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Anabaseine Is a Potent Agonist on Muscle and Neuronal *Alpha*-Bungarotoxin-Sensitive Nicotinic Receptors[1]

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ABSTRACT

We assessed the pharmacological activity of anabaseine, a toxin found in certain animal venoms, relative to nicotine and anabasine on a variety of vertebrate nicotinic receptors, using cultured cells, the *Xenopus* oocyte expression system, contractility assays with skeletal and smooth muscle strips containing nicotinic receptors and *in vivo* rat prostration assay involving direct injection into the lateral ventricle of the brain. Anabaseine stimulated every subtype of nicotinic receptor that was tested. It was the most potent frog skeletal muscle nicotinic receptor agonist. At higher concentrations it also blocked the BC^3H1 (adult mouse) muscle type receptor ion channel. The affinities of the three nicotinoid compounds for rat brain membrane *alpha*-bungarotoxin binding sites and their potencies for stimulating *Xenopus* oocyte homomeric *alpha*7 receptors, expressed in terms of their active monocation concentrations, displayed the same rank order, anabaseine>anabasine>nicotine. Although the maximum currents generated by anabaseine and anabasine at *alpha*7 receptors were equivalent to that of acetylcholine, the maximum response to nicotine was only about 65% of the acetylcholine response. At *alpha*4-*beta*2 receptors the affinities and apparent efficacies of anabaseine and anabasine were much less than that of nicotine. Anabaseine, nicotine and anabasine were nearly equipotent on sympathetic (PC12) receptors, although parasympathetic (myenteric plexus) receptors were much more sensitive to anabaseine and nicotine but less sensitive to anabasine. These differences suggest that there may be different subunit combinations in these two autonomic nicotinic receptors. The preferential interactions of anabaseine, anabasine and nicotine with different receptor subtypes provides molecular clues that should be helpful in the design of selective nicotinic agonists.

Neuronal nicotinic receptors have attracted much interest during the past few years, largely due to the discovery that the Alzheimer's brain loses many of its nicotinic receptors by the time of death, whereas muscarinic receptors are much less affected (Kellar *et al.*, 1987; Araujo *et al.*, 1988). So far, therapeutic approaches directed toward cholinergic systems in the brain have focused on stimulation of postsyaptic muscarinic cholinergic receptors, either directly with muscarinic agonists or indirectly by cholinesterase inhibition. Unfortunately these two strategies have thus far yielded only modest improvements in the cognitive functions of Alzheimer's patients. Stimulation of brain nicotinic receptors has been shown to enhance cognitive function in lower mammals (Woodruff-Pak *et al.*, 1994; Arendash *et al.*, 1995a, b; Decker *et al.*, 1995; Bjugstad *et al.*, in press), consistent with the idea that these nicotinic receptors may be potential targets treatment of Alzheimer's and other dementias (Newhouse *et al.*, 1993).

Molecular biological studies have revealed a plethora of nicotinic receptor subunits in the vertebrate brain (Papke, 1993; McGehee and Role, 1995; Lindstrom, 1996; Albuquerque *et al.*, 1997). Although there is still little understanding of the functional consequences of this receptor multiplicity, several labs are investigating the pharmacological properties of the predominant nicotinic receptor subtypes occurring in the nervous system to provide a rational basis for the design of compounds selective for particular nicotinic receptor subtypes that influence particular mental or motor functions (Decker *et al.*, 1995; de Fiebre *et al.*, 1995). Flores *et al.* (1992) have shown that the major receptor subtype displaying high nicotine, cytisine and methylcarbamyl-choline affinity in the rat brain is the alpha4-beta2 combination. A major receptor subtype showing low affinity for nicotine but high affinity for BTX contains *alpha*7 subunits (Wonnacott, 1986; Luetje *et al.*, 1990). *Alpha*7-containing receptors have been implicated in cognitive processes affected by hippocampal function, including sensory gating and spatial memory (Luntz-Leybman *et al.*, 1992; Bjugstad *et al.*, in press).

Pharmacological investigations of nicotinic receptors have been facilitated by the availability of many potent natural

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ABBREVIATIONS: ACh, acetylcholine; BTX, *alpha*-bungarotoxin; DHBE, dihydro-B-erythroidine; i.c.v., intracerebroventricular; ^{3}H-MCC; ^{3}H-methylcarbamylcholine; TC, *d*-tubocurarine; TXX, tetrodotoxin.

toxins, including curare, the erythrina alkaloids, the algal toxin anatoxin-a (Swanson and Albuquerque, 1992), the frog toxin epibatidine (Badio and Daly, 1994; Alkondon and Albuquerque, 1995), the flowering plant toxin methyllcaconitine (Ward *et al.*, 1990), and of course, nicotine. The pharmacological properties of some other potent nicotinic toxins, including leptodactyline (Erspamer, 1959) and anabaseine, have not yet been reported in much detail.

Anabaseine (fig. 1) was initially isolated from a marine worm, but has subsequently been found in certain species of ants (Kem *et al.*, 1971; Wheeler *et al.*, 1981). It is as toxic as nicotine when injected in mice (Kem *et al.*, 1976), stimulates acetylcholine release from rat brain cortical minces (Meyer *et al.*, 1987) and elevates cortical ACh and norepinephrine levels in the intact rat (Summers *et al.*, 1997). As with nicotine, anabaseine enhances passive avoidance behavior in nucleus basalis-lesioned rats (Meyer *et al.*, 1994). At the molecular level anabaseine differs from nicotine and anabasine by having a tetrahydropyridyl ring whose imine double bond is electronically conjugated with the 3-pyridyl ring (fig. 1). This causes its two rings to be approximately coplanar in their relative orientation, whereas the two rings in the tobacco alkaloids nicotine and anabasine are almost perpendicular to each other (Whidby and Seeman, 1976; Seeman, 1984).

Because some anabaseine derivatives have been shown to enhance a variety of cognitive behaviors (Meyer *et al.*, 1994; Woodruff-Pak *et al.*, 1994; Arendash *et al.*, 1995b), we examined the pharmacological properties of anabaseine on a variety of vertebrate, mostly mammalian, nicotinic receptors. To quantitatively compare anabaseine with the tobacco alkaloids, we measured the potencies and binding affinities of all three compounds on the same receptors under identical experimental conditions. Several important pharmacological differences were found to exist between the three compounds. Each displays a unique spectrum of action upon the various nicotinic receptors. Our data indicate that these compounds will be useful molecular models to design agonists selective for particular nicotinic receptors. Portions of this study were previously reported in abstract form (Kem and Papke, 1992; Kem *et al.*, 1994a).

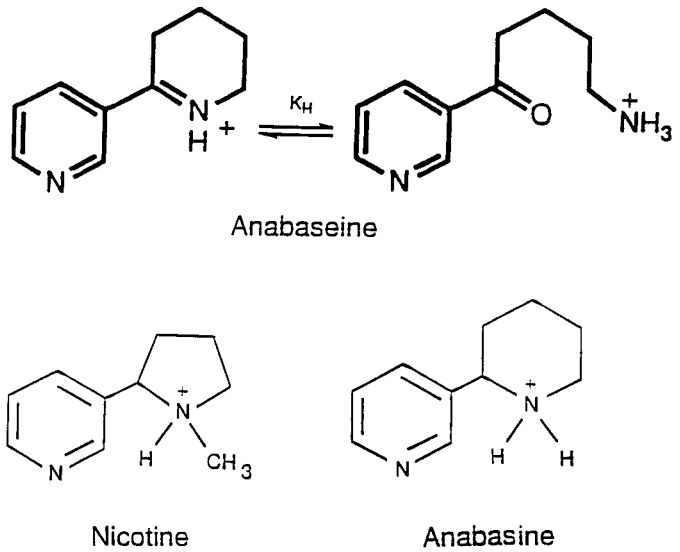

Fig. 1. Structures of the three nicotinic alkaloids. Anabaseine (A) is an animal toxin, whereas nicotine (C) and anabasine (D) are plant alkaloids. Under physiological conditions anabaseine occurs in three almost equally populated forms (Zoltewicz *et al.*, 1989; Bloom, 1990): cyclic iminium (A), cyclic imine and ammonium-ketone (B). Only the cyclic iminium form of anabaseine possesses significant nicotinic agonist activity (Kem *et al.*, 1994b; Kem *et al.*, submitted).

Materials and Methods

Chemicals. Anabaseine was synthesized according to Bloom (1990). The fully ionized, synthetic ammonium-ketone dihydrochloride salt (MW 251) was used in all of our experiments. DMAB-anabaseine dihydrochloride was synthesized as previously described (Kem, 1971; Zoltewicz *et al.*, 1989). Stock solutions of anabaseine, anabasine, nicotine and DMAB-anabaseine were kept in the dark at 5°C for a maximum of 1 wk to avoid deterioration of the alkaloids. (S)-Anabasine free base and reagents used to synthesize anabaseine were obtained from Aldrich (Nukwayjee, WI); (S)-nicotine free base, mecamylamine and other experimental drugs from Sigma Chemical Co. (St. Louis, MO); BTX and TTX from Boehringer-Mannheim (Indianapolis, IN). Radioisotopically labeled compounds, ^{3}H-MCC, ^{125}ICl and ^{86}RbCl were purchased from Du Pont-New England Nuclear (Boston, MA).

Frog skeletal muscle contractility. The two symmetrical rectus abdominis muscles of each frog (*Rana pipien*s, purchased from Nasco, Ft. Atkinson, WI) were used so that anabaseine potency relative to nicotine or anabasine could be measured on muscles from the same animal. Each muscle was mounted in a 10-ml tissue bath containing frog saline (115 mM NaCl, 5.0 mM KCl, 7.0 mM $CaCl_2$ and 2.0 mM sodium phosphate buffer, pH 7.2) which was continuously bubbled with oxygen at room temperature. The resting tension was initially adjusted to 1.0 g. After 30 min the muscles were briefly (20 sec) contracted with isotonic KCl saline (NaCl replaced with KCl) to measure the maximum isometric force of contracture. After complete recovery, each muscle was challenged with a sequence of 9 or 10 increasing concentrations of agonist until a maximum contractural force was observed. After each application the muscles were washed twice with normal saline and allowed to recover at least 30 min before the next contracture, due to the relatively slow relaxation after exposure to the three alkaloids. After the various agonist concentrations were tested, the final contractility of each muscle was again measured with isotonic KCl saline. A concentration-response curve for each muscle was constructed and expressed as a percentage of the average KCl-induced contracture force. The concentration-response data for each compound was fitted to the Hill equation using SigmaPlot and the EC_{50} and its S.E. were calculated from the computer-fitted curve.

Patch clamp experiments with neuromuscular type nicotinic receptors. BC3H-1 cells were cultured according to Covarrubias *et al.* (1989). During single channel recordings they were bathed in a saline containing 140 mM NaCl, 5.4 KCl, 10 mM NaHEPES, 1.8 mM $CaCl_2$ and 2.0 mM $MgCl_2$ titrated to pH 7.4. Single channels were recorded from cell-attached patches. The pipette saline containing agonist was otherwise identical to the bath saline. In most cases, cells were incubated for 5 to 12 min with 48 nM BTX before recordings to reduce the number of available channels in a patch. Single channel records were stored on videotape using a digital audioprocesser (20 kHz bandwidth). For computer analysis of single channel records, recordings were replayed and digitized at 50 kHz with analog filtering to yield a bandwidth of 5 kHz. Single channel events were analyzed with standard half amplitude threshold crossing methods (Auerbach and Lingle, 1987).

AChR activity was typically examined with agonist concentrations of 5 μM or higher. At such concentrations, channel openings and closings occur in groups that predominantly represent the behavior of single nicotinic receptors as they exit from relatively long-lived desensitized states (Sakmann *et al.*, 1980; Sine and Steinbach, 1984; Auerbach and Lingle, 1987) and that provide information about the true EC_{50} for half activation of current and the microscopic agonist

efficacy, without the complications of desensitization (Ogden and Colquhoun, 1985; Marshall *et al.*, 1991; Lingle *et al.*, 1992). From continuous records of channel activity, lists of channel transitions were subjected to a first pass sifting of groups of openings using an arbitrary group terminator, typically about 30 msec, to generate log-binned histograms (Sigworth and Sine, 1987). Based on the properties of closed intervals distributions, groups were then reselected with a new group terminator value. Group terminators were at least three to five times the closed interval identified as an activation closure (see "Results"), except at 5 μM where the group terminator was only 2-fold the activation closure. Group terminator values were 20 msec or longer for 50, 100 or 200 μM anabaseine, 50 or 100 msec for 20 μM anabaseine, and 100 msec for 5 or 10 μM anabaseine. For analysis of channel blockade by anabaseine, a simple and approximate two-state missed events procedure (Blatz and Magleby, 1986) was selected that only uses the fast component of closures and the longer open interval durations.

