Whiteaker P (2011) Progress and challenges in the study of alpha6-containing nicotinic acetylcholine receptors. *Biochem Pharmacol* **82**(8):862-872.
- Liu L, Zhao-Shea R, McIntosh JM, Gardner P and Tapper A (2012) Nicotine Persistently Activates Ventral Tegmental Area Dopaminergic Neurons Via Nicotinic Acetylcholine Receptors Containing alpha4 and alpha6 subunits. *Mol Pharmacol*. **81**(4):541-548
- Luo S, Nguyen TA, Cartier GE, Olivera BM, Yoshikami D and McIntosh JM (1999) Single-residue alteration in alpha-conotoxin PnIA switches its nAChR subtype selectivity. *Biochemistry* **38**(44):14542-14548.
- Mao D, Yasuda RP, Fan H, Wolfe BB and Kellar KJ (2006) Heterogeneity of nicotinic cholinergic receptors in rat superior cervical and nodose Ganglia. *Mol Pharmacol* **70**(5):1693-1699.
- Marriott AM, Cox BC, Yasuda RP, McIntosh JM, Xiao Y, Wolfe BB and Kellar KJ (2005) Nicotinic cholinergic receptors in the rat retina: simple and mixed heteromeric subtypes. *Mol Pharmacol* **68**(6):1656-1668.

MOL # 80853

- McIntosh JM, Azam L, Staheli S, Dowell C, Lindstrom JM, Kuryatov A, Garrett JE, Marks MJ and Whiteaker P (2004) Analogs of alpha-conotoxin MII are selective for alpha6-containing nicotinic acetylcholine receptors. *Mol Pharmacol* **65**(4):944-952.
- McIntosh JM, Plazas PV, Watkins M, Gomez-Casati ME, Olivera BM and Elgoyhen AB (2005) A novel alpha-conotoxin, PeIA, cloned from *Conus pergrandis*, discriminates between rat alpha9alpha10 and alpha7 nicotinic cholinergic receptors. *J Biol Chem* **280**(34):30107-30112.
- Moretti M, Vailati S, Zoli M, Lippi G, Riganti L, Longhi R, Viegi A, Clementi F and Gotti C (2004) Nicotinic acetylcholine receptor subtypes expression during rat retina development and their regulation by visual experience. *Mol Pharmacol* **66**(1):85-96.
- Papke RL, Buhr JD, Francis MM, Choi KI, Thinschmidt JS and Horenstein NA (2005) The effects of subunit composition on the inhibition of nicotinic receptors by the amphipathic blocker 2,2,6,6-tetramethylpiperidin-4-yl heptanoate. *Mol Pharmacol* **67**(6):1977-1990.
- Perez-Alvarez A, Hernandez-Vivanco A, McIntosh JM and Albillos A (2012) Native alpha6beta4* nicotinic receptors control exocytosis in human chromaffin cells of the adrenal gland. *FASEB J* **26**(1):346-354.
- Perez XA, Bordia T, McIntosh JM, Grady SR and Quik M (2008) Long-term nicotine treatment differentially regulates striatal alpha6alpha4beta2* and alpha6(nonalpha4)beta2* nAChR expression and function. *Mol Pharmacol* **74**(3):844-853.
- Pivavarchyk M, Smith AM, Zhang Z, Zhou D, Wang X, Toyooka N, Tsuneki H, Sasaoka T, McIntosh JM, Crooks PA and Dwoskin LP (2011) Indolizidine (-)-235B' and related structural analogs: discovery of nicotinic receptor antagonists that inhibit nicotine-evoked [3H]dopamine release. *Eur J Pharmacol* **658**(2-3):132-139.
- Pons S, Fattore L, Cossu G, Tolu S, Porcu E, McIntosh JM, Changeux JP, Maskos U and Fratta W (2008) Crucial role of alpha4 and alpha6 nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. *J Neurosci* **28**(47):12318-12327.
- Pucci L, Grazioso G, Dallanocce C, Rizzi L, De Micheli C, Clementi F, Bertrand S, Bertrand D, Longhi R, De Amici M and Gotti C (2011) Engineering of alpha-conotoxin MII-derived peptides with increased selectivity for native alpha6beta2* nicotinic acetylcholine receptors. *FASEB J* **25**(11):3775-3789.
- Quik M, Polonskaya Y, Gillespie A, Jakowec M, Lloyd GK and Langston JW (2000) Localization of nicotinic receptor subunit mRNAs in monkey brain by in situ hybridization. *J Comp Neurol* **425**(1):58-69.
- Rogers JP, Luginbuhl P, Shen GS, McCabe RT, Stevens RC and Wemmer DE (1999) NMR solution structure of alpha-conotoxin ImI and comparison to other conotoxins specific for neuronal nicotinic acetylcholine receptors. *Biochemistry* **38**(13):3874-3882.
- Vincler MA and Eisenach JC (2003) Immunocytochemical localization of the alpha3, alpha4, alpha5, alpha7, beta2, beta3 and beta4 nicotinic acetylcholine receptor subunits in the locus coeruleus of the rat. *Brain Res* **974**(1-2):25-36.
- Wooltorton JR, Pidoplichko VI, Broide RS and Dani JA (2003) Differential desensitization and distribution of nicotinic acetylcholine receptor subtypes in midbrain dopamine areas. *J Neurosci* **23**(8):3176-3185.
- Wu J, George AA, Schroeder KM, Xu L, Marxer-Miller S, Lucero L and Lukas RJ (2004) Electrophysiological, pharmacological, and molecular evidence for alpha7-nicotinic acetylcholine receptors in rat midbrain dopamine neurons. *J Pharmacol Exp Ther* **311**(1):80-91.
- Yang K, Hu J, Lucero L, Liu Q, Zheng C, Zhen X, Jin G, Lukas RJ and Wu J (2009) Distinctive nicotinic acetylcholine receptor functional phenotypes of rat ventral tegmental area dopaminergic neurons. *J Physiol* **587**:2:345-361.

