

RESEARCH PAPER

Interaction of benzylidene-anabaseine analogues with agonist and allosteric sites on muscle nicotinic acetylcholine receptors

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Background and purpose: Benzylidene-anabaseines (BAs) are partial agonists of the $\alpha 7$ nicotinic acetylcholine receptor (nAChR) but their mechanism(s) of action are unknown. Our study explores several possibilities, including direct interactions of BAs with the nAChR channel.

Experimental approach: Functional and radioligand-binding assays were used to examine the interaction of two BA analogues, 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA) and its primary metabolite 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine (4OH-DMXBA) with both agonist and non-competitive antagonist (NCA)-binding sites on muscle-type nAChRs.

Key results: Both BAs non-competitively inhibited ACh activation of human fetal muscle nAChRs and sterically inhibited the specific binding of the NCAs [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine ([³H]TCP) and [³H]dizocilpine to *Torpedo* nAChRs in the desensitized state. These compounds modulated [³H]tetracaine, [¹⁴C]amobarbital and [³H]TCP binding to resting nAChRs by allosteric mechanisms. Both BAs enhanced [³H]TCP binding when the nAChR was initially in the resting but activatable state, suggesting that both compounds desensitized the *Torpedo* nAChR. Although DMXBA failed to activate human fetal muscle nAChRs, 4OH-DMXBA was found to be a partial agonist. [³H]Nicotine competition-binding experiments confirmed that 4OH-DMXBA has higher affinity than DMXBA for the agonist sites, and that DMXBA is also a competitive antagonist.

Conclusions and implications: 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine is a partial agonist for human fetal muscle nAChRs, whereas DMXBA only has competitive and NCA activities. The NCA-binding site for BAs overlaps both the phencyclidine- and dizocilpine-binding sites in the desensitized *Torpedo* nAChR ion channel. The desensitizing property of BAs suggests another possible mode of non-competitive inhibition in addition to direct channel-blocking mechanisms.

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Abbreviations: [³H]TCP, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine); 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; α -BTx, α -bungarotoxin; BA, 3-(benzylidene)-anabaseine; CCh, carbamylcholine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; K_d , dissociation constant; K_i , inhibition constant; nAChR, nicotinic acetylcholine receptor; NCA, non-competitive antagonist; n_H , Hill coefficient; PCP, phencyclidine

Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of a superfamily of genetically linked ligand-gated ion channels that includes GABA_A, GABA_C, glycine and 5-HT₃ receptors

(nomenclature follows Alexander *et al.*, 2008). A variety of nAChRs have been found in mammals (Arias, 2006; Kalamida *et al.*, 2007). In the peripheral nervous system, the two most abundant nAChRs have traditionally been classified as muscle-type ($\alpha 1\beta 1\delta\gamma/e$; embryonic/adult) and ganglionic nAChRs (e.g. primarily $\alpha 3\beta 4$ subunit-containing receptors). The embryonic nAChR is similar to the *Torpedo* nAChR. In the central nervous system, nAChRs can be classified into two major subclasses: receptors that bind the competitive antagonist α -bungarotoxin (α -BTx) with high affinity but the agonist

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nicotine with low affinity, such as the primarily $\alpha 7$ -containing receptors, and nAChRs that bind nicotine with high affinity but α -BTx with low affinity, primarily those containing $\beta 2$ subunits expressed with $\alpha 4$ and/or $\alpha 6$ subunits. Over the past two decades, numerous studies have provided a much more detailed knowledge of the mechanisms by which nAChRs are activated or inhibited by various ligands, and there is currently much interest in the possible therapeutic utility of some of these compounds.

Anabaseine is a marine toxin that, like nicotine, potently stimulates a wide variety of nicotinic receptors (Kem *et al.*, 1997; 2006). In contrast to anabaseine, some 3-(benzylidene)-anabaseines (BAs), such as 3-(2,4-dimethoxybenzylidene)-anabaseine (DMXBA) and its hydroxy metabolites including 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine (4OH-DMXBA), are partial agonists only at $\alpha 7$ nAChRs (de Fiebre *et al.*, 1995; Meyer *et al.*, 1997; Meyer *et al.*, 1998; Papke *et al.*, 2000; Kem *et al.*, 2004). Phase I studies to evaluate DMXBA as a drug candidate for the treatment of cognitive dysfunction have been completed with promising results (Kitagawa *et al.*, 2003; Olincy *et al.*, 2006; Freedman *et al.*, 2008). As DMXBA and its primary phase I metabolite, 4OH-DMXBA, are lead compounds for the further development of therapies for several diseases in which $\alpha 7$ nAChR expression or function is abnormal, it is important to understand the mechanisms by which they affect this and other nAChRs. For example, the molecular basis of partial agonism by BAs at $\alpha 7$ nAChRs is not well understood. Partial agonism may be due to one or more mechanisms: (i) once bound to the nAChR agonist sites, BAs may not maximally increase the probability of channel opening; (ii) BAs may effectively induce the opening of the ion channel, but at the same time they may block the ion channel by binding to one or more non-competitive antagonist (NCA) site(s), the so-called agonist self-inhibitory mechanism (reviewed in Arias, 1996); and (iii) BAs may increase the desensitization process, through interactions either at the ACh-binding sites and/or by binding to NCA sites within the ion channel. Previous electrophysiological studies (de Fiebre *et al.*, 1995; Meyer *et al.*, 1998; Uteshev *et al.*, 2002) of BA action have provided some support for mechanism (ii), but non-competitive inhibition was not demonstrated.

In this study our primary goals were to: (i) demonstrate the NCA action of the two BAs upon muscle-type nAChRs (both human fetal and *Torpedo* nAChRs); (ii) characterize and locate the putative NCA-binding sites for the BAs on the *Torpedo* nAChR; and (iii) determine the influence of nAChR conformational state upon interaction of BAs with the NCA sites. We selected the *Torpedo* nAChR for this initial analysis of BA interaction because the $\alpha 7$ nAChR has not yet been expressed at levels that would allow us to measure binding to known NCA sites that have been well characterized for the *Torpedo* nAChR (Arias *et al.*, 2002a; 2006b; Arias and Bhumireddy, 2005).

Here, we initially demonstrate, using a cultured cell line (TE671) expressing human fetal muscle nAChRs, that 4OH-DMXBA is a partial agonist, whereas DMXBA only has competitive and non-competitive antagonistic activities. We compare the binding properties of BAs with the agonist sites by means of [3 H]nicotine competition experiments. We then characterize the binding of the BAs to *Torpedo* nAChR NCA-

binding sites by radioligand competition experiments using well-known NCAs such as [piperidyl-3,4- 3 H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine (3 H]TCP), a structural and functional analogue of phencyclidine (PCP) (Arias *et al.*, 2003; 2006a), [3 H]dizocilpine (Arias *et al.*, 2001a), [3 H]tetracaine (Middleton *et al.*, 1999; Gallagher and Cohen, 1999) and [14 C]jamobarbital (Arias *et al.*, 2001b).