The probability of being open within a cluster was determined directly from the closed and open interval durations and the numbers of each component in the histograms (Ogden and Colquhoun, 1985; Marshall *et al.*, 1991). Closures longer than the primary activation closure were considered to separate clusters and the putative blocking gap was also excluded from the p_o determination. All transmembrane potentials were calculated from the single channel current and the measured single channel conductance of 39 pA, assuming a reversal potential of 0 mV.

Xenopus oocyte expression and functional analysis of rat brain nicotinic receptors. Preparation of *in vitro* synthesized cRNA transcripts and oocyte injection have been described previously (de Fiebre *et al.*, 1995). Recordings were made 2 to 7 days after injections. Oocytes were placed in a Lucite recording chamber with a 0.6 ml total volume and perfused at room temperature with frog saline (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3) containing 1 μM atropine to block potential muscarinic responses. Calcium-activated chloride channels were not suppressed in our experiments, because their functional presence does not affect the agonist concentration-response relation (Papke *et al.*, 1977a). Drugs were diluted in perfusion solution and then applied after preloading of a 2.0 ml length of tubing at the terminus of the perfusion system. A Mariotte flask filled with Ringer saline was used to maintain a constant hydrostatic pressure for drug deliveries and washes. The rate (6 ml/min) of drug delivery was constant for all compound concentrations and receptor subtypes. Current responses to drug administration were measured using a two electrode voltage clamp with a holding potential of -50 mV. Recordings were made using a Warner Instruments oocyte amplifier interfaced with National Instruments LabView software.

Current electrodes were filled with 250 mM CsCl, 250 mM CsF and 100 mM EGTA, pH 7.3 and had resistances of 0.5 to 2.0 megaohms. Voltage electrodes were filled with 3 M KCl and had resistances of 1 to 3 megaohms. Only oocytes with resting membrane potentials more negative than -30 mV were used. Responses were normalized for the level of channel expression in each individual cell by measuring the response of the oocyte to an initial control ACh application 5 min before presentation of experimental solutions. The control ACh concentrations were 10 μM for *alpha*4-*beta*2 receptors and 500 μM for *alpha*7 receptors. Receptors expressed from *alpha*-7 cDNA often display an increased responsiveness after an initial application of agonist that subsequently stabilizes (de Fiebre *et al.*, 1995). Therefore, all *alpha*7 expressing oocytes received two control applications of ACh separated by 5 min at the start of recording. The second ACh response was then used to normalize the experimental response. For each experimental concentration the mean current response and S.E. were calculated from the normalized responses of at least four oocytes.

After the application of experimental drug solutions, the cells were washed with control Ringer's solution for 5 min and then evaluated for potential inhibition and response stability by measuring the oocyte response to another application of the ACh (control) solution. These second control responses were normalized to the initial ACh control responses measured 10 min earlier. If the second ACh response showed a difference of $> 25\%$ of the initial ACh control response the oocyte was not used for further analysis. If the postexperimental ACh control was within 75% of the preexperiment control ACh response, the second ACh application was allowed to serve as the control response to normalize the response of any subsequent experimental application.

Radioligand binding to nicotinic receptors. The steady-state binding of the three nicotinic compounds to neuromuscular type receptors was measured indirectly by assessing their ability to inhibit the rate of ^{125}I-BTX binding to *Torpedo californica* membranes prepared according to Eldefrawi *et al.* (1980).

The ability of anabaseine to displace the specific binding of 5 nM ^{3}H-MCC to rat cerebral cortex synaptosomal membranes was used to assess the ability of anabaseine and related nicotinoid compounds to bind to high nicotine affinity neuronal receptors, which in the rat are primarily of the *alpha*4-*beta*2 subtype (Flores *et al.*, 1992). The methods of Boksa and Quirion (1987) were followed with only minor modifications. Binding of ^{125}I-BTX (1 nM, 119 Ci/mmol) to rat brain membranes was performed in a total volume of 0.25 ml, essentially as described by Marks and Collins (1982). Membranes (0.4 mg protein per sample) were incubated with the radioligand at 37°C for 3 hr in the buffer indicated above, which also contained 2 mg/ml BSA. Displacement curves were analyzed by EBDA (Ligand) and K_i values were calculated using the Cheng-Prusoff (1973) equation.

Measurement of ganglionic nicotinic receptor activation. Rat pheochromocytoma (PC 12) cells grown in the absence of nerve growth factor on polylysine-coated plastic culture dishes were loaded overnight with ^{86}Rb preceding the efflux assays, which were carried out essentially as described by Lukas and Cullen (1988) at pH 7.4. After washing away extracellular rubidium three times, the agonist (in saline containing 10 μM atropine sulfate to inhibit muscarinic receptors and 0.5 mM ouabain to prevent rubidium reuptake by active transport) was added and 1 min later the released rubidium was removed for gamma counting. The rubidium efflux during agonist stimulation was expressed as a percent of total cellular rubidium released by 1 mM carbachol during the same time. All efflux estimates were corrected for spontaneous efflux in the absence of agonist. The amount of ^{86}Rb remaining after the 1 min test period in each cell sample was determined after exposure to 1.0 M NaOH for at least 1 hr. All measurements were done in quadruplicate.

A rat colon (Romano, 1981) preparation was used to assess the agonist activity of anabaseine. Longitudinal muscle strips with intact myenteric plexus were suspended in Tyrode saline (pH 7.4) and aerated with a continuous stream of oxygen bubbles. Each muscle was initially adjusted to a length which maintained a resting tension of 0.5g. Isometric contractions were recorded with a Grass FT.03 force displacement transducer connected to a Grass model 7 polygraph.

i.c.v. administration of anabaseine. When injected i.c.v. with a nicotinic agonist, rats rapidly become immobile with extended legs (Abood *et al.*, 1981). This prostration response was used to compare the central activity of anabaseine relative to nicotine. After implantation of a metal cannula into the third ventricle, 5 days were allowed for recovery from surgery. The rat received injections with 2 or 4 μl of the experimental compound dissolved in sterile 0.9% NaCl solution, pH 6.5. Prostration was judged to have occurred when all four legs of the Sprague-Dawley male rat (250–300 g) remained laterally extended for at least 10 sec. To detect prostration each rat was observed for at least 5 min after injection. All rats that were not prostrated after injection were killed after an additional i.c.v. injection of Evan's blue dye to ascertain that the cannula was operational; the result was not used if the dye was absent from the lateral ventricular space.

Results

Activation of frog neuromuscular nicotinic receptors by anabaseine. Anabaseine acted as a potent nicotinic agonist on frog rectus abdominis muscle. A wide variety of natural toxins and synthetic compounds have previously been tested on this preparation, which facilitated quantitative comparison of anabaseine with these other substances (table 1). When the median effective concentrations of the active, monocationic forms of each compound were compared, anabaseine was only 14- and 3.7-fold less potent, respectively, than epibatidine and anatoxin-a, which are the most potent nicotinic agonists thus far reported. Nicotine was 6.7-fold and cytisine 23-fold less potent than anabaseine.

The stimulatory action of anabaseine was competitively antagonized by the reversible nicotinic antagonist *d*-tubocurarine (fig. 2A) but noncompetitively antagonized by BTX, which due its extremely tight binding, acts essentially irreversibly on skeletal muscle type nicotinic receptors (fig. 2B). At higher concentrations both antagonists completely inhibited the effects of micromolar concentrations of anabaseine, which is consistent with the hypothesis that anabaseine action on the muscle membrane is mediated entirely through nicotinic receptors. The concentration-response curves for anabaseine, anabasine and nicotine are shown in figure 3. The contractures generated by all three of these weakly basic compounds were slow in onset as well as in reversal compared with carbamylcholine (results not shown).

The neuromuscular agonist potency of anabaseine on frog rectus abdominis muscles is compared with those of other naturally occurring nicotinic agonists in table 1. The estimated potency of the monocationic form of each agonist was calculated assuming that its nicotinic stimulatory potency was entirely due to its monocationic form. Much data support this assumption for nicotine (Barlow and Hamilton, 1962; Bartels and Podleski, 1964), anabaseine (Kem *et al.*, 1994b; Kem *et al.*, in preparation) and other nicotinic agonists. The cyclic iminium form of anabaseine was calculated to be approximately 7X more potent than monocationic nicotine.

TABLE 1

Relative potencies of nicotinic agonists on the frog rectus abdominis muscle

Compound	EC_{50} (μM)	pK_a	EC_{50I} (nM)	$EC_{50}Carb/EC_{50I}$
Epibatidine	0.018[a]	9.3[b]	0.018	410
(+)-Anatoxin-a	0.067[c]	9.3	0.066	112
Leptodactyline	0.12[d]	None	0.12	62
Anabaseine	0.74	NA[e]	0.25	30
Acetylcholine	0.53[c]	None	0.53	14
(S)-Nicotine	1.96	7.9[f]	1.63	4.5
Cytisine	6.70[c]	7.9[g]	5.56	1.3
Carbamylcholine	7.38	None	7.38	1.0
(S)-Anabasine	7.05	8.7[f]	6.83	0.93

In the column on the far right the potencies are calculated assuming that the mono-cationic (I) form of each compound is solely active. The EC_{50} values are calculated for a pH of 7.2.

[a] Bonhaus *et al.* (1995).

[b] Assumed value, in analogy with anatoxin.

[c] EC_{50} compared with Carb (Gund and Spivak, 1991) but using Carb EC_{50} determined in this article.

[d] EC_{50} compared with Nic (Barlow *et al.*, 1969) but using the nicotine EC_{50} determined in this paper.

[e] NA, Not applicable. The pK_a of anabaseine is insufficient for estimating the concentration of the cyclic iminium form of anabaseine, which was 34% of total anabaseine at pH 7.2 (Zoltewicz *et al.*, 1989).

[f] Yamamoto *et al.* (1962).

[g] Barlow and McLeod (1969).

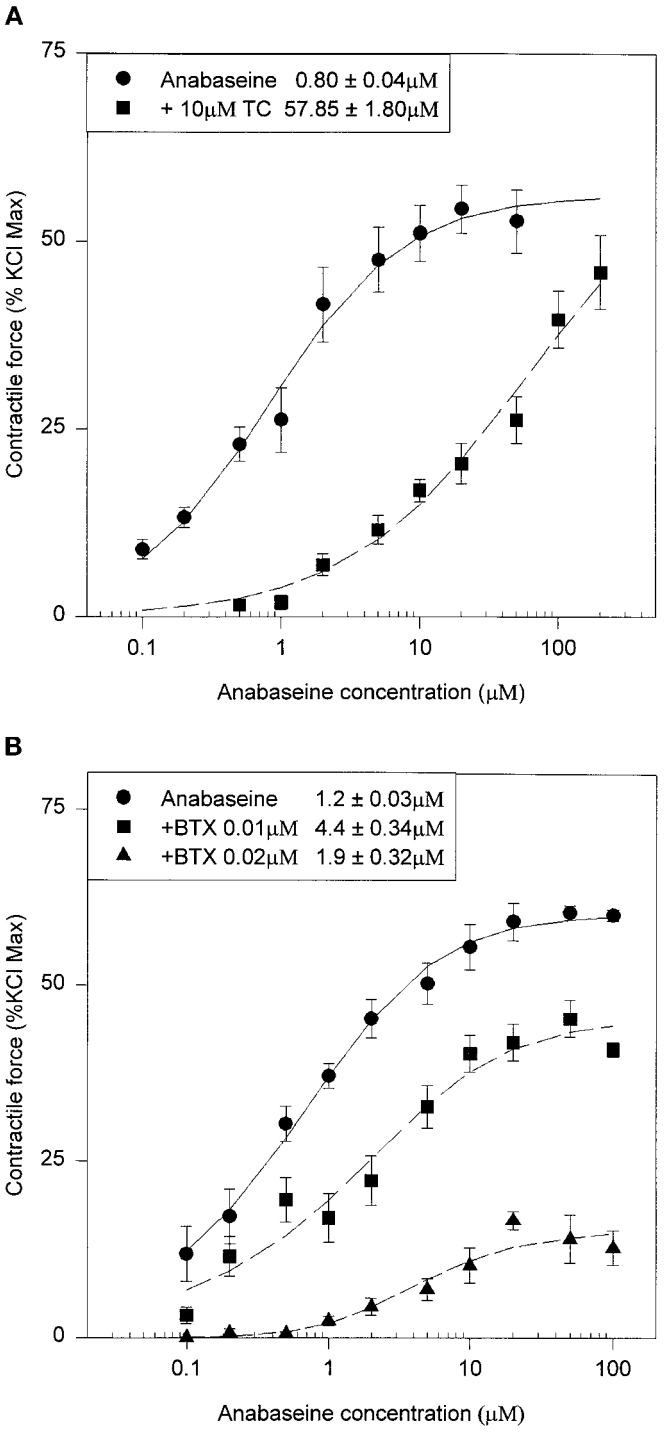

Fig. 2. Ability of muscle nicotinic receptor antagonists to affect the contracting action of anabaseine. (A) The reversible antagonist *d*-tubocurarine (10 μM) shifts the anabaseine concentration-response curve to the right. $n = 6$ muscles per point, except the lowest two concentrations of anabaseine alone, where $n = 4$. The EC_{50} for anabaseine in the presence of TC was calculated assuming that the maximum contractile response was the same as when anabaseine was applied alone. (B) BTX (30 minutes initial exposure) irreversibly reduces the response at all anabaseine concentrations. $n = 12$ muscles per point for anabaseine alone and $n = 6$ for points with BTX.

