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## Extending the analysis of nicotinic receptor antagonists with the study of alpha6 nicotinic receptor subunit chimeras

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### Abstract

Heterologous expression systems have increased the feasibility of developing selective ligands to target nicotinic acetylcholine receptor (nAChR) subtypes. However, the  $\alpha 6$  subunit, a component in nAChRs that mediates some of the reinforcing effects of nicotine, is not easily expressed in systems such as the *Xenopus* oocyte. Certain aspects of  $\alpha 6$ -containing receptor pharmacology have been studied by using chimeric subunits containing the  $\alpha 6$  ligand-binding domain. However, these chimeras would not be sensitive to an  $\alpha 6$ -selective channel blocker; therefore we developed an  $\alpha 6$  chimera ( $\alpha 4/6$ ) that has the transmembrane and intracellular domains of  $\alpha 6$  and the extracellular domain of  $\alpha 4$ . We examined the pharmacological properties of  $\alpha 4/6$ -containing receptors and other important nAChR subtypes, including  $\alpha 7$ ,  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ ,  $\alpha 3\beta 4$ ,  $\alpha 3\beta 2$ , and  $\alpha 3\beta 2\beta 3$ , as well as receptors containing  $\alpha 6/3$  and  $\alpha 6/4$  chimeras. Our data show that the absence or presence of the  $\beta 4$  subunit is an important factor for sensitivity to the ganglionic blocker mecamylamine, and that dihydro- $\beta$ -erythroidine is most effective on subtypes containing the  $\alpha 4$  subunit extracellular domain. Receptors containing the  $\alpha 6/4$  subunit are sensitive to  $\alpha$ -conotoxin PIA, while receptors containing the reciprocal  $\alpha 4/6$  chimera are insensitive. In experiments with novel antagonists of nicotine-evoked dopamine release, the  $\alpha 4/6$  chimera indicated that structural rigidity was a key element of compounds that could result in selectivity for noncompetitive inhibition of  $\alpha 6$ -containing receptors. Our data extend the information available on prototypical nAChR antagonists, and establish the  $\alpha 4/6$  chimera as a useful new tool for screening drugs as selective nAChR antagonists.

### Introduction

There are three major classes of nicotinic acetylcholine receptors (nAChR) in mammals: muscle-type receptors, heteromeric neuronal-type receptors, and homomeric neuronal receptors. All nAChRs are believed to form pentameric complexes. Important elements of the agonist binding sites are part of the alpha subunits, but the binding sites are located at the

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interface between alpha and non-alpha subunits, and the non-alpha subunits contribute importantly to the pharmacological properties of the receptors (Luetje and Patrick, 1991), including  $\beta 1$  in regard to the relative insensitivity of muscle type receptors to ganglionic blockers (Webster et al., 1999).

Eight alpha subunit genes have been cloned from mammalian neuronal tissue ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 8$ ,  $\alpha 9$ , and  $\alpha 10$ ), as well as three non-alpha subunits ( $\beta 2$ ,  $\beta 3$ , and  $\beta 4$ ). The simplest of the neuronal nAChR to study in the oocyte expression system is the homomeric subtype, which in mammalian brain is composed of only  $\alpha 7$  subunits (Gotti et al., 2006). The heteromeric neuronal type receptors form as various combinations of alpha and beta subunits, always containing at least one type of alpha (either  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ , or  $\alpha 6$ ) and at least one type of beta subunit. In the continued presence of agonist, heteromeric receptors convert to a desensitized state that binds nicotine and other agonists with high affinity; these receptors were first identified in radioligand binding studies with rat brain slices (Clarke et al., 1985).

Although there are many possible combinations of neuronal alpha and beta subunits, it has been shown that the majority of the high affinity nicotine binding sites in the brain contain combinations of just the  $\alpha 4$  and  $\beta 2$  subunits. Co-injections of RNA for these two subunits into *Xenopus* oocytes readily result in the expression of receptors with pharmacological properties consistent with those of the brain's high affinity receptors (Nelson et al., 2003). Likewise, co-expression of  $\alpha 3$  and  $\beta 4$  subunits, strongly expressed in the peripheral nervous system, yields receptors used to model ganglionic receptors. All pairwise combinations of  $\alpha 2$ ,  $\alpha 3$ , or  $\alpha 4$  with either  $\beta 2$  or  $\beta 4$  are easily characterized in the oocyte expression systems, and those results can be extended to model more complex subunit combinations through the analysis of the effects of systematic addition of other subunits (Gerzanich et al., 1998; Papke et al., 2007).

The first practical targeting of neuronal nicotinic acetylcholine receptors (nAChR) for a therapeutic indication came with the development of ganglionic blockers, such as hexamethonium and mecamylamine, for the treatment of hypertension (Stone et al., 1956). While the use of ganglionic blockers for the treatment of hypertension has been eclipsed by the subsequent development of more effective antihypertensive agents with better side-effect profiles, there is a renewed interest in antagonists that may inhibit nAChRs in brain (Dwoskin et al., 2004; Rose et al., 1994a; Rose et al., 1994b). Of necessity, for human studies this renewed interest has focused on the classical, and largely nonselective blocker, mecamylamine, since it is the only CNS active nAChR antagonist approved for use in humans. Mecamylamine has been shown to have usefulness as an adjunct therapy in the treatment of Tourette's syndrome (Sanberg et al., 1998) and, in combination with nicotine replacement therapy, to improve clinical effectiveness in smoking cessation therapy (Rose et al., 1994b).

As with any course of therapeutic development, the diversity of receptor subtypes associated with different indications has created a need for the development of subtype-selective drugs. Unfortunately, nAChRs in brain show diversity and subtlety of function that, arguably, is unsurpassed by any other receptor system in the CNS. Several subtype-selective agonists and partial agonists are now in clinical trials for various indications (Olincy et al., 2006; Potts and Garwood, 2007; Wilens et al., 2006). However, the development of subtype-selective antagonists has proven to be a more daunting challenge. One reason for this is that some complex subunit combinations that may exist *in vivo* are not easily recreated in artificial expression systems. This is particularly true for receptor subtypes containing the  $\alpha 6$  subunit (Dowell et al., 2003; Kuryatov et al., 2000). Nonetheless, because  $\alpha 6$ -containing receptors are likely involved in nicotine dependence (Azam et al., 2007; Azam and McIntosh, 2006), they have been proposed to be a potentially useful target for therapies using nicotinic receptor antagonists (Dwoskin et al., 2004; Zoli et al., 2002). We have extended the characterization of the commonly used nAChR antagonists mecamylamine and dihydro- $\beta$ -erythroidine (DH $\beta$ E),

and herein report a new chimera that may be useful for identifying  $\alpha 6$ -selective noncompetitive antagonists. We utilize that chimera to test a series of *bis*-quaternary ammonium compounds, identifying structural rigidity of the analogs as a potentially important feature for selective inhibition of receptors containing the  $\alpha 6$  intracellular and transmembrane domains.

## Methods

### Cloning of Chimeric subunits

Rat neuronal nAChR  $\alpha 4$  and  $\alpha 6$  clones were obtained from Dr. Jim Boulter (UCLA, Los Angeles, CA), and the rat  $\alpha 6/3$  chimera has been previously characterized (Dowell et al., 2003; Papke et al., 2005). All three clones were subcloned into the pSGEM vector, obtained from Dr. Michael Hollmann (Ruhr University, Bochum, Germany), which contains *Xenopus* beta-globin untranslated regions to aid *Xenopus* oocyte expression. The  $\alpha 4$  subunit was subcloned from pSP64 to pSGEM via Hind III. The  $\alpha 6$  subunit was first mutated to make a silent change; an EcoR I restriction site which was within the open reading frame was removed in order to use EcoR I for the subcloning from pBS(SK-) to pSGEM. The  $\alpha 6/3$  chimera was digested from pT7TS with Bgl II and BstE II to subclone into pSGEM digested with BamH I and BstE II.

The conserved amino acids isoleucine-arginine-arginine, located just before the first transmembrane domain (Table 3), are suitable for making the silent mutations to accommodate the BspE I restriction site. This is the same manner in which the  $\alpha 6/3$  chimera was created by Dowell et al. (2003). This restriction site was also introduced in  $\alpha 4$  and  $\alpha 6$ . Mutations were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene La Jolla, CA). Sequences were confirmed with automated fluorescent sequencing at the University of Florida core facility.

The  $\alpha 6/4$  chimera was created by digesting  $\alpha 6/3$  and  $\alpha 6$  with BspE I and Spe I and ligating the  $\alpha 6$  N-terminal portion with the  $\alpha 4$  carboxy terminal portion and vector.  $\alpha 4/6$  was created by digesting  $\alpha 4$  and  $\alpha 6$  with BspE I and Xba I and ligating the  $\alpha 4$  N-terminal portion with the  $\alpha 6$  carboxy terminal portion and vector.

Only two amino acids differ between  $\alpha 4$  and  $\alpha 6$  in the putative extracellular loop (ECL) between the second and third transmembrane domains (Table 3). These were exchanged using the QuikChange kit sequentially and confirmed with automated fluorescent sequencing to create  $\alpha 4_{(\alpha 6\text{ECL})}$  and  $\alpha 4/6_{(\alpha\text{ECL})}$ .

### Preparation of RNA

Additional rat nAChR clones were also obtained from Dr. Jim Boulter (UCLA). After linearization and purification of cloned cDNAs, RNA transcripts were prepared *in vitro* using the appropriate mMessage mMachine kit from Ambion Inc. (Austin, TX).

### Expression in *Xenopus* oocytes

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

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemical Corporation, Freehold, NJ) for 2 hours at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 15 mM HEPES (pH 7.6), 12 g/l tetracycline). Subsequently, stage 5 oocytes

were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 2 to 7 days after injection. Although the absolute magnitude of the evoked current responses increased over time, the normalized values of the experimental responses did not vary significantly over time.

## Chemicals

$\alpha$ -Conotoxin PIA was synthesized as previously reported (Dowell et al., 2003). The bis-azaaromatic quaternary ammonium compounds were synthesized at the University of Kentucky utilizing previously reported procedures (Ayers et al., 2002; Crooks et al., 2004; Zheng et al., 2007). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO). Fresh acetylcholine (ACh) stock solutions were made daily in Ringer's solution and diluted.

## Electrophysiology

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City CA) (Stokes et al., 2004). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Cells were automatically perfused with bath solution, and agonist solutions were delivered from a 96-well plate. Both the voltage and current electrodes were filled with 3 M KCl. The agonist solutions were applied via disposable tips, which eliminated any possibility of cross-contamination. Drug applications alternated between ACh controls and experimental applications. Flow rates were set at 2 ml/min for experiments with  $\alpha 7$  receptors and 4 ml/min for other subtypes. Cells were voltage-clamped at a holding potential of  $-60$  mV. Data were collected at 50 Hz and filtered at 20 Hz. Drug applications were 12 seconds in duration followed by a 181 second washout periods with  $\alpha 7$  receptors and 8 seconds with 241 second washout periods for other subtypes.

## Experimental protocols and data analysis

Each oocyte received two initial control applications of ACh, an experimental drug application, and then a follow-up control application of ACh. The control ACh concentrations were  $60 \mu\text{M}$  for  $\alpha 7$ ,  $10 \mu\text{M}$  for  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ , and  $\alpha 4/6\beta 4$  receptors and  $100 \mu\text{M}$  for the other subunit combinations tested. The peak amplitude and the net charge (Papke and Papke, 2002) of experimental responses were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. After each experimental measurement cells were rechallenged with ACh at the control concentrations. This allowed for the determination of residual inhibitory effects or rundown. Cells which showed more than a 25% variation between two consecutive ACh-evoked responses were not used for further experimental treatments. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. Unless otherwise noted, data presented are based on measurements of net charge for  $\alpha 7$  receptors and of peak currents for all other subtypes.

For concentration-response relationships, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated as the best fit of the average values to the Hill equation:

$$\text{Response} = \frac{I_{\max} [\text{agonist}]^n}{[\text{agonist}]^n + (EC50)^n}$$

where  $I_{\max}$  denotes the maximal response for a particular agonist/subunit combination, and  $n$  represents the Hill coefficient. For the calculation of  $IC_{50}$  values, negative Hill slopes were applied and  $I_{\max}$  was constrained to equal 1. Error estimates of the  $IC_{50}$  values are the standard errors of the parameters based on the Levenberg-Marquardt algorithm used for the generation

of the fits (Press, 1988). T-tests were used to determine statistical significance between antagonist activity at specific concentrations between pairs of nAChR subunit combinations.

