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Potencies and Channel Properties Induced by Semirigid Agonists at Frog Nicotinic Acetylcholine Receptors

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SUMMARY

Structure-activity relationships were investigated in a series of semirigid nicotinic agonists. Three of the agonists, (−)-ferruginine methiodide, arecoline methiodide, and its ketonic analogue arecolone methiodide, were cyclic analogues of anatoxin-a, a potent, naturally occurring, bicyclic alkaloid. Two other cyclic agonists, (−)-cytisine and (±)-muscarone, and the simplest agonist, the tetramethylammonium ion, were also tested. Arecolone methiodide and (−)-ferruginine methiodide have been tested as nicotinic agonists for the first time. Relative potency was assayed by contracture on the rectus abdominis muscle of the frog *Rana pipiens*. Natural, (+)-anatoxin-a, the most active of all of the agonists, was more than twice as potent as racemic anatoxin-a. Arecolone methiodide ranked after anatoxin-a in potency, being 8.6 times more potent than carbamycholine. A correlation between nicotinic potency and steric requirements probably involves the position of positively charged groups out of the plane defined by the carbonyl group and its two substituents. Channel properties induced by the agonists were evaluated by Fourier analysis of the end-plate current noise that resulted when the agonists were iontophoretically applied to frog sartorius muscle fibers. Average channel lifetimes were exponential functions of membrane potential, but the voltage sensitivity of channel lifetime seemed to vary among the agonists. Channel conductance, which was independent of membrane potential, also varied significantly among the agonists. The average charge traversing the membrane through each open channel, calculated from the product of average channel lifetime and current, did not correlate with potency. Therefore, the dominant component of potency is the frequency of channel opening. No clear relationship between the structure of the agonist and channel lifetime or conductance was evident.

INTRODUCTION

Nicotinic agonists are small, organic cations that, upon reacting with the recognition site of the AChR[4], permit the associated ion channel to open. As current crosses the muscle membrane, the end-plate region of the fiber depolarizes and, in multiply innervated fibers, the fiber undergoes contracture. Potencies of agonists are frequently compared by the force of contracture a given concentration will induce in frog rectus abdominis muscles or by the depolarization (measured by intracellular microelectrodes) it will induce in individual muscle fibers. These potencies in turn are composite functions of more elementary parameters, the opening frequency, the lifetime (τ), and the conductance (γ) of the AChR ion channels. The last two parameters, which can be estimated by Fourier analysis of end-plate current noise and by the patch-clamp technique, vary with the agonist (e.g., ref. 1). Our objectives were to seek interrelationships among some of the physiological responses one can measure as well as the relationships of these responses to agonist structure.

Most agonist molecules, especially the natural neurotransmitter ACh, are flexible structures capable of sev-

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[4] The abbreviations used are: AChR, denotes the entire glycoprotein complex comprising the recognition sites for agonists and the subunits that link these and form the cation channel. This abbreviation does not distinguish between the receptor of the sartorius and rectus abdominis muscles, although these receptors could be different; ACh, acetylcholine; AnTX-a, anatoxin-a; Carb, carbamylcholine; TMA, tetramethylammonium.

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eral conformations. Acetylcholine has been the subject of numerous investigations into its ground-state conformation and rotational energy barriers (e.g., see Discussion and citations in ref. 2). Since these investigations assume that certain environmental conditions (e.g., vacuum or bulk water) exist at the recognition site, such findings must still be viewed with caution. Completely rigid agonists would obviate this uncertainty, but very few are known. (+)-AnTX-a is a semirigid, bicyclic amine (Fig. 1) that is a potent nicotinic agonist (3–5). Its effect on channel properties resemble closely those induced by ACh (5). By testing analogues of (+)-AnTX-a, we hoped to find graded changes in potency and channel properties relating to alterations in structure. The thermodynamically preferred conformations of (+)-AnTX-a and its analogues are limited and governed by the (bi)cyclic ring system, the endocyclic double bond, and its influence on the rotational freedom of the conjugated carbonyl group. Two other cyclic agonists, (−)-cytisine and (±)-muscarone, were also tested. TMA served as a control because it is the simplest of the agonists and completely rigid.

MATERIALS AND METHODS

Solutions and drugs. The frog Ringer's solution had the following composition (millimolar): NaCl, 116; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4, 1.3; and NaH_2PO_4, 0.7. The pH was 7.1.