Interaction of nicotinoid compounds with electric fish muscle nicotinic receptors. The relative abilities of

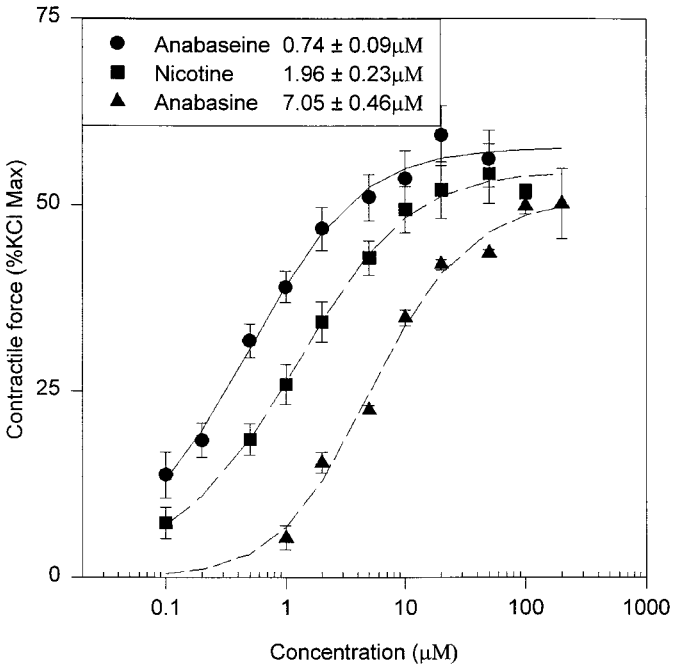

Fig. 3. Relative actions of anabaseine, anabasine and nicotine upon frog rectus abdominis muscle at pH 7.2 For anabaseine, $n = 8$ muscles per point; for nicotine, $n = 6$ muscles per point except the highest concentration where $n = 4$; for anabasine, $n = 6$ except for the two highest concentrations where $n = 4$.

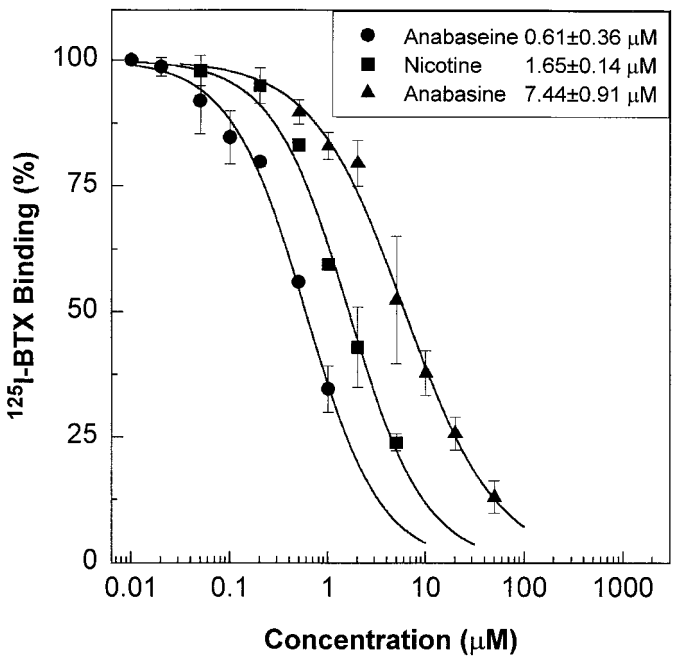

Fig. 4. Nicotinoid displacement of ^{125}I-BTX binding to *Torpedo* electric organ membranes. Membranes containing 10 μg protein by Lowry assay were preincubated 15 min with anabaseine in buffer (50 mM Tris-HCl, pH 7.4) containing 2 mg/ml bovine serum albumin and then incubated for 1 hr with ^{125}I-BTX in a final volume of .25 ml. Nonspecific binding was measured in the presence of 1 mM nicotine. After incubation samples were diluted with 1.2 ml of ice cold buffer and bound radioligand was separated from free ligand by filtration under vacuum through glass fiber filters (Whatman GF/C) at 4°C. The filters were presoaked for 15 min in 0.5% (v/v) polyethyleneimine containing 0.25 mg/ml BSA and washed with 2.5 ml buffer before filtration. The membranes were washed twice on the filters with 4 ml of ice cold buffer and then counted with a Biogamma counter. Each point is the average of triplicate measurements. Data were fitted with EBDA software (Ligand).

the three nicotinoid compounds to inhibit ^{125}I-BTX binding to *Torpedo* electric organ membranes are shown in figure 4. Anabaseine displayed the highest affinity of the three compounds although anabasine displayed the lowest affinity (table 2). The relative K_d for the electric fish muscle were very similar with the frog rectus muscle potency (EC_{50}) estimates shown in table 3.

Activation of mammalian neuromuscular nicotinic receptors by anabaseine. The ability of anabaseine to activate mouse embryonic neuromuscular type nicotinic receptors was examined using cell-attached single channel recordings from the clonal BC3H-1 cell line. The single channel conductance in the presence of anabaseine was indistinguishable from that obtained using ACh. As reported for other nicotinic agonists (Colquhoun and Sakmann, 1985; Sine and Steinbach, 1986; Papke *et al.*, 1988), a low anabaseine concentration (40 nM) caused two types of open channel activity: short duration (<500 μsec) bursts resulting primarily from single brief openings and long duration (>3 msec) bursts that were often interrupted by brief closures (results not shown). Histograms of burst durations revealed two components. The average duration of the longer component was somewhat shorter than for bursts activated by ACh, although the voltage-dependence of the burst durations was similar for both agonists. In some cases three components better described the distribution of burst durations, a characteristic also noted for bursts activated by ACh (Colquhoun and Sakmann, 1985).

To better compare the relative effectiveness of anabaseine and ACh as agonists, the behavior of nicotinic receptors activated by 5 μM or higher anabaseine was then examined. The appearance of groupings of channel openings and closings activated by either ACh or anabaseine is shown in figure 5 for three concentrations of each agonist. For both agonists, as the concentration of the agonist increased, the average duration of closures within the periods of activity became shorter and the average time the channel is open within clusters increased. Typically, open interval histograms were best fit with two exponential components, although at the highest anabaseine concentrations only a single open interval component was observed. Closed interval histograms

TABLE 2

Relative affinity (K_i) estimates for anabaseine, anabasine and nicotine on different vertebrate nicotinic receptors, expressed in terms of the active monocationic species

Receptor Type	$K_i \pm$ S.E. (μM)		
	Anabaseine	Nicotine	Anabasine
Skeletal muscle[a] (Fish)	0.21 ± 0.12	1.4 ± 0.12	7.2 ± 0.88
Alpha7[b] (Rat)	0.058 ± 0.007	0.40 ± 0.035	0.058 ± 0.027
Alpha4-Beta2[c] (Rat)	0.032 ± 0.004	0.0041 ± 0.001	0.26 ± 0.059

All three binding assays were done at pH 7.4, where the monocationic concentrations were: anabaseine, 29% of total; nicotine, 76% of total; anabasine, 95% of total.

[a] *Torpedo* electric organ values are IC_{50}s, since inhibition of the rate of irreversible binding of ^{125}I-BTX was measured.

[b] K_is were determined from monocationic IC_{50}s (fig. 10) using a ^{125}I-BTX K_i of 0.4 nM, calculated from a Scatchard plot (data not shown).

[c] K_is were determined from monocationic IC_{50}s (fig. 12) using a ^{3}H-MCC K_i of 11 nM obtained from the data shown in figure 11.

TABLE 3
Relative potency estimates for anabaseine, anabasine and nicotine on different vertebrate nicotinic receptors

Receptor Type	$EC_{50} \pm$ S.E. (μM)		
	Anabaseine	Nicotine	Anabasine
Skeletal muscle[a] (Frog)	0.25 ± 0.03	1.63 ± 0.19	6.83 ± 0.44
Alpha7[b] (Rat)	6.7 ± 0.55	47 ± 6.4	16.8 ± 3.8
Alpha4-Beta2[b] (Rat)	4.2 ± 1.3	14 ± 7.1	
Chromaffin cell[c] (Rat PC12)	21 ± 1.6	22 ± 5.0	34 ± 6.1
Parasym.ganglion[c] (Rat Colon)	0.66 ± 0.03	0.89 ± 0.06	9.9[d]

The EC_{50} values are expressed in terms of the monocation concentration of each agonist.
[a] The frog muscle assays were done at pH 7.2, where the monocationic concentrations were: anabaseine, 34% of total; nicotine, 72% of total; anabasine, 97% of total.
[b] These *Xenopus* expression assays were done at pH 7.3, where the monocationic concentrations were anabaseine, 32% of total concentration; nicotine, 76% of total; anabasine, 95% of total concentration.
[c] These assays were done at pH 7.4, where the monocationic concentrations were: anabaseine, 29% of total; nicotine, 76% of total; anabasine, 95% of total.
[d] Value reported by Romano (1981).

were best described by four or five components. Examination of closed interval distributions revealed one component that occurred with a frequency of about 0.08 to 0.11 per msec open time. This component became shorter in duration with increases in anabaseine concentration and is therefore a strong candidate for a closure reflecting reopenings of a channel from closed states directly leading to channel opening. We term this an activation closure. Support for this idea arises from the fact that the average time between such closures is comparable to the mean burst duration observed at low agonist concentrations. The time constant of the activation closure observed in these histograms was as follows (at -80 to -100 mV): 5 μM: 5.9 ± 1.2 msec (mean S.D.), $n = 4$ patches; 10 μM: 27.8 ± 3.1 msec, $n = 5$; 20 μM: 1.2 ± 3.6 msec, $n = 8$; 50 μM: 3.9 ± 1.9 msec, $n = 7$; 100 μM 2.4 ± .9 msec, $n = 6$; 200 μM: 0.8 ± 0.5 msec, $n = 5$. Closed histograms in many cases contained components at particular agonist concentrations that would be likely to overlap or contaminate these activation closures. However, such additional components in all cases occurred at frequencies considerably less than 0.05 per msec open time. Thus, although these additional closures may bias the properties of the component arising primarily from activation closures, they only minimally alter our estimates of duration and frequency of the activation closures.

A notable characteristic of the openings activated by anabaseine is that, as anabaseine concentration is increased, there is a shortening of the apparent time a channel stays open before closing and an increase in frequency of the short lived closure. This short-lived closure of about 80 to 120 μsec duration was the primary component in the closed interval histograms particularly at 20 μM anabaseine and higher. A short-lived closure of similar duration was also observed at 40 nM anabaseine, but with a frequency of only 0.07 ± 0.04 per msec open time. From the total open time in a record and the number of detected short gaps, a blockage frequency plot (see Ogden and Colquhoun, 1985) was generated (fig. 6A), which possessed a slope of 1.2×10^7 M^{-1} sec^{-1}. This linear dependence of the short gap frequency on anabaseine concentration is consistent with simple channel block behavior, as described for numerous channel blockers (Ogden and Colquhoun, 1985; Marshall *et al.*, 1991). Figure 6B shows the dependence of open interval duration and duration of the fast gap on anabaseine concentration. Values in figure 6B were corrected for missed events. The fit of a simple blocking model to the open interval durations yielded a drug blocking rate of 1.8×10^7 M^{-1} sec^{-1}. Considering the correction for missed events in the latter case, this blocking rate is comparable to that from analysis of the blocking gap frequency. The duration (fig. 6C) of the corrected blocking gap increased with membrane hyperpolarization (e-fold per 66 mV), although the frequency per unit open time of the fast gap (fig. 6D) exhibited only a slight increase (e-fold per 150 mV) with hyperpolarization. Analysis of the voltage-dependence of open interval durations also indicates that the forward rate of blockade is less voltage-dependent than the unblocking rate.

The relative effectiveness of ACh and anabaseine as agonists was examined by comparing cluster p_o values over a similar concentration range. Cluster p_os were determined as described in "Materials and Methods," based on identification of the activation closure described above. After correction for the occurrence of channel block, 20 μM anabaseine resulted in a p_o of 0.59 ± 0.12 (eight patches). In comparison, 20 μM ACh resulted in a cluster p_o of 0.90 ± 0.02 (eight patches). Estimates of p_o for multiple patches over a range of anabaseine concentrations are plotted in figure 7. A fit using the modified Hill equation (see fig. 7 legend) to the p_o values suggests that the limiting p_o for anabaseine is about 0.75 ± 0.08 (± 90% confidence limit) with half-activation at 9.6 ± 2.3 μM. The EC_{50} with ACh was somewhat less than 5 μM (not shown). The limiting p_o value qualitatively indicates that the efficacy of anabaseine in opening this channel is somewhat less than for ACh. Taking into consideration that only 29% of the anabaseine molecules are in the active cyclic iminium form at pH 7.4, the potency (EC_{50}) of the anabaseine cyclic iminium for activating the receptor was about twice that of ACh.