## Results

### Evaluation of the subtype selectivity of mecamylamine and dihydro- $\beta$ -erythroidine (DH $\beta$ E)

Mecamylamine is one of the most commonly used neuronal nAChR antagonists, believed to have some selectivity for ganglionic (i.e.,  $\beta$ 4-containing) receptors (Papke et al., 2001b), while DH $\beta$ E is a competitive antagonist, reportedly selective for  $\alpha$ 4-containing receptors (Chavez-Noriega et al., 1997). We tested these antagonists on the basic models for ganglionic ( $\alpha$ 3 $\beta$ 4) nAChR, and brain heteromeric ( $\alpha$ 4 $\beta$ 2) and homomeric ( $\alpha$ 7) nAChRs. As shown in Figure 1, experiments confirmed that  $\alpha$ 3 $\beta$ 4 was the most sensitive to mecamylamine inhibition and  $\alpha$ 4 $\beta$ 2 was the most sensitive to DH $\beta$ E inhibition (IC<sub>50</sub> values are given in Table 1).

### Chimeric subunits with the $\alpha$ 6 extracellular domain

We adopted and extended the approach introduced by Dowell *et al.* (2003) to identify the effects of these classic antagonists on receptors containing the  $\alpha$ 6 extracellular domain by utilizing chimeric subunits. The first chimera, shown in Figure 2, is the previously published  $\alpha$ 6/3 chimera (Dowell et al., 2003; Papke et al., 2005). To further confirm that the  $\alpha$ 6 extracellular domain alone is sufficient to produce  $\alpha$ 6-like pharmacology regarding agonists and competitive antagonists, we constructed a second  $\alpha$ 6 chimera,  $\alpha$ 6/4, incorporating the transmembrane and intracellular domains of the rat  $\alpha$ 4 subunit. A human  $\alpha$ 6/4 chimera has also been described (Evans et al., 2003; Kuryatov et al., 2000). To provide a novel tool for evaluating the potential  $\alpha$ 6 selectivity of noncompetitive antagonists, we constructed an  $\alpha$ 4/6 chimera containing the extracellular domain of  $\alpha$ 4 and the transmembrane and intracellular domains of  $\alpha$ 6.

We first tested the hypothesis that the differing transmembrane and intracellular regions of the  $\alpha$ 6/3 and  $\alpha$ 6/4 would have relatively little effect on the concentration response relationships to agonists. Since some native  $\alpha$ 6 receptors assemble with both  $\beta$ 2 and  $\beta$ 3 subunits (Gotti et al., 2006), the  $\alpha$ 6/3 and  $\alpha$ 6/4 chimeras were co-expressed with  $\beta$ 2 and  $\beta$ 3. The receptors formed had essentially identical concentration-response relationships to the agonists ACh and nicotine (Figure 3). The ACh EC<sub>50</sub> values for both chimeras were approximately 20  $\mu$ M while we have previously reported (Papke et al., 2007) that the ACh EC<sub>50</sub> for  $\alpha$ 3 $\beta$ 2 $\beta$ 3 receptors is approximately 90  $\mu$ M. Our study of mecamylamine and DH $\beta$ E was then extended to systematically evaluate the effects the  $\beta$ 3 subunit and the  $\alpha$ 6 extracellular domains on the inhibitory effects of these compounds. Comparison of the responses of  $\alpha$ 3- and  $\beta$ 2 expressing cells to those additionally expressing  $\beta$ 3 indicated that the  $\beta$ 3 subunit increased sensitivity to mecamylamine (Figure 4). However, cells expressing the  $\alpha$ 6/3 chimera along with  $\beta$ 2 and  $\beta$ 3 had lower sensitivity to mecamylamine than those expressing  $\alpha$ 3 $\beta$ 2 $\beta$ 3, suggesting an interaction between the way in which the  $\alpha$ 6 extracellular domain affected ACh activation and subsequent use-dependent block by mecamylamine. As shown in Figure 4, the  $\alpha$ 6 extracellular domain also led to a small increase in sensitivity to DH $\beta$ E, while  $\beta$ 3 expression did not affect the potency of this competitive antagonist.

### A chimeric subunit with the $\alpha$ 6 transmembrane and intracellular domains

The nicotinic noncompetitive antagonist mecamylamine has been shown to have some utility at increasing success in smoking cessation (Rose et al., 1994b). The selectivity of mecamylamine for neuronal nAChR compared to muscle-type nAChR is based on specific sequence elements in the transmembrane domains (Webster et al., 1999). However, as our current study shows, mecamylamine is most effective at inhibiting ganglionic ( $\alpha$ 3 $\beta$ 4-containing) receptors, and these are not likely to be the best targets for treating nicotine dependence. The fact that  $\alpha$ 6 expression is typically high in dopaminergic neurons associated

with reward suggests that antagonists with a selectivity for  $\alpha 6$ -containing receptors might be useful drugs for blocking nicotine-mediated reward in the brain. While the  $\alpha 6/3$  chimera has been useful in identifying the selective competitive inhibition of  $\alpha 6$ -containing receptors by select conus toxins (Dowell et al., 2003; McIntosh et al., 2004), conus toxins are not good drug candidates. Moreover, it is unlikely that chimeras containing the  $\alpha 6$  extracellular domain (i.e.  $\alpha 6/3$  and  $\alpha 6/4$ ) will be at all useful for identifying noncompetitive antagonists with selectivity for  $\alpha 6$ , due to the fact that the effectiveness of noncompetitive antagonists is expected to be dependent on sequence in the transmembrane domains, as was shown for mecamylamine. Therefore, we created an expressible construct ( $\alpha 6/4$ ) that contained the  $\alpha 6$  transmembrane and intracellular domains in combination with the  $\alpha 4$  extracellular domain (see Methods).

### Functional properties of the $\alpha 4/6$ chimera

The  $\alpha 4/6$  chimera was co-expressed with various other subunits to identify a useful beta subunit partner. The  $\alpha 4/6$  subunit did not function readily when co-expressed with  $\beta 2$  and  $\beta 3$  (data not shown) but did form functional receptors after several days when co-expressed with  $\beta 4$ . Thus, we used  $\alpha 4\beta 4$  receptors for comparisons to determine the pharmacological effects associated with the presence of the  $\alpha 6$  transmembrane and intracellular domains. Co-expression of the  $\alpha 4/6$  chimera with  $\beta 4$  had the additional advantage that functional receptors formed from this simple pairwise subunit combination reduced the variables that might arise with many different possible combinations of three subunits. The responses of  $\alpha 4\beta 4$  and  $\alpha 4/6\beta 4$  receptors were measured over a range of ACh concentrations (Figure 5). The  $EC_{50}$  for ACh with  $\alpha 4\beta 4$  receptors was  $15 \pm 1 \mu M$ . The responses of  $\alpha 4/6\beta 4$  receptors were fit with an  $EC_{50}$  of  $6.3 \pm 1.5 \mu M$ .

In order to confirm that the receptors containing the  $\alpha 4/6$  subunit retain  $\alpha 4$ -like functionality with competitive antagonists, we compared  $\alpha 4\beta 4$  receptors to  $\alpha 4/6\beta 4$  and  $\alpha 6/4\beta 4$  receptors in regard to their sensitivity to DH $\beta$ E and the  $\alpha 6$ -selective  $\alpha$ -conotoxin PIA (Dowell et al., 2003). As shown in Figure 6,  $\alpha 4/6\beta 4$  receptors were nearly as sensitive as  $\alpha 4\beta 4$  receptors to DH $\beta$ E ( $IC_{50}$  values in Table 1), while the  $\alpha 6/4\beta 4$  receptors were more than 10-fold less sensitive. In contrast, when receptors were pre-incubated in 100 nM  $\alpha$ -conus PIA toxin and then tested for their ACh responses in the continued presence of the toxin, both  $\alpha 4/6\beta 4$  and  $\alpha 4\beta 4$  receptors were relatively insensitive (Figure 6), while the  $\alpha 6/4\beta 4$  receptors were effectively blocked in a slowly reversible manner.

Two known nAChR noncompetitive antagonists, mecamylamine and the local anesthetic tetracaine, were tested on  $\alpha 4\beta 4$  and  $\alpha 4/6\beta 4$  receptors. As shown in Figure 7, cells with receptors containing the transmembrane and intracellular domains of  $\alpha 6$  were less sensitive to mecamylamine than cells expressing wild-type  $\alpha 4\beta 4$ . Tetracaine is a relatively unique noncompetitive antagonist, which has been shown to block both open and closed channels of muscle-type nAChR (Papke and Oswald, 1989). Cells with receptors containing the transmembrane and intracellular domains of  $\alpha 6$  appeared somewhat more sensitive to tetracaine than cells expressing wild-type  $\alpha 4\beta 4$  (Figure 7).

### Evaluation of compounds active at inhibiting nicotine-evoked dopamine release with the $\alpha 4/6$ chimera

Several classes of *bis*-azaaromatic quaternary ammonium compounds have recently been described which can produce potent partial inhibition of nicotine-evoked dopamine release from striatal slices (Crooks et al., 2004). One of the best characterized compounds from this large family of compounds is bPiDDB (Figure 8A), which has been shown to be active at decreasing dopamine-mediated behaviors including nicotine self-administration in rats (Neugebauer et al., 2006), and when delivered to the ventral tegmentum is able to inhibit the release of dopamine in the nucleus accumbens stimulated by the systemic delivery of nicotine.

The inhibitory activity of bPiDDB has been investigated on numerous AChR subtypes expressed in *Xenopus* oocytes (Rahman et al., 2008). In oocyte experiments, bPiDDB was most potent for inhibiting muscle type and  $\beta 4$ -containing receptors ( $IC_{50}$  values 200 – 500 nM) and less potent for  $\beta 2$ -containing receptors ( $IC_{50}$  values 10 – 20  $\mu$ M). The mechanism of bPiDDB inhibition of nAChR expressed in *Xenopus* oocytes is apparently noncompetitive since the inhibition of  $\alpha 4\beta 4$  responses evoked by high concentrations of ACh is roughly comparable to the inhibition of responses evoked by low concentrations of ACh. In the upper plot of Figure 8A, 10  $\mu$ M bPiDDB produced a 40% reduction in the  $I_{max}$  for ACh and only a relatively small change in the apparent  $EC_{50}$  values ( $16 \pm 1 \mu$ M and  $56 \pm 11 \mu$ M in the absence and presence of bPiDDB, respectively). Similar results have been obtained with the inhibition of  $\alpha 4\beta 2$  and  $\alpha 6/3\beta 2\beta 3$  receptors by bPiDDB (data not shown).

It has previously been reported (Rahman et al., 2008) that there is no significant difference in the  $IC_{50}$  values for the bPiDDB inhibition of  $\alpha 3\beta 2\beta 3$  and  $\alpha 6/3\beta 2\beta 3$  receptors ( $IC_{50} = 20 \pm 2.5$  and  $34 \pm 10 \mu$ M, respectively). We therefore used the  $\alpha 4/6$  chimera to determine if bPiDDB would selectively inhibit receptors based on the presence or absence of the  $\alpha 6$  transmembrane and intracellular domains. As shown in Figure 8A (lower plot), bPiDDB was more potent (Table 1B) at inhibiting receptors containing the complete  $\alpha 4$  subunit than at inhibiting those which contained the  $\alpha 4/6$  chimera.