Methods

Functional analysis of nAChR inhibition using cultured cells

TE671 cells naturally expressing human fetal muscle nAChRs obtained from the ATCC were cultured and used in 96-well FlexStation experiments, as previously described (Lee *et al.*, 2006). A membrane potential-sensing Molecular Devices proprietary dye (blue dye kit) was used to measure the kinetics of membrane depolarization resulting from sudden addition of 11 different concentrations of ACh alone or in the presence of one of six concentrations of DMXBA or 4OH-DMXBA. TE671 cells were maintained in a culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U·mL⁻¹ penicillin and 100 μ g·mL⁻¹ streptomycin, and grown in 75 cm² culture flasks housed in a humidified incubator at 37°C in an atmosphere of 5% CO₂. They were seeded at a density of roughly 3–6 \times 10⁴ cells per well into 96-well flat-bottom black-walled culture plates that had been coated with 50 μ L per well of 50 μ g·mL⁻¹ poly-D-lysine (70–150 kDa) and grown to near confluence overnight in 75 μ L of culture medium. The fluorescent dye solution was prepared by dissolving one vial of the dye kit into 36 mL Hanks' balanced salt solution (HBSS) supplemented with 20 mmol·L⁻¹ HEPES buffer (pH 7.4). The 96-well cell culture plates were equilibrated to room temperature for 10 min, and then the cells were washed once with 100 μ L HBSS- HEPES before adding 30 μ L of the membrane potential dye solution to each well. The cells were incubated with the dye for 45 min before agonist addition and recording. Serial dilutions of a compound for concentration-response analysis were prepared in 96-well V-bottom plates by addition of the required volume of a methanolic stock solution. After evaporation of the methanol, the compound in each well was redissolved in HBSS- HEPES (pH 7.4).

Changes in membrane potential resulting from sudden addition of ACh or KCl were measured in a FlexStation fluorimeter (Molecular Devices). Excitation and emission wavelengths were set to 530 nm and 565 nm respectively; the cut-off wavelength was 550 nm. The lipophilic anionic bis-oxonol dye crosses the membrane when it is depolarized, resulting in a significant enhancement of its fluorescence. A reading was taken every 1.44 s for 200 s, a total of 139 readings per well. The first 16 s were used as a basal reading. At 17 s, 30 μ L of a test compound was added to assess agonist activity. A 30 μ L pulse of concentrated KCl (to attain a final concentration of 40 mmol·L⁻¹ KCl) in saline was added to identical wells of cells not exposed to ACh or either BA to serve as a depolarizing calibrant and to correct for differences in dye loading and cell counts between experiments. Peak fluorescent (depolarizing) response (*F*) was initially normalized to the maximal calibrant (KCl, final concentration

40 mmol·L⁻¹) response: $F = (F_{\text{Max,Compound}} - F_{\text{Basal}})/(F_{\text{Max,Calibrant}} - F_{\text{Basal}})$. These F values were then expressed relative to the maximum response generated by ACh alone. Normalized F values were then fitted to a four-parameter logistic equation with variable slope using the Prism software (GraphPad Software, San Diego, CA, USA), for fitting sigmoidal concentration-response curves to estimate the BA concentration that produces 50% inhibition of agonist activity (IC₅₀) and maximal degree of activation for each agonist curve.

Preparation of *Torpedo* membranes for radioligand-binding experiments

Receptor rich membranes were prepared from frozen *Torpedo californica* electric organs obtained from Aquatic Research Consultants (San Pedro, CA, USA) by differential and sucrose density gradient centrifugation, as described previously (Pedersen *et al.*, 1986). Total *Torpedo* nAChR membrane protein was determined using the bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA) with bovine serum albumin as standard. Specific activities of these membrane preparations were determined by the decrease in dansyltrimethylamine (6.6 μmol·L⁻¹) fluorescence produced by the titration of suberyldicholine into receptor suspensions (0.3 mg·mL⁻¹) in the presence of 100 μmol·L⁻¹ PCP and ranged from 1.0 to 1.2 nmol of suberyldicholine-binding sites (mg total protein)⁻¹ (0.5–0.6 nmol nAChR mg protein⁻¹). The nAChR membrane preparations were stored at –80°C in 12% sucrose.

Benzylidene-anabaseine-induced inhibition of agonist and NCA binding to nAChRs

In order to determine the binding affinity of DMXBA and 4OH-DMXBA for the agonist sites, [³H]nicotine-binding competition experiments were performed. To determine the NCA-binding site location for BAs, we also studied the effect of these BAs on the binding of several NCAs including [³H]dizocilpine, [³H]TCP, [³H]tetracaine and [¹⁴C]amobarbital to nAChRs in different conformational states. In this regard, *Torpedo* membranes were suspended in binding saline buffer containing 50 mmol·L⁻¹ Tris-HCl, 120 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ KCl, 2 mmol·L⁻¹ CaCl₂, 1 mmol·L⁻¹ MgCl₂, pH 7.4, with 6.9 nmol·L⁻¹ [³H]nicotine (in the absence of any other ligand), or alternatively with 10 nmol·L⁻¹ [³H]dizocilpine or 5 nmol·L⁻¹ [³H]TCP [in presence of 1 mmol·L⁻¹ carbamylcholine (CCh) to stabilize the desensitized state], or 6 nmol·L⁻¹ [³H]tetracaine, 13 μmol·L⁻¹ [¹⁴C]amobarbital, or 5 nmol·L⁻¹ [³H]TCP (either in the absence of any ligand, or in the presence of 1 μmol·L⁻¹ α-BTx to stabilize the resting state), and preincubated for 1 h. α-BTx is a competitive antagonist that maintains the nAChR in the resting state (Moore and McCarthy, 1995). Non-specific binding was determined in the presence of 1 mmol·L⁻¹ CCh ([³H]nicotine experiments), 100 μmol·L⁻¹ PCP ([³H]TCP experiments), 500 μmol·L⁻¹ dizocilpine ([³H]dizocilpine experiments), 100 μmol·L⁻¹ tetracaine ([³H]tetracaine experiments) or 400 μmol·L⁻¹ amobarbital ([¹⁴C]amobarbital experiments). The membrane suspension was aliquoted equally to tubes and increasing concentrations of DMXBA or 4OH-DMXBA (i.e. 0.01 nmol·L⁻¹–500 μmol·L⁻¹;

depending on the experiment type) were then added. After incubating 2 h, nAChR-bound radioligand was then separated from free radioligand by a filtration assay using a 48-sample harvester system with GF/B Whatman filters (Brandel Inc., Gaithersburg, MD, USA), previously soaked with 0.5% polyethylenimine for 30 min. The membrane-containing filters were transferred to scintillation vials with 3 mL of Bio-Safe II (Research Product International Corp, Mount Prospect, IL, USA), and the radioactivity was determined using a Beckman LS6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

The concentration–response data were curve-fitted by non-linear least squares analysis using the Prism software, where the corresponding IC₅₀ values were calculated using the following equation:

$$\theta = 1/[1 + ([L]/IC_{50})^{n_H}] \quad (1)$$

where θ is the fractional amount of the radioligand bound in the presence of inhibitor at a concentration $[L]$ compared with the amount of the radioligand bound in the absence of inhibitor (total binding). IC₅₀ is the inhibitor concentration at which $\theta = 0.5$ (50% bound), and n_H is the Hill coefficient. The observed IC₅₀ values from the competition experiments described above were transformed into inhibition constant (K_i) values using the Cheng–Prusoff relationship (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/\{1 + ([\text{ligand}]/K_d^{\text{ligand}})\} \quad (2)$$

where $[\text{ligand}]$ and K_d^{ligand} are the initial concentration and the dissociation constant (K_d) for [³H]nicotine (Middleton and Cohen, 1991). The calculated K_i and n_H values for DMXBA and 4OH-DMXBA binding to the agonist sites are summarized in Table 1.

Taking into account that the nAChR presents one binding site for [³H]dizocilpine (Arias *et al.*, 2001a) and [³H]TCP (Arias *et al.*, 2003) in the desensitized state, the observed IC₅₀ values were transformed into K_i values using eq. 2 (Cheng and Prusoff, 1973). In this case, $[\text{ligand}]$ is the initial concentration of [³H]dizocilpine or [³H]TCP, and K_d^{ligand} is the K_d for [³H]dizocilpine (4.8 μmol·L⁻¹; Arias *et al.*, 2001a) or [³H]TCP (0.25 μmol·L⁻¹; Pagán *et al.*, 2001) in the desensitized state. The calculated K_i and n_H values for DMXBA and 4OH-DMXBA binding to the NCA sites are summarized in Table 2.