Rat brain neuronal nicotinic receptors: *Xenopus* oocyte experiments. At *alpha*7 receptors anabaseine and anabasine displayed very similar efficacies, although nicotine was significantly less efficacious (fig. 8A). Anabaseine and anabasine displayed the highest potencies for homomeric *alpha*7 receptor. The concentration dependence of recovery in responsiveness to ACh to each compound displayed the same concentration dependence as its agonist activity (fig. 8B).

Anabaseine and anabasine were only weak partial agonists upon the *alpha*4-*beta*2 receptor, displaying 8 and 4%, respectively, of the maximal current elicited by ACh (fig. 9). Nicotine displayed a much higher efficacy at this receptor. The EC_{50}s of the three nicotinoid compounds, corrected for their differing degrees of ionization, are presented in table 3. It was previously reported that anabaseine-activated currents in the oocyte were prolonged in comparison with ACh, in their rates of activation and desensitization (de Fiebre *et al.*, 1995). The anabasine and nicotine responses observed in this study also were prolonged relative to those generated by ACh.

Rat brain neuronal nicotinic receptors: radioligand binding experiments. Next we examined the ability of anabaseine, anabasine and nicotine to bind to rat brain neuronal receptors using two radioligand binding assays, involv-

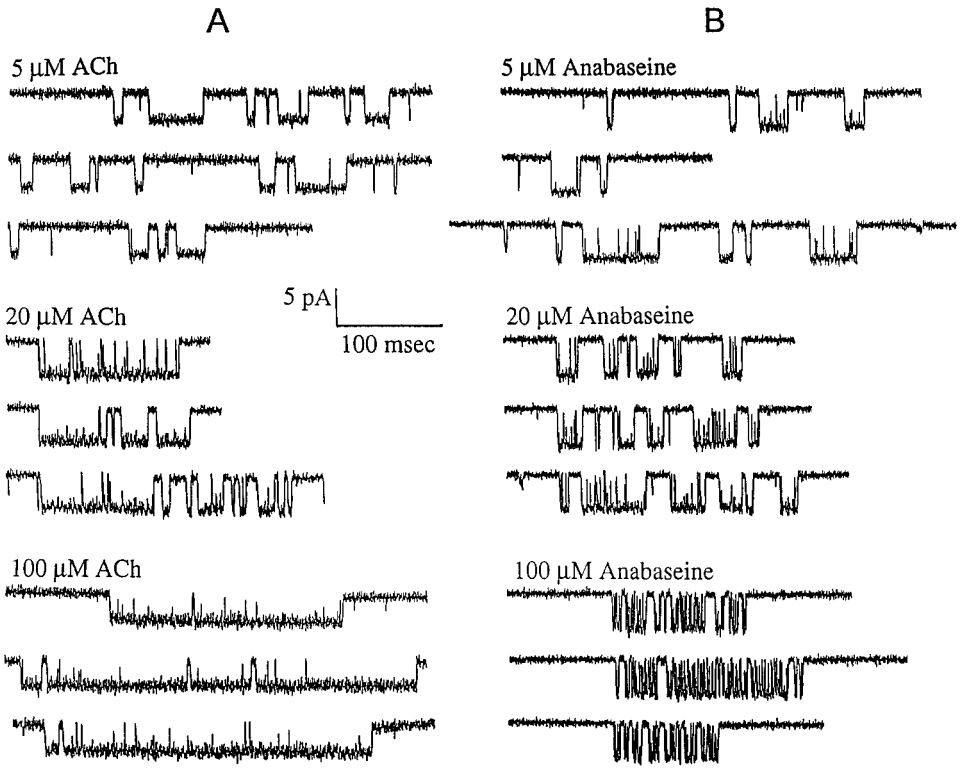

Fig. 5. Nicotinic agonist activity of anabaseine and ACh upon BC3H-1 cells. Data were recorded using the cell-attached voltage clamp method. Groupings of openings activated by either ACh (A) or anabaseine (B) are shown. Groups for analysis were selected as defined in "Materials and Methods." Qualitatively, closed intervals within groups of openings become shorter with increases in agonist concentration. With anabaseine, open intervals become shorter with increases in agonist concentration as a result of channel block by anabaseine, and an increase in frequency of a short duration gap is apparent.

ing displacement of ^{125}I-BTX and ^{3}H-MCC binding. Displacement of the first radioligand predominantly measures interaction with *alpha*7-containing receptors although displacement of the second one measures *alpha*4-*beta*2 receptor binding (Flores *et al.*, 1992).

The relative abilities of the three compounds to displace ^{125}I-BTX binding from brain receptors are shown in figure 10. When the K_i of anabaseine was expressed in terms of its cyclic iminium form the affinity of anabaseine for these sites was nearly twice that of anabasine and seven times that of nicotine (table 2). The displacement data were well fitted by the EBDA single binding site model.

Anabaseine previously was reported to displace the binding of tritiated cytisine to rat brain membranes (Meyer *et al.*, 1994). However, the nature of this inhibition was not determined. We investigated the effect of anabaseine on specific binding of ^{3}H-MCC to rat brain high nicotine affinity receptors. Scatchard analysis of ^{3}H-MCC binding in the absence and presence of anabaseine indicated that competitive inhibition occurred, as the slope decreased in the presence of anabaseine while the Bmax was not affected (fig. 11).

Our next goal was to assess the affinity of anabaseine, relative to the two tobacco alkaloids. The displacement curves are shown in figure 12 and the K_is for the monocationic forms are expressed in table 2. The K_i for nicotine displacement of ^{3}H-MCC from the *alpha*4-*beta*2 receptor subtype was about 100X less than for the neuronal BTX binding site, although the affinities of anabaseine and anabasine for these two major brain receptors were very similar.

Anabaseine stimulation of autonomic nicotinic receptors. All three nicotinic agonists acted as high efficacy nicotinic receptor agonists on PC12 cells (table 3). The major ganglionic nicotinic receptor (Rogers *et al.*, 1992) in this transformed cell line consists of *alpha*3 and *beta*4 subunits, and was previously shown to possess relatively low affinity for nicotine and ACh in functional assays (Lukas and Cullen, 1988; Lukas, 1989; Wong *et al.*, 1995). Because maximum nicotinic stimulation of the cells by the three alkaloids and carbamylcholine caused the release of only a small (usually less than 10%) fraction of the internal rubidium, our efflux measurements with this ion should reflect the average extent of receptor activation over the time (1 min) of the measurement. All three compounds acted as full agonists relative to carbachol (concentration-response curves not shown). When concentration was expressed in terms of the active cationic form of each compound, the potencies of the three nicotinoid compounds were very similar (table 3).

In terms of monocationic concentration, the rat colon relaxing potency of anabaseine was very similar to that of nicotine, but significantly greater than that of anabasine (table 3). The action of anabaseine on this preparation was non-competitively inhibited by tetrodotoxin, as would be expected if it acts by exciting myenteric plexus neurons (fig. 13).

Intracerebroventricular administration of anabaseine: rat prostration experiments. On a mole basis, anabaseine was approximately 2.7-fold less potent than nicotine in causing prostration when administered into the lateral ventricle of the unanesthetized rat (fig. 14). If only the monocationic forms of the two compounds are active, this equipotent mole ratio would become nearly one. Nicotine was less potent in our experiments than was previously reported by Abood *et al.* (1981). This may have been due to our use of a slightly more stringent behavioral endpoint for assessing prostration, as described in the Methods section. Mecamylamine and DHBE both inhibited the prostrating action of anabaseine. A large dose (80 μg) of DMAB-anabaseine failed to prostrate rats but did partially inhibit the prostrating action of nicotine.

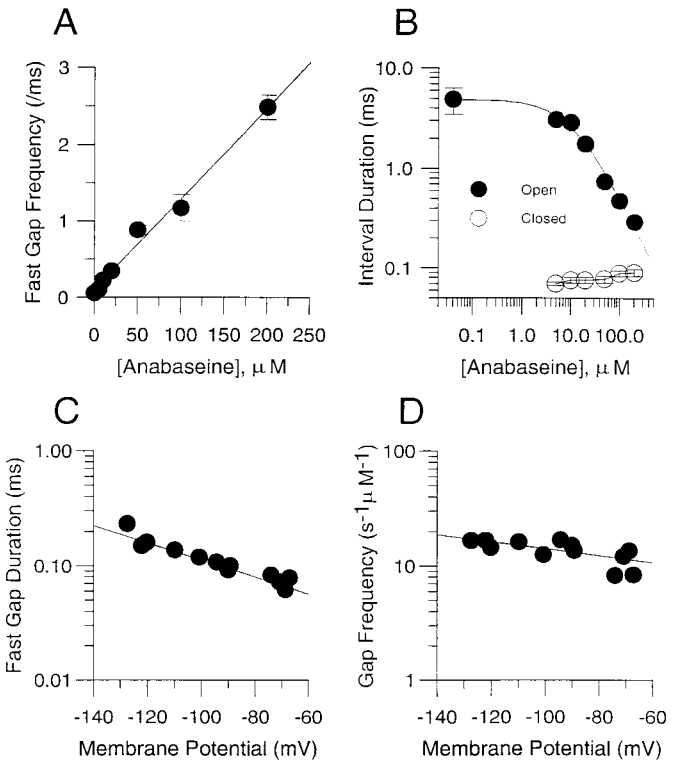

Fig. 6. Rapid channel block of BC3H-1 receptor channels by anabaseine. In A, the frequency of occurrence (events per msec open time) of a closed interval component of duration of about 100 msec is plotted as a function of anabaseine concentration. Patches with calculated membrane potential between -75 and -100 were included in this analysis. Error bars are S.E. for 6, 4, 7, 7, 9, 8 and 5 files for 0.04, 5, 10, 20, 50, 100 and 200 μM, respectively. The fitted line has a slope of 1.2×10^7 M^{-1} sec^{-1}, with intercept of 102 $\pm$ 88/s. In B, the blocking gap duration (open circles) and longer open time component (filled circles) are plotted as a function of anabaseine concentration. Error bars are S.E.s. Values were corrected for missed events. The open durations were fit with t([Anabaseine])= 1/(a * f[Anabaseine]), where a is the channel closing rate at low [Anabaseine]. The fitted values were a = 210 $\pm$ 10 sec^{-1}, with f = 1.8 ($\pm$ .3) $\times$ 10^7 M^{-1} sec^{-1}. In C, the duration of fast gaps corrected for missed events is plotted as a function of membrane potential. Only values obtained with 100 μM anabaseine were included in both C and D. The fitted line is described by $t_c(V) = t_c(0)^* e^{(A^*V)}$ with $t_c(0)$ = 0.02 $\pm$ .01 msec and A = -0.017 $\pm$ 0.003/mV. In D, the frequency of fast gaps scaled by the anabaseine concentration (100 μM) is plotted as a function of membrane potential. The 0-voltage frequency was 7.13 $\pm$ 2.56 sec^{-1} mM^{-1}, with a voltage-dependence of -0.007 $\pm$ 0.003/mV.

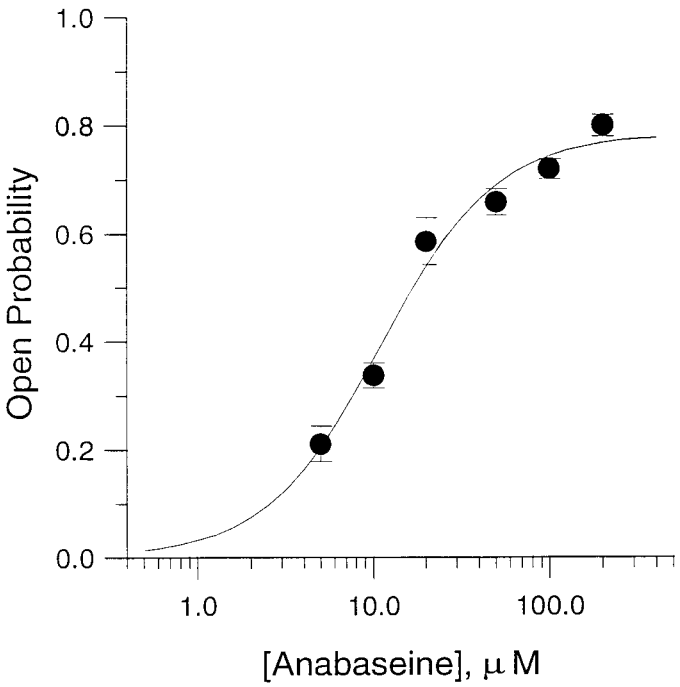

Fig. 7. Dependence of BC3H-1 nicotinic receptor channel cluster open probability on anabaseine. Channel open probability was calculated from the interval distributions from selected groups as described in "Materials and Methods." Closures attributed to channel block or short-lived desensitized states were excluded by this method. Each point is the mean of 4, 5, 8, 7, 6, and 5 values for 5, 10, 20, 50, 100 and 200 μM anabaseine, respectively, with error bars showing the S.E. The points were fit to $p_o([Anabaseine]) = p_o(max)/(1+(EC_{50}/[Anabaseine])^n)$ with $p_o(max)$ = 0.75 $\pm$ 0.08, EC_{50} = 9.6 $\pm$ 2.3, and n = 1.6 $\pm$ .5.

