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Effects at a distance in α7 nAChR selective agonists: benzylidene substitutions that regulate potency and efficacy

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Abstract

Anabaseine is a marine worm toxin that is a relatively non-selective nicotinic agonist, activating both muscle-type and neuronal nicotinic acetylcholine receptors (nAChR) with varying efficacy. While anabaseine has significant activity with muscle-type and neuronal α3β4 and α4β2 receptors, benzylidene anabaseine (BA) derivatives have high selectivity for the α7 receptor subtype. Two BA compounds with substituents at the 2 and 4 positions of the benzylidene ring, GTS-21 and 4OH-GTS-21, may have therapeutic potential for treating neuropathological disorders such as Alzheimer's disease due to their α7 selectivity. In this study, we specifically investigated the influence of the benzylidene attachment to anabaseine on α7 nicotinic receptor selectivity, as well as the effects of specific substituents at the 4- position of the benzylidene moiety. We demonstrate that benzylidene-attachment alone is sufficient to confer α7 selectivity to anabaseine. Increased potency and receptor binding affinity was obtained with a 4-hydroxyl substitution. Two other 4-substituted benzylidene anabaseines, 3-(4′-methylthiobenzylidene)anabaseine (4-MeS-BA) and 3-(4-trifluoromethylbenzylidene) anabaseine (4-CF_3-BA), offered very little agonist activity for any nicotinic receptors and instead were antagonists for both α7 and neuronal α3β4 and α4β2 receptors. Since the relative amounts of agonist and antagonist activities for specific BA compounds vary with the specific drug/receptor combinations, benzylidene anabaseines provide valuable tools for nAChR drug-receptor structure–function relationships.

Keywords: Xenopus oocytes; Voltage clamp; Alzheimer's disease; Partial agonists; Net charge analysis

1. Introduction

Members of the family of mammalian nicotinic acetylcholine receptor (nAChR) subunit genes (Le Novere et al., 2002) fall into three functional subfamilies: 1) those which code for subunits of the muscle-type receptor (α1, β1, γ1, δ, and ϵ), 2) those which code for subunits of the beta subunit-containing nAChR in nerve cells and bind nicotine with high affinity (α2, α3, α4, α5, α6, β2, β3, and β4), and 3) those that code for neuronal receptors that do not require beta subunits and are blocked by α-bungarotoxin (α-Btx) (α7, α9, and α10). Of all the α-Btx- sensitive receptors, the most abundant and well-studied is the α7 subtype. The α7-type nAChR is expressed throughout the brain and peripheral nervous system and, interestingly, is also found in nonexcitable cells such as vascular endothelium (Sharma and Vijayaraghavan, 2002) and macrophages (Wang et al., 2003).

Abbreviations: BA, the class of benzylidene anabaseine compounds; 3-BA, 3-benzylidene anabaseine; GTS-21, 3-(2,4-dimethoxybenzylidene)anabaseine; 4OH-GTS-21, 3-(2-methoxy,4-hydroxybenzylidene)anabaseine; 4OH-BA, 3-(4-hydroxybenzylidene) anabaseine; 4-MeS-BA, 3-(4-methylthiobenzylidene)anabaseine; 4-CF3-BA, 3-(4-trifluoromethylbenzylidene)anabaseine

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The α7-type nAChR has high calcium permeability, but under conditions of strong stimulation, current through these receptors is limited by both inward rectification and a unique form of concentration-dependent fast desensitization (Uteshev et al., 2002). It has been proposed that these features, particularly concentration-dependent desensitization, make this receptor ill-suited for synaptic transmission but responsive to low level signals, with potential impact on calcium homeostasis. Documented effects of prolonged low-level α7 receptor activation include synaptic facilitation in the ventral tegmental area and hippocampus (Radcliffe and Dani, 1998; Ji and Dani, 2000; Ji et al., 2001; Zhou et al., 2001; Mansvelder et al., 2002) and cytoprotection in various models of cytotoxic stress (Martin et al., 1994; Meyer et al., 1998b, c; Shimohama et al., 1998; Li et al., 2000). Choline is an efficacious, though low potency, agonist for α7 receptors and is likely to be an important modulator of these receptors in vivo (Uteshev et al., 2003).

The study of α7-type nAChR has been aided by the identification of the antagonists, methyllycaconitine (MLA) and α-Btx. While muscle-type nAChR are blocked at least as well as α7 receptors by α-Btx, α-Btx does discriminate between α7 receptors and the predominant neuronal types of beta subunit-containing neuronal nAChR, such as the α4β2 subtype. While the selectivity of low nanomolar concentrations of MLA for α7 receptors is an important tool, other nAChR subtypes are affected at MLA concentrations $\geq$ 100 nM (Lopez et al., 1998).

Another important advance in the study of α7 receptors has been the identification of α7-selective agonists, including the endogenous factor, choline, and experimental agents such as GTS-21 and AR-R-17779 (Papke et al., 1996; Meyer et al., 1998a; Mullen et al., 2000). GTS-21 (3-(2,4-dimethoxybenzylidene)anabaseine or DMXBA) is derived from the marine worm toxin, anabaseine, which is a relatively nonselective mixed agonist/antagonist for multiple types of nAChR (Kem et al., 1997). It is unknown to what extent the α7 selectivity of GTS-21 derives from the addition of the benzylidene moiety versus other substituents at the 2 or 4 position. In this study, we investigated this question with combinations of receptor binding and functioning assays with various nicotinic receptor subtypes, particularly α7 receptors and the beta subunit containing α3β4 and α4β2 receptor subtypes. Realistically, there are too many possible subunit combinations that are beta subunit containing to be tested in a comprehensive fashion. However, the receptors which contain α3 and β4 have very distinct pharmacological and physiological properties from those which contain α4 and β2 and these are two pairwise combinations of great physiological significance (in the PNS and CNS, respectively). Properties in common to these two subtypes (i.e. failure to be activated by benzylidene anabaseines) are likely to be common to all beta subunit-containing receptors, and therefore we can use these specific combinations as putative models for the broader class of beta subunit-containing receptors. Our results indicate that while the benzylidene derivation of anabaseine is sufficient to produce selective agonist activity for the α7 receptor subtype, 4-substitutions on the benzylidene ring modulate both the agonist and antagonist properties.

2. Methods

2.1. Preparation of RNA

Rat neuronal nAChR clones and mouse muscle nAChR cDNA clones were used. The clones were 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).

2.2. Animals

All animals used for these experiments were housed in approved University of Florida animal facilities and all experimental procedures were reviewed and approved by the University's Institutional Animal Care and Use Committee.

2.3. Expression in Xenopus oocytes

Mature (>9 cm) female *Xenopus laevis* frogs (Nasco, Ft. Atkinson, WI) were used as a 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) for 30 min. Oocytes were removed from an incision made in the abdomen.

In order to remove the follicular cell layer, harvested oocytes were treated with 1.25 mg/ml collagenase from Worthington Biochemical Corporation (Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 10 mM HEPES pH 7.6, 0.33 mM MgS04, 0.1 mg/ml gentamicin sulfate). Subsequently, stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) each of the appropriate subunit cRNAs. Recordings were made 5–15 days after injection.

2.4. Chemicals

Anabaseine was provided by Dr. Bill Kem (University of Florida). 3-benzylidene anabaseine (3-BA), 3-(4-hydroxybenzylidene)anabaseine (40H-BA), 3-(4-methylthiobenzylidene)anabaseine (4-MeS-BA), and 3-(4-trifluoromethylbenzylidene)anabaseine (4-CF_3-BA)

were synthesized in the laboratory of Dr. John Zoltewicz (University of Florida). All other chemicals for electrophysiology were obtained from Sigma Chemical Co. (St. Louis, MO). Fresh acetylcholine stock solutions were made daily in Ringer's solution and diluted.

2.5. Electrophysiology

The majority of experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City, CA). 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. The bath volumes were 200 μl. Flow rates were set at 2 ml/min for experiments with α7 receptors and 4 ml/min for other subtypes. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Agonist applications were 12 s in duration followed by 181-s washout periods with α7 receptors and 8 s with 241-s wash periods for other subtypes. For some experiments, particularly under conditions where residual inhibition precluded making repeated measurements from single cells (see below), manual oocyte recordings were made as previously described (Papke and Papke, 2002). In brief, Warner Instruments (Hamden, CT) OC-725C oocyte amplifiers were used, and data were acquired with a Minidigi or a Digidata 1200A with pClamp9 software (Axon Instruments). Sampling rates were between 10 and 20 Hz and the data were filtered at 6 Hz. Cells were voltage clamped at a holding potential of −50 mV. Data obtained with these methods were comparable to those obtained with OpusXpress.