In screens of other *bis*-azaaromatic quaternary ammonium compounds at a probe concentration of 1  $\mu$ M, we identified bPiPyB (Figure 8B) as an analog which produced more inhibition of  $\alpha 4/6\beta 4$  receptors than  $\alpha 4\beta 4$ . This selectivity between  $\alpha 4/6\beta 4$  and  $\alpha 4\beta 4$  receptors was confirmed with concentration-response studies (Figure 8B). Both bPiDDB and bPiPyB contain two 3-picolinium head groups separated by a linker containing 12 carbons. The most salient differences between the two compounds are in regard to the rigidity of the linkers and the likely conformational orientation (i.e. the intra-molecular distance between the head groups). In the case of bPiPyB, the molecule is constrained to an angular or ‘folded’ conformation as a result of the presence of the structurally rigid 1,2-diynylphenyl moiety. In the bPiDDB molecule, the rigid 1,2-diynylphenyl moiety is absent, and the molecule is conformationally very flexible, due to the presence of the *N,N'*-1,12-dodecanyl linker; this allows bPiDDB to adopt a more extended or ‘linear’ conformation than the bPiPyB molecule. We investigated the relative significance of these conformational differences between bPiDDB and bPiPyB by testing three other related *bis*-azaaromatic quaternary ammonium compounds: bIQDDB (Figure 9A) and bIQPyB (Figure 9B), which are homologs of bPiDDB and bPiPyB, respectively, except that they incorporate isoquinolinium rather than 3-picolinium head groups into their structure, and bIQPB (Figure 9C) is an intermediate structure in which the 1,2-diynylphenyl moiety in bPiPyB has been replaced with the more conformationally flexible 1,2-diethylphenyl moiety. Thus, bIQPB lacks the triple bonds in the linker units, which confer significant rigidity to both bPiPyB and bIQPyB.

As shown in Figure 9, neither bIQDDB nor bIQPB showed any significant difference in their inhibition of  $\alpha 4\beta 4$  and  $\alpha 4/6\beta 4$  receptors. However, like bPiPyB, bIQPyB produced significantly more inhibition of receptors containing the  $\alpha 4/6$  chimera. This observation suggested that conformational rigidity in the linker unit *per se* might be more important for inhibition of nAChRs containing the  $\alpha 6$  transmembrane and intracellular domains than the presence of a centrally placed 1,2-substituted phenyl moiety in the linker unit. We therefore tested two additional analogs of bPiDDB, bPiBB and bPiByB (Figure 10), in which the linker incorporated a central 1,4-diethylphenyl (bPiBB) and 1,4-diynylphenyl (bPiByB) moiety to afford a more linear analog comparable to an extended conformation of the pPiDDB molecule. As shown in Figure 11, bPiBB showed some selectivity for the  $\alpha 4/6$ -containing receptors, and this selectivity was further improved by the presence of the rigid triple bonds in bPiByB.

A potentially important subdomain within the  $\alpha 6$  portion of the  $\alpha 4/6$  chimera is the putative extracellular loop (ECL) between the second and third transmembrane domains (Table 3). This region has been shown to be of crucial importance for the coupling of agonist binding to channel activation (Bouzat et al., 2004; Lummis et al., 2005) and may be accessible to noncompetitive antagonists. There are two amino acids that differ between  $\alpha 4$  and  $\alpha 6$  in the ECL (Table 3). At the start of the ECL  $\alpha 4$  has an isoleucine where  $\alpha 6$  has a threonine and 12 residues down  $\alpha 4$  has an isoleucine where  $\alpha 6$  has a valine. To determine whether these differences had significance for inhibition by bPiByB, we mutated  $\alpha 4$  to have the  $\alpha 6$  sequence at these sites ( $\alpha 4_{(\alpha 6\text{ECL})}$ ) and the  $\alpha 4/6$  chimera to have the  $\alpha 4$  sequence at these sites ( $\alpha 4/6_{(\alpha 4\text{ECL})}$ ). These mutations did appear to reduce the selectivity of bPiByB, but not to reverse it.

## Discussion

Recent work with transgenic animals (Cordero-Erausquin et al., 2000) and new pharmacological tools (Nai et al., 2003) have been informative with respect to the complex nature of the nAChR subtypes involved in nicotine self-administration, and the  $\alpha 6$  subunit is part of this rich mix. This gene is expressed at high levels in the ventral tegmental area and, compared to  $\alpha 4$ ,  $\alpha 6$  has a very restricted pattern of expression in the brain, being associated almost exclusively with dopaminergic neurons, including those that are the source of the nicotine reinforcement (Charpantier et al., 1998; Goldner et al., 1997; Le Novere et al., 1996). Toxins shown to have some selectivity for  $\alpha 6$ -containing receptors in heterologous expression systems, are also effective at blocking a significant fraction of agonist-evoked dopamine release from synaptosomes (Salminen et al., 2007; Salminen et al., 2004), providing strong support for the hypothesis that  $\alpha 6^*$  receptors maybe an useful target for blocking the dopamine-mediated aspects of nicotine dependence. The conus toxins with  $\alpha 6$  selectivity are competitive antagonists and therefore could be characterized with chimeras that contain the  $\alpha 6$  extracellular domain (Dowell et al., 2003; McIntosh et al., 2004). However, since these molecules are proteins, they clearly are not good drug candidates. Alternative nAChR antagonists that do penetrate the brain when given systemically, such as mecamylamine and 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH) are noncompetitive antagonists (Damaj et al., 2005). Not only are these noncompetitive agents active in the CNS when given systemically, they may discriminate between nAChR subtypes based on single amino acid differences in the subunit sequences (Papke et al., 2005; Webster et al., 1999). These observations provide the rationale for seeking  $\alpha 6$ -selective noncompetitive antagonists.

Unfortunately, the pharmacological properties of  $\alpha 6$ -containing receptors have been difficult to characterize, since this subunit expresses poorly in heterologous systems and may even act as a dominant negative factor when expressed in combination with other subunits (Papke, unpublished observation). One successful approach for identifying properties of the  $\alpha 6$  agonist binding site has been the chimeric subunit ( $\alpha 6/3$ ), containing the  $\alpha 6$  extracellular domain and the transmembrane and intracellular domains of  $\alpha 3$  (Kuryatov et al., 2000) This chimera functions reasonably well when co-expressed with  $\beta 2$  and  $\beta 3$ . A rat  $\alpha 6/3$  chimera was used to confirm that the  $\alpha$ -conotoxin PIA, which blocks a significant fraction of nicotine-evoked dopamine release from synaptosomes (Azam and McIntosh, 2006), is in fact selective for  $\alpha 6$ -containing nAChRs (Dowell et al., 2003).

In this paper, we report two new  $\alpha 6$  chimeras based on combinations with the rat  $\alpha 4$  subunit. We show that the  $\alpha 6/4$  chimera is comparable to the  $\alpha 6/3$  chimera in regard to the pharmacology of agonists and competitive antagonists, while the  $\alpha 4/6$  chimera responds to these agents like the wild-type  $\alpha 4$ . However, when co-expressed with  $\beta 4$ , the  $\alpha 4/6$  chimera shows a pattern of sensitivity to antagonists different from that of  $\alpha 4\beta 4$  receptors, suggesting that this chimera will provide a useful tool in the further study of new potential drugs for treating nicotine dependence.

Our work supports and expands upon previous studies of the established pharmacological tools, mecamylamine, DH $\beta$ E, and  $\alpha$ -conotoxin PIA. High sensitivity to DH $\beta$ E is clearly related to the presence of the extracellular domain of  $\alpha$ 4. We confirm, with a larger number of subtypes than previously tested, that mecamylamine shows selectivity for  $\beta$ 4-containing receptors over  $\beta$ 2-containing receptors. This was true regardless of which alpha subunit was co-expressed with  $\beta$ 4. While the selectivity of mecamylamine between ganglionic and muscle-type receptors can be attributed to specific residues at the 6' and 10' sites of the second transmembrane domain (numbering within the second transmembrane domain alpha helix (TM2) as per Miller et al. (Miller, 1989), it is possible that the selectivity observed between  $\beta$ 2 and  $\beta$ 4 containing receptors may be related to a single site difference at the 13' position (Table 2).

Several observations support the validity of using separate chimeras like  $\alpha$ 6/3,  $\alpha$ 6/4 and  $\alpha$ 4/6 to independently determine pharmacological properties provided by the ligand-binding and pore-forming domains. For example, the ACh and nicotine concentration-response curves shown in Figure 3 show essentially no difference that may be attributed to the  $\alpha$ 3 or  $\alpha$ 4 transmembrane and intracellular domains. Likewise, the competitive antagonists shown in Figure 6 effectively discriminate subtypes based on the presence or absence of the  $\alpha$ 6 extracellular domain.

We saw significant systematic differences in the activity of the noncompetitive antagonists tested with  $\alpha$ 4 $\beta$ 4 and  $\alpha$ 4/6 $\beta$ 4 receptors, confirming that this model system is sufficiently sensitive to aid in the development of truly  $\alpha$ 6-selective antagonists. Sequence identity between  $\alpha$ 4 and  $\alpha$ 6 is nearly 90% in the transmembrane domains, but the differences are enough to significantly impact the hydrophobicity profiles around the mouth of the TM2 and through the extracellular loop that connects TM2 and TM3 (Figure 2). Even single amino acid differences in these domains can result in very large differences in the responses to noncompetitive antagonists (Palma et al., 1998; Papke et al., 2005; Papke et al., 2001a; Webster et al., 1999).

Mecamylamine and the lidocaine derivative QX-222 are known to bind directly in the nAChR channel (Papke et al., 2001a; Webster et al., 1999), and these molecules are not much larger than the head groups of the *bis*-azaaromatic quaternary ammonium compounds in the present study, so it is perhaps a reasonable hypothesis that these compounds are binding at alternative sites above the membrane's electric field, as was shown to be the case for the similar bifunctional compound BTMPS, which contains two sterically hindered amino groups on piperidine rings joined by a 10-carbon aliphatic linker (Francis et al., 1998). The degree of conformational rigidity in the *bis*-azaaromatic quaternary ammonium molecules seemed to be a key factor for selective inhibition of receptors containing  $\alpha$ 6 transmembrane and intracellular domains. If in fact, these bifunctional agents bind to multiple sites in the receptor, our data suggest that the sites might be closer together in  $\alpha$ 4 $\beta$ 4 receptors than in  $\alpha$ 4/6 $\beta$ 4, requiring greater flexibility in the linker to effectively allow the putatively active cationic head groups to bind. Likewise, if the putative binding sites are further apart in  $\alpha$ 4/6 $\beta$ 4 receptors than in  $\alpha$ 4 $\beta$ 4 receptors, then an increase in the rigidity of the linkers might promote binding if the molecules are constrained to more fully extended or linear conformations. The observation that molecules restricted to a fully extended conformation, are favored for inhibiting  $\alpha$ 4/6 suggests that the head group separation distance in the extended conformation might be close to the distance between the sites on the separate subunits in the receptor. If this is so, then it is a somewhat confounding observation that rigidity is favored for both the linear and the folded molecules, since they cannot equally be templates for positioning the head groups to the same sites. However, if there are three  $\alpha$ 4/6 subunits per receptor, then these subunits and their associated binding sites will be juxtaposed as either adjacent subunits, or as subunits opposite to each other. It could be the case that if the head groups are binding to pairs of sites, each in single subunits, compounds with a folded conformation (bIQPyB and bIQPB) might bind best to sites in adjacent subunits within the pentamer, while *bis*-azaaromatic quaternary ammonium

compounds with the extended linear conformation (bPiBB and bPiByB) might bind best to sites in subunits that are opposite to each other. Our results with the ECL mutants suggest that this portion of the protein may modulate inhibition by the relatively selective compound bPiByB, but that the sequence differences in this domain are in themselves insufficient to account for the selectivity. The ECL domain may partially contribute to a binding site or alternatively, the ECL differences may affect other domains of the protein important for the inhibition of function. The latter possibility seems likely, considering that the ECL is believed to be a hinge point affecting both the ligand binding and pore forming domains (Bouzat et al., 2004; Lummis et al., 2005).

While one effect of incorporating conformational rigidity into the structure of the *bis*-azaaromatic quaternary ammonium compounds will be to better define the relative spatial positions of the two head groups, alternatively, the linkers themselves may also contribute to the biological activity of the compounds. TMPH is a very potent and selective nAChR antagonist which contains a simple tetramethylpiperidine (TMP) head group attached to a long aliphatic chain. Although tetramethylpiperidine itself is a ganglionic blocker, the addition of the lipophilic aliphatic chain had the effect of significantly increasing the potency of TMPH compared to TMP (Papke et al., 1994).

Unfortunately, high resolution models of the membrane spanning and intracellular domains of neuronal nAChRs are not available; thus, future studies may have to rely on traditional site-directed mutations for further investigation of the significance of both the single residue differences and functional subdomains of the receptors. In the meantime, as demonstrated in the current study, the insertion of the several points of sequence divergence between  $\alpha 4$  and  $\alpha 6$  into the  $\alpha 4/6$  chimera has made it a useful tool for the further development of agents which may selectively inhibit  $\alpha 6$  receptors, and therefore ultimately prove useful as therapeutic agents for treating nicotine dependence.