Discussion

Anabaseine selectively stimulates nicotinic receptors. Although anabaseine stimulated all of the nicotinic receptor preparations that we investigated, its high potency upon neuromuscular and *alpha*7 nicotinic receptors is particularly noteworthy. On the skeletal muscle membrane, anabaseine appears to work entirely on nicotinic receptors, because its action could be completely blocked by the insurmountable antagonist BTX. Nerve action potentials were unaffected by this compound, even at millimolar concentrations (Kem, 1971). However, an effect at nerve terminals cannot yet be ruled out, as some mammalian motoneuron terminals seem to possess nicotinic receptors. Anabaseine affected neither rat brain muscarinic receptors nor plasma cholinesterase, except at very high concentrations (>100 μM) where nonspecific membrane effects often occur (Kem *et al.*, 1994c). Stimulation of the rat brain 5-HT_3 receptor, which possesses a subunit sequence homologous with nicotinic receptor subunits, was only inhibited by 24% in the presence of 100 μM anabaseine (Machu *et al.*, 1996). Thus, anabaseine is expected to selectively act on nicotinic receptors at concentrations of less than 100 μM.

Anabaseine actions upon single neuromuscular channels. The results show that the characteristics of openings and groups of openings activated by anabaseine at both low and high concentrations share many similarities to properties of openings activated by ACh. Based on the total concentration (~10 μM) of anabaseine at which a half maximal channel open probability is achieved, anabaseine activates the mouse embryonic neuromuscular nicotinic receptor with an apparent affinity slightly less than that of ACh (2–10 μM; Sine and Steinbach, 1987; C. Lingle, unpublished results). However, because the cyclic minimum concentration of anabaseine is only 29% of its total concentration at pH 7.4, this active form of anabaseine is probably slightly more potent than ACh on this mammalian receptor, as at the amphibian neuromuscular receptor (table 1). In our experiments the intrinsic activity or true efficacy (after correcting for its channel-blocking action) of anabaseine displayed a limiting open probability of less than 0.8 compared with values in excess of 0.9 for ACh (Sine and Steinbach, 1987; Zhang *et al.*, 1995). A higher limiting p_o value for the compound might have been achieved if higher anabaseine concentrations had been tested.

Anabaseine was a more effective open channel blocker than ACh (Ogden and Colquhoun, 1985; Sine and Steinbach,

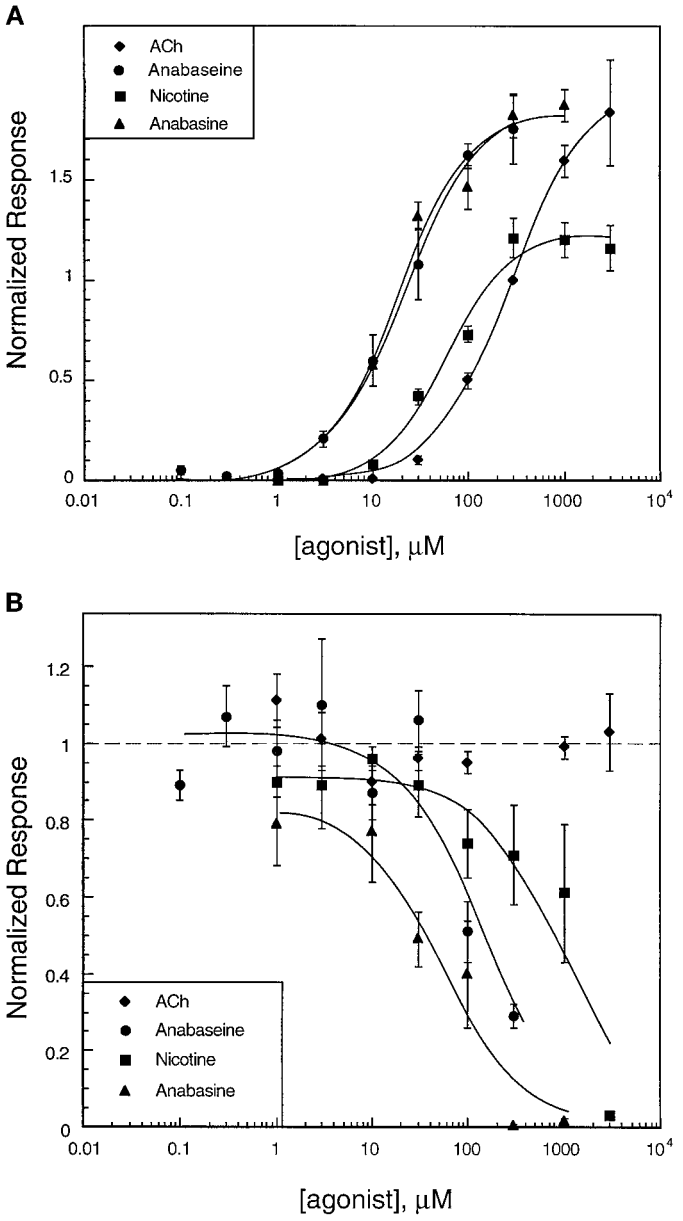

Fig. 8. Full agonist actions of anabaseine and anabasine upon rat brain *alpha*7 homomeric receptors expressed in the *Xenopus* oocyte. Agonist responses were normalized to the individual oocyte's response to a control ACh (500 μM) application made 5 min before the experimental applications. Each point represents the average response ($\pm$S.E.) of at least four oocytes to that agonist concentration.(A) concentration-response curves for activation of the receptor. (B) Concentration-response curves showing residual inhibitory activity 5 min after washing away the nicotinic agonist. The ACh data, previously reported in Papke *et al.* (1994), are included for comparison with the three alkaloids.

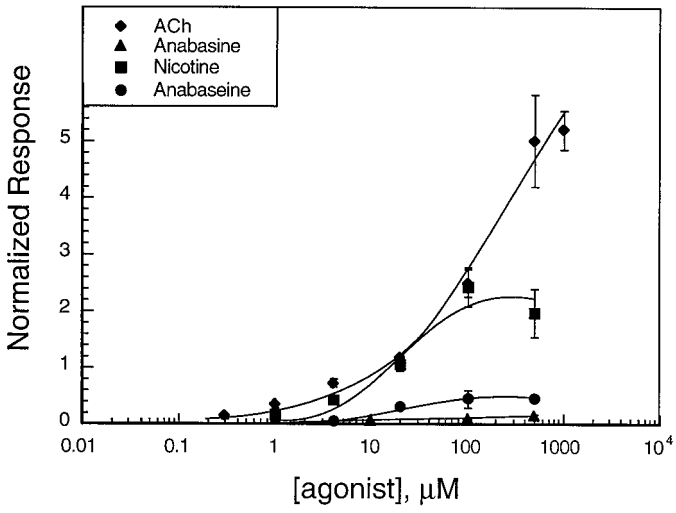

Fig. 9. Weak partial agonist actions of anabaseine and anabasine upon *alpha*4-*beta*2 receptors expressed in the *Xenopus* oocyte. Agonist responses were normalized to the individual oocyte's response to a control ACh (500 μM) application made 5 min before the experimental applications. Each point represents the average response ($\pm$S.E.) of at least four oocytes to that agonist concentration. The ACh data, previously reported in Papke *et al.* (1994), are included for comparison with the three alkaloids.

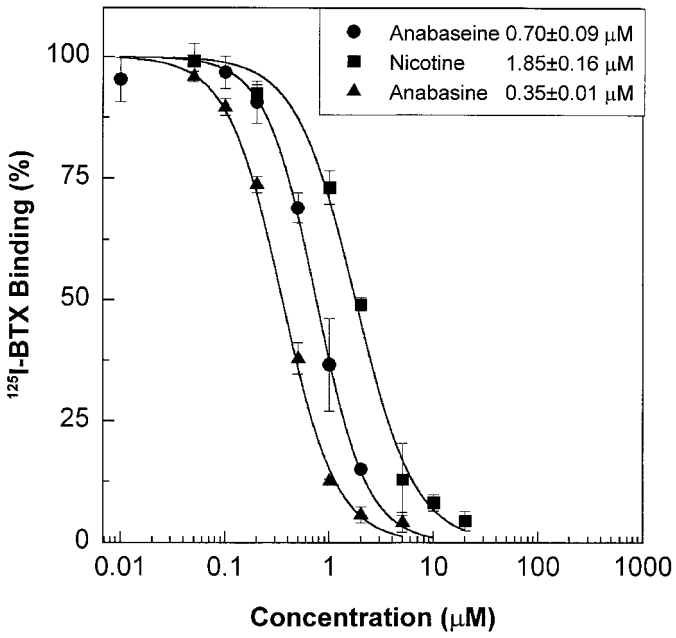

Fig. 10. Nicotinic agonist displacement of specific [^{125}I]-*alpha*-bungarotoxin binding to rat brain membranes. The BTX concentration was 1 nM. Membranes were equilibrated with the radioligand for 1 hr at 37 C before washing and filtration with ice-cold saline. Nonspecific binding was determined in the presence of 1 mM nicotine. Each point represents the mean value for triplicate samples. The IC_{50}s of the three alkaloids, determined by EBDA software, are shown in the box.

1984). This conclusion is largely based on the longer duration of the blocking interval produced by anabaseine. Qualitatively, the voltage-dependence of block and the linear dependence of blocking event frequency on anabaseine concentration are both consistent with the idea that anabaseine blocks the nicotinic receptor pore by a simple channel blocking mechanism. For a simple block model, the microscopic affinity of anabaseine for its blocking site is describable by $K_d(V) = K_d(0) * \exp^{A*V}$ where $K_d(0)$ is the 0-voltage affinity and A describes the voltage-dependence of the block. From the values derived from fitting the blocking and unblocking rates shown in figure 6C and D, $K_d(0) = 7$ mM with A = 0.024/mV, which corresponds with the movement of a single charged species a little more than halfway through the electric field. However, despite the somewhat stronger channel blocking effect of anabaseine, the affinity of anabaseine for block of the open channel was still much lower than the concentrations effective at activating the receptor. Over the physiological

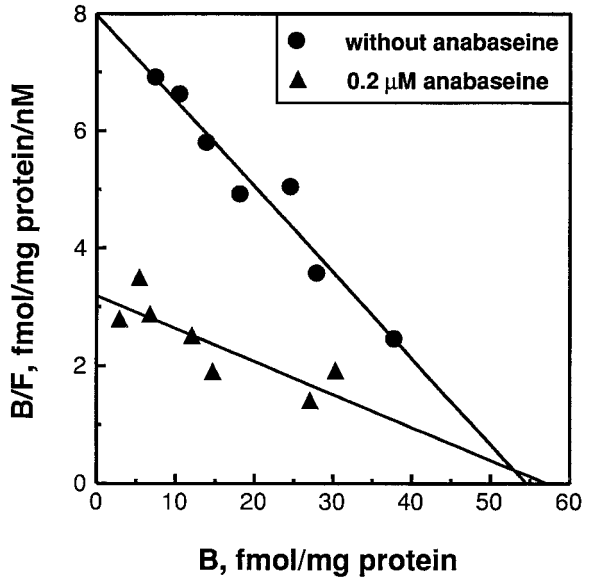

Fig. 11. Scatchard plot showing anabaseine effect upon binding of [^{3}H]-methylcarbamylcholine to rat brain high nicotine affinity receptors. Each point represents the mean value for triplicate samples. The K_d of [^{3}H]-MCC was calculated to be 11 nM.

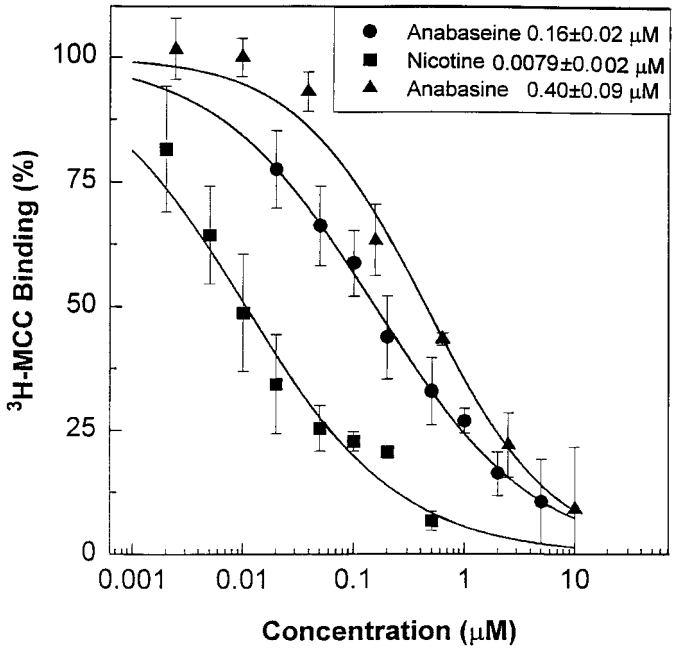

Fig. 12. Nicotinic agonist displacement of specific [^{3}H]-MCC binding to rat cerebral cortex membranes. The tritiated MCC concentration was 5 nM and the saline pH was 7.4. Nonspecific binding was determined in the presence of 10 μM carbamylcholine. Each point represents a mean value obtained from triplicate estimates. The IC_{50}s shown in the box were determined with EBDA software.

range of membrane potentials, the effective K_ds for channel block exceeded 500 μM anabaseine, so even at concentrations in excess of about 20 μM the reduction of macroscopic current by channel block would be rather minor.