Table 1 Binding affinity of benzylidene-anabaseine analogues for the *Torpedo* nAChR agonist-binding sites

Benzylidene-anabaseine analogue	K_i^a (μmol·L ⁻¹)	n_H^b
DMXBA	10.6 ± 0.8	1.27 ± 0.11
4OH-DMXBA	2.7 ± 0.2	1.07 ± 0.06

Data shown are the means ± SD, from two to three experiments each performed in triplicate.

^aInhibition constants were calculated from Figure 3 using eq. 2.

^bHill coefficients.

4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor.

Table 2 Effect of benzylidene-anabaseines on [³H]dizocilpine, [³H]TCP, [³H]tetracaine and [¹⁴C]amobarbital binding to the nAChR in different conformational states

Radioligand	Conformational state	DMXBA		4OH-DMXBA	
		K_i^a or IC_{50}^b ($\mu\text{mol}\cdot\text{L}^{-1}$)	n_H^c	K_i^a or IC_{50}^b ($\mu\text{mol}\cdot\text{L}^{-1}$)	n_H^c
[³ H]TCP	Desensitized	13 ± 1 ^a	0.91 ± 0.06	48 ± 5 ^a	0.90 ± 0.08
[³ H]Dizocilpine	Desensitized	39 ± 8 ^a	0.92 ± 0.16	84 ± 11 ^a	0.70 ± 0.07
[³ H]Tetracaine	Resting	262 ± 10 ^b	2.1 ± 0.2	0.79 ± 0.50 ^d	~1.3
[¹⁴ C]Amobarbital	Resting	95 ± 9 ^b	>3	46 ± 32 ^b	~0.4

Data shown are the means ± SD, from 2–3 experiments each performed in triplicate.

^aInhibition constants were calculated from Figures 4A ([³H]TCP), 4B ([³H] dizocilpine), 5A ([³H]tetracaine) and 5B ([¹⁴C]amobarbital), using eq. 2.

^bDrug concentration to produce 50% binding inhibition. These values were determined by non-linear least-squares fit according to eq. 1.

^cHill coefficients.

^dThis apparent EC₅₀ value is obtained from the 4OH-DMXBA-induced enhancement of [³H]tetracaine binding to the resting AChR.

[³H]TCP, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine; 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor.

Schild-type plots for DMXBA-induced inhibition of [³H]TCP binding

In order to have a better indication whether BAs inhibit [³H]TCP binding to the nAChR by a steric or allosteric mechanism, DMXBA-induced inhibition of [³H]TCP-binding experiments was performed at increasing initial concentrations of unlabelled PCP (i.e. 3.1, 6.2 and 9.3 $\mu\text{mol}\cdot\text{L}^{-1}$). The rationale of this experiment is based on the expectation that, for a higher initial concentration of the radioligand, a higher concentration of the competitor will be necessary to produce a total inhibition of radioligand binding. This is consistent with Schild-type analysis (Schild, 1949). From these competition curves, the apparent IC₅₀ values were obtained according to eq. 1. Then, we plot the ratio between the IC₅₀ values for DMXBA determined at different initial concentrations of unlabelled PCP under the IC₅₀ control values (no PCP added) versus the initial PCP concentration ($IC_{50}^{\text{PCP}}/IC_{50}^{\text{control}}$ vs. $[PCP]_{\text{initial}}$) (for more details, see the study by Arias *et al.*, 2002b). A linear relationship from this modified Schild-type plot would indicate a competitive interaction, whereas a non-linear relationship would suggest an allosteric mechanism of inhibition (Schild, 1949; Arias *et al.*, 2002b).

Drugs, chemical reagents and other materials

[Piperidyl-3,4-³H(N)]-N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine ([³H]TCP) (42–45 Ci·mmol⁻¹), (+)-[³H]dizocilpine (21.7 Ci·mmol⁻¹) and [³H]nicotine (77 Ci·mmol⁻¹) were obtained from PerkinElmer Life Sciences Products, Inc. (Boston, MA, USA). [¹⁴C]Amobarbital (50 Ci·mol⁻¹) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). [³H]Tetracaine (36 Ci·mmol⁻¹) was a gift from Dr Jonathan Cohen (Harvard Medical School, Boston, MA, USA). All radioligands were stored in ethanol at -20°C. CCh chloride, acetylcholine chloride (ACh), suberyldicholine dichloride and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [1-(Dimethylamino)naphthalene-5-sulphonamido] ethyltrimethylammonium perchlorate (dansyltrimethylamine) was purchased from Pierce Chemical Co. (Rockford, IL, USA). α -BTx was obtained from Molecular Probes (Eugene, OR, USA). (+)-Dizocilpine

maleate was purchased from Tocris (Ellisville, MO, USA). DMXBA dihydrochloride (DMXBA.2HCl) and 4OH-DMXBA (4OH-DMXBA.2HCl) were synthesized as described elsewhere (Kem *et al.*, 2004). Amobarbital hydrochloride and PCP hydrochloride were obtained through the National Institute on Drug Abuse (NIDA) (NIH, Bethesda, MD, USA). Fetal bovine serum and penicillin/streptomycin were obtained from Media Tech, Inc. (Herndon, VA, USA), Dulbecco's modified Eagle's medium was purchased from the American Type Culture Collection (Manassas, VA, USA) and the fluorescence dye kit was purchased from Molecular Devices (Sunnyvale, CA, USA). Salts were of analytical grade.

Results

Inhibition of ACh-mediated depolarization of TE671 cells

3-(2,4-dimethoxybenzylidene)-anabaseine and 4OH-DMXBA inhibited the TE671 cell response to ACh in a non-competitive manner (Figure 1). The entire ACh concentration-response curve was depressed by each BA concentration tested. The effects of DMXBA or 4OH-DMXBA on the ACh EC₅₀ values were concentration independent, as would be expected for non-competitive inhibition. The IC₅₀ values for BA inhibition of ACh response obtained from several experiments were 6.6 ± 1.2 $\mu\text{mol}\cdot\text{L}^{-1}$ for DMXBA and 10.0 ± 2.8 $\mu\text{mol}\cdot\text{L}^{-1}$ for 4OH-DMXBA (Figure 1C).

Although DMXBA failed to show any depolarizing action on the TE671 cells when tested over a wide range of concentrations (2 nmol·L⁻¹–200 $\mu\text{mol}\cdot\text{L}^{-1}$, data not shown), HPLC-pure 4OH-DMXBA consistently activated TE671 cell nAChRs with an EC₅₀ ~2 $\mu\text{mol}\cdot\text{L}^{-1}$ and a maximal effect that was 40–50% of the ACh response (Figure 2A). This indicates that although DMXBA binds to the agonist sites (see Figure 3), it does not present agonist properties. The depolarizing action elicited by 4OH-DMXBA was blocked by 10 nmol·L⁻¹ α -BTx in an insurmountable manner, as this toxin binds in a quasi-irreversible manner to skeletal muscle nAChRs and thus prevents competition by ACh during the short period of ACh exposure (Figure 2B). The stimulatory effect of 4OH-DMXBA was unexpected, as a 100 $\mu\text{mol}\cdot\text{L}^{-1}$ concentration was previ-