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#### References

- Ayers JT, Dvoskin LP, Deaciuc AG, Grinevich VP, Zhu J, Crooks PA. *bis*-Azaaromatic quaternary ammonium analogues: ligands for  $\alpha 4\beta 2^*$  and  $\alpha 7^*$  subtypes of neuronal nicotinic receptors. *Bioorganic Medicinal Chemistry Letters* 2002;12(21):3067–3071. [PubMed: 12372503]
- Azam L, Chen Y, Leslie FM. Developmental regulation of nicotinic acetylcholine receptors within midbrain dopamine neurons. *Neuroscience* 2007;144(4):1347–1360. [PubMed: 17197101]
- Azam L, McIntosh JM. Characterization of nicotinic acetylcholine receptors that modulate nicotine-evoked [3H]norepinephrine release from mouse hippocampal synaptosomes. *Molecular Pharmacology* 2006;70(3):967–976. [PubMed: 16735605]
- Bouzat C, Gumilar F, Spitzmaul G, Wang HL, Rayes D, Hansen SB, Taylor P, Sine SM. Coupling of agonist binding to channel gating in an ACh-binding protein linked to an ion channel. *Nature* 2004;430(7002):896–900. [PubMed: 15318223]
- Charpentier E, Barneoud P, Moser P, Besnard F, Sgard F. Nicotinic acetylcholine subunit mRNA expression in dopaminergic neurons of the rat substantia nigra and ventral tegmental area. *Neuroreport* 1998;9(13):3097–3101. [PubMed: 9804323]
- Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliott KJ, Johnson EC. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h  $\alpha 2\beta 2$ , h  $\alpha 2\beta 4$ , h  $\alpha 3\beta 2$ , h  $\alpha 3\beta 4$ , h  $\alpha 4\beta 2$ , h  $\alpha 4\beta 4$  and h  $\alpha 7$

- expressed in *Xenopus* oocytes. *Journal of Pharmacology and Experiment Therapeutics* 1997;280(1):346–356.
- Clarke PBS, Schwartz RD, Paul SM, Pert CB, Pert A. Nicotinic binding in rat brain: autoradiographic comparison of [<sup>3</sup>H] acetylcholine [<sup>3</sup>H] nicotine and [<sup>125</sup>I]-alpha-bungarotoxin. *Journal of Neuroscience* 1985;5:1307–1315. [PubMed: 3998824]
- Cordero-Erausquin M, Marubio LM, Klink R, Changeux JP. Nicotinic receptor function: new perspectives from knockout mice. *Trends in Pharmacological Sciences* 2000;21(6):211–217. [PubMed: 10838608]
- Crooks PA, Ayers JT, Xu R, Sumithran SP, Grinevich VP, Wilkins LH, Deaciuc AG, Allen DD, Dwoskin LP. Development of subtype-selective ligands as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release. *Bioorganic Medicinal Chemistry Letters* 2004;14(8):1869–1874. [PubMed: 15050618]
- Damaj MI, Wiley JL, Martin BR, Papke RL. In vivo characterization of a novel inhibitor of CNS nicotinic receptors. *European Journal of Pharmacology* 2005;521(1–3):43–48. [PubMed: 16181622]
- Dowell C, Olivera BM, Garrett JE, Staheli ST, Watkins M, Kuryatov A, Yoshikami D, Lindstrom JM, McIntosh JM. Alpha-conotoxin PIA is selective for alpha6 subunit-containing nicotinic acetylcholine receptors. *Journal of Neuroscience* 2003;23(24):8445–8452. [PubMed: 13679412]
- Dwoskin LP, Sumithran SP, Zhu J, Deaciuc AG, Ayers JT, Crooks PA. Subtype-selective nicotinic receptor antagonists: potential as tobacco use cessation agents. *Bioorganic Medicinal Chemistry Letters* 2004;14(8):1863–1867. [PubMed: 15050617]
- Evans NM, Bose S, Benedetti G, Zwart R, Pearson KH, McPhie GI, Craig PJ, Benton JP, Volsen SG, Sher E, Broad LM. Expression and functional characterisation of a human chimeric nicotinic receptor with alpha6beta4 properties. *European Journal of Pharmacology* 2003;466(1–2):31–39. [PubMed: 12679139]
- Francis MM, Choi KI, Horenstein BA, Papke RL. Sensitivity to voltage-independent inhibition determined by pore-lining region of ACh receptor. *Biophysical Journal* 74 May;:2306–2317.
- Gerzanich V, Wang F, Kuryatov A, Lindstrom J. alpha 5 Subunit alters desensitization, pharmacology, Ca<sup>++</sup> permeability and Ca<sup>++</sup> modulation of human neuronal alpha 3 nicotinic receptors. *Journal of Pharmacology and Experimental Therapeutics* 1998;286(1):311–320. [PubMed: 9655874]
- Goldner FM, Dineley KT, Patrick JW. Immunohistochemical localization of the nicotinic acetylcholine receptor subunit alpha6 to dopaminergic neurons in the substantia nigra and ventral tegmental area. *Neuroreport* 1997;8(12):2739–2742. [PubMed: 9295110]
- Gotti C, Zoli M, Clementi F. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends in Pharmacological Sciences* 2006;27(9):482–491. [PubMed: 16876883]
- Kuryatov A, Olale F, Cooper J, Choi C, Lindstrom J. Human alpha6 AChR subtypes: subunit composition, assembly, and pharmacological responses. *Neuropharmacology* 2000;39(13):2570–2590. [PubMed: 11044728]
- Le Novere N, Zoli M, Changeux JP. Neuronal nicotinic receptor alpha 6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *European Journal of Neuroscience* 1996;8(11):2428–2439. [PubMed: 8950106]
- Luetje CW, Patrick J. Both  $\alpha$ - and  $\beta$ -subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *Journal of Neuroscience* 1991;11(3):837–845. [PubMed: 1705971]
- Lumms SC, Beene DL, Lee LW, Lester HA, Broadhurst RW, Dougherty DA. Cistrans isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. *Nature* 2005;438(7065):248–252. [PubMed: 16281040]
- McIntosh JM, Azam L, Staheli S, Dowell C, Lindstrom JM, Kuryatov A, Garrett JE, Marks MJ, Whiteaker P. Analogs of alpha-conotoxin MII are selective for alpha6-containing nicotinic acetylcholine receptors. *Molecular Pharmacology* 2004;65(4):944–952. [PubMed: 15044624]
- Miller C. Genetic manipulation of ion channels: a new approach to structure and mechanism. *Neuron* 1989;2(3):1195–1205. [PubMed: 2483110]
- Nai Q, McIntosh JM, Margiotta JF. Relating neuronal nicotinic acetylcholine receptor subtypes defined by subunit composition and channel function. *Molecular Pharmacology* 2003;63(2):311–324. [PubMed: 12527802]

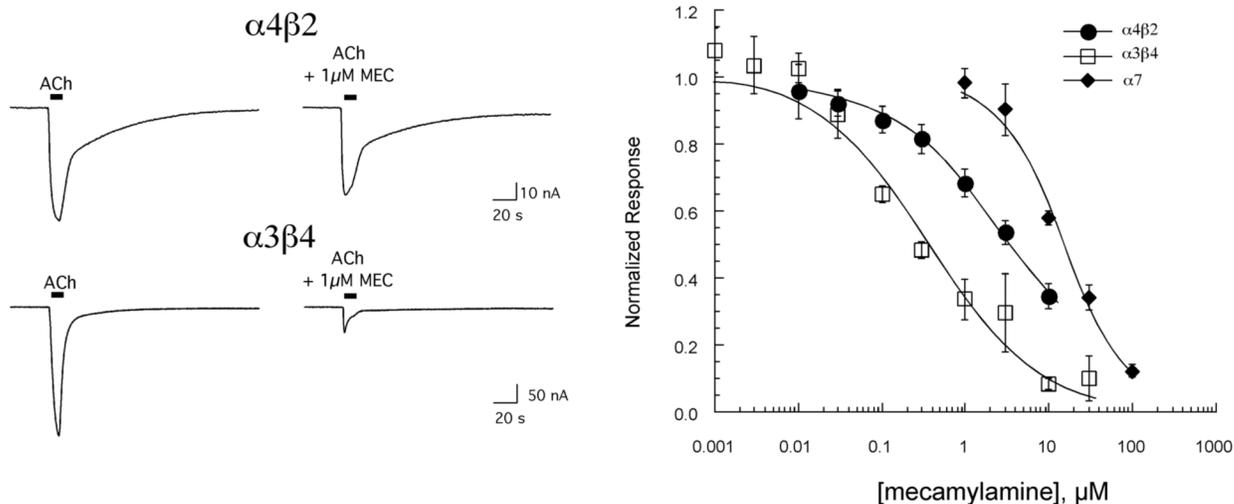
- Nelson ME, Kuryatov A, Choi CH, Zhou Y, Lindstrom J. Alternate stoichiometries of alpha4beta2 nicotinic acetylcholine receptors. *Molecular Pharmacology* 2003;63(2):332–341. [PubMed: 12527804]
- Neugebauer NM, Zhang Z, Crooks PA, Dwoskin LP, Bardo MT. Effect of a novel nicotinic receptor antagonist, N,N'-dodecane-1,12-diyl-bis-3-picolinium dibromide, on nicotine self-administration and hyperactivity in rats. *Psychopharmacology* 2006;184(3–4):426–434. [PubMed: 16220336]
- Olinicy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D, Ellis J, Zerbe GO, Leonard S, Stevens KE, Stevens JO, Martin L, Adler LE, Soti F, Kem WR, Freedman R. Proof-of-concept trial of an alpha7 nicotinic agonist in schizophrenia. *Archives of General Psychiatry* 2006;63(6):630–638. [PubMed: 16754836]
- Palma E, Maggi L, Mileli R, Eusebi F. Effects of Zn<sup>2+</sup> on wild and mutant neuronal alpha7 nicotinic receptors. *Proceedings of the National Academy of Science, U. S. A* 1998;95(17):10246–10250.
- Papke RL, Buhr JD, Francis MM, Choi KI, Thinschmidt JS, Horenstein NA. The effects of subunit composition on the inhibition of nicotinic receptors by the amphipathic blocker 2,2,6,6-tetramethylpiperidin-4-yl heptanoate. *Molecular Pharmacology* 2005;67(6):1977–1990. [PubMed: 15761116]
- Papke RL, Craig AG, Heinemann SF. Inhibition of nicotinic acetylcholine receptors by BTMPS, (Tinuvin® 770), an additive to medical plastics. *Journal of Pharmacology and Experimental Therapeutics* 1994;268:718–726. [PubMed: 8113983]
- Papke RL, Dwoskin LP, Crooks PA. The pharmacological activity of nicotine and normicotine on nAChR subtypes: relevance to nicotine dependence and drug discovery. *Journal of Neurochemistry* 2007;101(1):160–167. [PubMed: 17241116]
- Papke RL, Horenstein BA, Placzek AN. Inhibition of wild-type and mutant neuronal nicotinic acetylcholine receptors by local anesthetics. *Molecular Pharmacology* 2001a;60(6):1–10.
- Papke RL, Oswald RE. Mechanisms of noncompetitive inhibition of acetylcholine-induced single channel currents. *Journal of General Physiology* 1989;93:785–811. [PubMed: 2472461]
- Papke RL, Papke JKP. Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. *British Journal of Pharmacology* 2002;137(1):49–61. [PubMed: 12183330]
- Papke RL, Sanberg PR, Shytle RD. Analysis of mecamylamine stereoisomers on human nicotinic receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics* 2001b;297(2):646–656. [PubMed: 11303054]
- Potts LA, Garwood CL. Varenicline: the newest agent for smoking cessation. *American Journal of Health-System Pharmacy* 2007;64(13):1381–1384. [PubMed: 17592002]
- Press, WH. *Numerical Recipes in C: The art of Scientific Computing*. Cambridge, United Kingdom: Cambridge University Press; 1988.
- Rahman S, Zhang Z, Papke RL, Crooks PA, Dwoskin LP, Bardo MT. Region-specific effects of bPiDDB on in vivo nicotine-induced increase in extracellular dopamine. *British Journal of Pharmacology*. 2008in press.
- Rose, J.; Levin, E.; Behm, F.; Westman, E.; Stein, R.; Lane, J.; Ripka, G. Concurrent mecamylamine/nicotine administration. In: Clarke, PBS.; Quik, M.; Thurau, K.; Adlkofer, F., editors. *International Symposium on Nicotine: The Effects of Nicotine on Biological Systems II*. Montreal, Canada: Birkhauser Verlag; 1994a. p. 122
- Rose JE, Behm FM, Westman EC, Levin ED, Stein RM, Ripka GV. Mecamylamine combined with nicotine skin patch facilitates smoking cessation beyond nicotine patch treatment alone. *Clinical Pharmacology & Therapeutics* 1994b;56(1):86–99. [PubMed: 8033499]
- Salminen O, Drapeau JA, McIntosh JM, Collins AC, Marks MJ, Grady SR. Pharmacology of alpha-conotoxin MII-sensitive subtypes of nicotinic acetylcholine receptors isolated by breeding of null mutant mice. 2007;71(6):1563–1571.
- Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, Grady SR. Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Molecular Pharmacology* 2004;65(6):1526–1535. [PubMed: 15155845]
- Sanberg PR, Shytle RD, Silver AA. Treatment of Tourette's syndrome with mecamylamine. *Lancet* 1998;352:705–706. [PubMed: 9728992]