Electrophysiological comparison of the three alkaloids on oocyte-expressed neuronal nicotinic receptors. Anabaseine (Papke *et al.*, 1994) and anabasine (table 3) both displayed low efficacies on the *Xenopus* oocyte expressed *alpha*4-*beta*2 receptor. A submaximal efficacy on this receptor has previously been observed with other potent nicotinic agonists, including cytisine, anatoxin-a, epibatidine, nicotine and the synthetic nicotinic agonist ABT-418 (Papke and Heinemann, 1994; Alkondon and Albuquerque, 1995; Papke *et al.*, in press). Apparently the ligand molecular requirements for activating this receptor subtype are even more stringent than those for high affinity binding. Because most of the agonists that display high affinity are larger, less flexible molecules, high efficacy may be related to an ability to bind within a relatively restricted space on this receptor. An alternative interpretation would be that the *alpha*4-*beta*2 receptor channel is more readily blocked by receptor agonists, which would be reflected in a smaller maximum response or efficacy (Papke *et al.*, 1997b). Patch-clamp analyses of the actions of these agonists are clearly needed to determine the basis for the reduced apparent efficacy of these compounds on *alpha*4-*beta*2 and *alpha*7 nicotinic receptors.

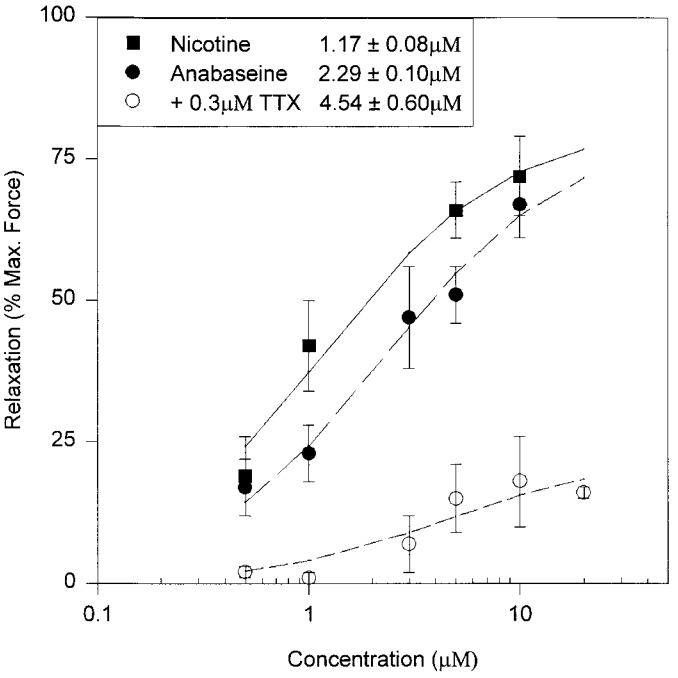

Fig. 13. Relaxing action of anabaseine and nicotine upon rat colon longitudinal muscle. The relaxing ability of a particular concentration of agonist was determined by adding it to the bath as soon as the peak contraction caused by 320 nM oxotremorine had been reached The amplitude of relaxation was expressed as a percentage of the oxotremorine-stimulated contractile force. TTX inhibited the relaxing effect of anabaseine in a noncompetitive fashion. $n = 6$ for both anabaseine and nicotine curves, although $n = 4$ for the anabaseine plus TTX curve.

We observed a similar rank order, anabaseine> anabasine>nicotine for *Xenopus* oocyte *alpha*7 receptor potency (table 3) as for rat brain *alpha*7 receptor affinity, as measured by BTX binding displacement (table 2). In both experiments the apparent affinities of anabaseine and anabasine for this nicotinic receptor were significantly higher than that of nicotine. Due to the rapid desensitization of *alpha*7 receptors, concentration-response curves for this receptor are quite dependent on the rate of agonist application (Papke *et al.*, 1997). Because differences in experimental methods for agonist application between laboratories prevented us from quantitatively comparing our present *alpha*7 data on anabaseine and anabasine with previously published

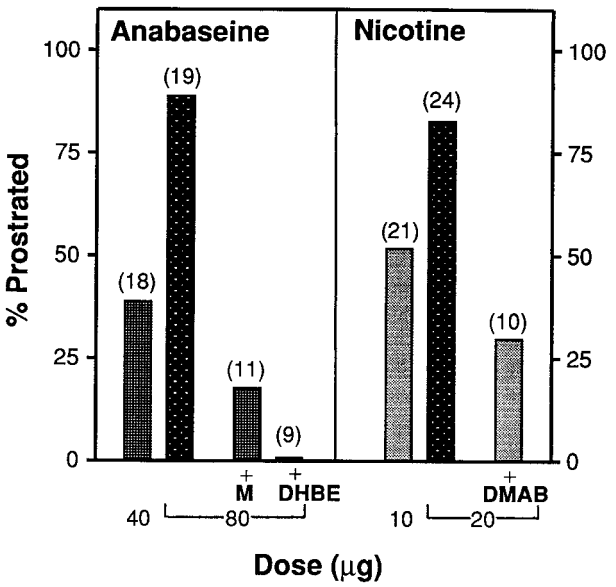

Fig. 14. Anabaseine causes immediate prostration in the rat. Also shown is the inhibition of 80 μg anabaseine prostration by pretreatment either with 40 μM mecamylamine or dihydro-B-erythroidine, and the inhibition of 20 μg nicotine prostration by 80 μg DMAB-anabaseine. The number of animals receiving injections (i.c.v.) at each dose is indicated within parentheses. Doses (μg) of anabaseine were based on its dihydrochloride form, whereas those of nicotine were based on its free base form.

data for nicotine, we again determined the concentration-response relation for nicotine under the same conditions. We found that the apparent efficacy (maximum current) of nicotine for stimulation of the homomeric *alpha*7 receptor was much less than that of anabaseine, anabasine or ACh (fig. 8).

Comparing the efficacies of the three alkaloids on the *alpha*7 and *alpha*4-*beta*2 receptors, it is readily apparent that anabaseine displays the greatest efficacy and affinity at *alpha*7 receptors, although at *alpha*4-*beta*2 receptors anabaseine and anabasine display a much lower efficacy and affinity than nicotine. This combination of properties predicts that the *in vivo* actions of anabaseine and anabasine on the brain are mostly mediated through *alpha*7 receptors, although the actions of nicotine are largely mediated through *alpha*4-*beta2* and possibly other high nicotine affinity receptors sharing a similar pharmacological profile in receptor binding and efficacy.

Anabaseine interaction with rat brain membrane nicotinic receptors. Our binding data with the naturally expressed BTX-binding nicotinic receptor is in agreement with our functional data on the oocyte-expressed homomeric *alpha*7 receptor. Quik *et al.* (1996) have reported an excellent correspondence between the ligand binding properties of the rat brain *alpha*7-containing receptor and those of the homomeric *alpha*7 receptor expressed in a transfected cell line, which is consistent with the notion that the *alpha*7 receptor in the rat brain may also be homomeric. However, Anand *et al.* (1993) have observed some pharmacological differences between the artificially expressed homomeric chick *alpha*7 receptor and brain receptors containing the *alpha*7 subunit, so at least in the chick brain the receptors are not the same.

We have shown that anabaseine acts as a competitive antagonist of ^{3}H-MCC, altering the apparent affinity of this radioligand without significantly affecting the receptor concentration available for binding (fig. 11). Compounds that act allosterically at sites other than the ACh recognition site would affect the Scatchard plot for MCC binding in a different manner (Takayama *et al.*, 1989). Although our results indicate that anabaseine primarily interacts with the ACh recognition sites of the receptor, it is possible that anabaseine might also bind to one or more allosteric sites that would not be detected by displacement of tritiated MCC. Indeed, our finding that anabaseine has a channel blocking action above 20 μM on BC^{3}H-1 cell nicotinic channels implies the existence of at least one allosteric site that might be detectable in electric organ membranes by measuring its ability to displace radiolabeled compounds which specifically bind to the nicotinic receptor ion channel (Eldefrawi *et al.*, 1980).

We carried out ^{3}H-MCC binding displacement experiments with all three compounds under identical conditions to facilitate quantitative comparisons between them. Although the rank order of binding affinities, nicotine$\gg$ anabaseine$>$ anabasine (table 2), were in excellent agreement with the rank order of potencies shown in table 3, the K_i and EC_{50} concentrations for each alkaloid were quite different. These differences arise from the fact that the steady-state binding assay measures the affinity of the desensitized receptor, whereas the functional assay measures the affinity of the activateable receptor. It is interesting that the ratio, EC_{50}/K_i for anabaseine was only 131, compared to a ratio of 3410 for nicotine.

Anabaseine actions on PC12 cells and parasympathetic neurons. On PC12 cell receptors anabaseine displayed a potency similar to nicotine and anabasine when the extent of ionization was taken into consideration, and the maximal responses (data not shown) were nearly identical with that of carbachol. The uncorrected EC_{50} value of 29 μM for nicotine stimulation of ^{86}Rb efflux is in excellent agreement with other reported nicotine EC_{50} values for these cells (29 μM, Kemp and Edge, 1987; 20 μM, Lukas, 1989). However, our observation that the maximal effect of nicotine on PC12 cells is comparable with the 1 mM carbamylcholine response differs from some previously reported data that indicated that nicotine's maximal effect was significantly less than the effect of 1 mM carbamylcholine (Lukas and Cullen, 1988; Lukas, 1989). Several factors, such as differences in the composition or degree of expression of nicotinic receptors between different PC12 cultures, could possibly contribute to such a difference. PC12 cells express other nicotinic receptor subunits besides *alpha*4 and *beta*3 (Rogers *et al.*, 1992). PC12 *alpha*7 receptors bind BTX, but probably have at most, only a small contribution to the rubidium fluxes we measured over a 1-min interval (Kemp and Edge, 1987; Rogers *et al.*, 1991).

Anabaseine apparently relaxes the colon smooth muscle indirectly by stimulating nicotinic receptors on paraympathetic neurons of the myenteric plexus, because TTX blocked its effect. TTX blocks the stimulatory effect of nicotine on guinea pig ileum smooth muscle by depressing the electrical excitability of myenteric plexus neurons (Torocsik *et al.*, 1991). Anabaseine and nicotine were much more potent than anabasine in relaxing the rat colon (table 3). Haefely (1974) also reported that anabasine was only about 4% as potent as nicotine in affecting the cat superior cervical ganglion preparation. The maximal relaxing effect of anabaseine was very

similar to that of nicotine, as shown in figure 13. Both anabaseine and nicotine displayed much higher potencies (32- and 25-fold, respectively) in relaxing the rat colon muscle relative to their potency in stimulating ^{86}Rb efflux from PC12 cells. Our data suggest that the nicotinic receptors in these two autonomic preparations are probably different in their subunit compositions, and warrants further investigation. Also, Bencherif *et al.* (1996) reported quite different nicotinoid affinities for PC12 cells and guinea pig ileum nicoinic receptors and suggested that myenteric plexus receptors are composed of *alpha*3, *beta*2 and possibly a third subunit.

Whole animal actions of anabaseine. At an initial stage of this investigation the rat prostration response to lateral ventricular injection of nicotinic agonists was selected as an *in vivo* bioassay for demonstrating neuronal nicotinic agonist activity of anabaseine. The *in vivo* agonistic and antagonistic activities of a variety of nicotinic compounds, neurotransmitters and toxins had previously been demonstrated using this assay (Abood *et al.*, 1981). The prostration response displayed pronounced stereo-specificity for the (S)-form of nicotine, as had been observed in radioligand binding experiments with the brain high nicotine affinity binding site. Because displacement of BTX binding to low affinity receptors shows little stereospecificity (Wonnacott, 1986), the existing data suggest that *alpha*7 type receptors do not play a major role in causing this prostration response. Abood *et al.* (1981) reported that i.c.v. injection of hexamethonium or mecamylamine immediately preceding nicotine administration partially inhibited its prostrating action. They also reported that preadministration of BTX or TC failed to inhibit the action of prostrating action of nicotine, although TC injected alone caused seizures.

