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Characterization of a Series of Anabaseine-Derived Compounds Reveals That the 3-(4)-Dimethylaminocinnamylidine Derivative Is a Selective Agonist at Neuronal Nicotinic $\alpha7$/^{125}I-α-Bungarotoxin Receptor Subtypes

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SUMMARY

Investigation of the naturally occurring, nicotinic agonist anabaseine and novel derivatives has shown that these compounds have cytoprotective and memory-enhancing effects. The hypothesis that these arise at least in part through actions on brain nicotinic receptors was evaluated by examining the ability of these compounds to displace the binding of nicotinic ligands and to affect the function of the $\alpha4\beta2$ and $\alpha7$ receptor subtypes expressed in *Xenopus* oocytes. The derivative 3-(4)-dimethylaminocinnamylidine anabaseine (DMAC) was found to be a selective $\alpha7$ receptor agonist; it was more potent than nicotine, acetylcholine, anabaseine, and other derivatives at activating the $\alpha7$ receptor subtype, while displaying little agonist activity at $\alpha4\beta2$ and other receptor subtypes. Compared with anabaseine and the other derivatives, DMAC was the most potent at displacing ^{125}I-α-bungarotoxin binding (putative $\alpha7$) and the least potent at displacing [^{3}H]cytisine binding (putative $\alpha4\beta2$) to brain membranes. Independently of agonist activities, all of the novel compounds displayed secondary inhibitory activity at both receptor subtypes. At the $\alpha4\beta2$ receptor subtype, inhibition by the 3-(2,4)-dimethoxybenzylidene derivative was enhanced by coapplication of acetylcholine, suggesting a noncompetitive form of inhibition. Anabaseine and nicotine prolonged the time course of activation of $\alpha4\beta2$ receptors, compared with acetylcholine, suggesting sequential channel-blocking activity. As selective agonists, anabaseine derivatives such as DMAC may be useful for elucidating the function of $\alpha7$ nicotinic receptors, including their potential role(s) in the cytoprotective and memory-enhancing effects of nicotinic agents.

Although nicotinic receptors at the neuromuscular junction have been extensively characterized, little is known about their counterparts in the CNS. Ligand binding studies reveal two classes of nicotinic binding sites in brain (for example, see Ref. 1), a high affinity site labeled by [^{3}H]cytisine, [^{3}H]nicotine, or [^{3}H]ACh binding and a site with lower affinity for nicotine that can be labeled by the binding of ^{125}I-BTX. In recent years, a number of neuronal α and β nicotinic receptor subunits have been cloned (for review, see Ref. 2). The neuronal receptor formed by $\alpha4$ and $\beta2$ ($\alpha4\beta2$ subtype) appears to be the primary form associated with [^{3}H]cytisine binding (3, 4), whereas the homo-oligomeric subtype formed by $\alpha7$ subunits appears to be associated with the ^{125}I-BTX site measurable in brain (5, 6).

The functional significance of the numerous, potential, CNS, nicotinic receptor subtypes formed by these subunits remains obscure. Studies conducted in *Xenopus* oocytes have been particularly useful in establishing that both the α and β subunits determine the single-channel properties and the whole-cell responses of nicotinic receptor subtypes to agonists (7–9) and antagonists (10). The discovery of novel agents that act with selectivity at given nicotinic receptor subtypes would facilitate probing their function even further, especially in complicated systems such as brain.

Anabaseine, a naturally occurring substance in nemertines, is an agonist at the neuromuscular junction (11) and is structurally related to nicotine (Fig. 1). The more well known agent anabasine (not used in these studies) is a weak nicotinic alkaloid found in tobacco that lacks the imine double bond present in anabaseine. In an effort to find more efficacious and less toxic agents than nicotine, we have been characterizing the short and long term actions of anabaseine and several novel analogs. Recently, we reported on the behavioral, memory-

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ABBREVIATIONS: CNS, central nervous system; DMAC, 3-(4)-dimethylaminocinnamylidine anabaseine; DMAB, 3-(4)-dimethylaminobenzylidene anabaseine; DMXB, 3-(2,4)-dimethoxybenzylidene anabaseine; ACh, acetylcholine; BTX, α-bungarotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

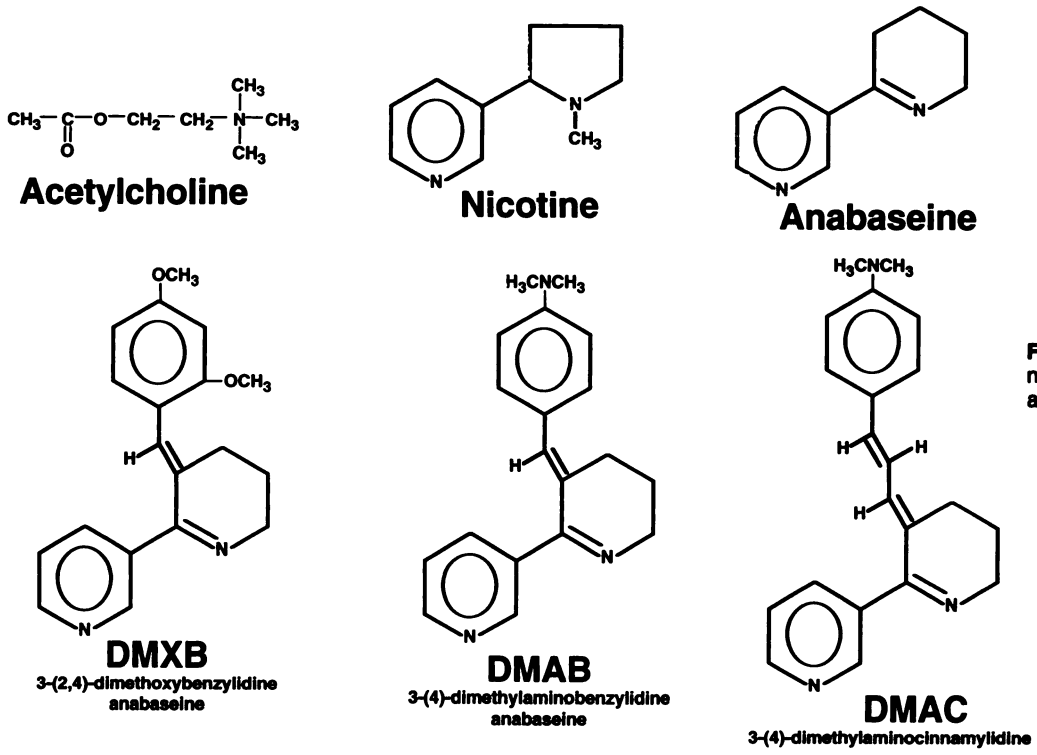

Fig. 1. Chemical structures of ACh, nicotine, anabaseine, DMXB, DMAB, and DMAC.