2.6. 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 for α1β1γδ, α3β4, α4β2, and α7 receptors were 30, 100, 10, and 300 μM, respectively. In other experiments (Papke et al., 2000; Papke and Papke, 2002) these concentrations were determined to be the EC_{74}, EC_{15}, EC_{22}, and EC_{100}, respectively. These concentrations were chosen since they gave the largest responses, for the respective subtypes, without producing significant rundown, desensitization, or residual inhibition.

Responses to experimental drug applications 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. For receptors other than α7, responses were measured as peak current amplitude. Drug responses were initially normalized to the internal ACh control response values and then adjusted to reflect the experimental drug responses relative to the ACh maximums. For example, if an α4β2 response to a test compound (or co-application) was 50% of the size of the 10 μM ACh control response that immediately preceded, the response relative to ACh maximum was calculated as 0.50 times 0.22 or 0.11, since the internal ACh control represented only 22% of the ACh maximum possible.

Responses for α7 receptors were calculated as net charge (Papke and Papke, 2002). In brief, for net charge measurement, a 90-s segment of data beginning 2 s prior to drug application was analyzed for each response. Data were first adjusted to account for any baseline offset by subtracting the average value of a 5-s period of baseline prior to drug application from all succeeding data points. Baseline reference was also corrected for possible drift using Clampfit 9.0 (Axon Instruments, Union City, CA). Net charge was then calculated by taking the sum of all the adjusted points. The normalized net charge values were calculated by dividing the net charge value of the experimental response by the net charge value calculated for the preceding 300 μM ACh control response. For nAChR subtypes other than α7, responses were calculated from the peak current amplitudes. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. The application of some experimental drugs caused the subsequent ACh control responses to be reduced, suggesting some form of residual inhibition (or prolonged desensitization). In order to measure the residual inhibitory effects, this subsequent control response was compared to the pre-application control ACh response. Whenever an ACh control that followed a drug application was $< 75\%$ of the previous ACh control response, the cell was not used for further testing.

For concentration–response relations, data derived from net charge analyses were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation

$$\text{Response} = \frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n + (\text{EC}_{50})^n}$$

where $I_{\max}$ denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. $I_{\max}$, n, and the EC_{50} were all

unconstrained for the fitting procedures. Negative Hill slopes were applied for the calculation of IC_{50} values.

Log *P* and molecular volumes were estimated within Chem3D Ultra (CambridgeSoft, Cambridge, MA).

2.7. Binding studies

Whole brains minus cerebellum were rapidly dissected from euthanized 4–5 month-old male Sprague Dawley albino rats and assayed for high affinity [^{125}I]-labeled α-Btx, [^{3}H]cytisine (de Fiebre et al., 1995), and [^{3}H]epibatidine binding (Marks et al., 1998) as described previously. Tissues were homogenized in 20 volumes of ice cold Krebs Ringer buffer (KRB; 118 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 20 mM HEPES; pH 7.5) with a Polytron (setting 4 for 15 s). After two 1-ml washes with KRB at 20,000 g, the membranes were incubated in 0.5 ml KRB with specified concentrations of each experimental compound and one of the following: (a) 1.2 nM [^{125}I]-labeled α-Btx for 120 min at $4^{o} \pm 5$ mM (−)nicotine; (b) 200 fM [^{3}H]epibatidine at room temperature for 60 min ± 100 nM, 10 M, or 5 mM (−)nicotine; or (c) 6 nM [^{3}H]cytisine for 2 h at $4^{\circ} \pm 5$ mM (−)nicotine. Tissues were washed three times with 5 ml cold KRB by filtration through Whatman GF/C filters that had been preincubated for 30 min with 0.5% polyethylenimine. They were assayed for radioactivity using liquid scintillation counting. For α-Btx and cytisine binding, nicotine-displaceable binding was calculated for each concentration of compound in triplicate in three separate experiments, from which IC_{50} values were determined using the Prism program (GraphPad Software, San Diego, CA). For epibatidine binding, a more complicated calculation was made involving three different concentrations of (−)nicotine, based on the observation that epibatidine binds to multiple receptor subtypes with apparently differential sensitivity to nicotine-displacement. It was reported (Marks et al., 1998) that the α3-containing subtype may be relatively insensitive to nicotine displacement compared to the α4-containing subtype, with epibatidine displacement occurring predominantly between 100 nM and 10 μM (−)nicotine in the presence of near Kd epibatidine concentrations. We therefore estimated the α3 component of epibatidine binding by subtracting binding in the presence of 10 μM (−)nicotine from that seen with 100 nM (−)nicotine (after first subtracting from all treatments the binding seen with 5 mM (−)nicotine, to eliminate non-specific binding). IC_{50} values for displacement of epibatidine over this nicotine concentration range were determined for each compound in three separate experiments, each conducted in triplicate.

3. Results

3.1. General profiles of agonist and antagonist activity for anabaseine and benzylidene anabaseine derivatives

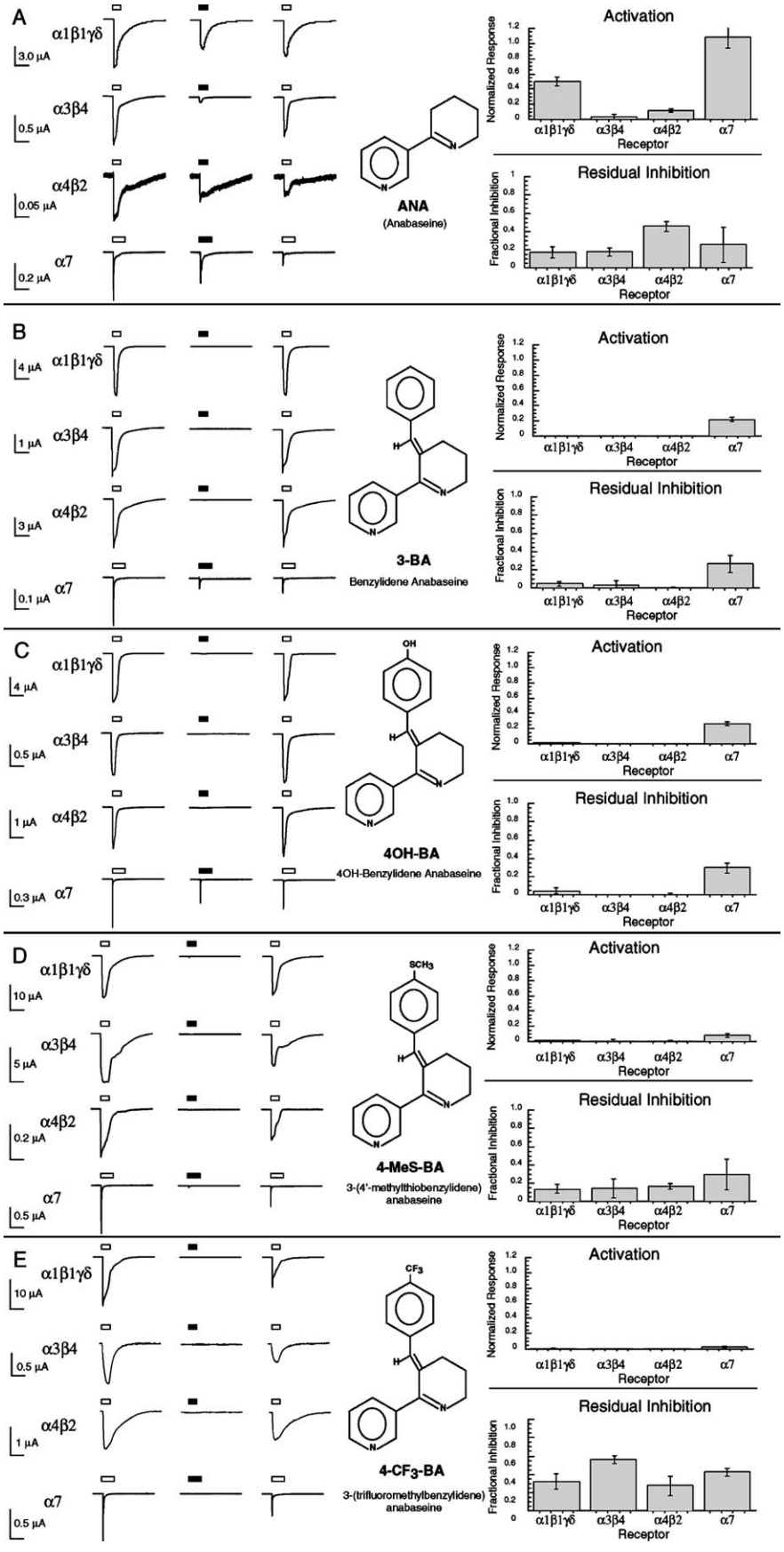
At 100 μM, anabaseine evoked large responses from muscle-type receptors (α1β1γδ) and α7 receptors, and readily detectable responses from α3β4 and α4β2 receptors, which are frequently used to model the high affinity beta subunit-containing receptors of the peripheral and central nervous systems, respectively (Fig. 1A). We have previously reported that anabaseine produces maximal responses of α4β2 receptors that are approximately 8% the ACh maximum responses with an EC_{50} of 4.2 ± 1.3 μM (Kem et al., 1997). For α3β4, α1β1γδ, and α7 receptors maximum responses to anabaseine were approximately 35, 60, and 100% of the relative ACh maxima, with EC_{50} values of 140, 30, and 20 μM, respectively (data not shown).