MOL # 80853

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.

MOL # 80853

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 $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$, $\alpha 6/\alpha 3\beta 4$, and $\alpha 9\alpha 10$ nAChRs with IC_{50} values of 9.73 (8.11-11.7) nM, 1.5 (1.3-1.7) μ M, 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 $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$, $\alpha 6/\alpha 3\beta 4$, and $\alpha 9\alpha 10$ 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 $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$, and $\alpha 6/\alpha 3\beta 4$ 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.

MOL # 80853

Figure 4. Concentration-response analysis of the activity of doubly substituted PeIA analogs on *Xenopus* oocyte expressed rat $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$, and $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 2\beta 3$ vs. $\alpha 6/\alpha 3\beta 4$ 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 $\alpha 6/\alpha 3\beta 4$ nAChRs (D-F). The error bars in A-C denote the SEM from 4 individual determinations and from 5 in D-F.

MOL # 80853

Figure 7. Block of $\alpha 6/\alpha 3\beta 2\beta 3$ and $\alpha 6/\alpha 3\beta 4$ nAChRs by PeIA[S9H,V10A,E14N]. Oocytes expressing $\alpha 6/\alpha 3\beta 2\beta 3$ (A) and $\alpha 6/\alpha 3\beta 4$ (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 $\alpha 6/\alpha 3\beta 2\beta 3$ subtype and from 6 expressing the $\alpha 6/\alpha 3\beta 4$ 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*.

MOL # 80853

Tables

TABLE 1
IC₅₀ values for inhibition of rat nAChRs expressed in *Xenopus* oocytes

	$\alpha 3\beta 2$	$\alpha 6/\alpha 3\beta 2\beta 3$	$\alpha 3\beta 4$	$\alpha 6/\alpha 3\beta 4$	$\alpha 6/\alpha 3\beta 4$ to $\alpha 6/\alpha 3\beta 2\beta 3$ ratio
PeIA	9.7 (8.1-11.7) nM	11.1 (8.2-15.0) nM	1.5 (1.3-1.7) μ M	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) μ M	302 (278-330) nM	126
PeIA[E14N]	149 (122-182) nM	22.0 (19.6-24.6) nM	1.9 (1.5-2.4) μ M	406 (385-427) nM	18

(), 95% CI.

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

	$\alpha 3\beta 2$				$\alpha 6/\alpha 3\beta 2\beta 3$			
	$k_{\text{off}} \text{ min}^{-1}$	$k_{\text{obs}} \text{ min}^{-1}$	$k_{\text{on}} \text{ min}^{-1} \text{ M}^{-1}$	$K_i (\text{M}^{-9})$	$k_{\text{off}} \text{ min}^{-1}$	$k_{\text{obs}} \text{ min}^{-1}$	$k_{\text{on}} \text{ min}^{-1} \text{ M}^{-1}$	$K_i (\text{M}^{-9})$
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)	$0.118 \pm 0.01 \times 10^9$ ^(a)	0.525 ^(b)	0.11 ± 0.01 (0.10-0.12)	0.31 ± 0.01 (0.29-0.34)	$0.200 \pm 0.01 \times 10^9$ ^(a)	0.550 ^(b)
PeIA[V10A]	0.456 ± 0.008 (0.440-0.472)	0.55 ± 0.04 (0.46-0.62)	$0.94 \pm 0.04 \times 10^8$ ^(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.
	$\alpha 3\beta 4$				$\alpha 6/\alpha 3\beta 4$			
	$k_{\text{off}} \text{ min}^{-1}$	$k_{\text{obs}} \text{ min}^{-1}$	$k_{\text{on}} \text{ min}^{-1} \text{ M}^{-1}$	$K_i (\text{M}^{-9})$	$k_{\text{off}} \text{ min}^{-1}$	$k_{\text{obs}} \text{ min}^{-1}$	$k_{\text{on}} \text{ min}^{-1} \text{ M}^{-1}$	$K_i (\text{M}^{-9})$
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)	$0.56 \pm 0.002 \times 10^6$ ^(a)	57 ^(b)	0.078 ± 0.001 (0.075-0.081)	0.21 ± 0.01 (0.184-0.240)	$0.132 \pm 0.01 \times 10^8$ ^(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 $k_{\text{obs}} = k_{\text{on}}[\text{Toxin}] + k_{\text{off}}$; ^(b), calculated from $K_i = k_{\text{off}}/k_{\text{on}}$; N.D., not determined; (), 95% CI;

\pm , SEM. from 3-9 oocytes.

MOL # 80853

TABLE 3
IC₅₀ values for inhibition of rat nAChRs by doubly substituted analogs of PeIA

	$\alpha 3\beta 2$	$\alpha 6/\alpha 3\beta 2\beta 3$	$\alpha 3\beta 4$	$\alpha 6/\alpha 3\beta 4$	$\alpha 6/\alpha 3\beta 4$ to $\alpha 6/\alpha 3\beta 2\beta 3$ 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.