- Stokes C, Papke JKP, McCormack T, Kem WR, Horenstein NA, Papke RL. The structural basis for drug selectivity between human and rat nicotinic  $\alpha 7$  receptors. *Molecular Pharmacology* 2004;66(1): 14–24. [PubMed: 15213292]
- Stone C, Torchiana M, Navarro A, Beyer K. Ganglionic blocking properties of 3-methylaminoisocamphane hydrochloride (mecamylamine): a secondary amine. *Journal of Pharmacology and Experimental Therapeutics* 1956;117:169–183.
- Webster JC, Francis MM, Porter JK, Robinson G, Stokes C, Horenstein B, Papke RL. Antagonist activities of mecamylamine and nicotine show reciprocal dependence on beta subunit sequence in the second transmembrane domain. *British Journal of Pharmacology* 1999;127:1337–1348. [PubMed: 10455283]
- Wilens TE, Verlinden MH, Adler LA, Wozniak PJ, West SA. ABT-089, a neuronal nicotinic receptor partial agonist, for the treatment of attention-deficit/hyperactivity disorder in adults: results of a pilot study. *Biological Psychiatry* 2006;59(11):1065–1070. [PubMed: 16499880]
- Zheng G, Sumithran SP, Deaciuc AG, Dwoskin LP, Crooks PA. tris-Azaaromatic quaternary ammonium salts: Novel templates as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release. *Bioorganic Medicinal Chemistry Letters* 2007;17(24):6701–6706. [PubMed: 17977723]
- Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, Gotti C. Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *Journal of Neuroscience* 2002;22(20):8785–8789. [PubMed: 12388584]

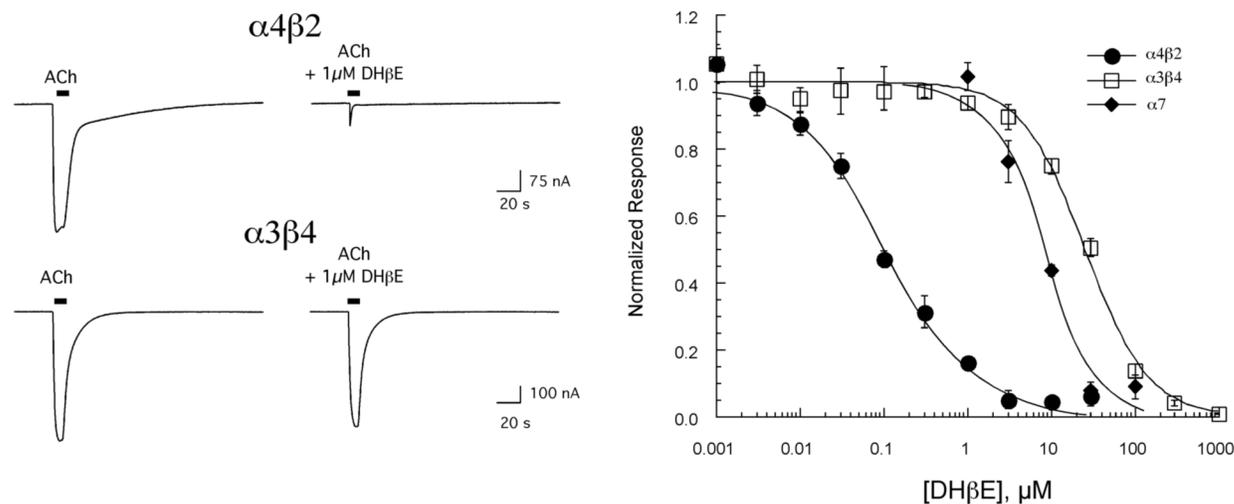
## Abbreviations

DH $\beta$ E, dihydro- $\beta$ -erythroidine; bPiDDB, *N,N'*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide; bPiPyB, 1,2-*bis*-[5-(3-picolinium)-pent-1-ynyl]-benzene dibromide; bIQDDB, *N,N'*-dodecane-1,12-diyl-*bis*-3-isoquinolinium dibromide; bIQPB, 1,2-*bis*-(5-isoquinolinium-pentyl)-benzene dibromide; bIQPyB, 1,2-*bis*-(5-isoquinolinium-pent-1-ynyl)-benzene dibromide; bPiBB, 1,4-*bis*-[4-(3-picolinium)-butyl]-benzene dibromide; bPiByB, 1,4-*bis*-[4-(3-picolinium)-butyl-1-ynyl]-benzene dibromide.

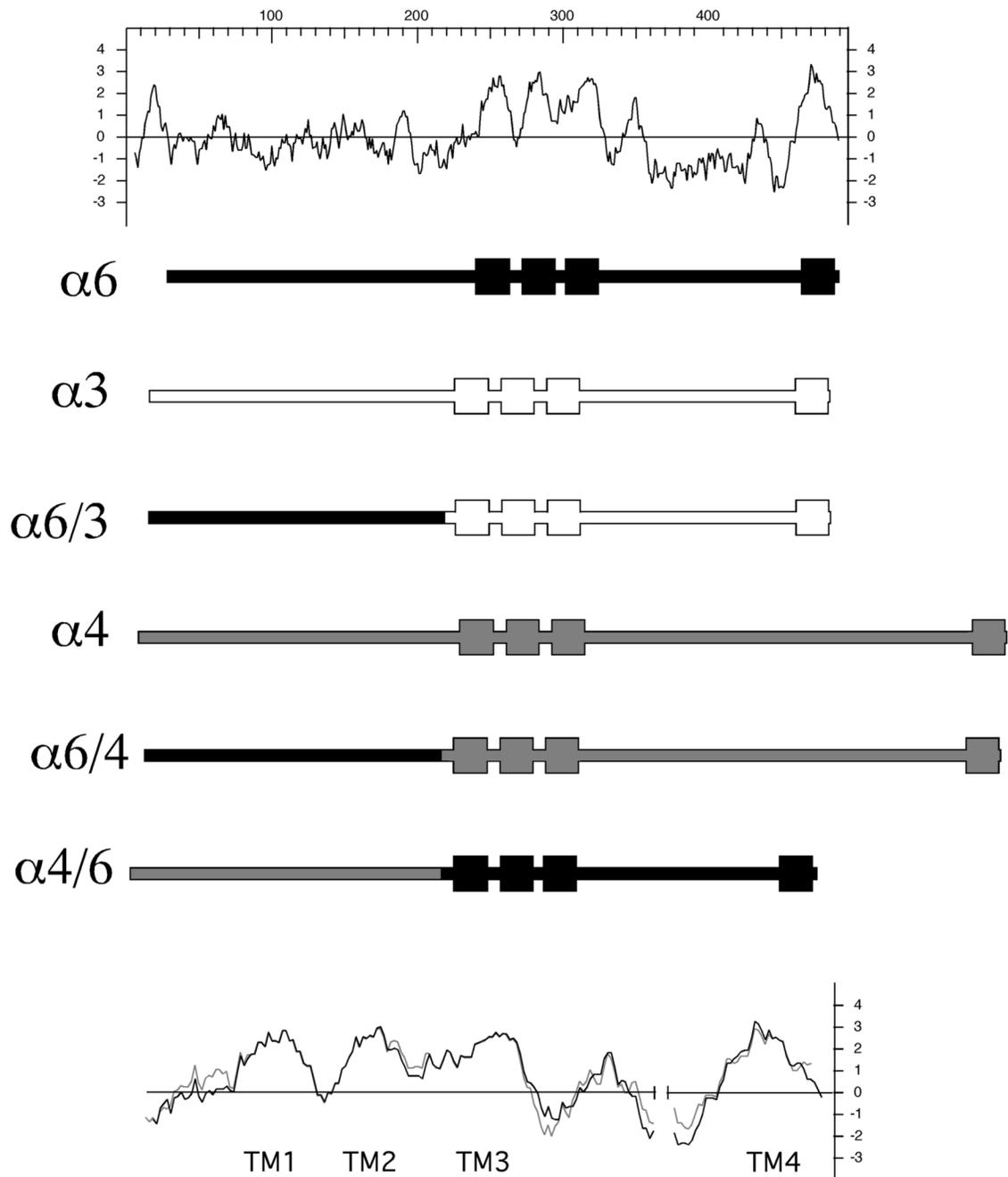
# Mecamylamine



# DH $\beta$ E

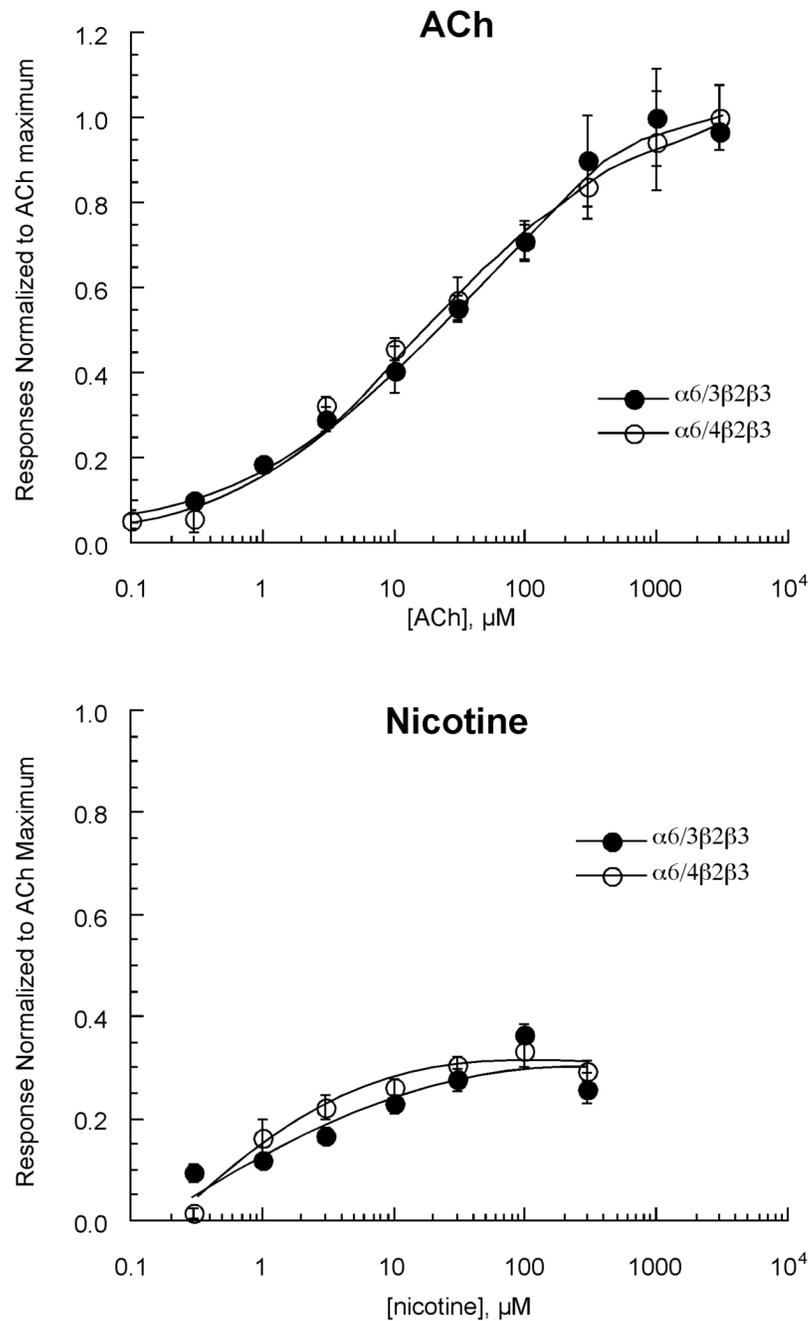


**Figure 1.** Inhibition of responses obtained from oocytes expressing rat neuronal nAChR subunits by mecamylamine (upper panel) or DH $\beta$ E (lower panel). Each panel shows raw data traces for  $\alpha 4\beta 2$  and  $\alpha 3\beta 4$  receptors on the left and on the right the averaged normalized responses ( $\pm$  SEM,  $n \geq 4$ ) from oocytes expressing  $\alpha 4\beta 2$ ,  $\alpha 3\beta 4$ , or  $\alpha 7$  subunits to the co-application ACh and increasing concentrations of antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used).