After the administration of anabaseine alone, subsequent control ACh responses were somewhat depressed for all the subtypes tested, suggesting that this agent produced residual inhibition or desensitization. This effect was most pronounced for α4β2 receptors.

Addition of the benzylidene moiety to anabaseine resulted in a compound, 3-BA that only activated α7 receptors at a high concentration, 100 μM (Fig. 1B). The application of 3-BA alone also produced less residual inhibition of non-α7 receptors than anabaseine did, but similar residual inhibition of α7.

When applied at a concentration of 100 μM, 3-(4-hydroxybenzylidene)anabaseine (4OH-BA) produced a pattern of activation and residual inhibition that was similar to that of 3-BA (Fig. 1C), with effects essentially restricted to the α7 subtype. In contrast, 3-(4-methylthiobenzylidene)anabaseine (4-MeS-BA)

Fig. 1. The effects of (A) anabaseine (ANA), (B) 3-BA, (C) 40H-BA, (D) 4-MeS-BA, and (E) 4-CF_3-BA on nAChR subtypes. Oocytes expressing either the mouse muscle α1β1γδ subunit or the rat neuronal α3β4, α4β2, or α7 subunits were tested for their responses to control (C1) ACh applications (open bars), and then, following a 5-min wash, the experimental agonists were applied at a concentration of 100 μM (solid bars). Subsequent to the application of the experimental agonists, the cells were washed for an additional 5 min and re-challenged with control ACh applications (C2). Control ACh concentrations for α1β1δε, α3β4, α4β2, and α7 receptors were 30, 100, 30, and 300 μM, respectively. Representative raw data traces are shown on the left. The vertical scale bars are as indicated and for all traces the horizontal scale bars represent 20 s. The upper graph in the right panels (“Activation”) shows the average response to the experiment agonists, normalized to the ACh control and expressed relative to the ACh maximum response determined in previous experiments (see Methods). The lower graph on the right side of the panel (“Residual Inhibition”) shows the average decrease in the control ACh responses after the application of the experimental agonist. Inhibition was calculated as 1 minus the ratio of C2 to C1. Each bar represents the average of at least four oocytes ± SEM.


A
α1β1γδ
3.0 μA
α3β4
0.5 μA
α4β2
0.05 μA
α7
0.2 μA
ANA
(Anabaseine)
Activation
Normalized Response
α1β1γδ α3β4 α4β2 α7
Receptor
Residual Inhibition
Fractional Inhibition
B
4 μA
1 μA
3 μA
0.1 μA
3-BA
Benzylidene Anabaseine
C
4 μA
0.5 μA
1 μA
0.3 μA
OH
4OH-BA
4OH-Benzylidene Anabaseine
D
10 μA
5 μA
0.2 μA
0.5 μA
SCH3
4-MeS-BA
3-(4'-methylthiobenzylidene) anabaseine
E
10 μA
0.5 μA
1 μA
0.5 μA
CF3
4-CF3-BA
3-(trifluoromethylbenzylidene) anabaseine

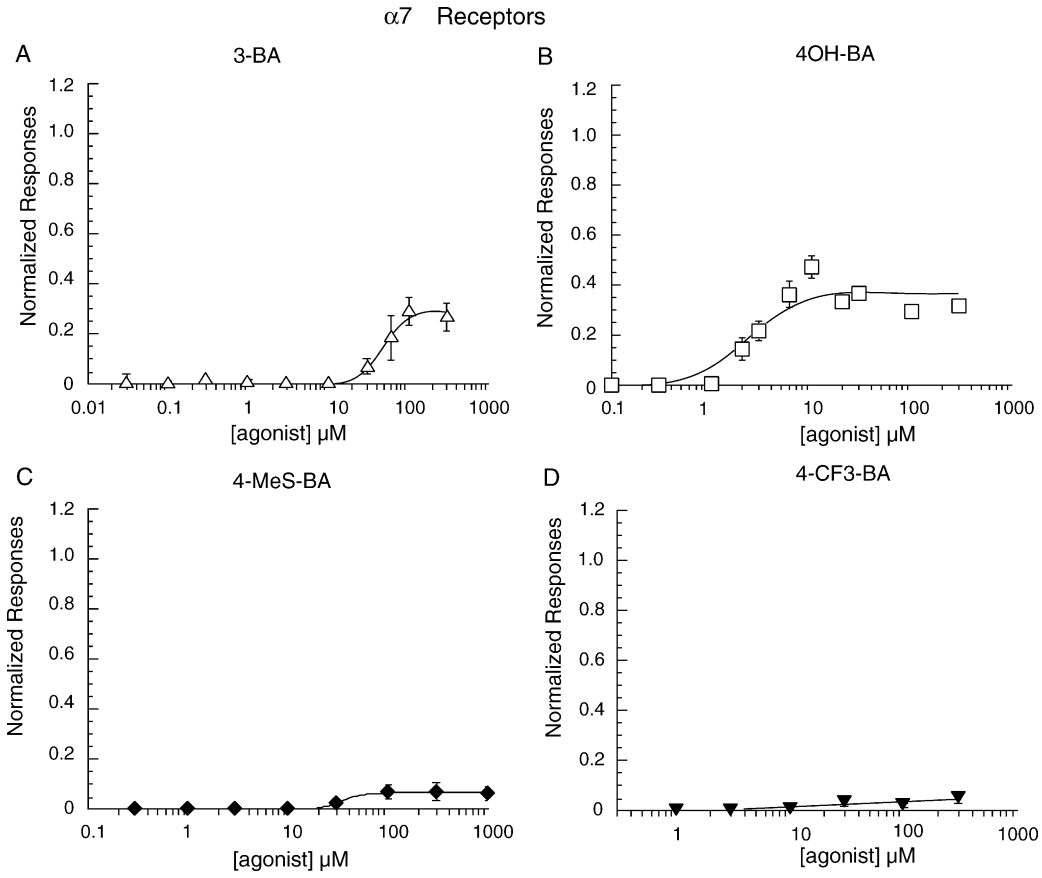


Fig. 2. Concentration–response studies of (A) 3-BA, (B) 40H-BA, (C) 4-MeS-BA, and (D) 4-CF_3-BA for the activation of rat α7 nAChR. Experiment protocols similar to those illustrated in Fig. 1 were used, with application of experimental agonists at varying concentrations, both preceded and followed by control responses to 300 μM ACh. Responses were calculated as the net charge accumulated over a period of 200 s. Each point represents the average of at least four oocytes ± SEM. The Hill slopes for the 3-BA and 4OH-BA curves were 2.0 and 3.1, respectively. The responses for 4-MeS-BA, and 4-CF_3-BA were too small for reliable curve fits.

applied at 100 μM produced relatively little activation of any nAChR subtype tested (Fig. 1D). There was only detectable agonist activity for α7 receptors. However, both muscle-type and α7 receptor subtypes showed residual inhibition. The anabaseine derivative 3-(4-trifluoromethylbenzylidene)anabaseine (4-CF_3-BA) also had little or no agonist activity for any of the receptors at 100 μM but produced significant residual inhibition of all subtypes tested (Fig. 1E).

3.2. *Dose–response studies of α7 receptors with benzylidene anabaseine derivatives*

Fig. 2 shows the responses (measured as net charge) of rat α7 receptors to the anabaseine compounds across a range of compounds. The addition of the hydroxyl group at the 4 position of benzylidene ring had little effect on the amplitude of the maximum net charge responses. The I_{max} values were 29 ± 2 and $36 \pm 3\%$ of the ACh maximum for 3-BA and 4OH-BA, respectively. However, the addition of a hydroxyl group at the 4 position of benzylidene ring lead to a decreased EC_{50} by a factor of at least 10. The EC_{50} values for 3-BA and 4OH-BA, were 45 ± 4 and 2.3 ± 0.5 μM, respectively. Consistent with the data in Fig. 1, both 4-MeS-BA and 4-CF_3-BA produced barely detectable α7 receptor activation.