In our prostrations assays anabaseine was approximately equipotent with nicotine, when expressed in terms of the total μmol amount of cyclic iminium form of anabaseine injected. This suggests that the nicotinic receptor mediating prostration behavior is not the *alpha*4-*beta*2 type, because this receptor subtype displays much higher (>10X) affinity for nicotine than for anabaseine (table 2). Both DHBE and mecamylamine antagonized the prostrating action of i.c.v. anabaseine. DMAB-anabaseine and other 3-substituted anabaseine derivatives are *alpha*7 partial agonists but antagonists at *alpha*3-*beta*4 and other nicotinic receptors (Kem and Papke, 1992; Papke *et al.*, 1994; de Fiebre *et al.*, 1995). Although the large dose of DMAB-anabaseine failed to cause prostration, it did inhibit the prostrating action of nicotine (fig. 14). Thus, our results and those of Abood *et al.* (1981) suggest that the nicotinic cholinergic receptors mediating the nicotinic prostration are neither the *alpha*7 nor the *alpha*4-*beta*2 types. The *alpha*3-*beta*4 subtype is a candidate receptor for mediating prostration in the rat, because nicotine and anabaseine were found to be of very similar potency (table 3) in stimulating PC12 cell rubidium efflux through nicotinic receptor channels, which are generally considered to be predominantly the *alpha*3-*beta*4 combination. This autonomic receptor subtype also displays stereospecificity in its interactions with the two isomers of nicotine (Madhok and Sharp, 1992). Other receptor subunit combinations such as *alpha*3-*beta*2 are also possibly involved. Further experiments with compounds selective for particular nicotinic receptor subtypes may assist in the identification of the nicotinic receptors mediating this behavior.

Preferential actions of the three alkaloids upon particular nicotinic receptors. To compare nicotinic agonists in molecular terms, it is necessary to quantitatively express potencies in terms of the concentration of the active form of each compound. This can be estimated with knowledge of the bulk pH of the saline and the pKa of the ionizable group. Fixed negative charges might alter the local pH at the ACh recognition site, so that it may differ from that of the bulk pH (Stauffer and Karlin, 1994). For instance, a slightly lower local pH at the ACh recognition site would enhance agonist ionization and increase the estimated potencies of secondary and tertiary amine compounds relative to a quaternary ammonium salt like carbamylcholine. Correction for the local pH effect would probably not greatly affect the potency comparisons of the non-quaternary compounds in table 1.

Our examination of the relative potencies and affinities of these closely related compounds provides useful insights for designing nicotinic compounds selective for a particular receptor subtype. Among the three compounds, the anabaseine structure seems optimal for strong neuronal *alpha*7 and neuromuscular agonist activities, although the nicotine structure seems optimal for designing *alpha*4-*beta*2 selective compounds. The anabasine structure, because of its low neuromuscular potency, would serve as a good model for designing *alpha*7-selective compounds.

Structural comparison of anabaseine with the two tobacco alkaloids. Both nicotine and anabasine possess a tetrahedral chiral carbon at position 2 of the saturated ring, whereas the same carbon atom in anabaseine is part of a trigonal imine bond whose pi electrons are conjugated with those of the pyridyl ring. Conformational analyses predict that the saturated ring is twisted approximately 90 degrees out of the plane of the pyridyl ring in nicotine and anabasine (Whidby and Seeman, 1976; Seeman, 1984), although the two rings of anabaseine are coplanar (Prokai *et al.*, in preparation).

The electropositive N-methyl group of nicotine will be in the same plane as the 3-pyridyl ring, which makes its presumed receptor-facing surface more similar to that of anabaseine. However, anabasine is predicted to lack a positive charged group in the same plane as its pyridyl ring. Analyses of anabaseine indicate that its two rings are coplanar. The neuromuscular nicotinic receptor ACh recognition sites (there are two, one for each *alpha* subunit) apparently interact most readily with a positively charged group that resides within the same plane as the pyridyl ring. The neuronal *alpha*7 ACh recognition sites apparently do not have this coplanarity requirement. Anabaseine and anabasine readily activate this receptor type relative to nicotine, perhaps because their cationic, unmethylated nitrogens are able to make more intimate contact with the *alpha*7 receptor ACh recognition sites.

Because anabasine and nicotine are thought to possess similar preferred conformations (ring twists) in solution, their differing *alpha*4-*beta*2 affinity is probably due to some other chemical differences between the two molecules. We suggest that optimal receptor binding to this receptor occurs when the ligand possesses an N-methyl substituent. Also, other experiments have shown that the greater size of the piperidine ring in anabasine relative to the pyrrolidine ring

of nicotine also reduces its interaction with this receptor (Kem *et al.*, unpublished results).

The ligand binding requirements we observed for the parasympathetic-type nicotinic receptors in the rat colon myenteric plexus most closely resembled the neuromuscular receptor requirements. Both receptors displayed relatively strong affinities for nicotine and anabaseine, but a much lower affinity for anabasine.

Comparison of anabaseine with 3-substituted anabaseines. Our study now provides a foundation for understanding the pharmacological properties of the benzylidene and cinnamylidene derivatives of anabaseines, including DMAB-, DMXB- and DMAC-anabaseines, which preferentially stimulate neuronal nicotinic receptors containing *alpha*7 subunits (Kem *et al.*, 1994c; Meyer *et al.*, 1994; Papke *et al.*, 1994; de Fiebre *et al.*, 1995) and enhance cognitive behavior (Woodruff-Pak *et al.*, 1994; Meyer *et al.*, 1994; Arendash *et al.*, 1995b; Bjugstad *et al.*, in press). As with anabaseine, these compounds display high affinity and efficacy on *alpha*7 receptors but low affinity and efficacy with *alpha*4-*beta*2 receptors. Thus, the 3-substitution of anabaseine merely increases further an *alpha*7 *vs. alpha*4-*beta*2 preferential activity already present in anabaseine. 3-Substituted anabaseines also lack significant agonist activity on peripheral nicotinic receptors of the autonomic and neuromuscular types (Kem *et al.*, 1994c). It is extremely interesting that the addition of a 3-substituent to anabaseine seems to diminish its peripheral nervous system and *alpha*4-*beta*2 stimulation without reducing central *alpha*7 stimulation. This is probably the major pharmacodynamic advantage of the 3-substituted anabaseines over the parent toxin. One of these derivatives, DMXB-anabaseine (also known as GTS-21; Kem *et al.*, 1996), is currently in clinical trials for possible treatment of Alzheimer's dementia. Further studies are needed to fully understand the nature of the molecular differences between the 3-substituted derivatives of anabaseine and the natural toxin.

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References

Abood, L. G., Reynolds, D. T., Booth, H. and Bidlack, J. M.: Sites and mechanisms for nicotine's action in the brain. Neurosci. Behav. Rev. **5:** 479–486, 1981.

Anand, R., Peng, X. and Lindstrom, J.: Homomeric and native alpha7 acetylcholine receptors exhibit remarkably similar but nonidentical pharmacolgical properties suggesting that the native receptor is a heteromeric protein complex. FEBS Lett. **327:** 241–246, 1993.

Albuquerque, E. X., Alkondon, M., Pereira, E. F. R., Castro, N. B., Schrattenholz, A., Barbosa, C. T. F., Bonfante-cabarcas, R., Aracava, Y., Eisenberg, H. M. and Maelicke, A.: Properties of neuronal nicotinic acetylcholine receptors: Pharmacological characterization and modulation of synaptic function. J. Pharmacol. Exp. Ther. **280:** 1117–1136, 1997.

Alkondon, M. and Albuquerque, E. X.: Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. III. Agonist actions of the novel alkaloid epibatidine and analysis of type II current. J. Pharmacol. Exp. Ther. **274:** 771–782, 1995.

Araujo D. M., Lapchak, P. A., Robitaille, Y., Gauthier, S. and Quirion, R.: Differential alteration of various cholinergic markers in cortical and subcortical regions of human brain in Alzheimer's disease. J. Neurochem. **50:** 1914–1923, 1988.

Arendash G. W., Sanberg, P. R. and Sengstock, G. J.: Nicotine enhances the learning and memory of aged rats. Pharmacol. Biochem. Behavior **52:** 517–523, 1995a.

Arendash G. W., Sengstock, G. J., Sanberg, P. R. and Kem, W. R.: Improved learning and memory in aged rats with chronic administration of the nicotinic receptor agonist GTS-21. Brain Res. **674:** 252–259, 1995b.

Auerbach, A. and Lingle, C. J.: Activation of the primary kinetic modes of large- and small-conductance cholinergic ion channels in *Xenopus* myocytes. J. Physiol. **393:** 437–466, 1987.

Badio, B. and Daly, J. W.: Epibatidine, a potent analgetic and nicotinic agonist. Mol. Pharmacol. **45:** 563–569, 1994.

Barlow, R. B. and Hamilton, J. T.: The effects of pH on the activity of nicotine and nicotine monomethiodide on the rat diaphragm preparation. Br. J. Pharmacol. **18:** 543–549, 1962.

Barlow, R. B. and McLeod, L. J.: Some studies on cytisine and its methylated derivatives. Br. J. Pharmacol. **35:** 161–174, 1969.

Barlow, R. B., Thompson, G. M. and Scott, N. C.: The affinity and activity of compounds related to nicotine on the rectus abdominis muscle of the frog (*Rana pipiens*). Br. J. Pharmacol. **37:** 555–584, 1969.

Bartels, E. and Podleski, T. R.: Action of nicotine on the electroplax and difference of potency between ionized and unionized forms. Biochim. Biophys. Acta **79:** 11–520, 1964.

Bencherif, M., Lovette, M. E., Fowler, K. W., Arrington, S., Reeves, L., Caldwell, W. S. and Lippiello, P. M.: RJR-2403: A nicotinic agonist with CNS selectivity I. In vitro characterization. J. Pharm. Exp. Ther. **279:** 1413–1421, 1996.

Bjugstad, K. B., Mahnir, V. M., Kem, W. R. and Arendash, G. W.: Long-term treatment with GTS-21 or nicotine enhances water maze performance in aged rats without affecting the density of nicotinic receptor subtypes in neocortex. Drug Dev. Res. **39:** 19–28, 1996.

Blatz, A. L. and Magleby, K. L.: Correcting single channel data for missed evens. Biophys. J. **49:** 967–980, 1986.

Bloom, L. B.: Influence of solvent on the ring-chain hydrolysis equilibrium of anabaseine and syntheses of anabaseine and nicotine analogues. Ph.D. Dissertation, Gainesville, University of Florida, pp. 1–206, 1990.

Bonhaus, D. W., Bley, K. R., Broka, C. A., Fontana, D. J., Leung, E., Lewis, R., Shieh, A. and Wong, E. H. F.: Characterization of the electrophysiological, biochemical and behavioral actions of epibatidine. J. Pharmacol. Exp. Ther. **272:** 1199–1203, 1995.

Boksa, P. and Quirion, R.: [^{3}H]N-methyl-carbamylcholine, a new radioligand specific for nicotinic acetylcholine receptors in brain. Eur. J. Pharmacol. **139:** 323–333, 1987.

Cheng, Y-C. and Prusoff, W. H.: Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50% inhibition(IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. **22:** 3099–3108, 1973.

Covarrubias, M., Kopta, C. and Steinbach, J. H.: Inhibitors of asparagine-linked oligosaccharide processing alter the kinetics of the nicotinic acetylcholine receptor. J. Gen. Physiol. **93:** 765–783, 1989.

Colquhoun, D. and Sakmann, B.: Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. J. Physiol. **369:** 501–557, 1985.

Decker, M., Brioni, J., Bannon, A. and Arneric, S.: Diversity of neuronal nicotinic acetylcholine receptors: Lessons from behavior and implications for CNS therapeutics. Life Sci. **56:** 545–570, 1995.

de Fiebre, C. M., Meyer, E. M., Henry, J. C., Muraskin, S. I., Kem, W. R. and Papke, R. L.: Characterization of a series of anabaseine-derived compounds reveals that the 3-(4)-dimethylaminocinnamylidine derivative is a selective agonist at neuronal nicotinic a7/^{125}I-a-bungarotoxin receptor subtypes. Mol. Pharmacol. **47:** 164–171, 1995.

Eldefrawi, M. E., Aronstam, R. S., Bakry, N. M., Eldefrawi, A. T. and Albuquerque, E. X.: Activation, inactivation, and desensitization of acetylcholine receptor channel complex detected by binding of perhydrohistrionicotoxin. Proc. Natl. Acad. Sci. U.S.A. **77:** 2309–2313, 1980.

Erspamer, V.: Isolation of leptodactyline (m-hydroxyphenylethyltrimethylammonium) from extracts of *Leptodactylus* skin. Arch. Biochem. Biophys. **82:** 431–438, 1959.

Flores, C. M., Rogers, S. W., Pabreza, L. A., Wolfe, B. and Kellar, K. J.: A subtype of nicotinic cholinergic receptor is composed of alpha4 and beta2 subunits and is up-regulated by chronic nicotine treatment. Mol. Pharmacol. **41:** 11–37, 1992.