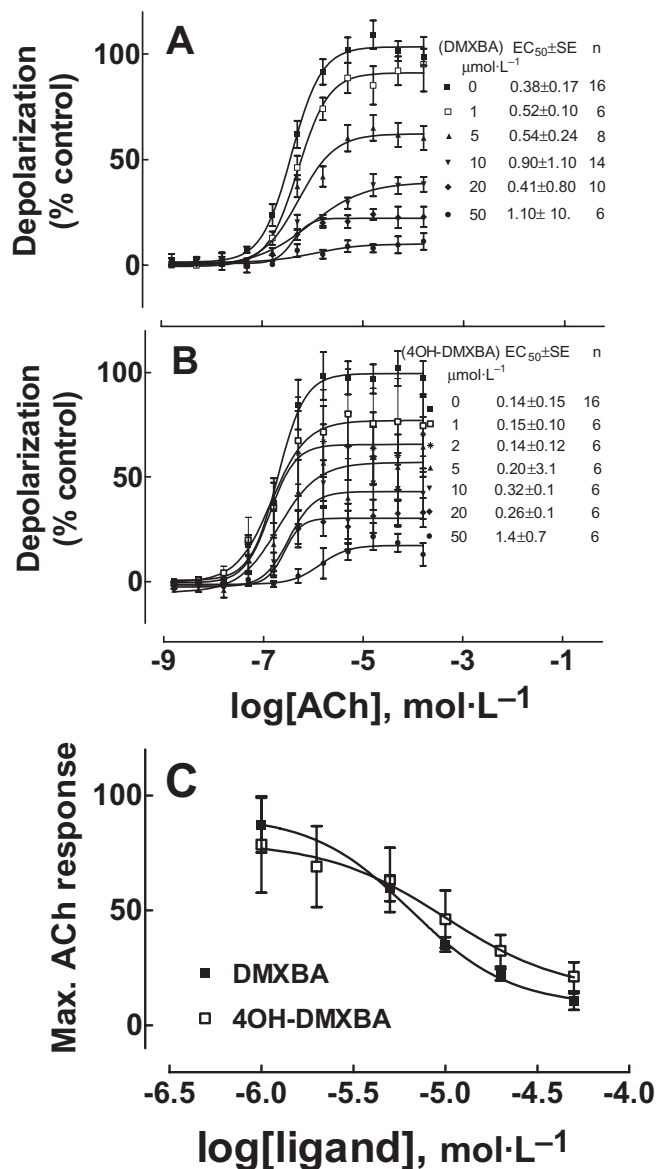


Figure 1 Non-competitive inhibition of ACh-induced membrane depolarization in TE671 cells expressing the human fetal muscle nAChR by DMXBA (A) and 4OH-DMXBA (B). Each curve was fitted using Prism software as described in Methods section. The drug was manually added to the cells in programmed wells at least 10 min before ACh was added by the FlexStation program (*n*, number of wells tested at each ACh concentration). The ACh response at high concentrations in the control curve is less than maximal due to the desensitizing effect being greater at high concentrations. (C) Maximal ACh response for varying concentrations of DMXBA [taken from (A)] and 4OH-DMXBA [taken from (B)] were plotted to estimate the IC₅₀ values for DMXBA ($6.6 \pm 1.2 \mu\text{mol}\cdot\text{L}^{-1}$) and 4OH-DMXBA ($10.0 \pm 2.8 \mu\text{mol}\cdot\text{L}^{-1}$). Data shown are means \pm SEM from the number of assays shown. 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor.

ously reported to lack agonist activity at the adult mouse skeletal muscle nAChR expressed in the *Xenopus* oocyte (Meyer *et al.*, 1998). However, we subsequently learned that the fetal-type nAChR, but not the adult type, is activated by d-tubocurarine and some other competitive antagonists of the

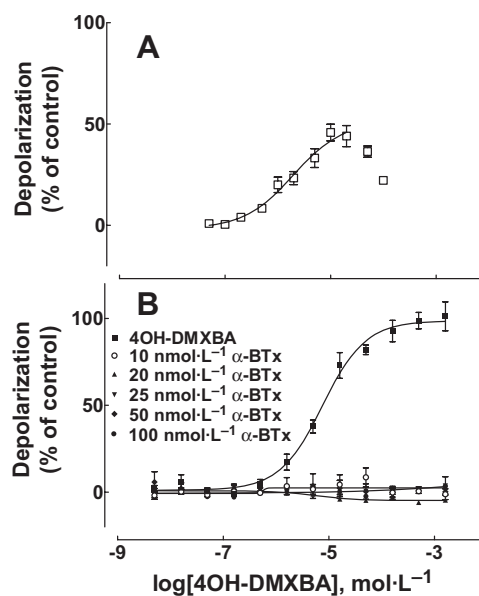


Figure 2 (A) Intrinsic agonist effect of 4OH-DMXBA on human fetal muscle nAChR-containing TE671 cells (*n* = 14; where the error bars are SD). A depression of the response was observed at the highest concentrations and is likely due to desensitization and/or channel block by 4OH-DMXBA. The curve shown was calculated with Prism where the points obtained with the two highest concentrations were omitted from the analysis. The obtained EC₅₀ value was $1.98 \pm 0.15 \mu\text{mol}\cdot\text{L}^{-1}$, and $n_H = 0.94 \pm 0.23$. (B) The agonist activity of 4OH-DMXBA was inhibited by preincubation with α -BTx. Here, 4OH-DMXBA effects are normalized with respect to its maximal effect, rather than to the maximal ACh effect as in (A). Data shown are means \pm SD from four separate assays. 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; α -BTx, α -bungarotoxin; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor; n_H , Hill coefficient.

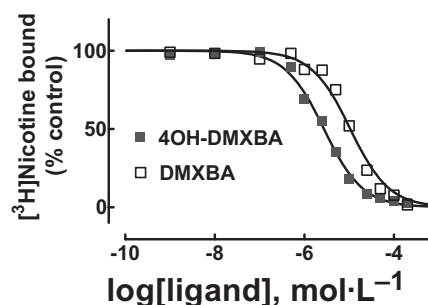


Figure 3 Inhibition of [³H]nicotine binding to *Torpedo* nAChRs induced by BA analogues. nAChR-rich membranes ($0.3 \mu\text{mol}\cdot\text{L}^{-1}$ nAChR) were equilibrated (2 h) with $6.9 \text{ nmol}\cdot\text{L}^{-1}$ [³H]nicotine, and increasing concentrations (i.e. $1 \text{ nmol}\cdot\text{L}^{-1}$ – $200 \mu\text{mol}\cdot\text{L}^{-1}$) of DMXBA or 4OH-DMXBA respectively. Non-specific binding was determined in the presence of $1 \text{ mmol}\cdot\text{L}^{-1}$ CCh. The IC₅₀ and n_H values were obtained by non-linear least-squares fit according to eq. 1. Subsequently, the *K_i* values were calculated using eq. 2. The calculated *K_i* and n_H values are summarized in Table 1. Values shown are the averages of two different experiments. 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; BA, 3-(benzylidene)-anabaseine; CCh, carbamylcholine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor; n_H , Hill coefficient.

adult, ϵ -subunit expressing muscle receptor (Ziskind and Dennis, 1978; Kopta and Steinbach, 1994; Fletcher and Steinbach, 1996).

Benzylidene-anabaseine inhibition of [³H]nicotine binding to *Torpedo* nAChRs

To determine the binding affinity of each analogue for the *Torpedo* nAChR agonist sites, [³H]nicotine-binding competition experiments were performed (see Figure 3). The results indicate that 4OH-DMXBA has higher affinity than DMXBA for the agonist-binding sites (Table 1). The fact that the calculated n_H values for DMXBA and 4OH-DMXBA are close to one (Table 1) indicates that both analogues inhibit [³H]nicotine binding in a non-cooperative manner. This suggests that BAs do not discriminate between the high- and low-affinity agonist-binding sites and thus, BAs bind to both agonist sites in the same concentration range. In addition, our data indicate that the two BAs possess much lower binding affinities for the *Torpedo* nAChR relative to the rat brain $\alpha 7$ nAChR, where the DMXBA and 4OH-DMXBA K_i values determined by [¹²⁵I] α -BTx displacement experiments were ~ 0.13 and ~ 0.24 $\mu\text{mol}\cdot\text{L}^{-1}$ respectively (Kem *et al.*, 2004).