In contrast, all of the BA derivatives in this study produced residual inhibition of α7 receptors (Fig. 3). With the partial agonists 3-BA and 4OH-BA, the inhibitory effects were mostly observed at concentrations significantly higher than the EC_{50}s for receptor activation (see Table 1). The compounds which showed the least agonist activity, 4-MeS-BA and 4-CF_3-BA, produced residual inhibition in roughly the same concentration range in which they generated small but detectable currents. Note that two of the compounds (3-BA and 4-CF3-BA) did not appear to give complete residual inhibition. This incomplete effect may be the result of applying insufficiently high concentration to achieve full residual inhibition. However, another important factor may be the reversibility of this form of inhibition. By definition, residual inhibition is measured after a 5-min washout period. Without specifically determining recovery rates, it is unclear whether 60% residual inhibition, measured after 5 min of wash,

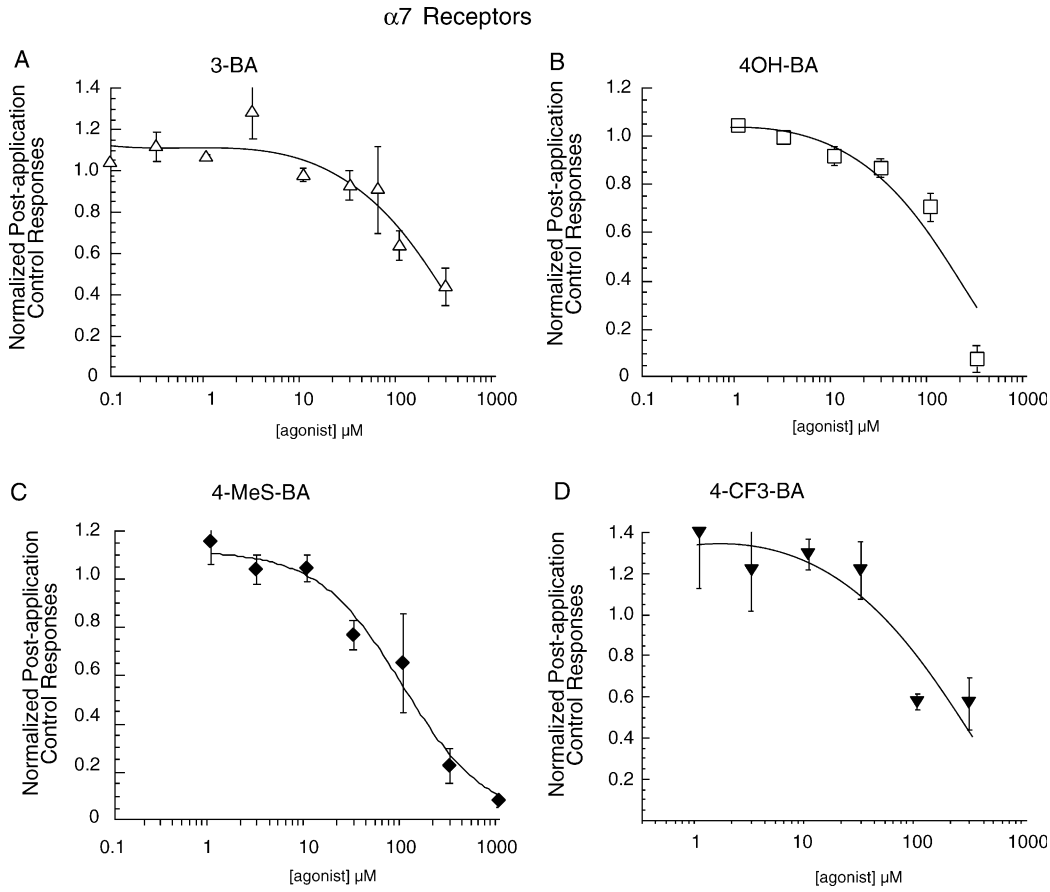


Fig. 3. Concentration–response studies of (A) 3-BA, (B) 40H-BA, (C) 4-MeS-BA, and (D) 4-CF_3-BA for the residual inhibition of rat α7 nAChR. Experiment protocols similar to those illustrated in Fig. 1 were used, with application of experimental agonists at varying concentrations, both preceded and followed by control responses to 300 μM ACh. The plots show the magnitude of the control response which followed the application of the experimental agonist, relative to the control ACh response which preceded the application of the experimental agonist. Each point represents the average of at least four oocytes ± SEM. The Hill slopes for all of the curves shown were fixed at −1, and the IC_{50} values were 175 ± 43, 111 ± 48, 98 ± 20, and 135 ± 50 μM for 3-BA, 4OH-BA, 4-MeS-BA, and 4CF_3-BA, respectively.

represents incomplete inhibition when the drug was applied or partial recovery after the drug has been washed out.

3.3. The inhibition of rat α7 receptors by 4-MeS-BA and 4-CF_3-BA in co-application experiments

The residual inhibition illustrated in Fig. 3 represents effects of the BA derivatives on ACh responses that were produced by application of the drugs and persisted after the drugs were washed from the chamber. In order to determine whether the effects of 4-MeS-BA and 4-CF_3-BA were use-dependent, we examined the effects of these drugs when they were delivered in co-application with the full agonist ACh. As shown in Fig. 4A, in co-application experiments 4-MeS-BA and 4-CF_3-BA produced inhibition of the ACh responses (see Table 2 for IC_{50} values). However, the highest 4-MeS-BA concentration tested (100 μM) was not sufficient to fully inhibit (i.e. out-compete) the response to 300 μM ACh. Interestingly, the residual inhibition following co-application of the drugs and ACh was considerably less than when the drugs were applied alone (Fig. 4B and Table 2). This suggests that the inhibition of α7 receptors by these drugs might be due to competitive effects at the ACh binding site(s). In order to test this, competition curves were generated in which ACh concentrations were increased in co-application experiments with fixed concentrations of 4-MeS-BA or

Table 1
α7 inhibition with and without agonist co-application

IC_{50} values (μM)			
	Co-application response	Residual inhibition	
		Applied alone	After co-application
4-MeS-BA	68 ± 32	98 ± 20	360 ± 270
4-CF_3-BA	17 ± 4.6	135 ± 50	185 ± 91

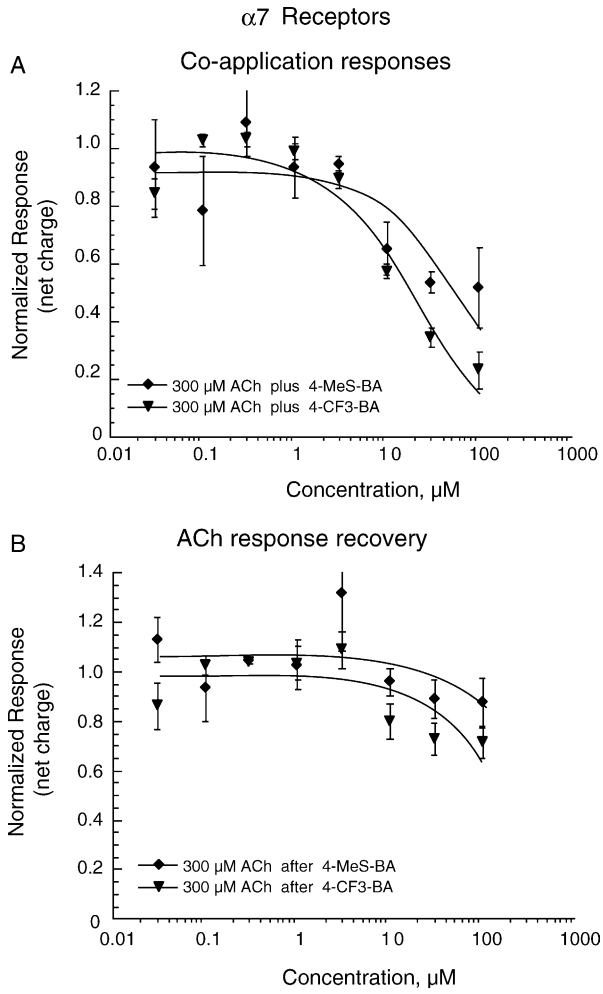


Fig. 4. Concentration–response studies of the inhibition of ACh responses by the co-application of 300 μM ACh with varying concentrations of either 4-MeS-BA, or 4-CF_3-BA on rat α7 nAChR. Co-application of ACh and the experimental agonists at varying concentrations were both preceded and followed by control responses to 300 μM ACh. (A) Co-application responses, calculated as the net charge accumulated over a period of 200 s and normalized to the net charge of the responses to 300 μM ACh alone. (B) Responses to 300 μM ACh alone following the co-application of either 4-MeS-BA or 4-CF_3-BA at the indicated concentrations. Each point represents the average of at least four oocytes ± SEM. The Hill slopes for all of the curves shown were fixed at −1.