MOL # 80853

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 $\alpha 6/\alpha 3\beta 2\beta 3$)
r $\alpha 3\beta 2$	335 pM	264-423	1.5
r $\alpha 3\beta 4$	441 nM	370-527	1,970
r $\alpha 4\beta 2$	209 nM	169-258	937
r $\alpha 4\beta 4$	37.6 μ M	32.9-43.0	>10,000
r $\alpha 6/\alpha 3\beta 2\beta 3$	223 pM	186-266	1
m $\alpha 6/\alpha 3\beta 2\beta 3$	470 pM	393-652	2.0
h $\alpha 6\beta 2\alpha 4\beta 2\beta 3$	759 pM	685-842	3.4
r $\alpha 6\beta 4$	66.0 nM	51.4-84.7	296
r $\alpha 6/\alpha 3\beta 4$	65.4 nM	43.4-98.6	293
m $\alpha 6/\alpha 3\beta 4$	75.6 nM	61.1-96.3	339
r $\alpha 7$	421 nM	356-498	1,880
r $\alpha 9\alpha 10$	27.8 μ M	15.5-50.0	>10,000

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

Figure 1

A

PeIA **G****C****C**S**H**P**A****C**SVNH**P**E**L****C**
MII **G****C****C**S**N**P**V****C**H**L**E**H**S**N**L**C**
PnIA **G****C****C**S**L**P**P****C**A**A**N**N**P**D****Y****C**