ACh chloride, Carb chloride, and tetrodotoxin were purchased from Sigma Chemical Company (St. Louis, Mo.); tetramethylammonium bromide from Aldrich Chemical Company (Milwaukee, Wisc.); and (−)-cytisine from Koch-Light Laboratories (Colnbrooks, Bucks., England). Arecoline methiodide was synthesized from arecoline and iodomethane. (+)-AnTX-a, (−)-ferruginine methiodide, and arecolone[5] methiodide were synthesized as described by Campbell *et al.* (6). Racemic AnTX-a was obtained by one of us (B.W.). (±)-Normuscarone was synthesized as described previously (7) and quaternized with iodomethane. Acetylcholinesterase, partially purified from *Torpedo ocellata*, was kindly provided by Dr. M. Eldefrawi (University of Maryland School of Medicine).

The potency ratios of the agonists, defined as reciprocals of equipotent molar ratios, were estimated by measuring the force of contracture of frog (*Rana pipiens*) rectus abdominis muscles at 22°. Isometric tension was measured by Grass FT.03 force displacement transducers. The muscles equilibrated under 1–2 g of resting tension for usually 1 h before adding an agonist solution. Muscles were bathed in the agonist solutions for 5 min or until tension reached a peak, whichever came first. Then they were washed three or more times for >30 min before the next concentration was tested. The protocol followed that of a symmetrical (3 + 3) assay using concentrations of a standard agonist (usually Carb at 10, 20, and 40 μM) and three nearly equivalent concentrations of the agonist under test, all of which were administered in random order. Potency ratios and their confidence intervals were calculated as described by Colquhoun (8).

[5] The trivial name arecolone is used to denote 1-methyl-3-acetyl-1,2,5,6-tetrahydropyridine.

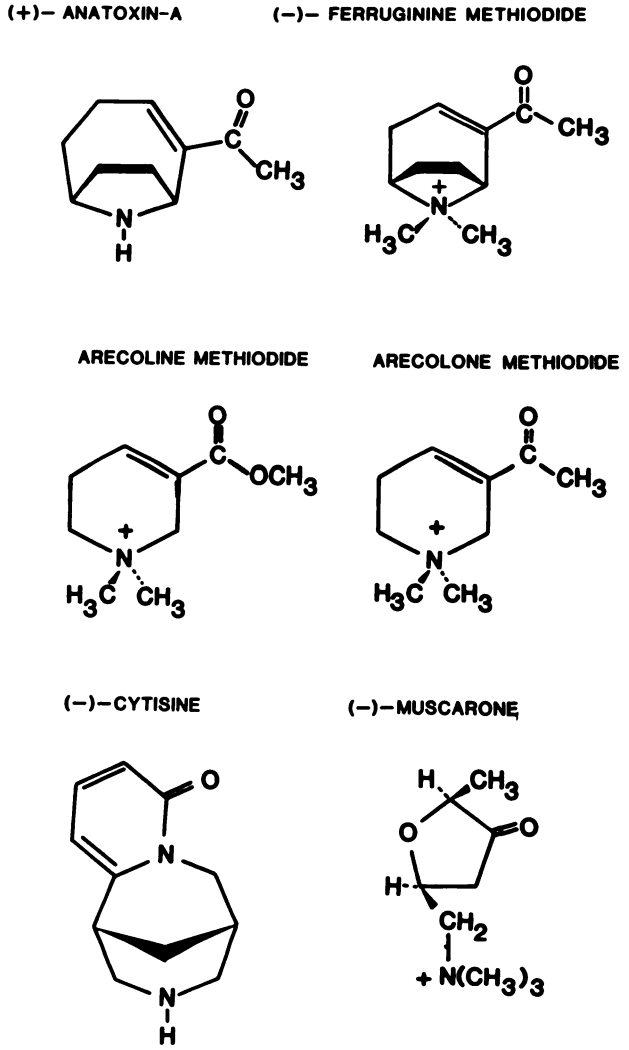

FIG. 1. *Structures of the cyclic agonists studied.* These projections were drawn to show the family resemblance among the top four agonists and the similar placement of the carbonyl and amine groups in the bottom two agonists. Although (−)-muscarone is depicted, racemic muscarone was used. In addition to these cyclic agonists, tetramethylammonium was also tested.

Before comparing the natural enantiomer to racemic AnTX-a, the concentrations of the two stock solutions were reconciled spectrophotometrically (λ_{max} = 228 nm). This precaution (which led to a 15% correction) was necessary because the small amounts of toxins were obtained preweighed.