enhancing, and cytoprotective effects of several of these agents (12, 13). Here, we report on the ability of these compounds to inhibit [^{3}H]cytisine and ^{125}I-BTX binding and on the functional properties of these agents at $\alpha4\beta2$ and $\alpha7$ nicotinic receptor subtypes expressed in *Xenopus* oocytes. Some of these agents act as selective agonists for the $\alpha7$ nicotinic receptor subtype, with DMAC displaying the greatest $\alpha7$ subtype selectivity.

Materials and Methods

Chemicals. Anabaseine, DMAC, DMAB, and DMXB (see structures in Fig. 1) were synthesized as their dihydrochloride salts (14). [^{3}H]Cytisine was purchased from New England Nuclear Corp.; ^{125}I-BTX was purchased either from Amersham Corp. or from New England Nuclear Corp. All other chemicals and drugs were purchased from Sigma Chemical Co., unless noted otherwise.

Receptor binding assays. [^{3}H]Cytisine binding was measured using a modification of the method of Pabreza *et al.* (15), as described previously (13). ^{125}I-BTX binding was measured using the method of Marks and Collins (16). K_d values for each of these ligands were calculated by linear regression analysis of Scatchard plots. The ability of anabaseine, DMAB, DMAC, and DMXB to inhibit the binding of these ligands to membranes prepared from whole rat brain (minus cerebellum and hindbrain) was determined by the simultaneous addition of varying concentrations of each compound to assay tubes with the radioligand. The concentrations of [^{3}H]cytisine and ^{125}I-BTX used in these inhibition assays were approximately 1 and 2 nM, respectively. IC_{50} values were calculated and K_i values were estimated by the equation of Cheng and Prusoff (17).

***Xenopus* oocyte expression and recording.** The preparation of *in vitro* synthesized cRNA transcripts and oocyte injection have been described previously (18); however, instead of mechanical dissection, a modified collagenase treatment was used for the removal of the ovarian follicular cell layer. Briefly, ovaries were cut open to expose oocytes and were treated with collagenase in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.6, 0.33 mM $MgSO_4$, 0.1 mg/ml gentamicin sulfate) for 2 hr at room temperature. Stage 5 oocytes were then isolated and were injected with 5 ng each of $\alpha4$ and $\beta2$ cRNAs or 5 ng of $\alpha7$ cRNA on the day after harvesting. Recordings were made 2–4 days after injections.

For electrophysiological recordings, oocytes were placed in a Lucite recording chamber with a total volume of 0.5 ml and were perfused at room temperature with frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, pH 7.3, 1.8 mM $CaCl_2$) plus 1 μM atropine to block potential muscarinic responses. Drugs were diluted in perfusion solution and were applied after the preloading of a 1.8-ml length of tubing at the terminus of the perfusion system. This system permitted a bolus drug application of approximately 10-sec duration. A Mariotte flask filled with Ringer's solution was used to maintain a constant hydrostatic pressure for drug deliveries and washes. Current responses to drug administration were studied under two-electrode voltage-clamp conditions at a holding potential of −50 mV, using a Warner Instruments amplifier interfaced with National Instruments' LabView software, and were measured to the nearest 1 nA. Current electrodes were filled with 250 mM CsCl, 250 mM CsF, 100 mM EGTA, pH 7.3, and had resistances of 0.5–3 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of 1–3 MΩ. Oocytes with resting membrane potentials of less than −30 mV were rejected.

For oocytes expressing $\alpha4$ and $\beta2$ cRNAs and for oocytes expressing $\alpha7$ cRNA, drug responses were normalized to the responsiveness of the oocyte to 10 μM and 500 μM ACh, respectively, applied 5 min before drug application. Because in our hands receptors expressed from $\alpha7$ cDNA display increased responsiveness after an initial application of agonist, which subsequently stabilizes,[1] all $\alpha7$-expressing oocytes received two applications of ACh, separated by 5 min, at the start of recording. The second ACh application served as a reference for subsequent drug applications. ACh (10 or 500 μM for $\alpha4\beta2$ and $\alpha7$, respectively) was again applied 5 min after drug application, and current response was measured and normalized to the response of the oocyte to ACh delivered 10 min earlier. This second application of ACh also served as a reference for any subsequent drug applications. A minimum of three concentrations of each drug (20, 100, and 500 μM) were tested at each receptor subtype.

[1] R. L. Papke, unpublished observations.

It is not unusual to see some decreased responsiveness in an oocyte after repeated agonist applications, especially after application of high agonist concentrations (for example, see Ref. 9). Such decreases may be due to the desensitizing or antagonist properties of these agents. If the decrease in ACh responses after agonist application was >25%, oocytes were not used for further analysis. However, the dramatic decreases in responsiveness commonly reported after agonist application have recently been demonstrated to be potentially due to use-dependent inhibition by additives (Tinuvin 770 and Tinuvin 765) in the plastics used in many oocyte perfusion systems (19). These experiments were designed to minimize the possibility of contamination by these agents. Specifically, Monoject syringes were used and all solutions were mixed in glass beakers.