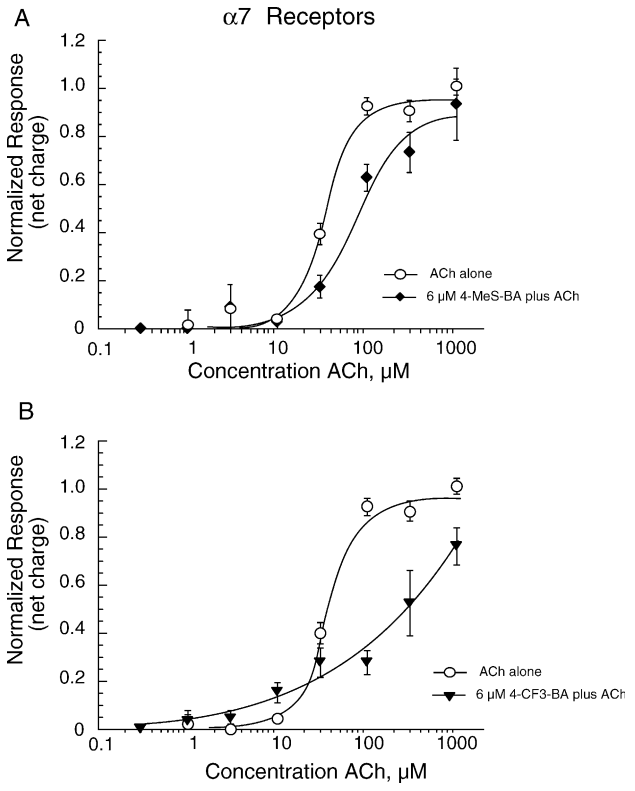


Fig. 5. The effects of (A) 4-MeS-BA, and (B) 4-CF_3-BA on rat α7 nAChR ACh responses. The experimental agonists were co-applied at a fixed concentration of 6 μM with varying concentrations of ACh (filled symbols) These responses are compared to the responses of ACh alone (open circles). Co-application responses were calculated as the net charge accumulated over a period of 200 s and normalized to the net charge of the responses to 300 μM ACh alone. Each point represents the average of at least four oocytes ± SEM. The data for ACh alone were fit with an I_{max} of 0.97 ± 0.02, an EC_{50} of 34± 2 μM, and a Hill slope of 2.6 ± 0.5. For ACh plus 4-MeS-BA the curve fit had an I_{max} of 0.90 ± 0.07, an EC_{50} of 68 ± 15 μM, and a Hill slope of 1.5 ± 0.4. The data for ACh plus 4-CF_3-BA were not well fit with the Hill equation since a realistic I_{max} could not be defined.

4-CF_3-BA. The results, shown in Fig. 5, indicate that the inhibitory effects of these drugs decreased at the highest ACh concentrations. However, note that while inhibition by 4-MeS-BA looks surmountable, inhibition by 4-CF3-BA may not be. The curve for the latter compound is also very shallow and perhaps even biphasic, suggesting that this compound may work at multiple sites to inhibit receptor response.

Table 2
Inhibition of function or radioligand binding

IC_{50} (μM)						
Co-application experiments				Binding experiments		
	α7	α3β4	α4β2	α-Btx	Epi	Cyt
3-BA	175 ± 43	184 ± 65	72 ± 13	3.4 ± 1.0	5.3 ± 0.8	9.7 ± 1.9
4OH-BA	111 ± 48	63 ± 32	19 ± 6	0.9 ± 0.2	2.8 ± 0.9	4.0 ± 1.1
4-MeS-BA	68 ± 32	6.8 ± .8	28 ± 9	2.5 ± 0.7	0.3 ± 0.1	2.9 ± 0.4
4-CF_3-BA	17 ± 4.6	8.0 ± 2.	22 ± 3	5.8 ± 1.4	0.7 ± 0.1	2.1 ± 0.3

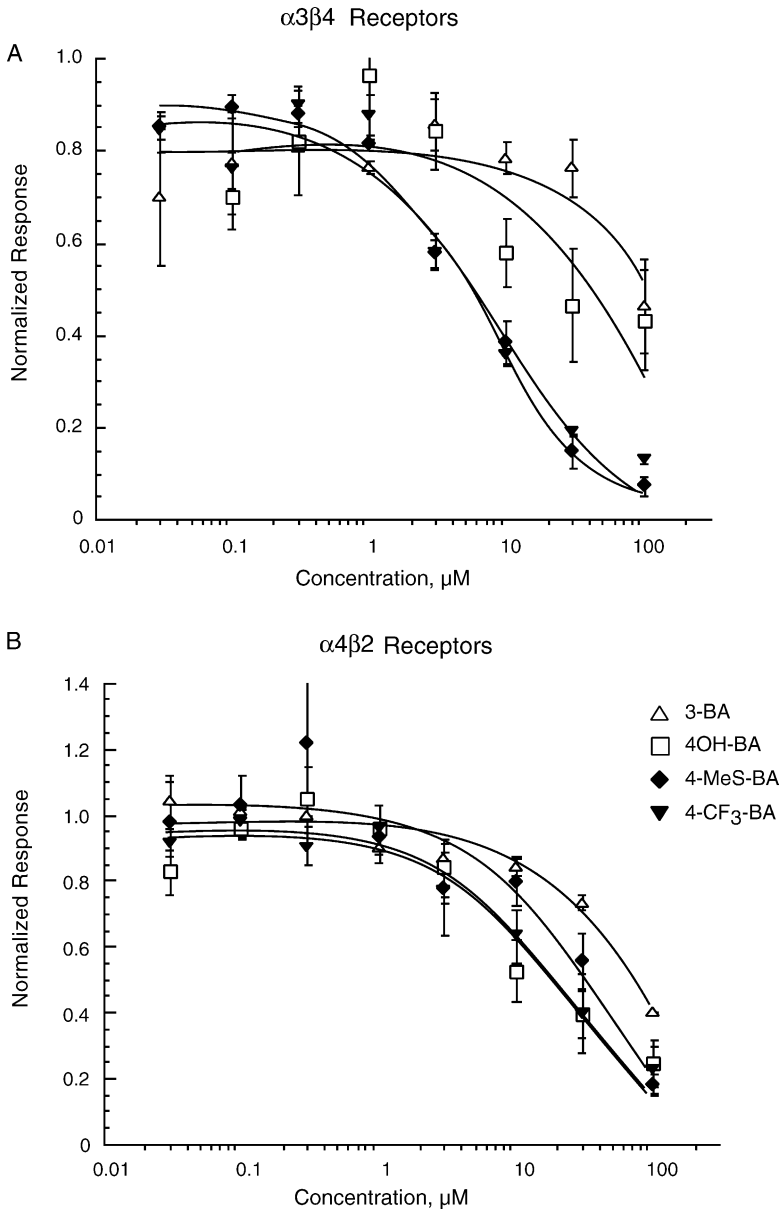


Fig. 6. Concentration–response studies of the inhibition of the ACh responses of α3β4 and α4β2 receptors by the co-application of ACh with varying concentrations of 3-BA, 40H-BA, 4-MeS-BA, and 4-CF_3-BA. (A) The inhibition of α3β4 receptors. (B) The inhibition of α4β2 receptors. Co-application of ACh and the experimental agonists at varying concentrations were both preceded and followed by control responses to ACh. Peak current amplitudes were used as the measure of response, and the data were normalized to ACh control responses obtained prior to the co-application responses. Each point represents the average of at least four oocytes ± SEM. The Hill slopes for all of the curves shown were fixed at −1.

3.4. The inhibition of α3β4 and α4β2 receptors by benzylidene anabaseine derivatives

The BA derivatives were co-applied with ACh at the control concentrations (see Section 2). The responses of oocytes expressing α3β4 receptors were most potently inhibited by 4-MeS-BA or 4-CF_3-BA, and rather less effectively by BA and 40H-BA (Fig. 6A and Table 2). The unsubstituted 3-BA was least potent for inhibiting α4β2 receptors, while 4OH-BA, 4-MeS-BA, and 4-CF_3-BA all inhibited the α4β2 ACh responses with IC_{50} values in the range of 20–30 μM (Fig. 6B and Table 2).