Estimation of channel lifetime (τ) and conductance (γ). Frog sartorius muscles from *R. pipiens*, pinned to a paraffin or Sylgard block, were bathed in frog Ringer's solution containing 0.3 μM tetrodotoxin to inhibit action potentials. Details of the recording and analysis of endplate current noise are described elsewhere (9, 10). Briefly stated, a two-microelectrode voltage clamp was applied to the junctional region of surface fibers. Microelectrodes contained 3 M KCl and had resistances of 2–5 Mohm. Agonists were delivered iontophoretically from micropipettes whose braking currents were so adjusted that they did not depolarize denervated (7–14 days) rat soleus muscles. Upon voltage clamping a fiber, noise reponses

were obtained at the initial resting potential of the fiber and then, at 3- to 5-min intervals, at hyperpolarizing potentials, increased in 10- or (usually) 20-mV increments until either the clamp failed or the d.c. offset became excessive. Then records were obtained at descending potentials in the same way, starting from the most hyperpolarizing potential. Iontophoretic currents were about 30 namp, and d.c. end-plate currents were usually around 40 namp. Usually two or three fibers were tested per muscle. The d.c. and high-gain a.c. (0.5–800 Hz bandpass) end-plate currents induced in the muscle fiber as well as the magnitude and duration of the microiontophoretic current were recorded on a Racal Store 4 DS FM tape recorder. For analysis, the noise from the a.c. channel was digitized (2 KHz), and a fast Fourier transform (11) was performed by a PDP 11/40 digital computer on 512-point samples. Baseline spectral density was subtracted from that induced by the agonist. Each spectrum was the average of 30 such pairs of samples. The cutoff frequency and the intercept, $S(0)$, were estimated from a Lorentzian function, fitted to the spectral density points by the MODFIT nonlinear regression program (12). Most of the noise studies were performed at a bath temperature of 10° (± 1.0°, range) so that channel lifetime would be prolonged sufficiently for the sampling and analysis routines.

RESULTS

Potency ratios. In the previous study (5), racemic AnTX-a was shown to be more potent than the commonly available agonists. The natural, (+)-enantiomer is now seen (Table 1) to be more than twice as potent as the racemic mixture. The 99% confidence interval for the potency ratio [(+) to (±)] was 2.1 to 3.1, suggesting that the (−)-enantiomer may even block the AChR.

Potency ratios for other pairs of agonists are shown in Table 1. Initially we used (±)-AnTX-a as a reference because of its similarity to ACh and its stability in the presence of cholinesterase. We switched to Carb as a reference because it is widely available and crystalline. The potency ratio for (−)-cytisine versus Carb may be calculated to be 1.1 from the potency ratio of (±)-AnTX-a to Carb of 12 (5) and the ratio of (−)-cytisine to (±)-AnTX-a in Table 1. Arecoline and arecoline methiodide have been reported to compete with ACh for acetylcholinesterase (13). If arecoline methiodide were rapidly hydrolyzed, its apparent potency would be decreased. To test for hydrolysis, we monitored pH changes in the presence of partially purified acetylcholinesterase (0.12 mg/ml). Arecoline methiodide (10 mM) was stable. Changes in pH (a few hundredths of a pH unit) were indistinguishable from a water blank. By contrast, much lower concentration of ACh (0.1 mM) was rapidly hydrolyzed under these conditions, decreasing pH by 0.8 unit in 1 min.

TABLE 1

Potency ratios for pairs of agonists tested at frog rectus abdominis muscles

Drug pair	No. of frogs	Potency ratio	95% confidence interval
Natural vs. (±)-AnTX-a	7	2.5	2.2–2.9
(−)-Cytisine vs. (±)-AnTX-a	6	0.088	0.075–0.104
TMA vs. Carb	5	0.20	0.17–0.23
(±)-Muscarone I vs. Carb	8	0.77	0.67–0.90
Arecoline methiodide vs. Carb	8	1.3	1.2–1.4
(−)-Ferruginine methiodide vs. Carb	8	3.3	2.7–4.0
Arecolone methiodide vs. Carb	9	8.6	7.5–10.0

Contractures testing TMA differed from those testing the other agonists. A total of 14 muscles was tested, and in 9 of these, the third point (highest concentration of TMA, 200 μM) produced an increase in muscle tension around 30% higher than expected based on extrapolating the line joining the other two points, which was nearly parallel with the line joining the Carb responses. This anomaly appeared in the analysis of variance as a highly significant ($p < 0.001$) deviation from parallelism between the two curves. However, the first two points of these curves supported the potency ratio of 0.2, as derived from the remaining five experiments (Table 1). For no other pair of agonists was deviation from parallelism seen.