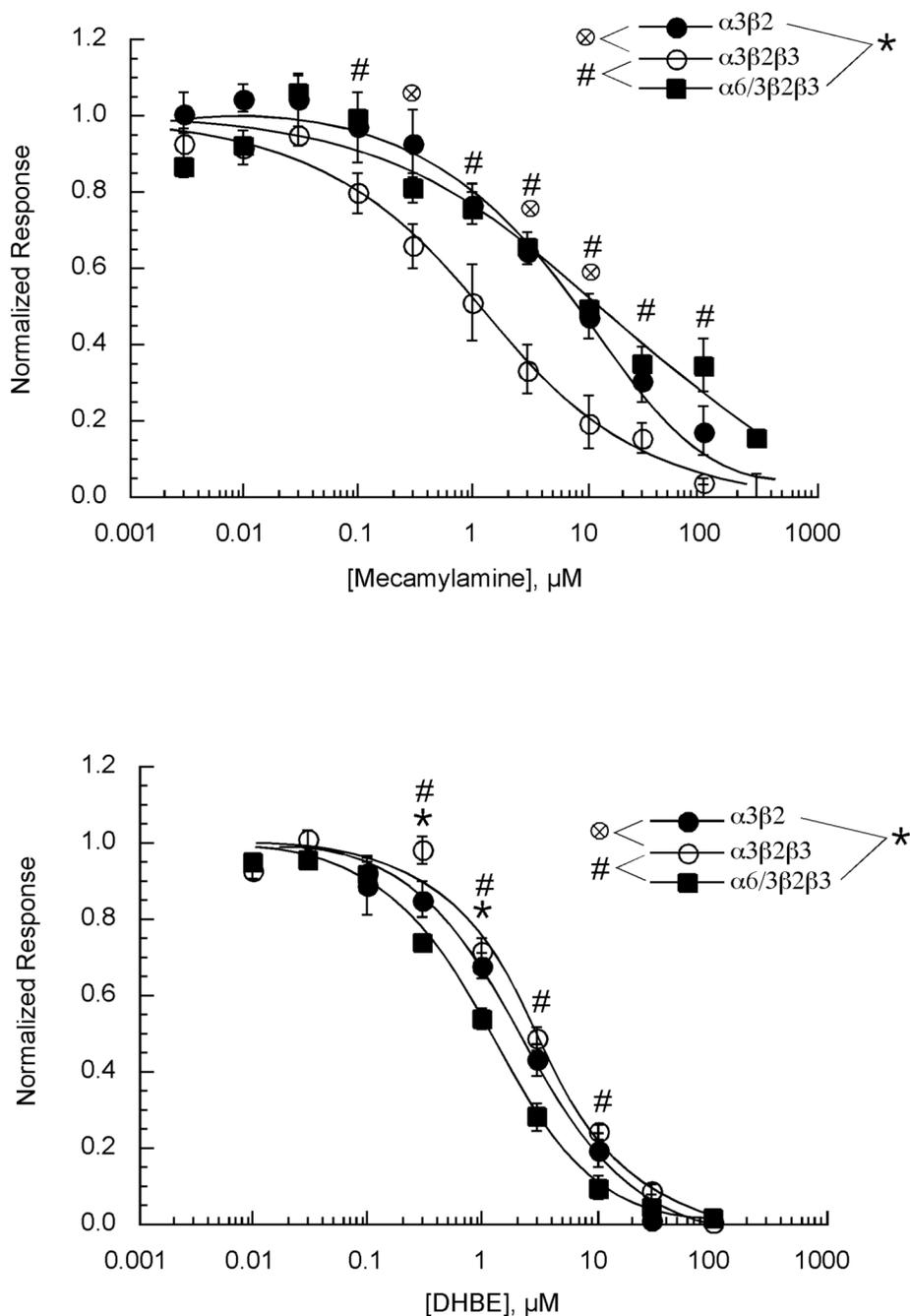


**Figure 2.**

Schematic representation of wild-type and chimeric subunits used to study the influence of  $\alpha 6$  subdomains of the pharmacological properties of neuronal nAChR. The putative transmembrane domains of  $\alpha 6$  are identified in the Kyte-Doolittle plot (11/1 generated by DNA Strider) at the top of the figure. These domains are identified by the wide bars in the schematics below. All the schematics are at the same scale as the Kyte-Doolittle plot but omit the signal sequences. An expansion and overlay of Kyte-Doolittle hydrophobicity plots for the transmembrane domains of  $\alpha 6$  (black) and  $\alpha 4$  (gray) is shown at the bottom of the figure.

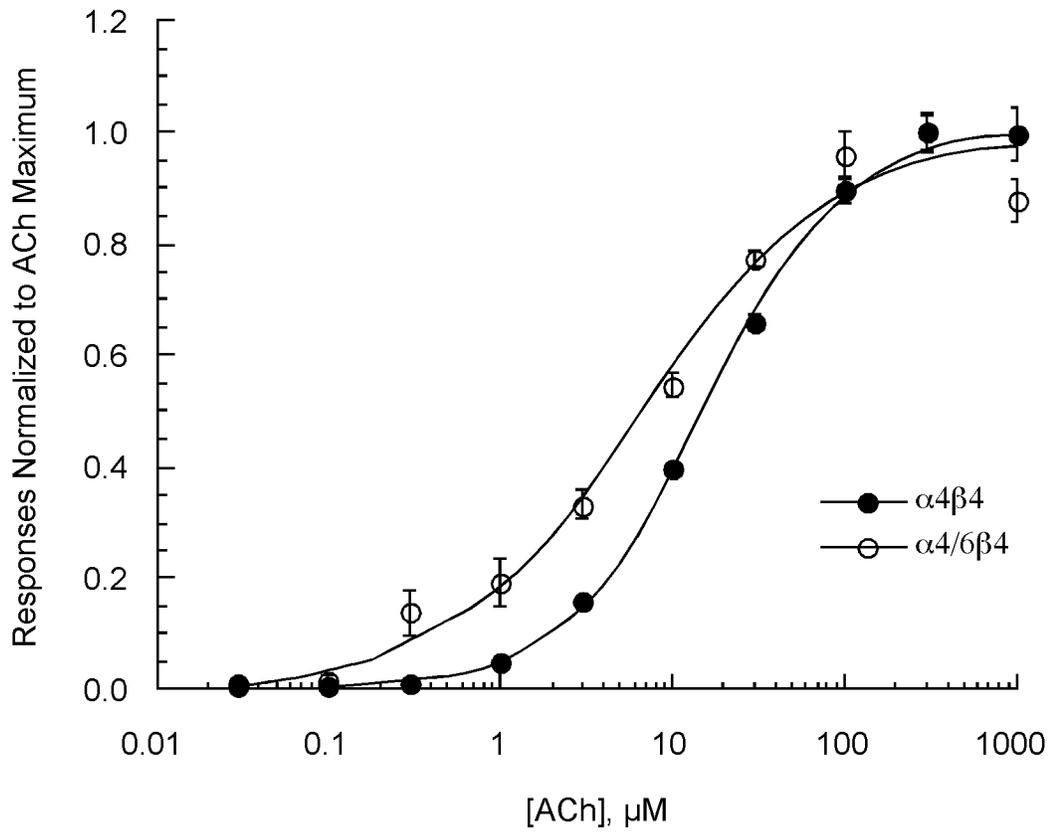


**Figure 3.** Concentration-response curves obtained from oocytes expressing rat neuronal nAChR  $\alpha 6/3\beta 2\beta 3$  or  $\alpha 6/4\beta 2\beta 3$  subunits given ACh (upper panel) or nicotine (lower panel). Each panel shows the averaged data ( $\pm$  SEM,  $n \geq 4$ ) normalized to the maximum ACh responses obtained from the same oocytes.

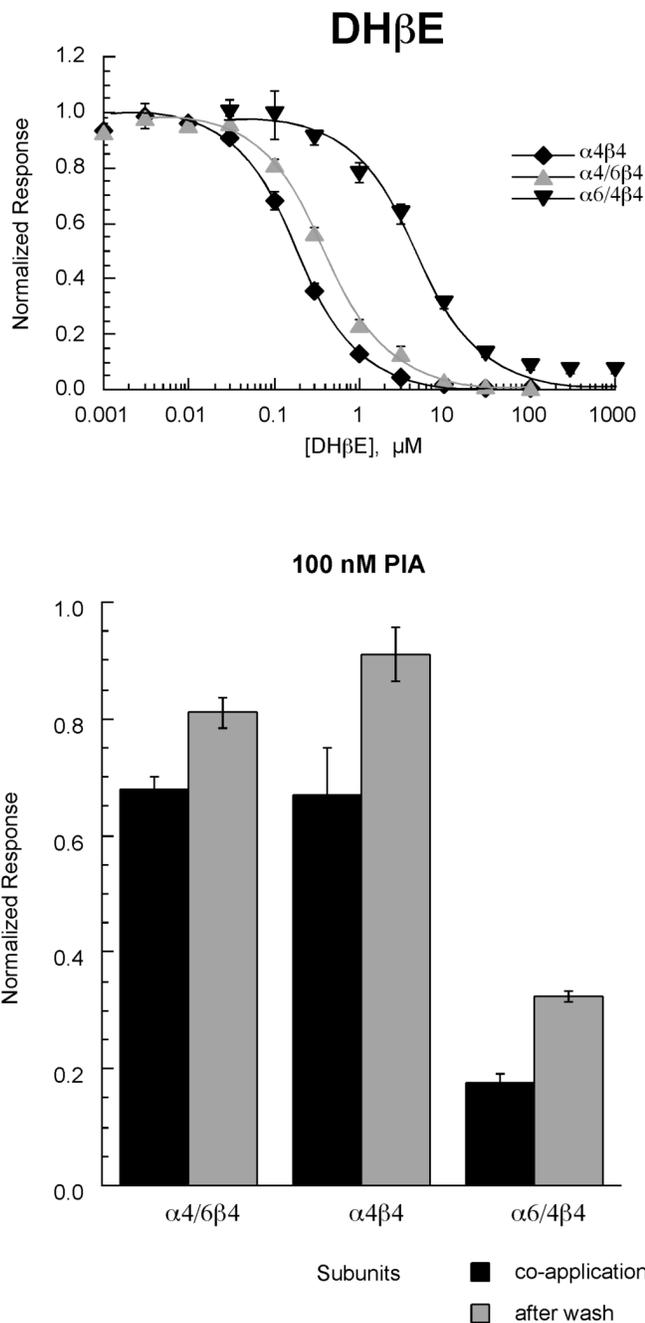


**Figure 4.** Inhibition of responses obtained from oocytes expressing rat neuronal nAChR subunits by mecamylamine (upper panel) or DHβE (lower panel). Each panel shows the averaged normalized data ( $\pm$  SEM,  $n \geq 4$ ) from oocytes expressing  $\alpha 3\beta 2$ ,  $\alpha 3\beta 2\beta 3$ , or  $\alpha 6/3\beta 2\beta 3$  subunits to the co-application of ACh and antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Significant ( $p < 0.05$ ) differences in inhibition were found between receptor subtypes at the concentrations indicated.  $\otimes$  represents significant difference between  $\alpha 3\beta 2$  and  $\alpha 3\beta 2\beta 3$  responses, # represents

significant difference between  $\alpha_3\beta_2\beta_3$  and  $\alpha_3/6\beta_2\beta_3$  responses, and \* represents significant difference between  $\alpha_3\beta_2$  and  $\alpha_3/6\beta_2\beta_3$  responses.