In order to determine whether the inhibition of ACh responses by co-application with the BA compounds was produced through a competitive mechanism, ACh concentration response curves were generated and compared to those recorded in the presence of a fixed concentration of the experimental compounds. As shown in Fig. 7, with α3β4 receptors inhibition was not surmounted by applying ACh at the highest concentrations tested. Since at concentrations of ACh higher than 3 mM open channel block by agonist becomes a limiting factor in ACh dose response studies, we can-

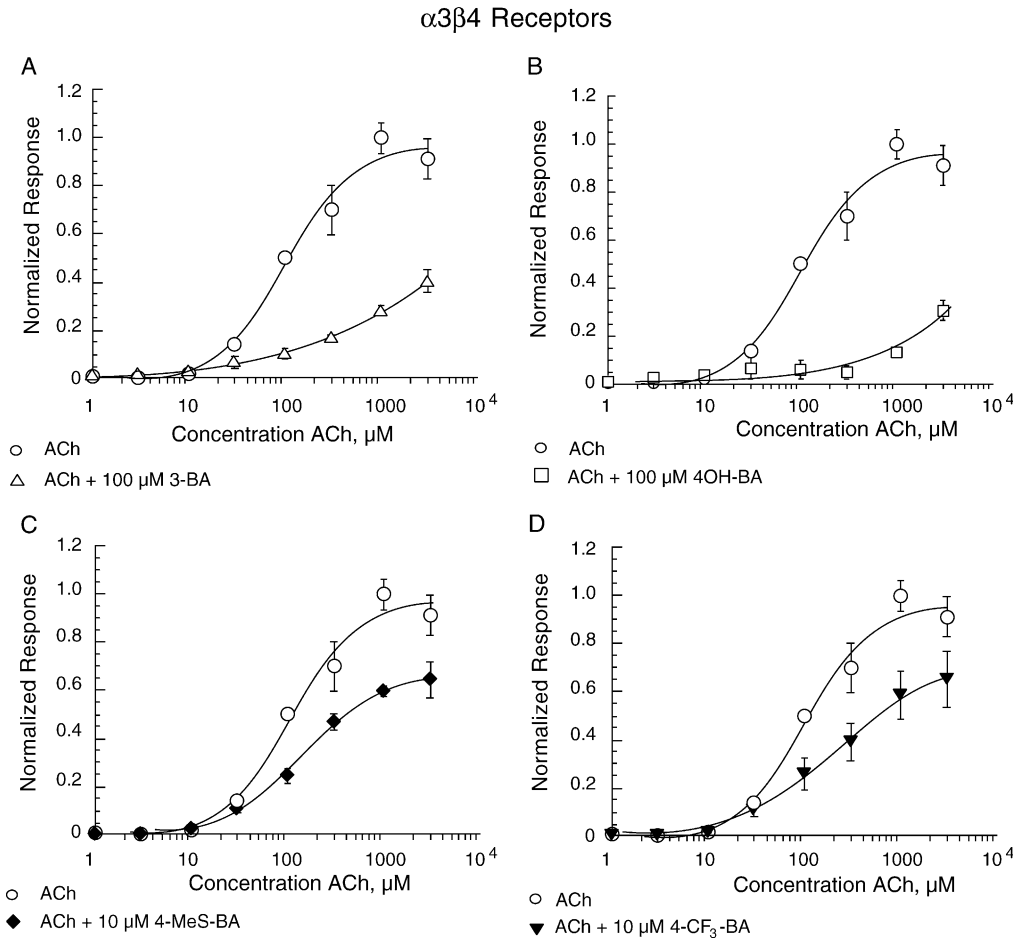


Fig. 7. Competition experiments with α3β4 receptors and benzylidene anabaseine compounds. The effects of (A) 3-BA, (B) 40H-BA, (C) 4-MeS-BA, and (D) 4-CF_3-BA on rat α3β4 nAChR ACh responses. Fixed concentrations of the experimental agonists were co-applied with varying concentrations of ACh. 3-BA and 40H-BA were used at a fixed concentration of 100 μM. 4-MeS-BA and 4-CF_3-BA were used at a fixed concentration of 10 μM. These responses are compared to the responses of ACh alone (open circles). Each point represents the average of at least four oocytes ± SEM. The data for ACh alone were fit with an I_{max} of 0.97 ± 0.05, an EC_{50} of 108 ± 19 μM, and a Hill slope of 1.3 ± 0.25. The data for ACh plus 3-BA or 4OH-BA were not well fit with the Hill equation since realistic I_{max} values could not be defined. For ACh plus 4-MeS-BA the curve fit had an I_{max} of 0.67 ± 0.01, an EC_{50} of 150 ± 10 μM, and a Hill slope of 1.14 ± 0.06. The data for ACh plus 4-CF_3-BA were fit with an I_{max} of 0.71 ± 0.04, an EC_{50} of 211 ± 35 μM, and a Hill slope of 0.90 ± 0.10.

not be sure whether inhibition by BA or 4OH-BA might have been surmountable at much higher ACh concentrations.

As shown in Fig. 8, inhibition of α4β2 receptors by 3-BA was surmounted at the highest ACh concentrations, while inhibition by the substituted BA compounds was not surmountable. The data for ACh co-applied with MeS-BA, and 4-CF_3-BA could be fit to the Hill equation, and those fits suggested approximately a 20% decrease in maximum response at the highest ACh concentrations.

3.5. Radioligand binding

Each BA was evaluated for its displacement of radioligand binding to α7 receptors using [^{125}I]-labeled α-Btx and to other nicotinic receptor subtypes using [^{3}H]cytisine and [^{3}H]epibatidine in rat whole brain membrane preparations (Fig. 9A, Table 2). Each of the BAs displaced 100% of the binding of the labeled ligands at sufficiently high concentrations. Generally, attachment of substituents to the 4 position of the benzylidene moiety increased or had no effect on IC_{50} binding values for all of the ligands. The range of IC_{50} values for nicotine-sensitive displacement of labeled α-Btx was from 0.9–5.8 μM, with 4OH-BA the most potent of these agents. This was consistent with 4OH-BA having the most potent binding-dependent action at α7 receptors among these drugs: an agonist EC_{50} of 3.4 μM. However, the linear regression correlation coefficient for IC_{50} values of displacement of [^{125}I]-labeled α-Btx displacement

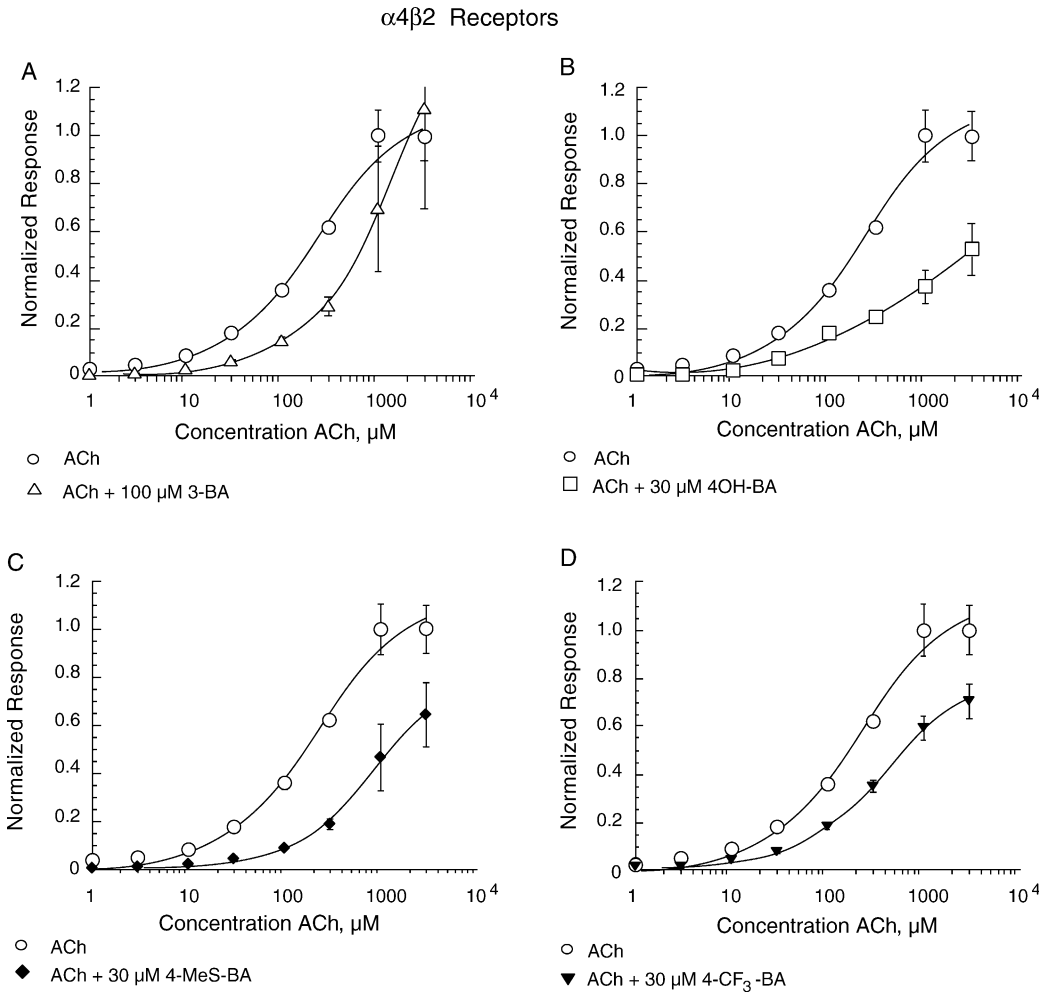


Fig. 8. Competition experiments with α4β2 receptors and benzylidene anabaseine compounds. The effects of (A) 3-BA, (B) 40H-BA, (C) 4-MeS-BA, and (D) 4-CF_3-BA on rat α3β4 nAChR ACh responses. Fixed concentrations of the experimental agonists were co-applied with varying concentrations of ACh. 3-BA was used at a fixed concentration of 100 μM. 40H-BA, 4-MeS-BA, and 4-CF_3-BA were used at a fixed concentration of 30 μM. These responses are compared to the responses of ACh alone (open circles). Each point represents the average of at least four oocytes ± SEM. The data for ACh alone were fit with an I_{max} of 1.14 ± 0.09, an EC_{50} of 212 ± 60 μM, and a Hill slope of 0.93 ± 0.16. The data for ACh plus 3-BA or 4OH-BA were not well fit with the Hill equation since realistic I_{max} values could not be defined. For ACh plus 4-MeS-BA the curve fit had an I_{max} of 0.80 ± 0.07, an EC_{50} of 800 ± 170 μM, and a Hill slope of 1.1 ± 0.1. The data for ACh plus 4-CF_3-BA were fit with an I_{max} of 0.83 ± 0.03, an EC_{50} of 400 ± 49 μM, and a Hill slope of 0.90 ± 0.06.