Because (+)-AnTX-a is a secondary amine, the effects of substitution on the nitrogen of the other agonists were of interest. (−)-Ferruginine and arecolone (tertiary amines) were tested in preliminary contracture experiments. Potency ratios (± about 25%) relative to Carb were 0.04 and 0.17, repsectively. Arecoline has been tested previously by others (see Discussion). In addition the planar, aromatic, quaternary analogue of arecolone, 3-acetylpyridine methiodide was also tested in preliminary experiments and found to be about 0.02 as potent as Carb.

Channel properties. Upon applying an agonist to a voltage-clamped muscle fiber, both the d.c. current and the fluctuations in the current ("noise") increase. Fourier analysis of the noise yields power density spectra, as exemplified in Fig. 2. From the cutoff frequency and intercept [$S(0)$], one estimates average channel lifetime (τ) and conductance (γ) by simple formulae (e.g., refs. 9 and 10).

The mean γ values for the various agonists are shown in Fig. 3. For each agonist the γ estimates are rather dispersed, and analysis of variance showed significant differences between cells. Therefore, in comparing the γ between agonists, nested analysis of variance (14) was used to account for differences between cells. From the analysis of variance table, the mean square deviation was 503 between agonists (5 degrees of freedom), 52.5 (88 degress of freedom) between cells, and 8.50 pS^2 (381 degrees of freedom) between tests (within cells). Thus, while there were significant differences between cells, there were also significant ($p < 0.0005$) differences between agonists. Histograms of γ within agonists were unskewed and unimodal; means agreed with medians. Of the 94 cells represented here, in only one was there a correlation between γ and membrane potential. The γ for ACh at 10° was estimated to be 10.1 ± 0.8 (SE) pS (22

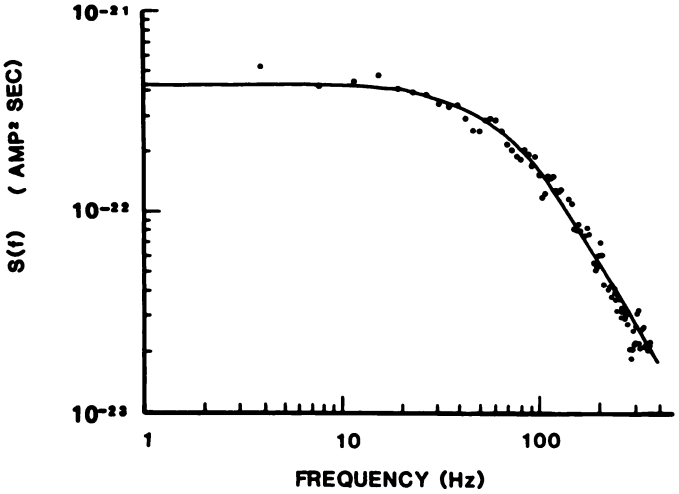

FIG. 2. *An example of a power density spectrum of end-plate current fluctuations in response to (−)-ferruginine methiodide*

This and all other spectra were obtained from surface fibers of frog sartorius muscles. In this example the holding potential was −65 mV and the end-plate current was −66 namp. From the cutoff frequency (76.1 Hz), τ was calculated to be 2.09 msec. Using the intercept [$S(0) = 4.31 \times 10^{-22}$ amp^2 sec), and the data given above, γ was calculated to be 15.7 pS.

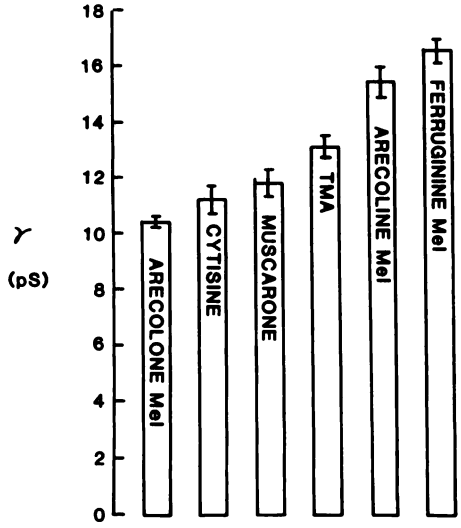

FIG. 3. *Average channel conductance, γ, induced by the agonists tested*

Means and standard errors are shown. The number of spectra averaged to obtain these values were as follows: arecolone methiodide, 93; (−)-cytisine, 80; (±)-muscarone, 59; TMA, 75; arecoline methiodide, 69; (−)-ferruginine methiodide, 99.

spectra). The γ for ACh at 22° was found to be 21.0 ± 0.1 (SE) pS (44 spectra).