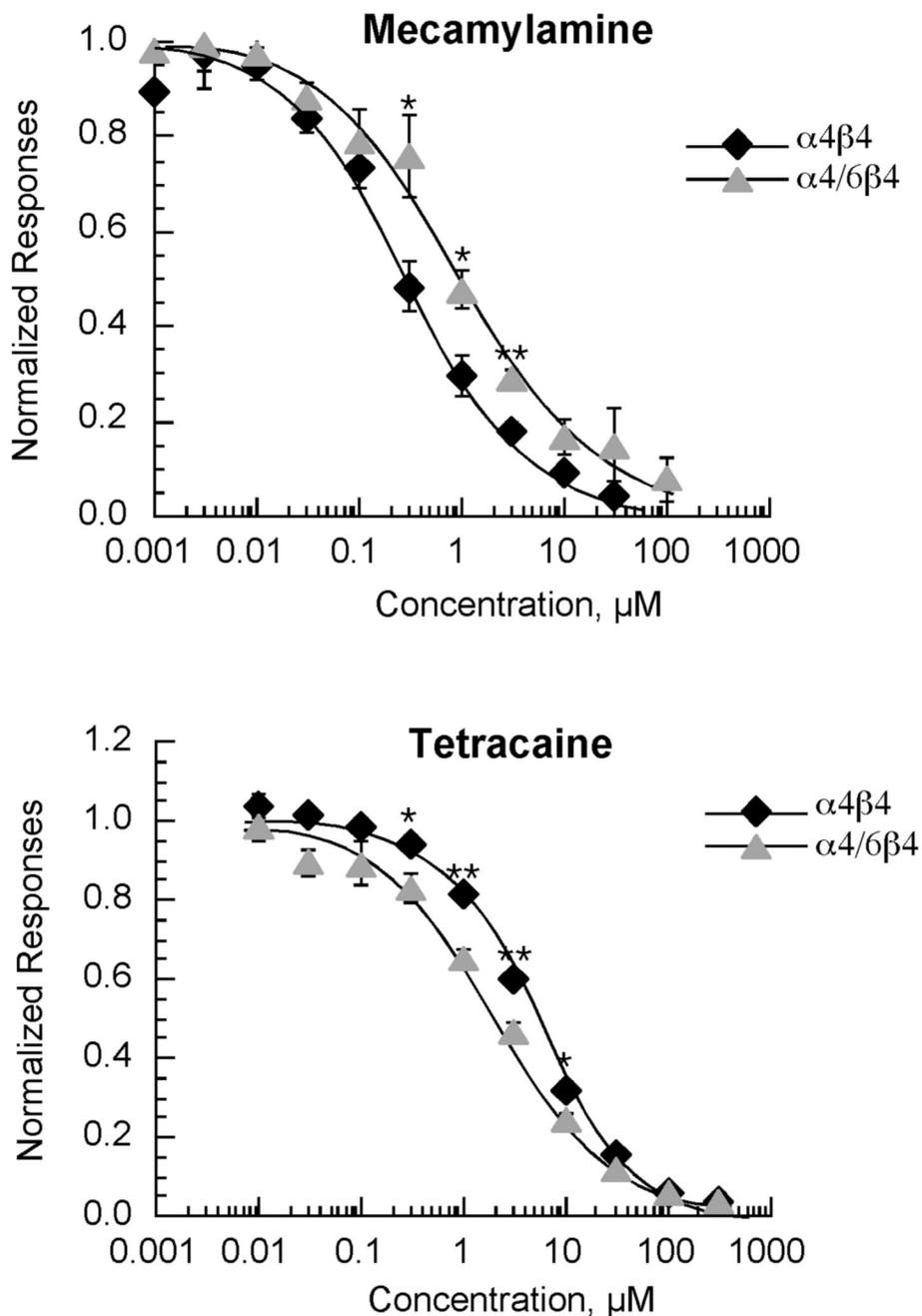


**Figure 5.** Concentration-response curves obtained from oocytes expressing rat neuronal nAChR  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  subunits given ACh. Shown are the averaged data ( $\pm$  SEM,  $n \geq 4$ ) normalized to the maximum ACh responses obtained from the same oocytes.



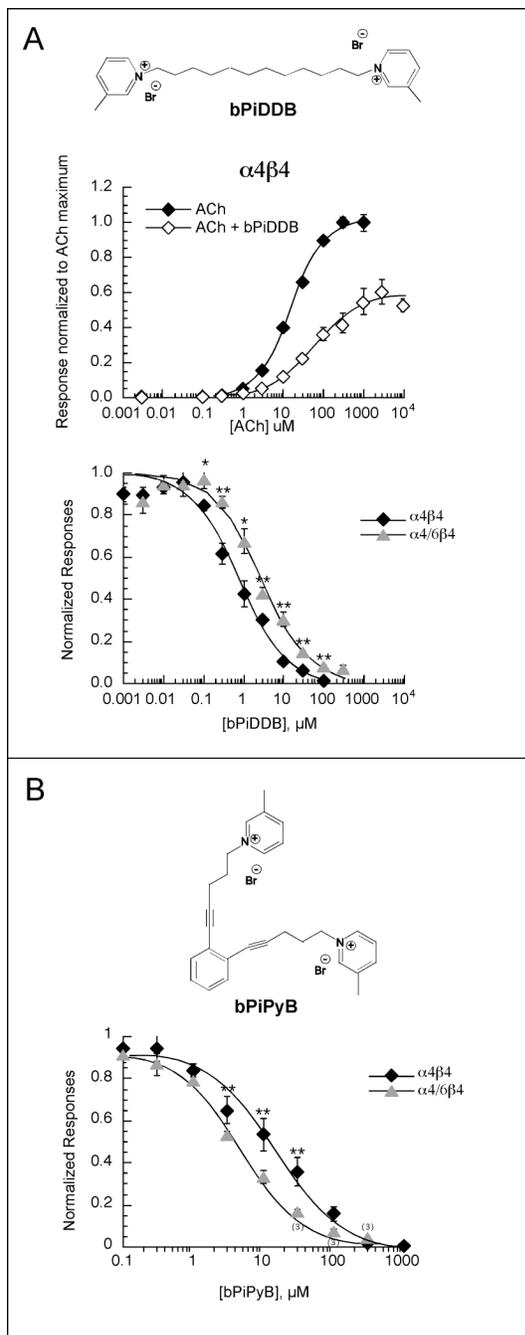
**Figure 6.** Inhibition of responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$ ,  $\alpha 6/4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits to competitive antagonists. The inhibition of responses to DH $\beta$ E are shown in the upper panel. The lower panel illustrates the effects of 100 nM  $\alpha$ -conotoxin PIA on ACh-evoked responses. Initial control responses to 100  $\mu$ M ACh were obtained, then cells were incubated for 5 min in 100 nM toxin (plus 0.1 mg/ml protease-free BSA), and then ACh was applied in the continued presence of the toxin. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Also shown are the Ach-

evoked responses obtained after a 5 min washout of the toxin. Each panel shows the averaged normalized data ( $\pm$  SEM,  $n \geq 4$ ) from oocytes.



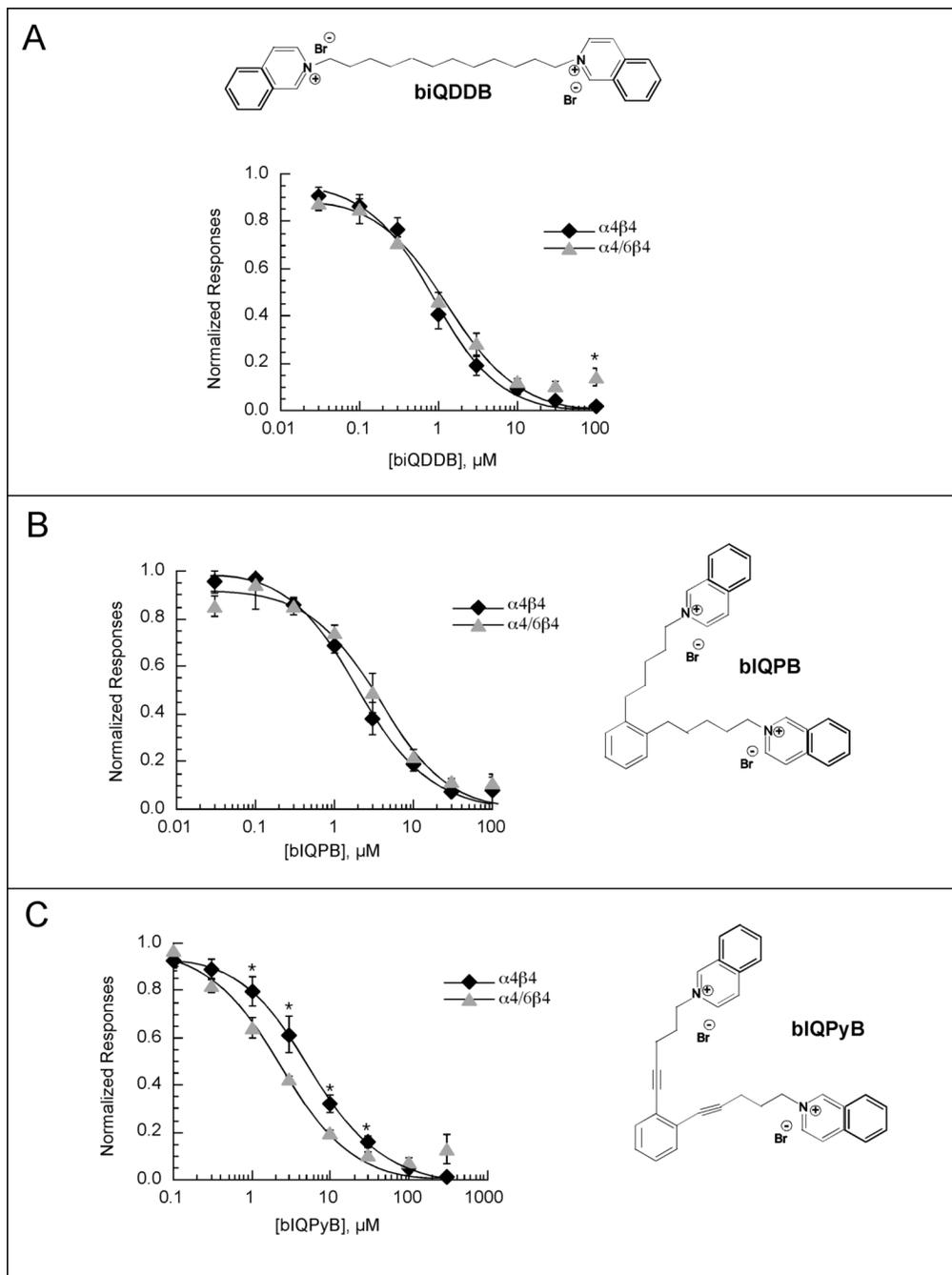
**Figure 7.**

Inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by mecamylamine (upper panel) or tetracaine (lower panel). Each panel shows the averaged normalized responses ( $\pm$  SEM,  $n \geq 4$ ) from oocytes expressing those subunits to the co-application of ACh and antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Significant differences in inhibition were found at the concentrations indicated (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).