Effect of benzylidene-anabaseines on [³H]dizocilpine, [³H]TCP, [³H]tetracaine and [¹⁴C]amobarbital binding to the *Torpedo* nAChR

The binding sites for [³H]dizocilpine (Arias *et al.*, 2001a) and [³H]TCP (Arias *et al.*, 2002b; 2003; 2006a) in the desensitized state, and for [³H]tetracaine (Middleton *et al.*, 1999), [¹⁴C]amobarbital (Arias *et al.*, 2001b) and [³H]TCP (Arias *et al.*, 2002b; 2003; 2006a) in the resting state, have been previously characterized. Thus, we sought to determine the BA-binding site location relative to these NCA-binding loci. To this end, we determined the effect of DMXBA and 4OH-DMXBA on either [³H]TCP, [³H]dizocilpine, [³H]tetracaine or [¹⁴C]amobarbital maximal binding to *Torpedo* nAChRs in different conformational states. Figure 4 shows the displacing effects of the two BAs on the binding of [³H]TCP (Figure 4A) and [³H]dizocilpine (Figure 4B), respectively, to the desensitized nAChR. The data obtained from these plots indicate that DMXBA has higher affinity than 4OH-DMXBA for both the TCP- and dizocilpine-binding sites. The fact that the calculated n_H values are close to unity (Table 2) suggests that BAs inhibit both [³H]dizocilpine and [³H]TCP binding in a non-cooperative manner. This further suggests that BAs interact with just a single binding site. Thus, we suggest that the BA-binding site overlaps both PCP and dizocilpine loci.

Figure 5 shows the effect of DMXBA and 4OH-DMXBA on [³H]tetracaine (Figure 5A) and [¹⁴C]amobarbital (Figure 5B) binding to the nAChR when the resting state predominates (in the presence of α -BTx). Considering that tetracaine and amobarbital bind preferably to the resting nAChR (Middleton *et al.*, 1999; Arias *et al.*, 2001b), we did not study the interaction of BAs to these binding sites in the desensitized state. DMXBA displaces 100% of [³H]tetracaine specific binding to the resting nAChR (see Figure 5A). The calculated IC_{50} value (262 ± 10 $\mu\text{mol}\cdot\text{L}^{-1}$; Table 2) for DMXBA is in the same concentration range as that obtained by DMXBA-induced inhibition of [³H]TCP-binding experiments in the resting state (172 ± 12 $\mu\text{mol}\cdot\text{L}^{-1}$; Table 3). However, the observed n_H value was higher than unity, indicating that DMXBA inhibits [³H]tetracaine binding in a cooperative manner. This suggests that DMXBA interacts with more than one binding site and that

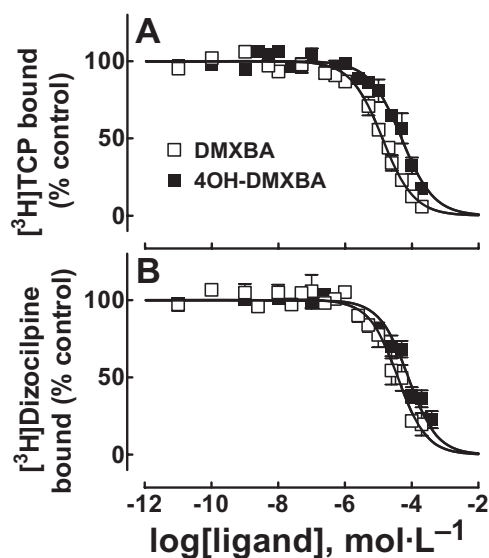


Figure 4 Effect of BAs on [³H]TCP (A) and [³H]dizocilpine (B) binding to *Torpedo* nAChRs in the desensitized state. Inhibition of [³H]TCP (A) or [³H]dizocilpine (B) binding to the desensitized nAChR elicited by DMXBA or 4OH-DMXBA respectively. nAChR-rich membranes (0.3 $\mu\text{mol}\cdot\text{L}^{-1}$ nAChR) were equilibrated (2 h) with 5 $\text{nmol}\cdot\text{L}^{-1}$ [³H]TCP or 10 $\text{nmol}\cdot\text{L}^{-1}$ [³H]dizocilpine, in the presence of 1 $\text{mmol}\cdot\text{L}^{-1}$ CCh (nAChRs are mainly in the desensitized state), and increasing concentrations of the BA (e.g. 0.01 $\text{nmol}\cdot\text{L}^{-1}$ – 500 $\mu\text{mol}\cdot\text{L}^{-1}$; depending on the experiment type). Non-specific binding was determined in the presence of 100 $\mu\text{mol}\cdot\text{L}^{-1}$ PCP or 500 $\mu\text{mol}\cdot\text{L}^{-1}$ dizocilpine respectively. The IC_{50} and n_H values were obtained by non-linear least-squares fit according to eq. 1. Subsequently, the K_i values were calculated using eq. 2. The calculated K_i and n_H values are summarized in Table 2. Values shown are the means \pm SD from two to three separate experiments, each one performed in triplicate. [³H]TCP, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine); 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; BA, 3-(benzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; K_i , inhibition constant; n_H , Hill coefficient.

DMXBA probably inhibits [³H]tetracaine binding through an allosteric mechanism. On the contrary, 4OH-DMXBA enhances [³H]tetracaine binding with apparent EC_{50} ~ 0.8 $\mu\text{mol}\cdot\text{L}^{-1}$ (Table 2). This result argues against the idea of any steric competition between these two molecules, and rules out the possibility that the 4OH-DMXBA locus overlaps the tetracaine-binding site in the resting ion channel. Moreover, this finding supports the hypothesis that the binding of 4OH-DMXBA to its locus provokes a local conformational change that allows the tetracaine molecule to fit more readily within its binding domain.

Regarding the [¹⁴C]amobarbital-binding experiments (Figure 5B), both DMXBA and 4OH-DMXBA displace only $\sim 50\%$ [¹⁴C]amobarbital binding at maximal concentrations, with apparent n_H values different from unity. Additional sigmoid curves fitting the experimental data with $n_H = 1$ are included for the sake of comparison (Figure 5B). In this regard, an unmistakable difference can be observed between both fitted curves. These results suggest that DMXBA and 4OH-DMXBA do not bind to the barbiturate locus in the resting ion channel. The possibility of two allosterically linked binding domains, the PCP and the barbiturate loci

bridged by the tetracaine domain, in the resting ion channel has been previously proposed (Gallagher *et al.*, 2001; Arias *et al.*, 2002b; 2003; 2006a). Thus, this result is in agreement with the previous experimental conclusions indicating that the binding site for DMXBA and 4OH-DMXBA does not overlap the tetracaine locus (Figure 5A).