B

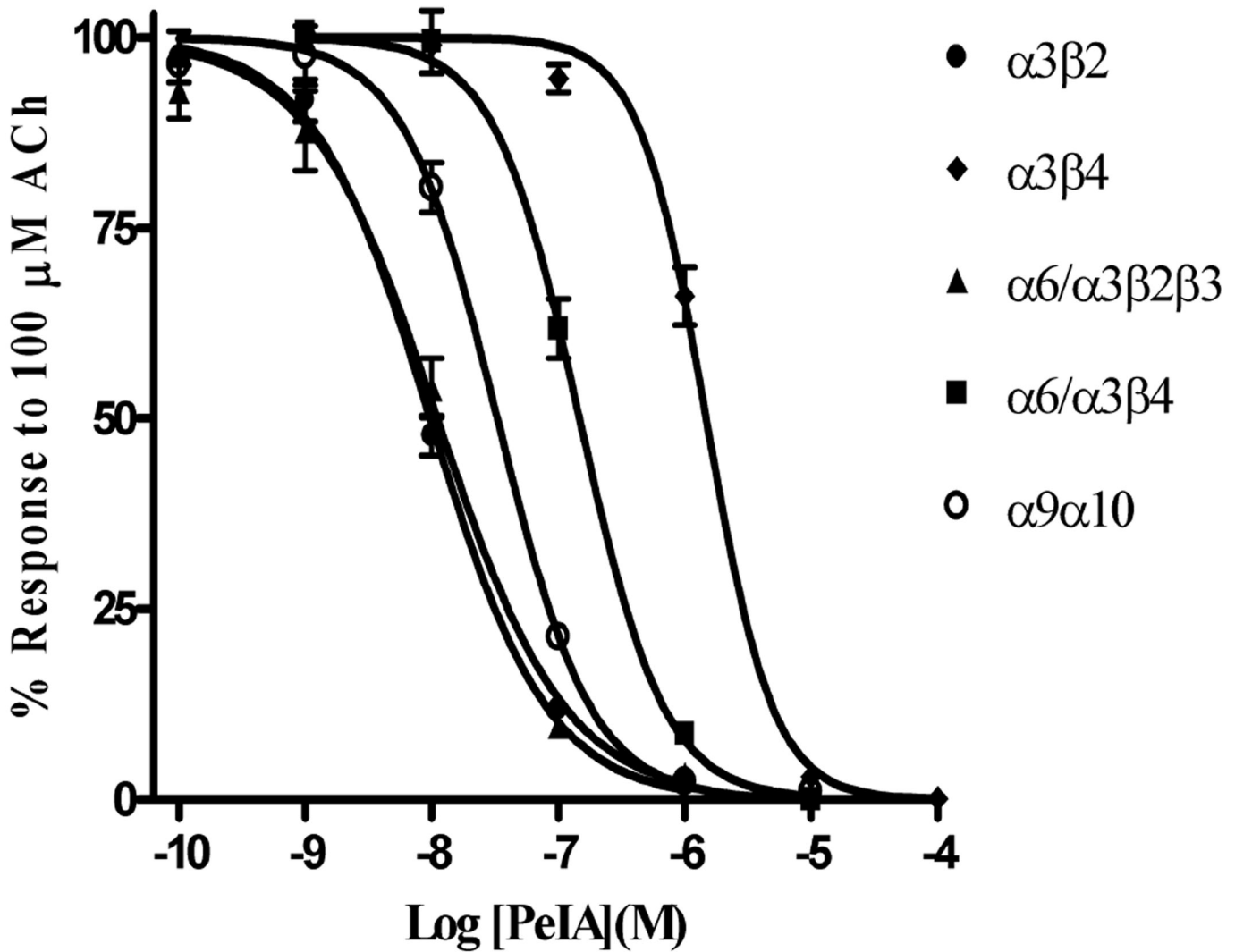


Figure 2

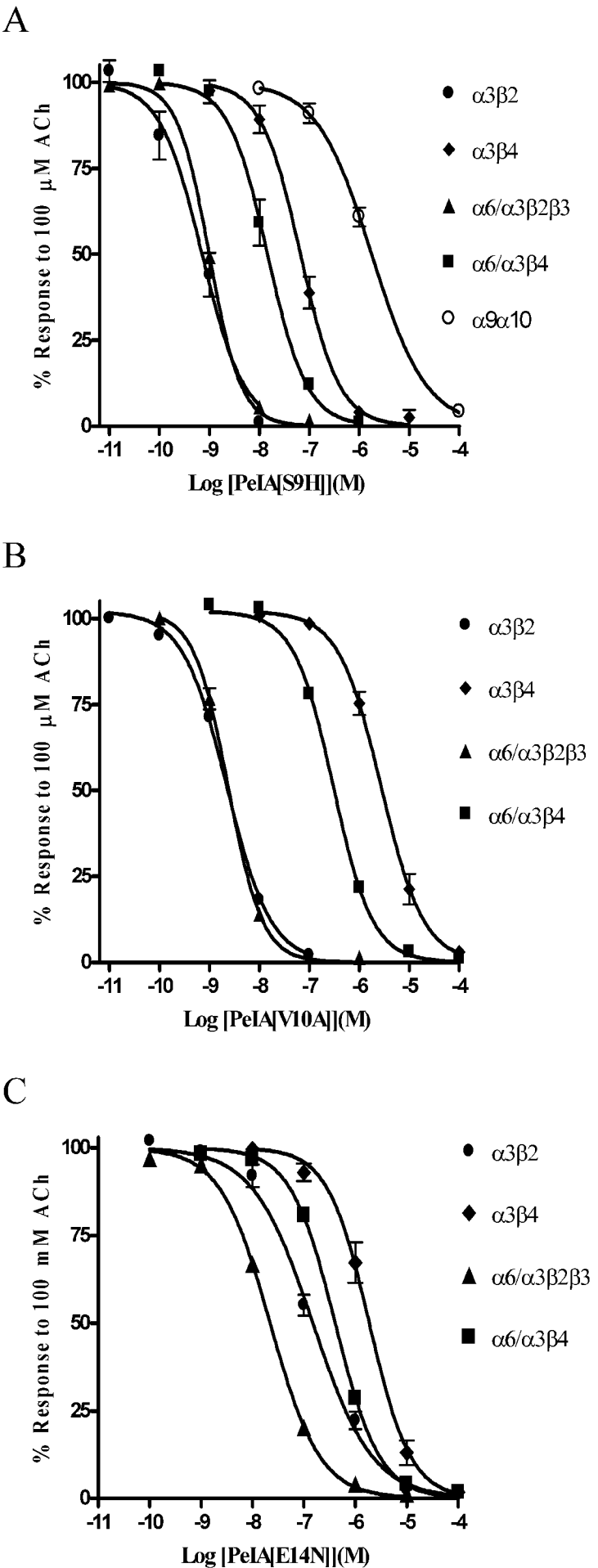