**Figure 8.**

**A)** The structure of bPiDDB (*N,N'*-dodecane-1,12-diyl-*bis*-3-picolinium dibromide), a novel *bis*-azaaromatic quaternary ammonium compound which functions as a partial antagonist of nicotine-evoked dopamine release (Crooks et al., 2004), is shown at the top of the figure. The upper plot is a competition experiment, comparing responses evoked by ACh or ACh plus 10  $\mu$ M bPiDDB. The lower plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4 \beta 4$  or  $\alpha 4 / 6 \beta 4$  nAChR subunits by bPiDDB. **B)** The structure of bPiPyB (1,2-*bis*-[5-(3-picolinium)-pent-1-ynyl]-benzene dibromide) is shown at the top of the panel. The plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4 \beta 4$  or  $\alpha 4 / 6 \beta 4$  nAChR subunits by bPiPyB. Each panel shows the

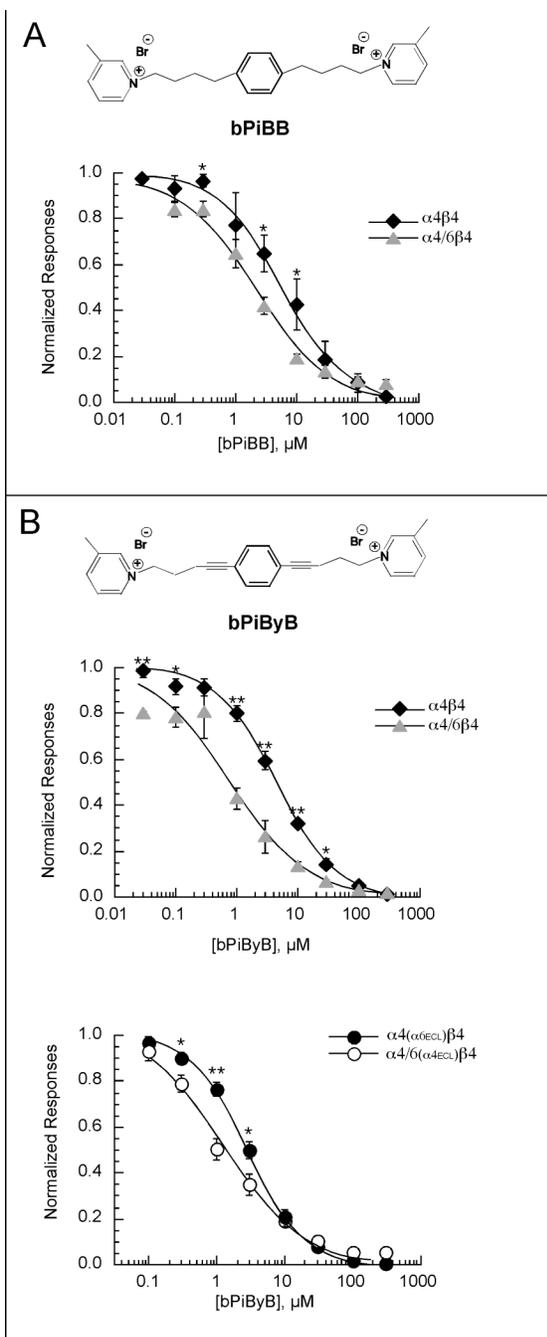
averaged normalized responses ( $\pm$  SEM,  $n \geq 4$ ) from oocytes expressing those subunits to the co-application of ACh and antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Significant differences in inhibition were found at the concentrations indicated (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).



**Figure 9.**

**A)** The structure of biQDDB (*N,N'*-dodecane-1,12-diyl-*bis*-3-isoquinolinium dibromide) is shown at the top of the panel. The plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by biQDDB. **B)** The structure of biQPB (1,2-*bis*-(5-isoquinolinium-pentyl)-benzene dibromide) is shown at the right of the panel. The plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by biQPB. **C)** The structure of biQPyB (1,2-*bis*-(5-isoquinolinium-pent-1-ynyl)-benzene dibromide) is shown at the right of the panel. The plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by biQPyB. The data in each

panel show the averaged normalized responses ( $\pm$  SEM, except where indicated,  $n \geq 4$ ) from oocytes expressing those subunits to the co-application of ACh and antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Significant differences in inhibition were found at the concentrations indicated (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).



**Figure 10.**

**A)** The structure of bPiBB (1,4-bis-[4-(3-picolinium)-butyl]-benzene dibromide) is shown at the top of the panel. The plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by bPiBB. **B)** The structure of bPiByB (1,4-bis-[4-(3-picolinium)-but-1-ynyl]-benzene dibromide) is shown at the top of the panel. The upper plot illustrates the inhibition of peak current responses obtained from oocytes expressing rat neuronal  $\alpha 4\beta 4$  or  $\alpha 4/6\beta 4$  nAChR subunits by bPiByB ( $n = 4$  for the  $\alpha 4\beta 4$  data and  $n = 3$  for the  $\alpha 4/6\beta 4$  data). The lower plot shows the inhibition produced by bPiByB of  $\alpha 4(\alpha 6\text{ECL})\beta 4$  or  $\alpha 4/6(\alpha 4\text{ECL})\beta 4$ , double mutants which contain in  $\alpha 4$  the two residues from  $\alpha 6$  which differ in the extracellular loop between TM2 and TM3 (see Table 3) and in the

$\alpha 4/6$  chimera, the two extracellular loop residues from  $\alpha 4$ , respectively. The data in each panel show the averaged normalized responses ( $\pm$  SEM) from oocytes expressing those subunits to the co-application of ACh and antagonist. Each response to the co-application of ACh and antagonist was normalized based on the response of the same cell to the control application of ACh alone (see Methods for control ACh concentrations used). Significant differences in inhibition were found at the concentrations indicated (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ ).

**Table 1**Table 1A: IC<sub>50</sub> values for DHβE and mecamylamine against various nAChR subunit combinations

subunits	DHβE	IC <sub>50</sub>	mecamylamine
α7	8 ± 1 μM		15.6 ± 1.4 μM
α4β2	0.10 ± 0.01 μM		3.6 ± 0.2 μM
α3β4	26 ± 2 μM		0.39 ± 0.10 μM
α3β2	2.0 ± 0.3 μM		7.6 ± 1.4 μM
α3β2β3	2.9 ± 0.3 μM		1.0 ± 0.1 μM
α6/3β2β3	1.1 ± 0.1 μM		11 ± 3 μM
α6/4β4	4.6 ± 0.5 μM		0.50 ± 0.40 μM
α4β4	0.19 ± 0.01 μM		0.33 ± 0.04 μM
α4/6β4	0.38 ± 0.03 μM		1.0 ± 0.1 μM

**Table 1B: IC<sub>50</sub> values for DHβE, mecamylamine, tetracaine and novel synthetic quaternary ammonium analogs against α4β4 and α4/6β4 subunit combinations and the ECL mutants.**

Drug	α4β4	α4/6β4	Range of IC <sub>50</sub> ratio*
tetracaine	5 ± 1 μM	2.3 ± 0.2 μM	1.60 – 2.90
bPiDDb	0.7 ± 0.1 μM	2.7 ± 0.5 μM	0.19 – 0.36
bPiPyB	14.5 ± 2.8 μM	5.2 ± 0.6 μM	2.02 – 3.76
bIQDDb	0.8 ± 0.1 μM	1.3 ± 0.3 μM	0.44 – 0.90
bIQPB	2.1 ± 0.3 μM	3.7 ± 0.7 μM	0.41 – 0.80
bIQPyB	5.4 ± 0.2 μM	2.3 ± 0.6 μM	1.79 – 3.29
bPiBB	6.0 ± 0.6 μM	2.0 ± 0.3 μM	2.35 – 3.88
bPiByB	4.4 ± 0.3 μM	0.8 ± 0.2 μM	4.10 – 7.83
	α4 <sub>(α6ECL)</sub> β4	α4/6 <sub>(α4ECL)</sub> β4	
bPiByB	3.0 ± 0.1 μM	1.4 ± 0.2 μM	1.81 – 2.58

\* Given the estimated IC<sub>50</sub> and their associated error estimates, this is the range for the likely ratios of IC<sub>50</sub> values. For example for DHβE, (0.19 – 0.01)/(0.38 ± 0.03) = 0.44 represents one bracket for the ratio range while (0.19 + 0.01)/(0.38 – 0.03) = 0.57 represents the other.

**Table 2**

TM2 domains

$\alpha$ 3	VTLCISVLLSLTVFLLVITE
$\alpha$ 4	VTLCISVLLSLTVFLLITE
$\alpha$ 6	VTLCISVLLSLTVFLLVITE
$\alpha$ 7	ISLGITVLLSLTVFMLLVAE
$\beta$ 2	MTLCISVLLALTVFLLISK
$\beta$ 4	MTLCISVLLALTFVLLISK
$\beta$ 3	LSLSTSVLVSLTVFLLVIEE

**Table 3**

Alpha4 and alpha6 Sequences

Signal sequence and extracellular domain	
Rat_alpha4	MANSGTGAPPPLLLLPLLLLGTGLLPASSHIETRAHAEERLLKRLFSGYNKWSRPVA 58
Rat_alpha6	MLNGWGRGDLRSGLCLWICGFLAFFKGSR-----GCVSEEQLFHTLFAHYNRFIRPVE 53
Rat_alpha4	NISDVVLRVFGLSIAQLIDVDEKNQMMTTNVVWKQEWHDYKLRWDPGDYENVTSIRIPSE 118
Rat_alpha6	NVSDPVTVHFELAITQLANVDEVNQIMETNLWLRHVWKDYRLCWDPTTEYDGIETLRVPAD 113
Rat_alpha4	LIWRPDIVLYNNADGDFAVTHLTKAHLFYDGRVQWTPPAIYKSSCSIDVTFPPFDQQNCT 178
Rat_alpha6	NIWKPDIVLYNNAVGDFQVEGKTKALLKYDGVITWTPPAIFKSSCPMDITFFPFDPHQNCS 173
Rat_alpha4	MKFGSWTYDKAKIDLVSMSRVDQLDFWESGEWVIVDAVGTYNTRKYE 226
Rat_alpha6	LKFGSWTYDKAEIDLLIGSKVDMNDFWENSEWEIVDASGYKHDIKYN 221
Rat_alpha4	CCA EIYPDITYAFI
Rat_alpha6	CCEIYTDITYSFY
Transmembrane* and intracellular domains	
Rat_alpha4	<b>IRRLPLFY</b> TINLI <b>IPCL</b> LISCLTVLVFYLPSECG-EK <b>VTL</b> CIS 282
Rat_alpha6	<b>IRRLPMFY</b> TINLI <b>PCL</b> FISFLTVLVFYLPSDCG-EK <b>VTL</b> CIS 277
Rat_alpha4	<b>VLLSLTV</b> <u>FLL</u> LITEIPSTSLVIPLI <b>GEYLL</b> <b>FTMIFV</b> TL <b>SIVITV</b> FVLNVHHRSPRTHT 341
Rat_alpha6	<b>VLLSLTV</b> <u>FLL</u> VITETIPSTSLVIPLI <b>GEYLL</b> <b>FTMIFV</b> TL <b>SIVVTV</b> FVLNIHYRTPATHT 336
Rat_alpha4	MPAWVRRVFLDIVPRLLFMKRPSVVKDNCRRLIESMHKMANAPRFWPEPVGEPGILSDIC 401
Rat_alpha6	MPKWVKTMFLQVFP SILMMRRPLD-----KT <b>KEMD</b> G <b>VKDPK</b> ----- 372
Rat_alpha4	NQGLSPAPTEFCNPTDTAVETQPTCRSPPLEVPDLK <b>TSEVEK</b> ASPCSPGSCPPPKSSSGA 461
Rat_alpha6	-----THTK <b>RP</b> AK <b>VK</b> FTHR <b>KEPKLL</b> KECRHCHKS----SEIAP--GK <b>RL</b> SQ <b>QA</b> Q <b>W</b> VTE 420
Rat_alpha4	PMLIKARSLSVQHVPSSQEAAEDGIRCRSRSIQYCVSQDGAASLADSKPTSSPTSLKARP 521
Rat_alpha6	-----
Rat_alpha4	SQLPVSDQAS <b>PCKCTCKE</b> SPVSPVTVLKAGG <b>TKAPPQHLPLSPAL</b> TRAVEG 573
Rat_alpha6	----- <b>NSEHPPDVEDVIDS</b> 434
Rat_alpha4	VQYIADHLKAEDTDFSVKEDWKYVAMVIDR <b>IFLW</b> <b>MFHIV</b> <b>CLL</b> GT <b>VG</b> L <b>FL</b> PP 624
Rat_alpha6	VQFIAENMKSHNETKEVEDDWKYMAMVVDR <b>VFLW</b> <b>VFHIV</b> <b>CV</b> FG <b>TV</b> G <b>L</b> FLQP 485
Rat_alpha4	WLAAC--- 629
Rat_alpha6	LLGNTGAS 493

\* Putative transmembrane domains are in bold. The two residues of sequence difference between  $\alpha$ 4 and  $\alpha$ 6 in the extracellular loop (ECL) between TM2 and TM3 are underlined. These residues were reversed in the ECL mutants (Figure 10).