Because of their high potency and efficacy, we conducted a more thorough dose-response analysis of the agonist effects of DMAC and DMXB (compared with ACh) at $\alpha 7$ receptors. Complete dose-response curves for these compounds were constructed and numerically fit to the following equation: $I = (I_{max} \times [\text{agonist}]^n)/([\text{agonist}]^n + EC_{50})$, where I_{max} and n are the maximum current and the Hill coefficient, respectively. For other compounds, dose-response curves generated were incomplete; therefore, curve fitting was not appropriate. Data for responses at $\alpha 4\beta 2$-expressing oocytes were also not subjected to curve fitting, because the very low efficacy of anabaseine derivatives at these receptors resulted in an almost undetectable dose-response relationship.

To further evaluate the selectivity of DMAC as an agonist for nicotinic receptors, additional subunit combinations were tested. Responses to DMAC were measured in oocytes coexpressing the $\alpha 4$ subunit and the alternative β subunit $\beta 4$ ($\alpha 4\beta 4$ subtype), as well as in oocytes coexpressing the $\beta 2$ subunit with either of the alternative α subunits, $\alpha 2$ or $\alpha 3$ ($\alpha 2\beta 2$ and $\alpha 3\beta 2$ subtypes, respectively). Muscle-type receptors ($\alpha 1\beta 1\gamma\delta$) were also tested.

For anabaseine, a more detailed analysis of agonist activity at $\alpha 4\beta 2$ receptors was conducted and compared with the agonist activities of ACh and nicotine at these receptors. Specifically, the net charge over a 5-min period commencing with the 10-sec application of drug (anabaseine, ACh, or nicotine) was estimated and compared with the peak current produced by this same drug application. Net charge was estimated by summing the individual calculated change (from base-line values) of currents measured at 250-msec intervals during the 5-min period. Both peak current and net charge were normalized to the corresponding aspect of the control response.

Results

Inhibition of [^{3}H]cytisine and ^{125}I-BTX binding. Anabaseine, DMAC, DMAB, and DMXB displaced the binding of [^{3}H]cytisine and ^{125}I-BTX from rat brain membranes (Fig. 2). Estimated K_i values for these compounds are presented in Table 1. Whereas anabaseine was the most potent of these compounds at displacing [^{3}H]cytisine binding, it was the least potent at displacing ^{125}I-BTX binding. DMAC was the most potent at displacing ^{125}I-BTX binding but the least potent at displacing [^{3}H]cytisine binding.

Agonist effects at $\alpha 4\beta 2$ receptors. The functional responses of anabaseine and its derivatives at $\alpha 4\beta 2$ receptor subtypes expressed in *Xenopus* oocytes are presented in Fig. 3. Anabaseine, which was the most potent of these compounds at displacing [^{3}H]cytisine binding, appeared to be a relatively strong partial agonist at these receptors. The derivatives displayed very weak agonist activity at $\alpha 4\beta 2$ receptors. This slight aggnist activity occurred at concentrations where ACh also produces small responses, suggesting that these compounds differ from ACh in efficacy but not potency. Such low efficacy, however, makes potency difficult to assess.

Agonist effects of anabaseine and analogs of anabaseine at $\alpha 7$ receptors. Unlike the activity of the anabaseine derivatives at $\alpha 4\beta 2$ receptors, all of these agents displayed significant agonist activity at $\alpha 7$ receptors (Fig. 4). DMAC, which was the most potent of these agents at displacing ^{125}I-BTX binding, was also the most potent at activating $\alpha 7$ receptors. Anabaseine had the lowest potency but the greatest efficacy at these receptors.

A more thorough dose-response analysis of the agonist effects

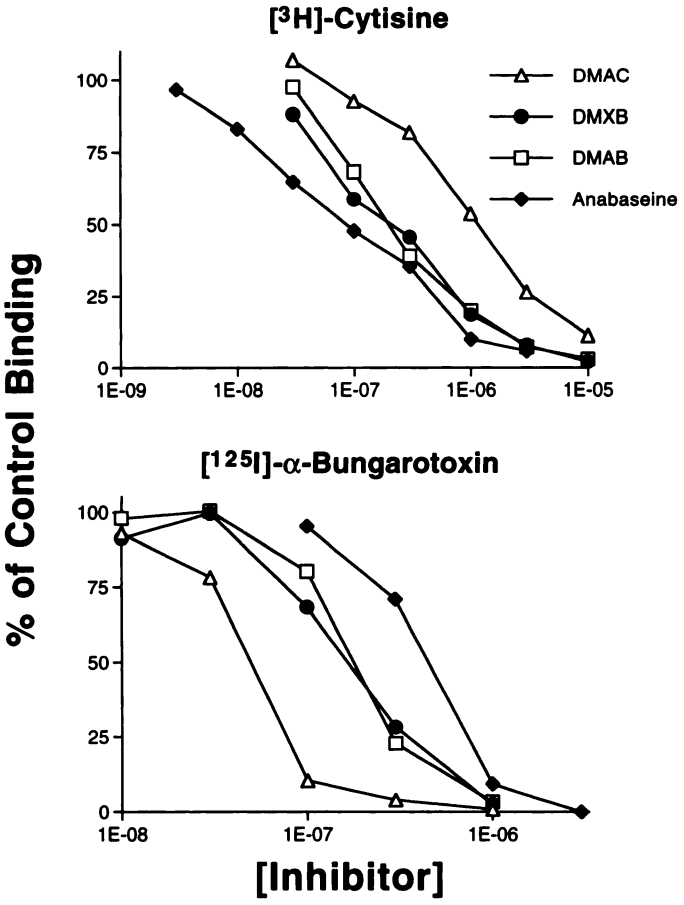

Fig. 2. Inhibition by anabaseine compounds of the binding of [^{3}H]cytisine and ^{125}I-BTX. Data presented are for duplicate determinations derived from a representative experiment, which was repeated three to five times. Each *point* represents the percentage of control binding at the indicated concentration of inhibitor.

TABLE 1

Inhibition of the binding of [^{3}H]cytisine and ^{125}I-BTX

Each value represents the mean ± standard error of three to five separate binding experiments.