Figure 3

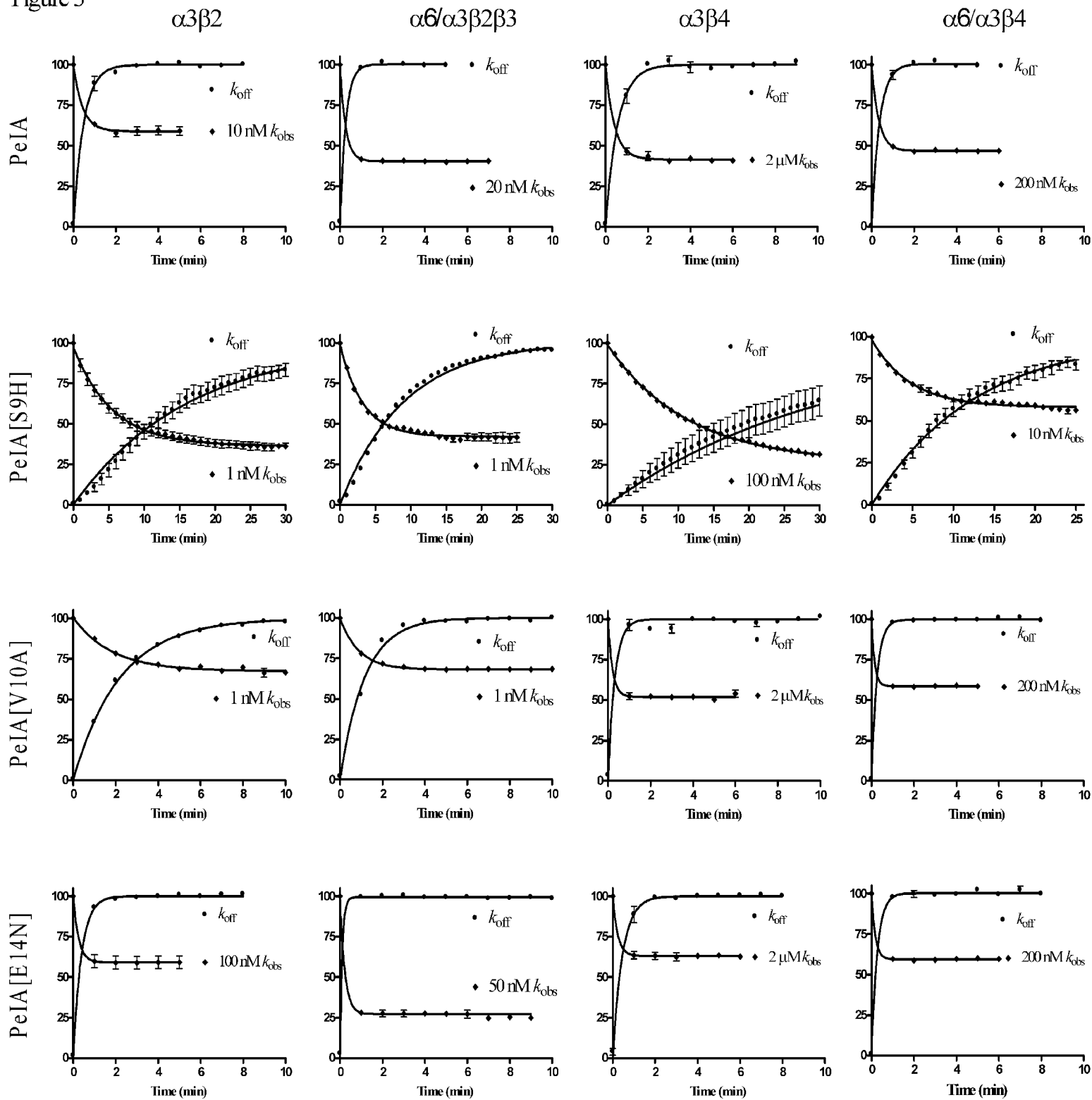


Figure 4