Gund, T. M. and Spivak, C. E.: Pharmacophore for nicotinic agonists. Methods Enzymol. **203:** 677–695, 1991.

Haefely, W.: The effects of various "nicotine-like" agents in the cat superior cervical ganglion *in situ*. N. S. Arch. Pharmacol. Pathol. **281:** 93–117, 1974.

Kellar, K. J., Whitehouse, P. J., Martino-Barrows, A. M., Marcus, K. and Price, D. L.: Muscarinic and nicotinic cholinergic binding sites in Alzheimer's disease cerebral cortex. Brain Res. **435:** 62–68, 1987.

Kem, W. R.: A study of the occurrence of anabaseine in *Paranemertes* and other nemertines. Toxicon **9:** 23–32, 1971.

Kem, W. R. and Papke, R. L.: Actions of anabaseine and DMAB-anabaseine upon neuronal alpha4-beta2 and PC12 cell nicotinic receptors (Abstract). Soc. Neurosci. **18:** 1358, 1992.

KEM, W. R., ABBOTT, B. C. AND COATES, R. M.: Isolation and structure of a hoplonemertine toxin. Toxicon **9:** 15–22, 1971.

KEM, W. R., MAHNIR, V. M., BLOOM, L. B. AND GABRIELSON, B. J.: The active form of the nicotinic receptor agonist anabaseine is the cyclic iminium cation. *In* Eleventh World Congress on Animal, Plant, and Microbial Toxins, Tel Aviv, p. 157, 1994b (Abstract).

KEM, W. R., MAHNIR, V. M. AND LIN, B.: Interaction of DMXBA (GTS-21), a cognition-enhancing compound, with cholinergic receptors. Soc. Neurosci. Abstr. **20:** 1134, 1994c (Abstract 467.2).

KEM, W. R., MAHNIR, V. M., LIN, B., LINGLE, C. J., PAPKE, R. L. AND PROKAI-TATRAI, K.: The marine toxin anabaseine is a potent nicotinic agonist. *In* Proceedings of the International Symposium on Nicotine, Montreal, p. 22, 1994a (Abstract).

KEM, W. R., MAHNIR, V. M., LIN, B. AND PROKAI-TARTRAI, K.: Two primary GTS-21 metabolites are potent partial agonists at alpha7 nicotinic receptors expressed in the Xenopus oocyte. Soc. Neurosci. Abstr. 22: 268, 1996 (Abstract 110.7).

KEM, W. R., SCOTT, K. N. AND DUNCAN, J. H.: Hoplonemertine worms—a new source of pyridine neurotoxins. Experientia **32:** 684–686, 1976.

KEMP, G. AND EDGE, M.: Cholinergic function and a-bungarotoxin binding in PC12 cells. Mol. Pharmacol. **32:** 356–363, 1987.

LINDSTROM, J.: Neuronal nicotinic acetylcholine receptors. *In* Ion Channels, ed. by T. Narahashi, pp. 377–450, Plenum Press, New York, 1996.

LINGLE, C. J., MACONOCHIE, D. AND STEINBACH, J. H.: Activation of skeletal muscle nicotinic acetylcholine receptors. J. Membrane Biol. **126:** 195–217, 1992.

LUETJE, C. W., WADA, K., ROGERS, S., ABRAMSON, S. N., HEINEMANN, S. AND PATRICK, J: Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptors. J. Neurochem. **55:** 632–640, 1990.

LUKAS, R. J.: Pharmacological distinctions between functional nicotinic acetylcholine receptors on the PC12 rat pheochromocytoma and the TE671 human medulloblastoma. J. Pharmacol. Exp. Ther. **251:** 175–182.

LUKAS, R. J. AND CULLEN, M. J.: An isotopic rubidium ion efflux assay for the functional characterization of nicotinic acethylcholine receptors on clonal cell lines. Anal. Biochem. **175:** 212–218, 1988.

LUNTZ-LEYBMAN, V., BICKFORD, P. C. AND FREEDMAN, R.: Cholinergic gating of response to auditory stimuli in rat hippocampus. Brain Res. **587:** 130–136, 1992.

MACHU, T. K., STRAHLENDORF, J. AND KEM, W. R.: Nicotinic receptor ligands antagonize 5-HT3 receptors expressed in *Xenopus* oocytes. Soc. Neurosci. **22:** 1780, 1996 (Abstr. 700.5).

MADHOK, C. AND SHARP, B. M.:Nerve growth factor enhance [^{3}H]nicotine binding to a nicotinic cholinergic receptor on PC12 cells. Endocrinology 130: 825–830, 1992.

MARKS, M. J., AND COLLINS, A. C.: Characterization of nicotine binding in mouse brain and comparison with the binding of alpha-bungarotoxin and quinuclidinyl benzilate. Mol. Pharmacol. **22:** 554–564, 1982.

MARSHALL, C. G., OGDEN, D. AND COLQUHOUN, D.: Activation of ion channels in the frog endplate by several analogues of acetylcholine. J. Physiol. **433:** 73–93, 1991.

MCGEHEE, D. S. AND ROLE, L. W.: Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Ann. Rev. Physiol. **57:** 521–546, 1995.

MEYER, E. M., ARENDASH, G. W., JUDKINS, J. H., YING, L., WADE, C. AND KEM, W. R.: Effects of nucleus basalis lesions on the muscarinic and nicotinic modulation of [^{3}H]acetylcholine release in the rat cerebral cortex. J. Neurochem. **49:** 1758–1762, 1987.

MEYER, E. M., DE FIEBRE, C. M., HUNTER, B. E., SIMPKINS, C. E., FRAUWORTH, N. AND DE FIEBRE, N. E.: Effects of anabaseine-related analogs on rat brain nicotinic receptor binding and on avoidance behaviors. Drug Dev. Res. **31:** 127–134, 1994.

NEWHOUSE, P. A., POTTER, A. AND LENOX, R. H.: The effects of nicotinic agents on human cognition: possible therapeutic applications in Alzheimer's and Parkinson's diseases. Med. Chem. Res. **2:** 628–642, 1993.

OGDEN, D. C. AND COLQUHOUN, D.: Ion channel block by acetylcholine, carbachol, and suberyldicholine at the frog neuromuscular junction. Proc. Roy. Soc. B **225:** 329–355, 1985.

PAPKE, R. L.: The kinetic properties of neuronal nicotinic receptors: genetic basis of functional diversity. Prog. Neurobiol. **41:** 509–531, 1993.

PAPKE, R. L. AND HEINEMANN, S. F.: Partial agonist properties of cytisine on neuronal nicotinic receptors containing the B2 subunit. Mol. Pharmacol. **45:** 142–149, 1994.

PAPKE, R. L., MILLHAUSER, G., LIEBERMAN, Z. AND OSWALD, R.: E. Relationships of agonist properties to the single channel kinetics of nicotinic acetylcholine receptors. Biophys. J. **53:** 1–10, 1988.

PAPKE, R. L., DE FIEBRE, C. M., KEM, W. R. AND MEYER, E. M.: The subunit specific effects of novel anabaseine-derived nicotinic agents. *In* Alzheimer Disease: Therapeutic Strategies, ed. by E. Giacobini and R. Becker, eds., pp. 206–211, Birkhauser, Boston, 1994.

PAPKE, R. L., FRANCIS, M. M. AND THINSCHMIDT, J. S.: Techniques for improved resolution of kinetic data from macroscopic currents. Biophys. J. **72:** Abstr. M-Pos86, 1997.

PAPKE, R. L., THINSCHMIDT, J. S., MOUTON, B. A., MEYER, E. M. AND POIRIER, A.: Activation and inhibition of rat neuronal nicotinic receptors by ABT-418. Br. J. Pharmacol. **120:** 429–438, 1997.

QUIK, M., CHOREMIS, J., KOMOURIAN, J., LUKAS, R. J. AND PUCHACZ, E.: Similarity between rat brain nicotinic alpha-bungarotoxin receptors and stably expressed alpha-bungarotoxin binding sites. J. Neurochem. **67:** 145–154, 1996.

ROGERS, S. W., GAHRING, L. C., PAPKE, R. L. AND HEINEMANN, S.: Identification of cultured cells expressing ligand-gated cationic channels. Prot. Exp. Purif. **2:** 108–116, 1991.

ROGERS, S. W., MANDELZYS, A., DENERIS, E. S., COOPER, E. AND HEINEMANN, S.: The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. J Neurosci. **12:** 4611–4623, 1992.

ROMANO, C.: Nicotine action on rat colon. J. Pharmacol. Exp. Ther. **217:** 828–833, 1981.

SAKMANN, B., PATLAK, J. AND NEHER, E.: Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. Nature **287:** 447–449, 1980.

SEEMAN, J. I.: Recent studies in nicotine chemistry. Conformational analysis, chemical reactivity studies, and theoretical modeling. Heterocycles **22:** 165–193, 1984.

SIGWORTH, F. J. AND SINE, S. M.: Data transformations for improved display and fitting of single-channel dwell time histograms. Biophys. J. **52:** 1047–1054, 1987.

SINE, S. M. AND STEINBACH, J. H.: Agonists block currents through acetylcholine receptor channels. Biophys. J. **46:** 277–284, 1984.

SINE, S. M. AND STEINBACH, J. H.: Activation of acetylcholine receptors on clonal mammalian BC^{3}H-l cells by low concentrations of agonist. J. Physiol. **373:** 129–162, 1986.

SINE, S. M. AND STEINBACH, J. H.: Activation of acetylcholine receptors on clonal mammalian BC^{3}H-1 cells by high concentrations of agonist. J. Physiol. **385:** 325–359, 1987.

STAUFFER, D. A. AND KARLIN, A.: Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates. Biochemistry **33:** 6840–6849, 1994.

SUMMERS, K. L., KEM, W. R. AND GIACOBINI, E.: Nicotinic agonist modulation of neurotransmitter levels in the rat frontoparietal cortex. Jpn. J. Pharmacol. **74:** 139–146, 1997.

SWANSON, K. L. AND ALBUQUERQUE, E. X.: Nicotinic acetylcholine receptors and low molecular weight toxins. Handb. Exp. Pharmacol. **102:** 611–658, 1992.

TAKAYAMA, H., MAJEWSKA, M. D. AND LONDON, E. D.: Interactions of noncompetitive inhibitors with nicotinic receptors in the rat brain. J. Pharmacol. Exp. Ther. **253:** 1083–1089, 1989.

TOROCSIK, A., OBERFRANK, F., SERSHEN, H., LAJTHA, A., NEMESY, K. AND VIZI, E. S.: Characterization of somatodendritic neuronal nicotinic receptors located on the myenteric plexus. Eur. J. Pharmacol. **202:** 297–302, 1991.

WARD, J. M., COCKCROFT, V. B., LUNT, G. G., SMILLIE, F. S. AND WONNACOTT, S.: Methyllycaconitine: A selective probe for neuronal alpha-bungarotoxin binding sites. FEBS Lett. **270:** 45–48, 1990.

WHEELER, J. W., OLUBAJO, O., STORM, C. B. AND DUFFIELD, R. M.: Anabaseine: venom alkaloid of *Aphaenogaster* ants. Science **211:** 1051–1052, 1981.

WHIDBY, J. F. AND SEEMAN, J. I.: The configuration of nicotine. A nuclear magnetic resonance study. J. Org. Chem. **41:** 1585–1590, 1976.

WONG, E. T., HOLSTAD, S. G., MENNERICK, S. J., HONG, S. E., ZORUMSKI, C. F. AND ISENBERG, K. E.: Pharmacological and physiological properties of a putative ganglionic nicotinic receptor, alpha3-beta4, expressed in transfected eucaryotic cells. Mol. Brain Res. **28:** 101–109, 1995.

WONNACOTT, S.: Alpha-Bungarotoxin binds to low-affinity nicotine binding sites in rat brain. J. Neurochem. **47:** 1706–1712, 1986.

WOODRUFF-PAK, D. S., LI, Y-T. AND KEM, W. R.: A nicotinic receptor agonist (GTS-21), eyeblink classical conditioning, and nicotinic receptor binding in rabbit brain. Brain Res. **645:** 309–317, 1994.

YAMAMOTO, I., KAMIMURA, H., YAMAMOTO, R., SKAI, S., GODA, M.: Studies on nicotinoids as insecticides. I. Relation of structure to toxicity. Agr. Biol. Chem.(Tokyo) **26:** 709–716, 1962.

ZHANG, Y., CHEN, J. AND AUERBACH, A.: Activation of recombinant mouse acetylcholine receptors by acetylcholine, carbamylcholine, or tetramethylammonium. J. Physiol. **486:** 189–206, 1995.

ZOLTEWICZ, J. A., BLOOM, L. B. AND KEM, W. R.: Quantitative determination of the ring-chain hydrolysis equilibrium constant for anabaseine and related tobacco alkaloids. J. Org. Chem. **54:** 4462–4468, 1989.

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