	K_i values	
	[^{3}H]Cytisine	[^{125}I-BTX]
	nM	
Anabaseine	74.9 ± 16.1	347.1 ± 159.9
DMXB	84.46 ± 20.6	211.5 ± 78.2
DMAB	109.0 ± 18.5	141.1 ± 14.2
DMAC	347.2 ± 76.6	33.6 ± 11.3
L-Nicotine	3.47[a]	820[b]
Cytisine	0.46[a]	1400[b]
ACh	14.52[a]	4000[b]

[a] Mean K_i values for the inhibition of [^{3}H]cytisine binding by classical nicotinic agents were estimated from published IC_{50} values (15) by the equation of Cheng and Prusoff (17).

[b] Mean K_i values for the inhibition of ^{125}I-BTX binding by classical nicotinic agents are from published reports (1).

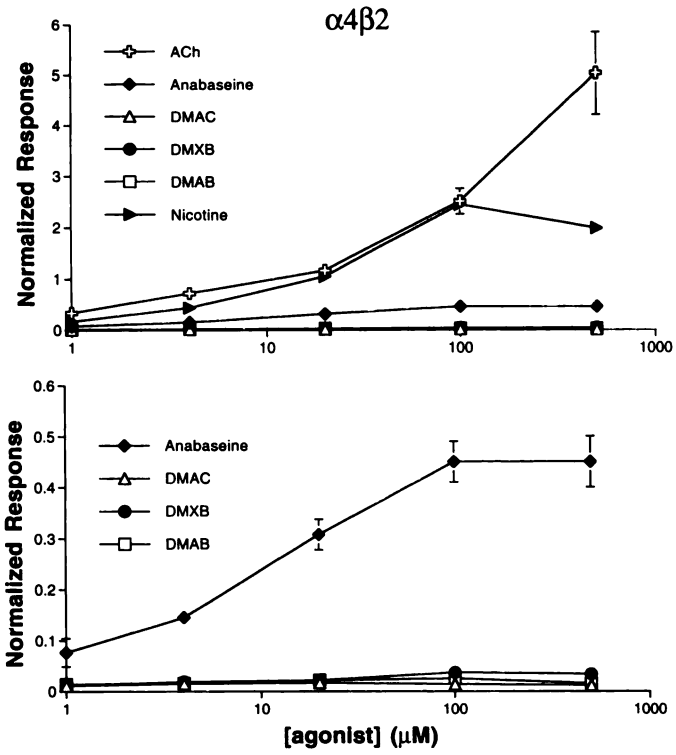

Fig. 3. Agonist activity of ACh, nicotine, and anabaseine compounds at the $\alpha4\beta2$ receptor subtype. Oocytes expressing $\alpha4\beta2$ nicotinic receptors were studied under two-electrode voltage-clamp conditions, for peak responses to classical and novel nicotinic agents. Responses were normalized to the responsiveness of the oocyte to 10 μM ACh applied 5 min earlier. Each *point* represents the mean ± standard error of the response from four oocytes, except for nicotine at 1 and 4 μM, where five oocytes were tested.

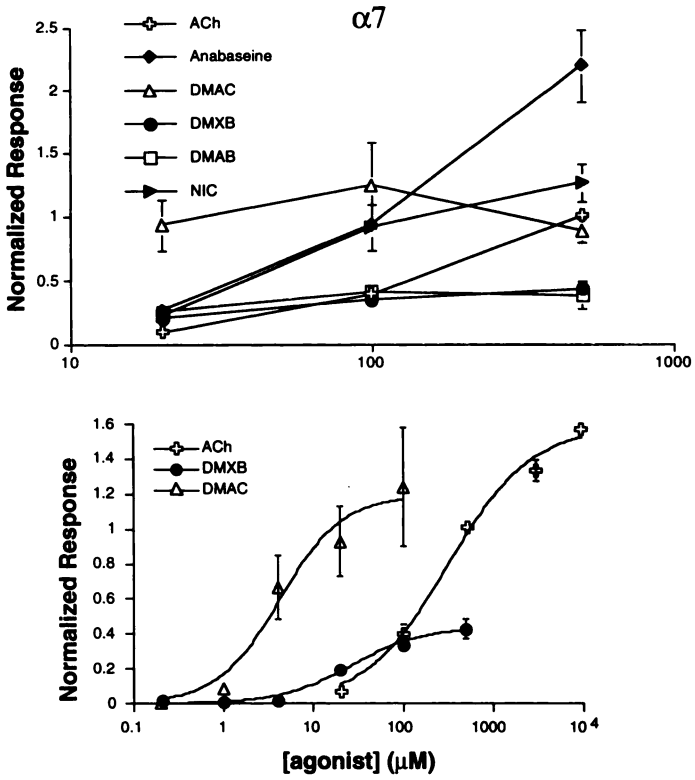

Fig. 4. Agonist activity of ACh, nicotine (*NIC*), and anabaseine compounds at the $\alpha7$ receptor subtype. Oocytes expressing $\alpha7$ nicotinic receptors were studied under two-electrode voltage-clamp conditions, for peak responses to classical and novel nicotinic agents. Responses were normalized to the responsiveness of the oocyte to 500 μM ACh applied 5 min earlier. *Bottom*, results of curve fitting with Kaleidagraph of dose-response data for DMAC, DMXB, and anabaseine. Each *point* represents the mean ± standard error of the response from four oocytes (DMAC, DMXB, and DMAB) or three oocytes (ACh, anabaseine, and nicotine).

of DMAC and DMXB (compared with ACh) at $\alpha7$ receptors was conducted (Fig. 4, *bottom*). Estimates of efficacy (I_{max}), potency (EC_{50}), and the Hill coefficient are presented in Table 2. DMAC displayed a nearly 100-fold greater potency than ACh, but with efficacy similar to that of ACh. Whereas DMXB also was a more potent agonist than ACh at $\alpha7$ receptors, its efficacy appeared to be lower than that of ACh, perhaps because of the antagonist properties of DMXB at $\alpha7$ receptors (see below). Similarly, the efficacy of DMAC may be an underestimate because of the antagonist properties of this compound, especially at high concentrations (note that the 500 μM concentration was not used in curve fitting for DMAC because of the antagonism seen at this high concentration).