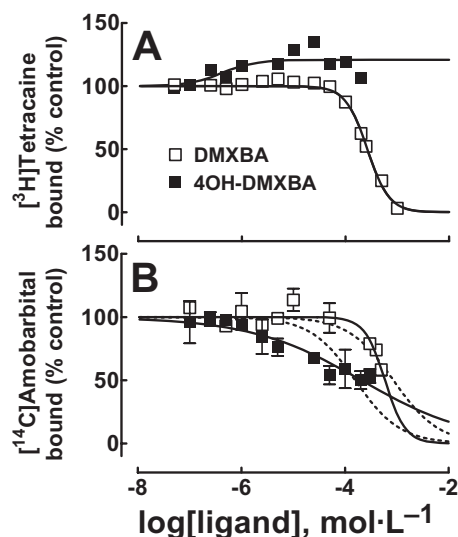


Figure 5 Effect of BAs on [³H]tetracaine (A) and [¹⁴C]amobarbital (B) binding to *Torpedo* nAChRs in the resting state. Inhibition of [³H]tetracaine (A) or [¹⁴C]amobarbital (B) binding to the resting nAChR elicited by DMXBA or 4OH-DMXBA respectively. nAChR-rich membranes (0.3 μmol·L⁻¹ AChR) were equilibrated (2 h) with 6 nmol·L⁻¹ [³H]tetracaine or 13 μmol·L⁻¹ [¹⁴C]amobarbital, in the presence of 1 μmol·L⁻¹ α-BTx (nAChRs are mainly in the resting state), and increasing concentrations of the BA (e.g. 0.1–1000 μmol·L⁻¹; depending on the experiment type). Non-specific binding was determined in the presence of 100 μmol·L⁻¹ tetracaine or 400 μmol·L⁻¹ amobarbital respectively. The apparent IC₅₀ values for DMXBA and 4OH-DMXBA, the EC₅₀ value for 4OH-DMXBA (A), and the respective n_H values were obtained by non-linear least-squares fit according to eq. 1. In the case of (B), a variable (–) or constant (n_H = 1) n_H value (---) was considered for the sake of comparison. The IC₅₀, EC₅₀ and n_H values are summarized in Table 2. Values shown are the result of one out of two experiments (A) or the mean ± SD from two experiments performed in triplicate (B). 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; α-BTx, α-bungarotoxin; BA, 3-(benzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor; n_H, Hill coefficient.

Schild-type plots for DMXBA-induced inhibition of [³H]TCP binding

Although competition-binding results suggest that BAs inhibit [³H]TCP (Figure 4A) binding to the desensitized nAChR in a steric fashion, we cannot rule out that this competition is produced by a potent allosteric mechanism as well. In this regard, we determined whether BAs inhibit [³H]TCP binding to the desensitized nAChR in a steric or allosteric manner by Schild-type analyses (Schild, 1949). Figure 6A shows the DMXBA-induced inhibition of [³H]TCP binding at different initial concentrations of unlabelled PCP. At increased initial PCP concentrations, the plots were shifted to the right, indicating that higher DMXBA amounts are required to inhibit the binding of [³H]TCP at increased PCP concentrations (see Figure 6A). By means of a modified Schild-type plot (Figure 6B), we found a linear relationship between the initial concentration of PCP and the extent of radioligand inhibition with goodness of fit values (*r*²) of 0.98 (Figure 6B). This suggests that DMXBA inhibits [³H]TCP by a steric mechanism. Thus, we may infer that the DMXBA locus overlaps the PCP-binding site in the desensitized state.

Modulation of [³H]TCP binding to *Torpedo* nAChRs elicited by benzylidene-anabaseines

In order to determine the mechanisms of inhibition elicited by BAs, we studied the effect of DMXBA and 4OH-DMXBA on the binding of the NCA [³H]TCP to *Torpedo* nAChRs in distinct conformational states. Figure 7 shows that in the absence of any ligand, where the nAChR is mainly in the resting (but activatable) state, both DMXBA and 4OH-DMXBA enhance [³H]TCP binding in the concentration range between 0.01 nmol·L⁻¹ and ~10 μmol·L⁻¹. Taking into account that [³H]TCP binds with ~3-fold higher affinity to the desensitized (Pagán *et al.*, 2001) than to the resting state (Arias *et al.*, 2003), we can conclude that BAs enhance [³H]TCP binding by inducing nAChR desensitization. To quantify this [³H]TCP-binding enhancement, we calculated the concentration to produce 50% binding enhancement (apparent EC₅₀) as a measure of the apparent desensitizing properties of the drug. The apparent EC₅₀ values for DMXBA and 4OH-DMXBA are 3.9 ± 1.2 and 0.21 ± 0.02 μmol·L⁻¹ (Table 3), respectively, indicating that 4OH-DMXBA is a more potent desensitizing agent than DMXBA. The fact that 4OH-DMXBA increases [³H]TCP binding by ~180%, whereas DMXBA increases [³H]TCP

Table 3 Modulation of [³H]TCP binding to *Torpedo* nAChRs elicited by benzylidene-anabaseine analogues

Benzylidene-anabaseine analogue	Apparent EC ₅₀ ^a (μmol·L ⁻¹)	n _H ^c	IC ₅₀ ^b (μmol·L ⁻¹)	n _H ^f
DMXBA	3.9 ± 1.2	0.79 ± 0.03	172 ± 12	2.04 ± 0.25
4OH-DMXBA	0.21 ± 0.02	1.37 ± 0.17	611 ± 65	1.91 ± 1.10

Data shown are the means ± SD, from two to three experiments each performed in triplicate.

^aBA concentration required to produce 50% binding enhancement as a measure of AChR desensitization. These values were obtained using nAChRs in the absence of any ligand (mostly in the resting but activatable state), from the portion of the plot where drugs enhance radioligand binding (Figure 7).

^bDrug concentration to produce 50% binding inhibition. These values were obtained using nAChRs in the resting state (in the presence of α-BTx), from the portion of the plot where drugs inhibit radioligand binding (Figure 7).

^cHill coefficients.

[³H]TCP, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine); 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; α-BTx, α-bungarotoxin; BA, 3-(benzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor.

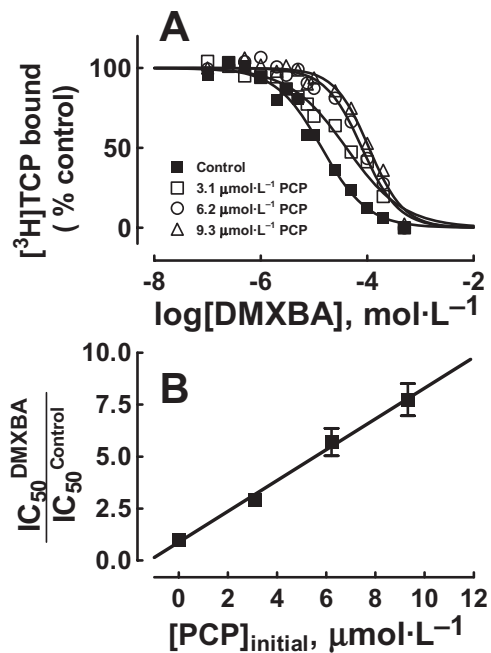


Figure 6 Schild-plot for DMXBA-induced inhibition of $[^3\text{H}]\text{TCP}$ binding to the desensitized *Torpedo* nAChR. (A) nAChR-rich membranes ($0.3 \mu\text{mol}\cdot\text{L}^{-1}$ nAChR) were equilibrated with $[^3\text{H}]\text{TCP}$, in the presence of $1 \text{ mmol}\cdot\text{L}^{-1}$ CCh (desensitized state), at initial PCP concentrations of 0 (control), 3.1 , 6.2 and $9.3 \mu\text{mol}\cdot\text{L}^{-1}$ respectively. The apparent IC_{50} values were calculated by non-linear least-squares fit according to eq. 1. Values shown are the averages of experiments performed in triplicate. (B) Modified Schild-plot for DMXBA-induced inhibition of $[^3\text{H}]\text{TCP}$ binding. The plot shows a linear relationship with a r^2 value of 0.98, suggesting that DMXBA inhibits $[^3\text{H}]\text{TCP}$ binding by a steric mechanism. Values shown are the means \pm SD from one to three experiments performed in triplicate. $[^3\text{H}]\text{TCP}$, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine); CCh, carbamylcholine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor; PCP, phencyclidine.

binding by $\sim 120\%$, also indicates that 4OH-DMXBA is a more efficacious desensitizing agent than DMXBA. Moreover, both drugs start inhibiting $[^3\text{H}]\text{TCP}$ binding at concentrations higher than $\sim 10 \mu\text{mol}\cdot\text{L}^{-1}$.