versus IC_{50} values for inhibition of co-applied agonist-induced α7 receptor activation was not significant (Fig. 9A; $p = 0.50$), perhaps because of the multiple actions observed with some of these compounds: agonist activity, competitive antagonism, and non-competitive antagonism. The IC_{50} values for the nicotine-insensitive component of [^{3}H]epibatidine binding among these compounds correlated significantly with the ability of these compounds to inhibit co-applied agonist to α3β4 receptors (Fig. 9B; $p < 0.05$, $r = 0.98$). Similarly, for labeled cytisine displacement, the IC_{50} range was from 2.1–9.9 μM, and there was a significant correlation between these IC_{50} values and the IC_{50} values for inhibition of co-applied agonist at α4β2 receptors, the predominant receptor type believed to bind to cytisine in brain membranes (Fig. 9B; $p < 0.05$, $r = 0.95$).

4. Discussion

In this paper we show that the addition of the benzylidene group to anabaseine is sufficient to account for the selective activation of α7 receptors that has been reported for GTS-21 and other BAs (de Fiebre et al., 1995; Meyer et al., 1998a). Our data support the hypothesis that, compared to other nAChR subtypes, the α7 homomeric receptor has relatively relaxed requirements for activation via the agonist binding site(s). It is possible that the presence of the benzylidene group creates a steric hindrance for the BA compounds to bind to the agonist binding site of α3β4 and α4β2 receptors, or that if they do bind, the benzylidene group prevents activation. It is interesting to note that not only are the large BAs more efficacious for α7 receptors than for

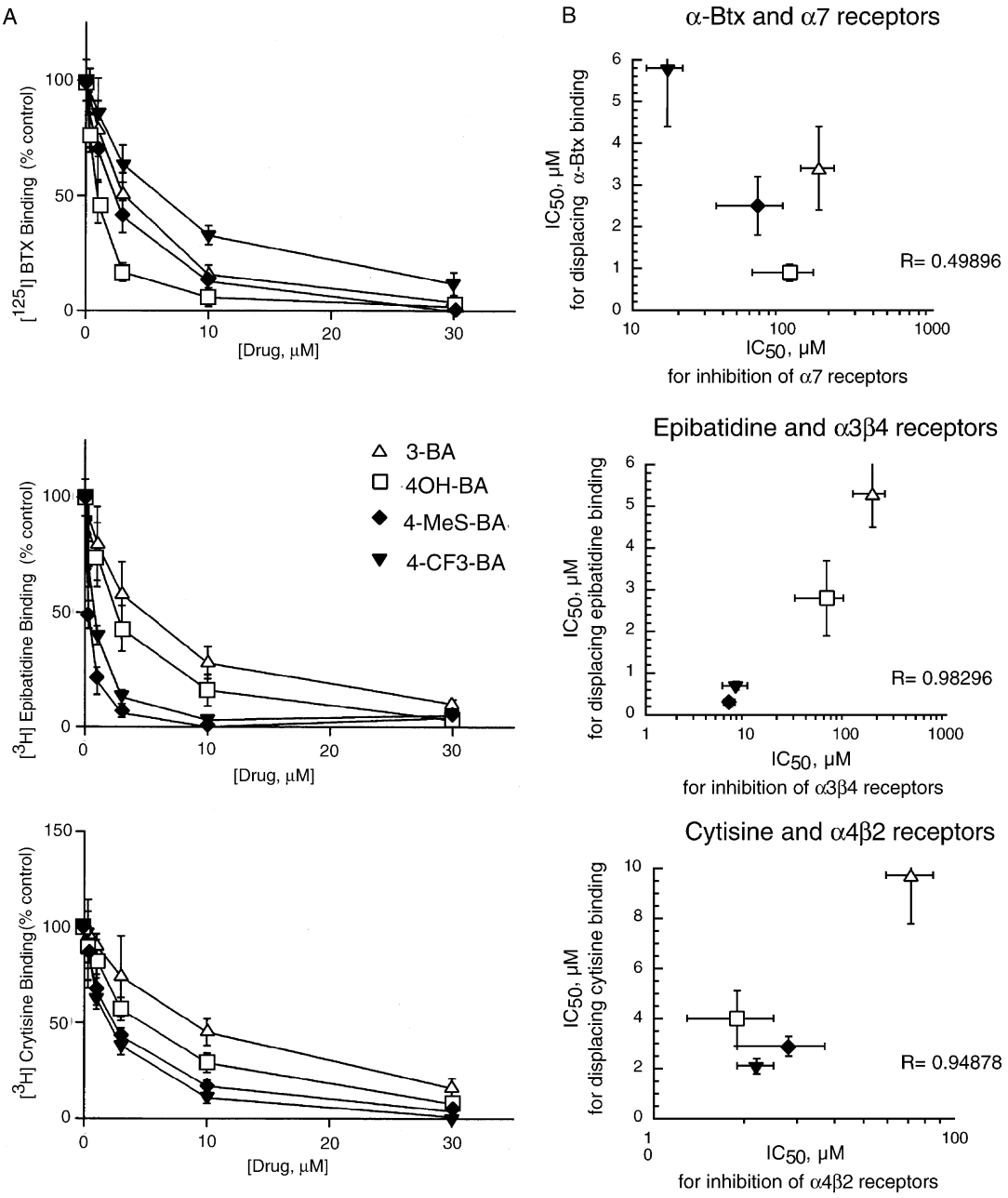


Fig. 9. (A) Displacement binding studies for the four BA compounds. The BA compounds were used at specified concentrations to displace labeled α-Btx, epibatidine, or cytisine binding in the upper, middle or lower panel, respectively, as described in the text. Values are means ± SEM of % control binding in the absence of any BA compound ($N = 3$ experiments, each in triplicate). (B) Scatter plots of the IC_{50} values for binding experiments and physiological evaluations of inhibition. As shown in the upper panel, there is poor correlation between α7 inhibition (in co-application experiments) and α-Btx displacement. However, as shown in the lower two panels, there appear to be strong correlations between α3β4 inhibition and nicotine-insensitive epibatidine binding, and α4β2 inhibition and cytisine binding.

α3β4 and α4β2 receptors, but α7 receptors are also more receptive to the ACh precursor, choline. We have previously shown that all neuronal nAChR subtypes may be activated to a significant degree by tetramethylammonium and ethyltrimethylammonium, suggesting that the hydrogen bond subsite of the classical nicotinic pharmacophore (Beers and Reich, 1970; Papke et al., 1996) is not strictly a requirement for the activation of neuronal nAChR. However, the activation of beta subunit-containing nAChR is largely eliminated with the addition of a hydroxyl group to ethyltrimethylammonium (i.e. in the form of choline) (Papke et al., 1996), suggesting that the hydroxyl group of choline may interact with residues in the agonist binding site of beta subunit-containing receptors (but not α7) in such a way as to stabilize the resting state. This is con-

sistent with the observation that choline can antagonize the ACh-evoked responses of α4β4 receptors (Zwart and Vijverberg, 2000).

While the requirements for the α7 pharmacophore are sufficiently relaxed compared to other nAChR that both choline and the relatively large BAs appear as selective agonists, there nonetheless appear to be specific constraints on what makes any particular BA derivative a strong agonist, a weak agonist, or even an antagonist. In the present study, our data indicate that the addition of the hydroxyl group to the benzylidene ring increases the potency and binding affinity to α7 receptors but not the efficacy. One possible reason why this might be is that the hydroxyl addition simply increases the affinity within the agonist binding pocket. This hypothesis is consistent with the increase in the apparent potency of 4OH-BA compared to 3-BA for the displacement of radiolabeled α-Btx (Table 2). It is also possible that the addition of the para-hydroxyl group on the benzylidene has a sufficient electron donating effect to decrease the pK_a of the imminium nitrogen of the core anabaseine. This would increase the percentage of the compound in the charged form at physiological pH. If, in fact, the charged form of the drug is the most effective at activating the channel, then a decrease in the pK_a could produce a corresponding decrease in the EC_{50}.