Antagonist effects of anabaseine and analogs of anabaseine at $\alpha4\beta2$ receptors. Although anabaseine and its analogs may have low agonist efficacy at $\alpha4\beta2$ receptor subtypes, they may be potent inhibitors of these receptors, as shown by a decrease in responsiveness to ACh applied 5 min after the application of these compounds (Fig. 5). This inhibition may be due to receptor desensitization or some form of noncompetitive inhibition. The efficacy of these compounds in inhibiting $\alpha4\beta2$ receptor subtypes was independent of the agonist efficacy of these agents (e.g., DMAC is a very weak partial agonist but is a potent inhibitor of later ACh activation of $\alpha4\beta2$ receptors).

Antagonist effects of anabaseine and analogs of anabaseine at $\alpha7$ receptors. Anabaseine and its derivatives also displayed antagonist properties at $\alpha7$ receptors, measured as a decrease in responsiveness to ACh applied 5 min after drug application (Fig. 6). DMAC, which displayed the greatest agonist potency at $\alpha7$ receptors, also appeared to be the most potent of these agents in producing inhibition. As with $\alpha4\beta2$ receptors, however, these antagonist properties appeared to be independent of agonist properties (e.g., DMAB is a weak agonist at $\alpha7$ receptors but a stronger inhibitor of $\alpha7$ receptors). Although responsiveness to ACh after nicotine appeared to follow a biphasic dose-response relationship, only responses to 100 and 500 μM ACh differed significantly from each other ($t_6 = 3.24$, $p < 0.5$).

Agonist effects of DMAC at additional nicotinic receptor subtypes. The selectivity of DMAC as an agonist for

TABLE 2

Estimation of the parameters describing the dose-response relationship for DMAC, DMXB, and ACh at $\alpha7$ receptors

Dose-response curves were analyzed by Kaleidagraph, and estimates of potency (EC_{50}), efficacy (I_{max}), and the Hill coefficient (n) were obtained for each of these compounds as described in Materials and Methods. Standard deviations of the estimates are presented as calculated by Kaleidagraph. (The 500 μM concentration was not used in the analysis of the DMAC dose-response curve.)

	EC_{50}	I_{max}	n
	μM	normalized response	
DMAC	4.19 ± 1.76	1.19 ± 0.15	1.24 ± 0.55
DMXB	26.21 ± 6.42	0.44 ± 0.03	1.11 ± 0.23
ACh	316.29 ± 82.90	1.58 ± 0.10	0.94 ± 0.18

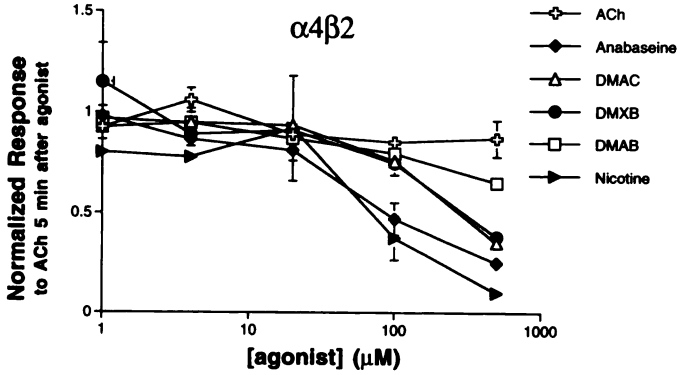

Fig. 5. Antagonist activity of ACh, nicotine, and anabaseine compounds at the $\alpha4\beta2$ receptor subtype. Oocytes expressing $\alpha4\beta2$ nicotinic receptors were studied under two-electrode voltage-clamp conditions, for peak responses to ACh (10 μM) 5 min after the application of classical and novel nicotinic agents. Responses were normalized to the responsiveness of the oocyte to 10 μM ACh applied 10 min earlier. Each *point* represents the mean ± standard error of the response from four oocytes, except for nicotine at 1 and 4 μM, where five oocytes were tested.

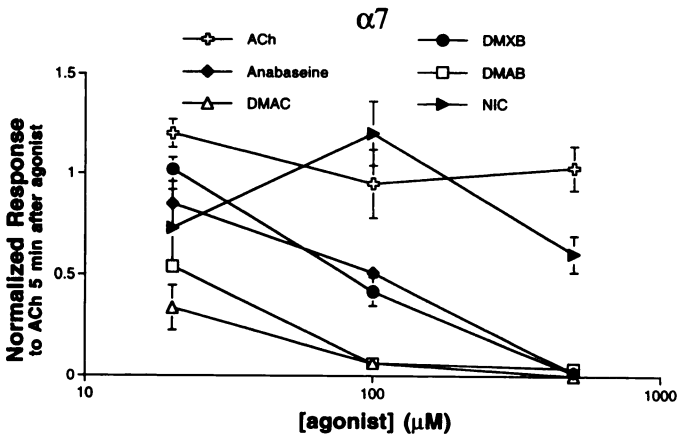

Fig. 6. Antagonist activity of ACh, nicotine (*NIC*), and anabaseine compounds at the $\alpha7$ receptor subtype. Oocytes expressing $\alpha7$ nicotinic receptors were studied under two-electrode voltage-clamp conditions, for peak responses to ACh (500 μM) 5 min after the application of classical and novel nicotinic agents. Responses were normalized to the responsiveness of the oocyte to 500 μM ACh applied 10 min earlier. Each *point* represents the mean ± standard error of the response from four oocytes (DMAC, DMXB, and DMAB) or three oocytes (ACh, anabaseine, and nicotine).

nicotinic receptors was assessed at additional receptor subtypes. Table 3 reports the responses of the $\alpha4\beta4$, $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha1\beta1\gamma\delta$ receptor subtypes to DMAC. For all of these subunit combinations, maximal responses to DMAC (1–500 μM) were less than approximately 1% of the ACh controls.

Mechanism of anabaseine- and analog-induced antagonism of $\alpha4\beta2$ receptors. Whereas DMXB application at $\alpha4\beta2$ receptors produced a potent inhibition of later activation by ACh, ACh application did not produce a similar inhibition (Fig. 5). As shown in Fig. 7, coapplication of DMXB and ACh (10 μM) enhanced the inhibition produced by DMXB alone. The observation that coapplication of ACh did not produce an attenuation of the inhibition produced by DMXB alone suggests a noncompetitive mode of antagonism.