Figure 7 also shows that neither DMXBA nor 4OH-DMXBA enhances $[^3\text{H}]\text{TCP}$ binding when the nAChR is stabilized in the resting state with a high concentration ($1 \mu\text{mol}\cdot\text{L}^{-1}$) of $\alpha\text{-BTx}$. This result indicates that BAs do not induce any additional conformational change when the nAChR is arrested in the resting state (in the presence of $\alpha\text{-BTx}$), and supports the above conclusion that BAs induce the desensitization process. Moreover, DMXBA inhibits $\sim 100\%$ $[^3\text{H}]\text{TCP}$ binding, whereas 4OH-DMXBA inhibits $[^3\text{H}]\text{TCP}$ binding only partially ($<50\%$), even at very high concentrations. The observed IC_{50} values are very high ($\sim 200\text{--}600 \mu\text{mol}\cdot\text{L}^{-1}$; see Table 3), suggesting that if BAs bind to the $[^3\text{H}]\text{TCP}$ -binding site in the resting state, they do so with very low affinity. The fact that the n_{H} values are higher than unity (see Table 3) indicates a cooperative interaction, supporting the idea that the BA-binding site in fact does not overlap the PCP locus in the resting state. Considering that the PCP locus partially overlaps the tetracaine-binding domain in the resting ion channel (Arias *et al.*, 2002b; 2003; 2006a); reviewed in (Arias *et al.*, 2002a; 2006b;

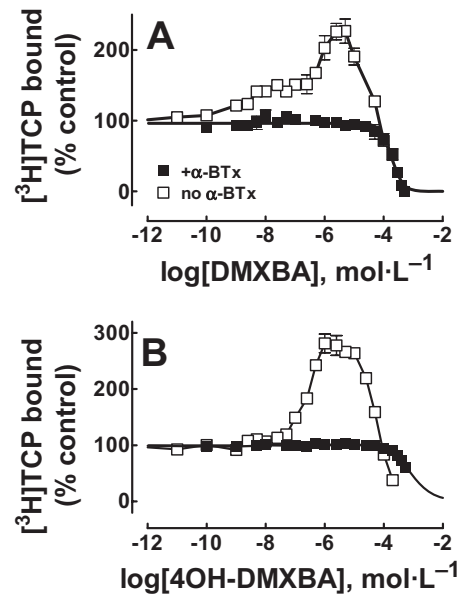


Figure 7 BA-induced modulation of $[^3\text{H}]\text{TCP}$ binding to *Torpedo* nAChRs in different conformational states. Modulation of $[^3\text{H}]\text{TCP}$ binding elicited by DMXBA (A) or 4OH-DMXBA (B) in the activatable (no $\alpha\text{-BTx}$) and in the resting ($+\alpha\text{-BTx}$) states respectively. nAChR native membranes ($0.3 \mu\text{mol}\cdot\text{L}^{-1}$ nAChR) were equilibrated (1 h) with $5 \text{ nmol}\cdot\text{L}^{-1}$ $[^3\text{H}]\text{TCP}$, in the absence (the nAChR is in the resting but activatable state) or in the presence of $1 \mu\text{mol}\cdot\text{L}^{-1}$ $\alpha\text{-BTx}$ (the nAChR is maintained resting state), and increasing concentrations of the BA (i.e. $0.01 \text{ nmol}\cdot\text{L}^{-1}\text{--}500 \mu\text{mol}\cdot\text{L}^{-1}$). The IC_{50} and n_{H} values were obtained by non-linear least-squares fit according to eq. 1, whereas the apparent EC_{50} values were obtained using an equation similar to eq. 1, where apparent EC_{50} instead of IC_{50} was used. The calculated apparent EC_{50} , IC_{50} and n_{H} values are summarized in Table 3. Shown is the mean \pm SD from one experiment performed in triplicate. $[^3\text{H}]\text{TCP}$, [piperidyl-3,4-3H(N)]-(N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine); 4OH-DMXBA, 3-(4-hydroxy-2-methoxybenzylidene)-anabaseine; $\alpha\text{-BTx}$, $\alpha\text{-bungarotoxin}$; BA, 3-(benzylidene)-anabaseine; DMXBA (or GTS-21), 3-(2,4-dimethoxybenzylidene)-anabaseine; nAChR, nicotinic acetylcholine receptor.

Arias and Bhumireddy, 2005), this latter conclusion also fits very well with the previous results indicating that the BA-binding site does not overlap the tetracaine locus (see Figure 5A).

Discussion and conclusions

The TE671 cell FlexStation data (Figure 1) of DMXBA and 4OH-DMXBA inhibition of the depolarizing effect of ACh indicates a non-competitive antagonism of nAChR function under the conditions of BA preincubation. Inhibition by DMXBA when administered simultaneously with ACh yielded essentially the same results (not shown). The micromolar concentrations at which inhibition by DMXBA was observed ($\text{IC}_{50}\text{s} \sim 7 \mu\text{mol}\cdot\text{L}^{-1}$) were much higher (>20 -fold) than the plasma concentrations attained by DMXBA in the preclinical animal tests (Mahnr *et al.*, 1998; Azuma *et al.*, 1999; Kem *et al.*, 2004) and various clinical tests (Kitagawa *et al.*, 2003; Olincy *et al.*, 2006; Freedman *et al.*, 2008). Furthermore, it is known that about 96% of DMXBA is bound in plasma (Kem *et al.*, 2004). Thus, the extent to which the DMXBA might

inhibit muscle-type, $\alpha 7$ -type and possibly other nAChRs *in vivo* is uncertain. A significant fraction of $\alpha 7$ nAChRs, the presumed clinical targets for DMXBA central actions, are known to be tonically active at concentrations significantly below those required for synchronous activation of all $\alpha 7$ receptors (Papke and Thinschmidt, 1998; Papke *et al.*, 2000; Uteshev *et al.*, 2002).

The significant partial agonist activity of 4OH-DMXBA on the human fetal muscle nAChR resembles the observed agonist activity of d-tubocurarine on fetal mouse muscle nAChRs (Ziskind and Dennis, 1978; Kopta and Steinbach, 1994; Fletcher and Steinbach, 1996). Indeed, both molecules contain hydroxyl groups which could conceivably form important H-bonds with amino acid side chains within the ACh-binding site. d-Tubocurarine does not stimulate adult muscle-type nAChRs which contain the ϵ subunit instead of the γ subunit (Ziskind and Dennis, 1978; Kopta and Steinbach, 1994). A clinical implication of our finding is that pregnant women should not be given either of these two BAs until their possible contracting effect on fetal muscle is further investigated.