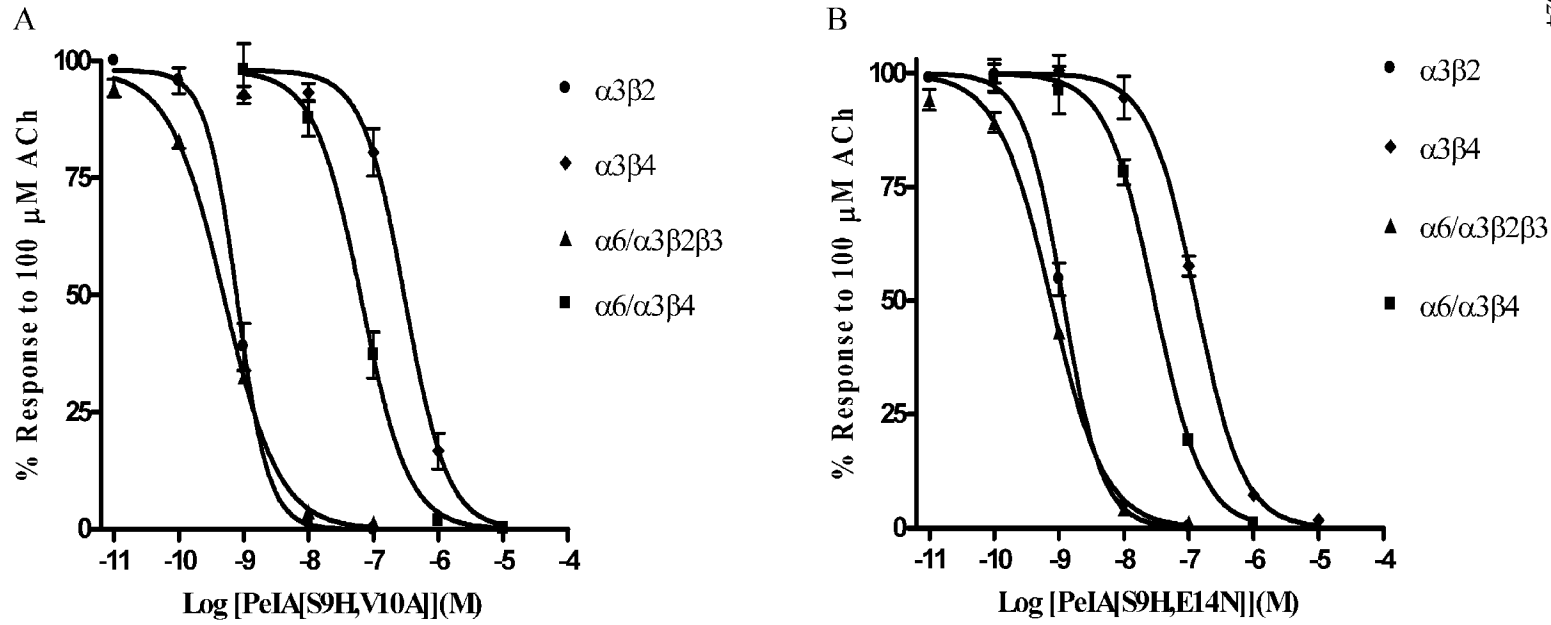
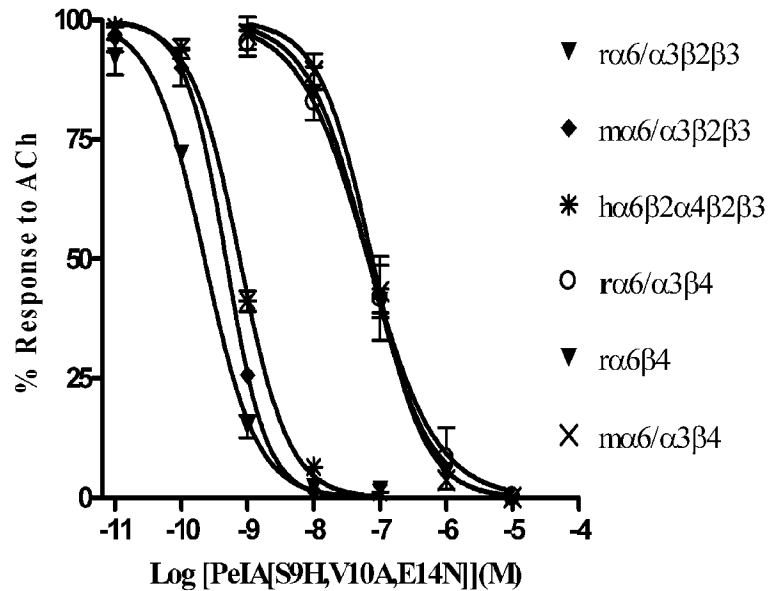