As shown in Fig. 8, the time course for current responses to return to base-line levels was much longer for anabaseine than for ACh. Because of these different time courses for current responses, a more detailed analysis of the agonist effects of anabaseine, nicotine, and ACh was conducted. Peak normalized current produced by drug application was measured and compared with the net normalized charge produced during the 5-min period commencing with the application of drug. The ACh concentration-response relationships for normalized peak current and net charge were nearly identical (Fig. 9). For anabaseine and nicotine, however, the normalized net charges were greater than normalized peak currents, especially at high agonist concentrations.

Discussion

The data presented here clearly demonstrate that derivatives of anabaseine in general, and specifically DMAC, are selective agonists at the $\alpha7$ neuronal nicotinic ACh receptor subtype. Although the parent compound anabaseine is an agonist at many receptor subtypes, including the $\alpha4\beta2$ and neuromuscular junction subtypes (11), DMAC is highly selective as an agonist at the $\alpha7$ subtype.

The data suggest that potency in inhibiting binding might be predictive of functional (agonist) potency in oocytes, especially at the $\alpha7$/^{125}I-BTX receptor, for structurally similar compounds (e.g., anabaseine derivatives). Of the compounds tested, the one that displays the greatest potency in inhibiting ^{125}I-BTX binding, namely DMAC, is also the most potent and selective of these compounds at activating $\alpha7$ receptors, the receptor subtype thought to be associated with ^{125}I-BTX binding in the CNS. Although it is a strong agonist at $\alpha7$ receptors, DMAC displays very weak partial agonist activity at $\alpha4\beta2$ receptors and is the weakest of these agents at displacing [^{3}H]-cytisine binding. The compound that displays the greatest potency in inhibiting [^{3}H]cytisine binding, anabaseine, is the most potent of these compounds at activating $\alpha4\beta2$ receptors, the subtype thought to be associated with [^{3}H]cytisine binding in the CNS. Anabaseine is the weakest of these compounds at displacing ^{125}I-BTX and has the weakest potency but the greatest efficacy at $\alpha7$ receptors. Although these data suggest that

TABLE 3

Normalized responses to DMAC of oocytes expressing the $\alpha4\beta4$, $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha1\beta1\gamma\delta$ nicotinic receptor subtypes

Subtype	Response				
	1 μM	4 μM	20 μM	100 μM	500 μM
$\alpha4\beta4$[a]	0.000	0.003 ± 0.002	0.002 ± 0.001	0.002 ± 0.001	0.005 ± 0.001
$\alpha2\beta2$[a]	0.003 ± 0.002	0.004 ± 0.001	0.002 ± 0.001	0.007 ± 0.001	0.008 ± 0.004
$\alpha3\beta2$[a]	0.000	0.000	0.000	0.007 ± 0.003	0.010 ± 0.001
$\alpha1\beta1\gamma\delta$[b]	0.003 ± 0.003	0.008 ± 0.003	0.005 ± 0.004	0.010 ± 0.002	0.007 ± 0.001

[a] DMAC responses (means ± standard error of three oocytes) are normalized to the response of the cells to 10 μM ACh applied 5 min earlier.
[b] DMAC responses (means ± standard errors of four oocytes) are normalized to the response of the cells to 1 μM ACh applied 5 min earlier.

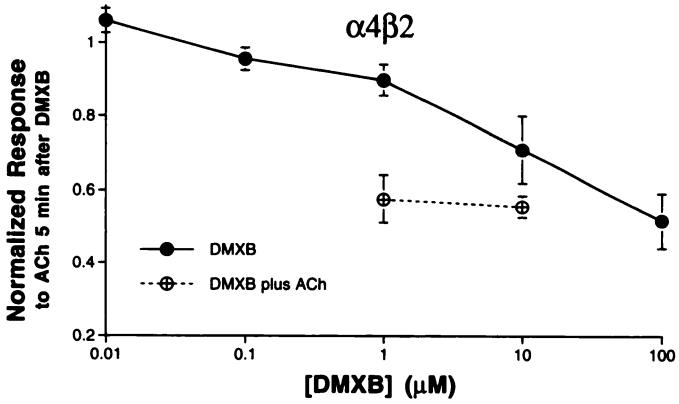

Fig. 7. Effect of coadministration of DMXB and ACh at the $\alpha4\beta2$ subtype on the antagonism of later activation by ACh. DMXB, with or without ACh (10 μM), was applied to oocytes expressing the $\alpha4\beta2$ receptor subtype, and the response to 10 μM ACh was measured 5 min later. Responses were normalized to the response of the oocyte to 10 μM ACh applied 5 min before the application of DMXB with or without ACh. Each *point* represents the mean ± standard error of the response of four oocytes. Data are from a separate series of experiments from those presented in Fig. 5.