Although the residual inhibition of α7 receptors by BA compounds appears to be competitive, since it is less when the BA compounds are co-applied with ACh than when they are applied alone, as shown in Fig. 9, there is poor correlation between the IC_{50} for the inhibition of the ACh responses in co-application experiments and the K_i values for the BA compounds' displacement of α-Btx. This is at least in part due to the fact that 3-BA and 4OH-BA have significant partial agonist activity and will only inhibit ACh responses to the extent that they are less efficacious than ACh, and then, probably only when they occupy nearly all the agonist binding sites. Since 4-MeS-BA and $4CF_3$-BA have little or no agonist activity, they may behave more like classical competitive antagonists. However, other possibilities should be considered. For example, the mechanism of residual inhibition of α7 receptors may be qualitatively different from the inhibition occurring during a co-application with ACh. That is, while residual inhibition may involve interactions at ACh binding sites, inhibition during an ACh-evoked response may be, at least for some of the compounds, use-dependent and noncompetitive. If that is the case, it could also explain the lack of correlation between α-Btx displacement and IC_{50}s calculated from co-application experiments (Fig. 9A). Curiously, although the IC_{50}s for inhibition during co-application vary by as much as a factor of 10, the IC_{50}s for residual inhibition when the drugs are applied alone are not significantly different among the 4 compounds (Fig. 3). The IC_{50}s values for residual inhibition also are not correlated to the α-Btx K_i values, even though residual inhibition of α7 receptors by the BA compounds is lessened by agonist co-application. One hypothesis for why this might be the case is that although these assays are affected by binding to the ACh activation site, they access the receptor in two different states, desensitized and activatable. The rank order potency of the BA compounds for binding to these two states may differ and therefore show no correlation. An alternative hypotheses is that the protection from residual inhibition provided by co-application with ACh may come, not from competition at the ACh binding site, but from ACh's ability to affect the conformational equilibrium of the receptor. The BA compounds might then bind noncompetitively but with lower affinity to the states promoted by ACh activation (i.e. desensitized states) than to the activable state predominating in the absence of ACh.

Although none of the BAs in this study had significant efficacy for α3β4 and α4β2 receptors, they nonetheless interacted with the receptors and inhibited ACh-evoked responses. High affinity cytisine binding to rat whole-brain membranes reflects primarily α4β2 binding (Zhang and Steinbach, 2003), so we expected the significant correlation we observed between IC_{50} values for displacement of this ligand and the IC_{50} values for blocking of agonist-induced responses at this receptor subtype. Similarly, while epibatidine binds with high affinity to multiple nicotinic receptor subtypes, including those containing α6, several reports suggest that a component of this binding is both relatively nicotine-insensitive (displaced by 100 nM – 10 μM nicotine) and likely to represent α3-containing receptors (Marks et al., 1998). This was consistent with the significant linear regression coefficient that was seen between nicotine-insensitive epibatidine binding and the inhibition of α3β4 agonist activity under competitive conditions. Taken together though, the data suggest that the inhibition of the α3β4 and α4β2 receptors may arise from both competitive and noncompetitive effects that vary with the specific drug/receptor combinations. While the competition studies with α3β4 and α4β2 receptors (Figs. 7 and 8, respectively) for the most part (with the exception of 3-BA's effects on α4β2 receptors) did not show that inhibition of these non-α7 receptors was fully surmountable at high ACh concentrations, the correlations shown in Fig. 9 nonetheless support the hypothesis that competitive interactions contribute to the inhibitory effects of these agents. Additionally there may be use-dependent effects as previously reported for GTS-21 on α4β2 receptors (de Fiebre et al., 1995). It has also been proposed that GTS-21 may inhibit α3β4 receptors by binding to allosteric inhibitor sites (Papke, 2002). The binding of the BA compound to these allos-

teric sites was decreased by the apparently competitive binding of TC-2403 to the same sites.

It remains an important question of whether there are qualitative differences between the inhibition observed during co-application and the "residual inhibition" of α3β4 and α4β2 observed in these studies and previously reported for anabaseine compounds and other agonists such as nicotine, epibatidine, and ABT-418 (de Fiebre et al., 1995; Papke et al., 1997, 2000; Meyer et al., 1998c). When 4-CF_3-BA was applied alone to α3β4 and α4β2 receptors it produced detectable residual inhibition (Fig. 1), which was quite pronounced in the case of α3β4 receptors. There was no increase in this residual inhibition when the same concentration of 4-CF_3-BA was co-applied with ACh, and, in fact, the residual inhibition of α3β4 receptors was essentially decreased to zero when the drug was co-applied with ACh ($p< 0.01$, data not shown), suggesting that the residual inhibition of α3β4 receptors by 4-CF_3-BA requires at least one step (e.g., initial receptor binding) that is competitive. However, while 3-BA's antagonism of α4β2 receptors appears largely competitive, other inhibitory interactions between benzylidene derivatives and α3β4 and α4β2 receptors appear to be, at least in part, noncompetitive. Therefore, it seems likely that while 3-BA and its 4-substituted analogs can all access the agonist binding site in α3β4 and α4β2 receptors, only the 4-substituted compounds interact at more distal sites to inhibit the receptor, presumably through the steric or ionic actions of this substitution. The observation that the BAs tested in the study were in many cases at least as potent in displacing high affinity cytisine and nicotine-insensitive epibatidine binding as they were at displacing α-Btx binding further supports the hypothesis that they have access to the agonist binding site of all the neuronal nAChR.

The addition of the benzylidene group causes anabaseine to lose its agonist activity for non-α7 receptors. We see that 4OH-BA retains α7 agonist activity, and we have previously reported other substituted benzylidene anabaseines that retain good α7 agonist activity. However, 4-MeS-BA and 4-CF_3-BA appear to lose much of their α7 agonist activity but retain their ability to displace α-Btx. This supports the idea that they might function as broad spectrum nAChR competitive antagonists. A comparative analysis of some of the molecular properties of the compounds employed in this study leads to the hypothesis that the shift from agonist to antagonist behavior might arise via the relative size and hydrophobicity of the group at the 4-position of BA. Table 3 presents a tabulation of the calculated molecular volumes and log of the partition coefficient between *n*-octanol and water for the BAs. While 4-OH BA is only 5 $Å^3$ greater in volume than 3-BA itself, the corresponding differences in volume for 4-CF_3-BA and 4-MeS-BA are respectively, 27 and 37 $Å^3$, relative to 3-BA. Further, the CF_3 and MeS substituents confer considerable hydrophobicity to the compounds, with log *P* values of 3.7 and 3.5 compared to a log *P* of 2.7 for 3-BA. A hypothesis consistent with these observations is that the larger substituents are unable to interact in the binding site so as to produce the agonist effect, but instead are better accommodated in a hydrophobic pocket that leads to antagonism. We note that α3β4 receptors distinguish between 3-BA and 4OH-BA in terms of potency compared to 4-MeS-BA and 4-CF_3-BA, while α4β2 receptors apparently do not. Possibly, the α3β4 subtype may interact more selectively with the 4-MeS and 4-CF_3 substituents due to a unique complementary hydrophobic pocket, whereas the α4β2 may be complementary to both non-polar and polar substituents at the 4-position of BAs. It has been previously published that there are numerous beta subunit-dependent differences in agonist and antagonist sensitivity for β2- and β4-containing receptors (Luetje and Patrick, 1991; Parker et al., 1998). Many of these differences are believed to be due to differences in amino acid sequence in the beta subunit portion of the agonist binding site interface (Fig. et al., 1992; Papke et al., 1993; Cohen et al., 1995).

Table 3
Physical parameters of the BA compounds

Compound	Volume ($Å^3$)	log *P*
3-BA	220	2.7
4OH-BA	225	2.3
4-MeS-BA	257	3.5
4-CF_3-BA	247	3.7

As drug development progresses with neuronal nAChR as therapeutic targets, it is important to consider the physiological significance of having drugs with mixed agonist/antagonist properties. Drugs may be found which will activate or inhibit α7 receptors selectively and spare the responses of other subtypes. Likewise, other drugs may "tune" the neuronal nAChR systems of the brain by activating one subtype and antagonizing another. The challenges still existing for drug development include evaluating whether such drugs will, in fact, be able to reach important sites in the brain and determining the optimal sort of receptor selectivity to be used to achieve specific therapeutic endpoints. These goals will be achievable by combining physiological characterizations of new drugs, such as those reported here, along with in vitro biochemical studies and in vivo studies of animal behavior. Using this approach, our results point to the importance of the 4-position of benzylidene anabaseines for having effects on the potency of this class of agonists for α7 receptors in particular, as well as the antagonist activity for other receptor subtypes.

Acknowledgements

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