Figure 5

A



B

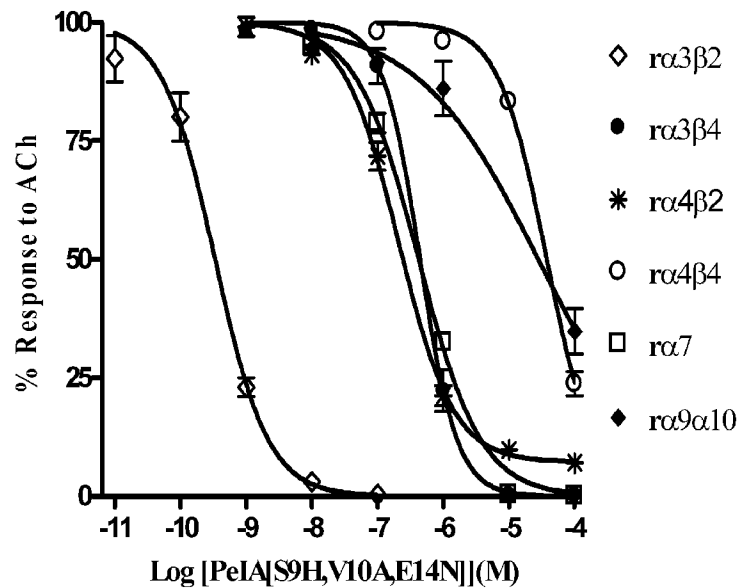


Figure 6

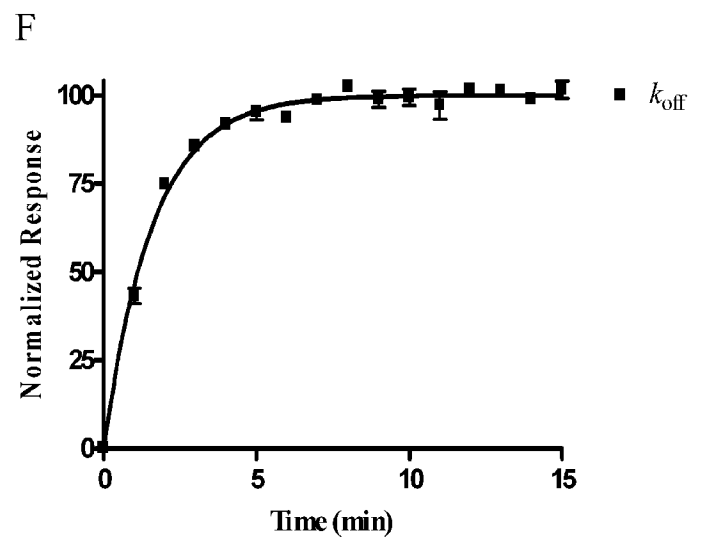
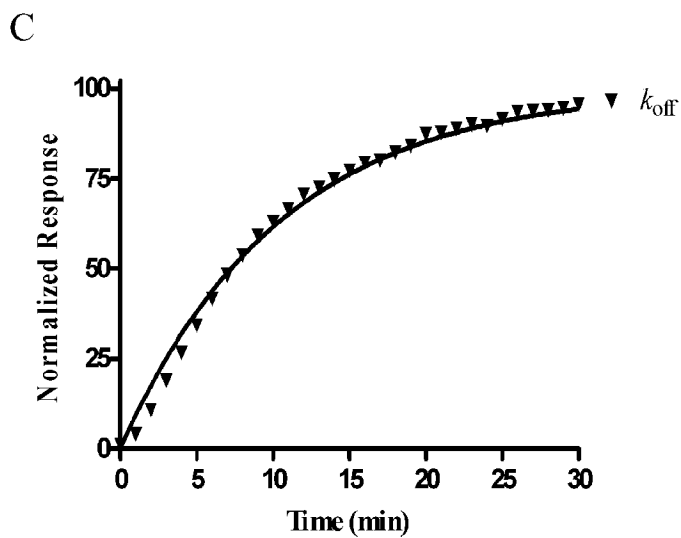
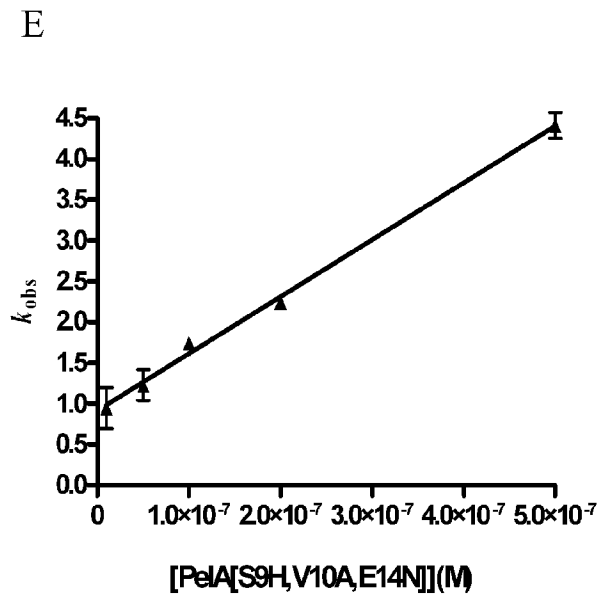
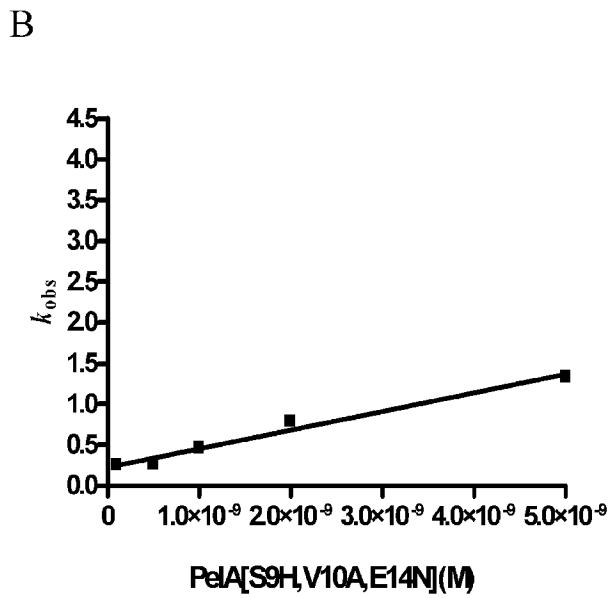
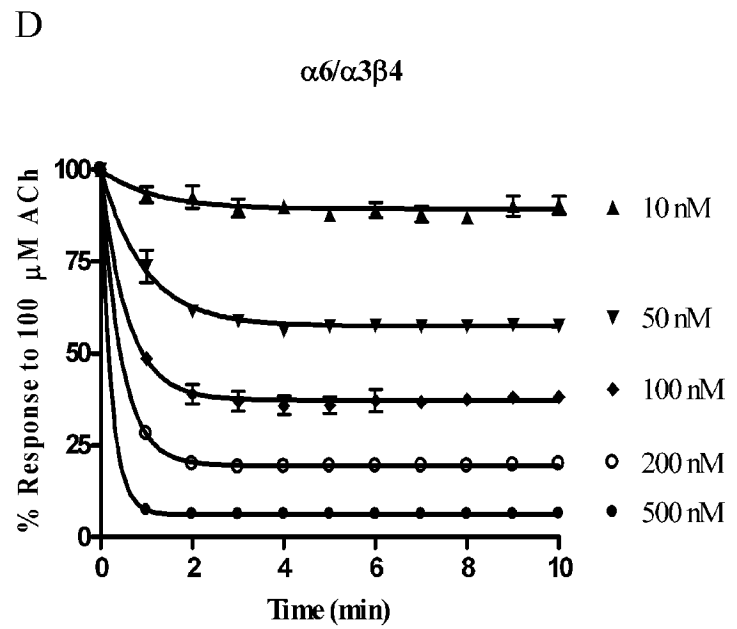
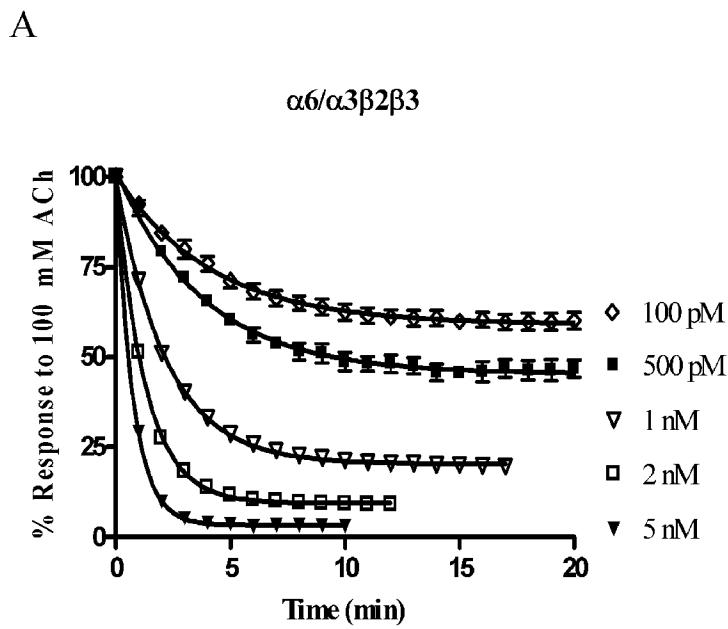