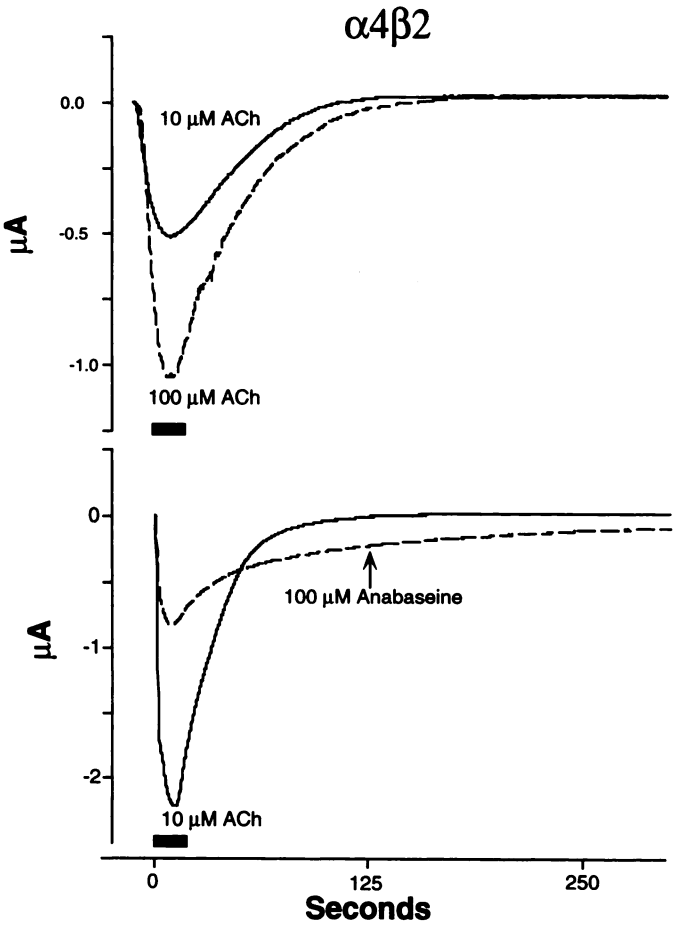

Fig. 8. Comparison of the time course of current responses to ACh or anabaseine in $\alpha4\beta2$-expressing oocytes. ACh or anabaseine was applied as a 10-sec pulse (*thick bars*), and holding current, expressed as a change in current from base-line values, was measured for a total of 5 min. *Top*, both current tracings are from the same oocyte; *bottom*, both tracings are from a second oocyte. Perfusion rate and the resulting washout rate were held constant both within and between oocytes.

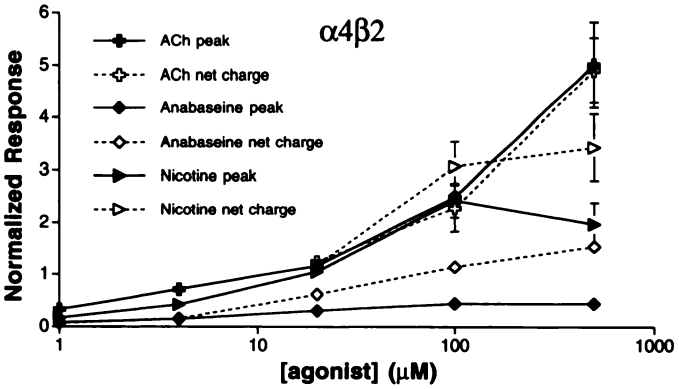

Fig. 9. Comparison of the normalized peak current and normalized net charge produced in response to ACh, nicotine, or anabaseine in $\alpha4\beta2$-expressing oocytes. Drugs were applied as a 10-sec pulse, and peak current was measured. Responses were normalized to the response of the oocyte to 10 μM ACh applied 5 min earlier. Net charge was estimated by summing the individual change from base-line currents measured every 250 msec for a total of 5 min, commencing at the time of drug application. This response was normalized to the net charge of the oocyte over 5 min in response to 10 μM ACh applied 5 min earlier. Each *point* represents the mean ± standard error of the response from four oocytes, except for nicotine at 1 and 4 μM, where five oocytes were tested.

potency in binding assays may be predictive of potency in oocytes, binding data may have little value in predicting efficacy. Inclusion of data for the classical nicotinic agonists nicotine and ACh reduces the apparent relationship between binding and functional potencies; therefore, potency in binding assays may be predictive of potency in oocytes only for structurally similar compounds.

Anabaseine derivatives, including DMXB, displace the binding of both ^{125}I-BTX and [^{3}H]cytisine. DMXB, however, does not displace the binding of the muscarinic ligand quinuclidinyl benzilate (data not shown). The potential ability of anabaseine derivatives to bind at other receptors (e.g., serotoninergic) remains to be assessed; however, our data suggest that the cytoprotective actions of DMXB can be blocked by the nicotinic antagonist mecamylamine (12).

Unlike ACh, application of anabaseine and its derivatives to oocytes expressing $\alpha4\beta2$ receptors results in oocytes that are less responsive to ACh applied 5 min later. If the mode of anabaseine derivative-induced inhibition of $\alpha4\beta2$ responsiveness to ACh were competitive, coapplication of ACh should decrease the later inhibition produced by these agents. The finding that coapplication of ACh with DMXB enhances this inhibitory effect clearly suggests that this inhibition is noncompetitive. The mechanism for enhancement, however, cannot be easily explained by these data.

The finding of a differential time course for currents to return to base-line values in $\alpha4\beta2$-expressing oocytes after application of either anabaseine or nicotine, compared with ACh, was unexpected (Figs. 8 and 9). This slow return to base-line values suggests that, after the large initial peak response usually associated with activation of these receptors by an agonist, anabaseine and nicotine can produce prolonged, albeit small, agonist activities at $\alpha4\beta2$ receptors. Such activity could be the result of sequential channel-blocking activity by anabaseine and nicotine, similar to that which has been described at neuromuscular junction nicotinic receptors (for example, see Ref. 20). In such a model, as shown below, n number of

molecules of nicotine or anabaseine (D) could bind to a closed form of the receptor (R), and their agonist activity would cause the channel to open (R*). Once the channel was open, an additional molecule of nicotine or anabaseine could bind to a site within the channel leading to a blocked form of the receptor (R′). An equilibrium between the DR*D and DR′D conformations could then produce the observed prolonged agonist activities of anabaseine and nicotine.

$$
\begin{array}{lcll}
nD + R & \leftrightarrows & D_nR & \text{closed} \\
 & & \updownarrow & \\
 & & D_nR^* + D \leftrightarrows D_nR^*D & \text{open} \\
 & & \updownarrow & \\
 & & D_nR'D & \text{blocked}
\end{array}
$$

The potential sequential channel-blocking activity of anabaseine and nicotine may provide insight into the mechanism by which coapplication of ACh produces an enhancement of the inhibition produced by DMXB alone. Our data indicate that DMXB is a weak partial agonist at $\alpha4\beta2$ receptors. If DMXB possesses channel-blocking activity, coapplication of a strong agonist such as ACh should open more channels than would be opened by DMXB alone. If DMXB then bound to more channels, there would be an enhancement of the inhibition of later responsiveness to ACh.