Our radioligand competition experiments indicate that BAs effectively inhibit the binding of [3 H]dizocilpine and [3 H]TCP (the structural and functional analogue of the dissociative general anesthetic PCP) to desensitized nAChRs (Table 2). Interestingly, DMXBA has ~2–4 times higher affinity for either NCA-binding site than 4OH-DMXBA. Practically, the same ratio was observed in our results from the non-competitive inhibition of ACh-induced nAChRs by BAs (see Figure 1). However, the opposite is true for the binding to the agonist sites, where 4OH-DMXBA has ~4-fold higher affinity than DMXBA (see Table 1). This result might explain why the 4OH metabolite has ~4-fold higher potency and ~2-fold higher efficacy to activate the human $\alpha 7$ nAChR than that for DMXBA, if a similar binding affinity situation holds for the $\alpha 7$ receptor. Considering that drug efficacy and potency properties are influenced by several mechanisms including binding, activation, gating and self-inhibition, it may be that 4OH-DMXBA has a lesser propensity to self-inhibition than DMXBA at the $\alpha 7$ nAChR ion channel and that this may be at least one reason for its higher efficacy at this receptor. While 4OH-DMXBA displays more favourable agonist properties than DMXBA at the $\alpha 7$ nAChR, its ability to activate human muscle-type nAChRs might lead to adverse effects, especially in the fetus.

Schild-type analyses indicate that DMXBA displaces [3 H]TCP binding from its high-affinity site in the desensitized nAChR by a steric (competitive) mechanism (Figure 6). These data suggest that the BA-binding site overlaps the high-affinity PCP locus in the desensitized state. Previous studies aimed at characterizing the high-affinity PCP locus have only been partially successful (reviewed in Arias *et al.*, 2002a; 2006b; Arias and Bhumireddy, 2005). For instance, photoaffinity labelling studies using [3 H]ethidium diazide, which binds with high affinity to the PCP locus, helped to determine the structural components of this site in the desensitized state (Pratt *et al.*, 2000). The results indicated that residues Leu²⁵¹ at position 9' (i.e. the leucine ring) and Ser²⁵² at position 10' from the $\alpha 1$ -M2 transmembrane segment as well as other unknown amino acids located in the M1 and M2 transmem-

brane segments from the δ subunit are structurally involved in this particular site. On the other hand, the dizocilpine locus was suggested to be in a domain different from that for the fluorescent NCAs, crystal violet and ethidium, based on Schild-plot analyses as well as [25 I]dizocilpine photoaffinity labelling studies (Arias *et al.*, 2001a). More recently, however, Schild-type analyses of dizocilpine-induced inhibition of [3 H]TCP binding indicate that dizocilpine and PCP bind to overlapping sites in the desensitized nAChR (Arias, unpublished experiments). Consequently, our data are in accord with a model where the desensitized *Torpedo* ion channel bears overlapping binding sites for BAs, PCP and dizocilpine. The fact that different mutations in the ion channel increase the self-inhibitory effect of DMXBA on nAChRs (Papke, 2002), supports the idea that certain amino acids in the ion channel are possible sites for the antagonist action of DMXBA.

Regarding the location of the BA-binding site in the resting state, our results indicate that DMXBA inhibits [3 H]tetracaine binding in an allosteric fashion, whereas 4OH-DMXBA enhances [3 H]tetracaine binding. These results rule out the possibility that the BA locus overlaps the tetracaine-binding site. Other experimental data (Papke, 2002) indicating that tetracaine does not protect the receptor from the inhibition produced by DMXBA is in accord with our results. In addition, both BAs partially inhibit [14 C]amobarbital and inhibit [3 H]TCP binding to nAChRs arrested in the resting state (in the presence of α -BTx) in an allosteric manner. These data indicate that BAs bind neither to the barbiturate nor to the PCP site in the resting state. The fact that BAs enhance [3 H]TCP binding when the nAChR is in the resting but activatable state (in the absence of α -BTx) (see Figure 7) also supports the same conclusion.

Based on photoaffinity labelling results using *Torpedo* nAChRs in the resting state, the tetracaine-binding site has been located in a long portion of the M2 transmembrane domain that involves residues from position 5' to 13' (i.e. the valine ring) (Gallagher and Cohen, 1999; Middleton *et al.*, 1999) and probably extending to the outer ring at position 20' (Arias *et al.*, 2002a). The tetracaine-binding domain partially overlaps the barbiturate locus in approximately the middle of the ion channel (between position 9' and 13') (Arias *et al.*, 2001b) as well as the PCP site, which is located after the valine ring (i.e. position 13') and close to the outer ring (i.e. position 20') (Arias *et al.*, 2001b; 2002b; 2003; 2006a; Gallagher *et al.*, 2001). Considering our data and the results from different laboratories, we can envision a model where the NCA-binding site for BAs in the resting *Torpedo* nAChR is located in a domain different from the tetracaine site (i.e. between position 5' and 20'). A possibility is that relatively lipophilic BAs allosterically modulate [3 H]tetracaine, [14 C]amobarbital and [3 H]TCP binding by interacting with hydrophobic sites at the lipid-protein interface, as different local anesthetics do (Horváth *et al.*, 1990; Mantipragada *et al.*, 2003).

Previous electrophysiological studies indicated that BAs, in addition to activating $\alpha 7$ nAChRs with differing potency, inhibit muscle- (e.g. $\alpha 1_2\beta 1\gamma\delta$) and neuronal-type nAChRs (e.g. $\alpha 4\beta 2$ and $\alpha 3\beta 4$) (Meyer *et al.*, 1998; Papke, 2002; Papke *et al.*, 2000; 2004; Uteshev *et al.*, 2002; 2003). One possible mechanism of BA nAChR inhibition, besides low affinity for the

open state upon binding to the agonist-binding site and/or direct ion channel block mechanisms, would be enhancement of desensitization. In fact, BAs may modulate the function of the $\alpha 7$ nAChR in tuberomammillary histamine neurons from the posterior hypothalamus by a combination of activation, blocking and desensitization processes (Papke *et al.*, 2000; Uteshev *et al.*, 2002; 2003). In this regard, the BA-induced desensitization observed as BA-enhanced [3 H]TCP (see Figure 7) binding to the *Torpedo* nAChR in the resting but activatable state (in the absence of α -BTx) supports these previous experiments. Our results using *Torpedo* nAChRs also indicate that 4OH-DMXBA is a more potent and efficacious desensitizing agent than DMXBA (see Table 3). Our present observation that 4OH-DMXBA is a rather potent partial agonist for the human fetal muscle nAChR, whereas DMXBA lacks agonist effects on this receptor subtype (see Figure 2), predicts that 4OH-DMXBA would be a more potent desensitizer of this nAChR, and maybe of the $\alpha 7$ nAChR. Direct interaction of BAs with the nAChR ion channel may also favour nAChR desensitization.

Another way of considering the inhibitory activity of DMXBA at the nAChR would be to compare its affinity for the different conformational states of the receptor. As the nAChR subtype in *Torpedo* electric organs and in the human TE671 cell line is the same as fetal muscle nAChRs ($\alpha 1\beta 1\gamma\delta$), we can assume that the structural and functional properties of these nAChRs are basically the same and thus, we can infer that DMXBA interacts with the muscle receptor ion channel with the following relative affinities: open ($IC_{50} \sim 7 \mu\text{mol}\cdot\text{L}^{-1}$; see Figure 1) > desensitized ($K_i = 13 \pm 1 \mu\text{mol}\cdot\text{L}^{-1}$; see Table 2) >> resting ($K_i = 172 \pm 12 \mu\text{mol}\cdot\text{L}^{-1}$; see Table 3). Considering these relative affinities, we can assume that under *in vivo* conditions, DMXBA would interact initially with the open ion channel, after the receptor is activated by ACh. Desensitization of the receptor could then occur, leading to a more profound functional inhibition. Further experiments are needed to determine which of the various possible inhibitory mechanisms discussed above operate to limit nAChR activation by DMXBA and other BAs. A better understanding of these non-competitive inhibitory phenomena should facilitate the design of more efficacious BA-type $\alpha 7$ nAChR agonists with lower activity on muscle-type nAChRs.

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Conflicts of interest

None.

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