Figure 7

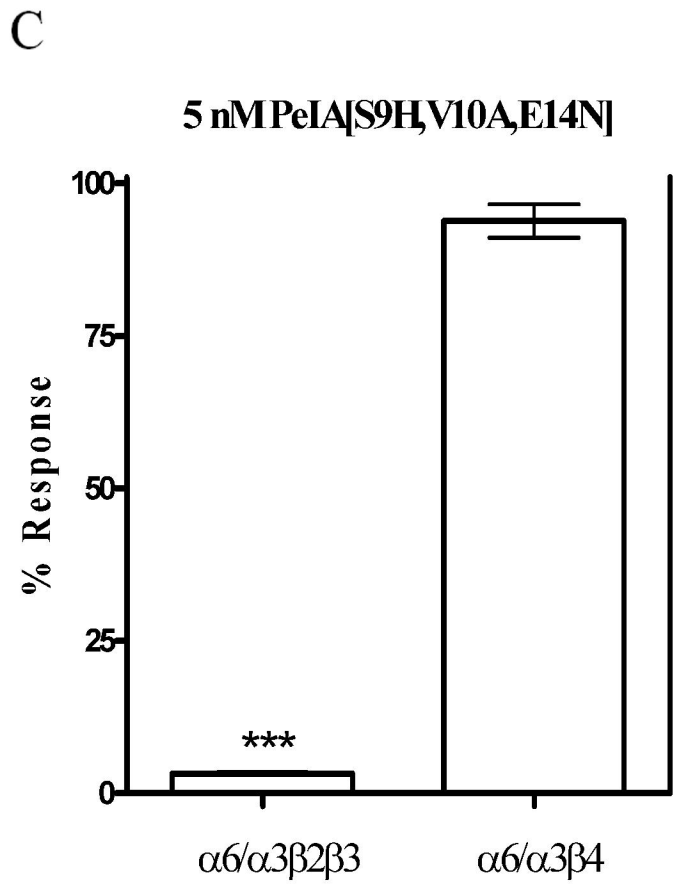
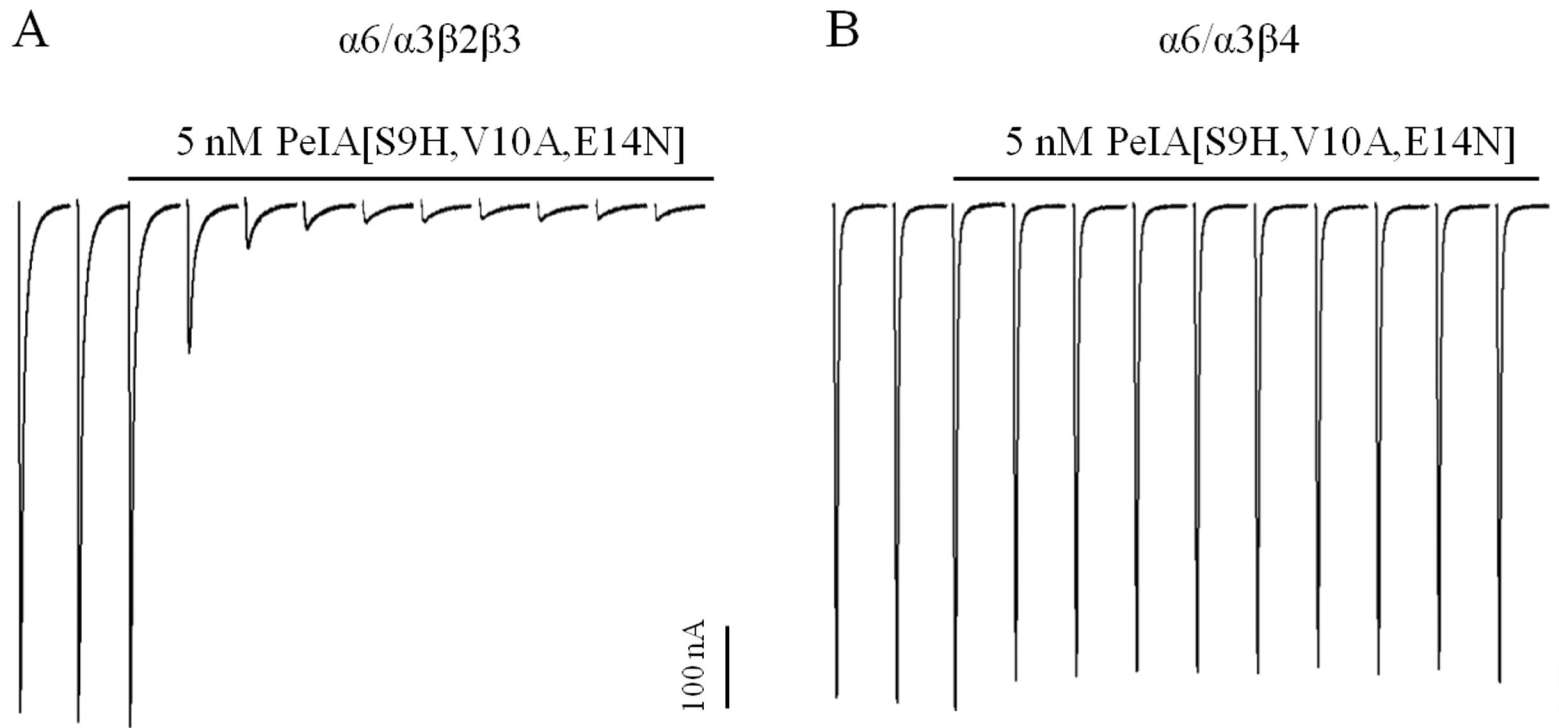


Figure 8