Further study, preferably using single-channel patch-clamp techniques, is necessary to confirm this and/or other models for the prolonged agonist activity of anabaseine and nicotine and/or the mechanism by which nicotine- and anabaseine-like compounds inhibit later responses of $\alpha4\beta2$ receptors to ACh. If these compounds are able to produce sequential channel blockade, a rethinking of the mechanism by which nicotine produces inhibition may be necessary. Specifically, some of the inhibitory properties of nicotine that have been attributed to desensitization may in fact be due to the channel-blocking activity of this compound.

The relatively high efficacy of the anabaseine compounds at the $\alpha7$ subtype makes it hard to determine whether coapplication with ACh enhances inhibition. Additionally, rapid and pronounced desensitization of $\alpha7$ receptors makes it difficult to determine whether these drugs affect the time course of currents, as is seen at $\alpha4\beta2$ receptors with anabaseine and nicotine. The use of mutant $\alpha7$ receptors that display less desensitization (21) may allow a more thorough examination of the mechanism of inhibition.

Although all of the anabaseine derivatives tested exhibit both agonist and antagonist properties at both $\alpha4\beta2$ and $\alpha7$ receptors, all are better agonists for the $\alpha7$ subtype than they are for the $\alpha4\beta2$ subtype. DMAC, the most selective agonist at $\alpha7$ receptors, displays very weak partial agonist activity not only at the $\alpha4\beta2$ subtype but also at $\alpha4\beta4$, $\alpha2\beta2$, and $\alpha3\beta2$ neuronal nicotinic receptor subtypes and at the $\alpha1\beta1\gamma\delta$ neuromuscular junction receptor (<1% of the efficacy of ACh). At least with regard to rodent nicotinic cholinergic receptors expressed in *Xenopus* oocytes, the agonist activity of DMAC is indeed highly selective for $\alpha7$ receptors. As such, DMAC may be an especially valuable tool for deciphering the functional consequences of selective $\alpha7$ receptor activation.

Historically, the $\alpha7/^{125}$I-BTX binding site in the CNS was thought to be a nonfunctional and potentially noncholinergic receptor. Evidence not only has shown this site to be cholinergic (1, 5) but also has implicated this site in the regulation of behavioral effects of nicotine (22). This receptor is also implicated in the deficit in auditory gating seen in schizophrenic patients (23). Our own evidence with anabaseine compounds suggests that the $\alpha7/^{125}$I-BTX binding site in the CNS may be important in the cytoprotective actions of nicotinic agonists after serum removal in PC-12 cells and after fimbria-fornix transections in rats (12), as well as in the memory-enhancing effects of these agents (13). The use of $\alpha7$-selective agents like DMAC may be valuable in elucidating the apparent numerous functions of this receptor.

It has been proposed that nicotine be used in the treatment of Alzheimer's disease because concentrations of [^{3}H]nicotine/[^{3}H]cytisine binding sites are consistently reduced in this disease (for example, see Ref. 24). Although the few reports on the efficacy of nicotine in treating Alzheimer's disease indicated that scores on several measures of cognitive function were improved, long term efficacy was not examined and toxic effects of nicotine were reported to limit its usefulness (25, 26). Because these toxic effects may be due to the lack of specificity of nicotine for nicotinic receptor subtypes, selective agonists may be as effective in treating the disease without producing the pronounced toxicity of nicotine. For example, treatment with agonists selective for the $\alpha4\beta2$ receptor subtype may be capable of counteracting the deleterious effects resulting from the reduction in the number of [^{3}H]nicotine ($\alpha4\beta2$) receptors without producing toxicity associated with the activation of other neuronal or peripheral receptor subtypes. Alternately, the large reduction in the number of [^{3}H]nicotine receptors might limit the usefulness of $\alpha4\beta2$-selective agents; $\alpha7$-selective agents such as anabaseine analogs may be more useful because ^{125}I-BTX binding does not appear to be affected in Alzheimer's disease (27).

Although DMAC and DMAC-like compounds might be useful for examining the properties of nicotinic $\alpha7$ receptors, compounds that possess selective agonist activity at the numerous other nicotinic subtypes that might be expressed in brain could also be very useful for deciphering the role of nicotinic systems in normal CNS function and in CNS pathologies. To date, several compounds including ABT-418 [(*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole] (28) and epibatidine (29)[2] have been reported to be selective agonists at non-$\alpha7$ receptors. The specificity of these agents for a single subtype among the numerous non-$\alpha7$ receptor subtypes, however, has yet to be demonstrated. In fact, preliminary data (not shown) suggest that epibatidine may not be selective for non-$\alpha7$ receptors and may be a full agonist at $\alpha7$ receptors.

In the studies presented here, the *Xenopus* oocyte gene expression technique has been used to try to determine at which nicotinic receptor subtypes novel agents act in the CNS. Although there is evidence that most of the [^{3}H]cytisine binding sites in brain are made up of only $\alpha4$ and $\beta2$ subunits (3, 4), reports suggest that more complex receptor subtypes that contain more than two different subunits including the so-called "silent" subunits (e.g., $\alpha5$) may exist *in vivo* (30). Another report suggests that native BTX sites in brain differ from $\alpha7$ homo-oligomeric receptors expressed in oocytes (31). Therefore, it is possible that the actions of DMAC and DMAC-like agents may be less selective in the CNS than would be predicted

[2] Personal communications with S. P. Arneric and S. W. Daly.

from oocyte studies. Experiments with native receptors will be necessary to address this possibility.

In conclusion, derivatives of anabaseine inhibit the binding of both [^{3}H]cytisine and ^{125}I-BTX. In *Xenopus* oocytes, these compounds are selective agonists at neuronal nicotinic α7 receptor subtypes and possess only very weak partial agonist activity at α4β2 subtypes. At both receptor subtypes, anabaseine-like compounds display antagonist properties that may be due to the ability of these agents to produce sequential channel blockade. Given their agonist selectivity for α7 neuronal nicotinic receptor subtypes, these compounds may be less toxic than nonselective compounds such as nicotine and therefore may be more useful in the treatment of human pathologies such as Alzheimer's disease. These compounds may also act as useful experimental probes for elucidating the function of α7 neuronal nicotinic ACh receptors.

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