Logarithms of channel lifetimes (τ) for the agonists are plotted against membrane potential in Fig. 4. Linear regression analysis of each plot yielded the estimates of slope and predicted τ at −90 mV shown in Table 2. Each slope differed significantly ($p \ll 0.01$) from zero. The slopes also differed from one another (Table 2). Analysis of variance (14) confirmed that the slopes were indeed heterogeneous ($p < 0.002$), apparently clustering at around $-9V^{-1}$ and $-15V^{-1}$ (logarithms to base e). ACh spectra (obtained at 10°) yielded a slope of 10.9 ± 3.2 V^{-1}

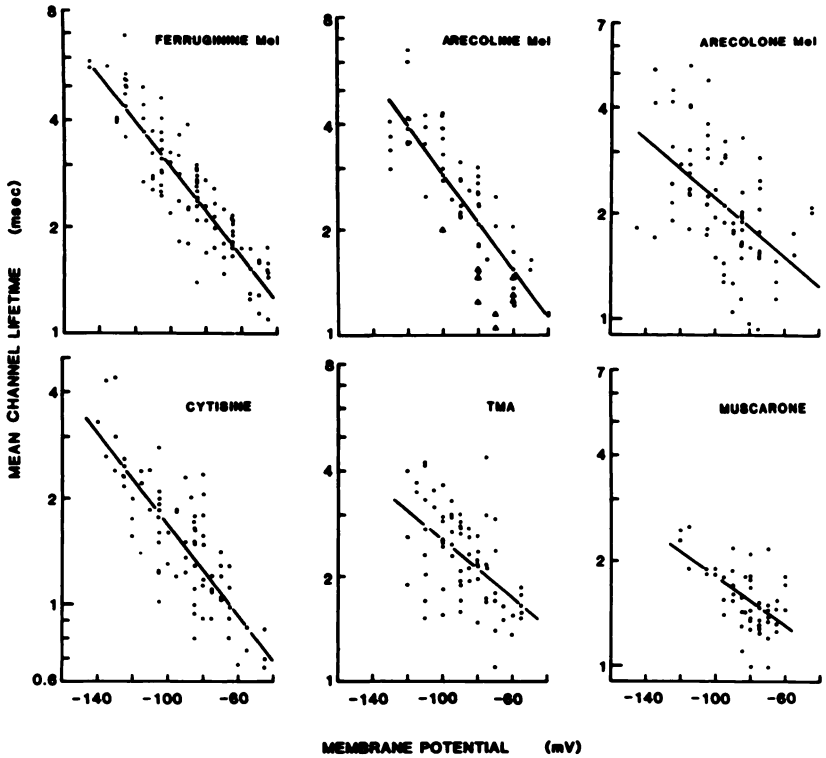

FIG. 4. *Semilogarithmic plots of average channel lifetime, τ, versus membrane potential for the agonists tested*

Each *point* represents τ estimated from one spectrum. In the plot for arecoline methiodide, *triangles* represent τ estimates obtained about 1.5 years earlier than the rest, and are nearly continuous with the other points. The lines shown were found from linear regression treatment of the logarithms of τ on membrane potential.

TABLE 2

Linear regression parameters from the plots of logarithm (base e) of average channel lifetime (τ) versus membrane potential

The τ values were estimated by fluctuation analysis of end-plate currents obtained from frog sartorius muscle fibers.

Agonist	No. of spectra	Slope V^{-1}	Predicted τ at −90 mV
(±)-Muscarone	53	8.1 ± 1.7	1.68 ± 0.05
TMA	75	9.5 ± 1.7	2.33 ± 0.07
Arecolone methiodide	77	9.6 ± 1.8	2.02 ± 0.08
(−)-Ferruginine methiodide	100	14.5 ± 0.7	2.61 ± 0.05
(−)-Cytisine	80	15.0 ± 1.3	1.45 ± 0.04
Arecoline methiodide	69	15.7 ± 1.4	2.48 ± 0.08

and predicted τ at −90 mV of 4.4 ± 0.3 msec. These values resulted from only 25 spectra and are therefore less precise than the others. At 22°, the plot of logarithm τ versus membrane potential for ACh had a slope of 7.5–3.5 V^{-1} and predicted τ at −90 mV of 1.28 ± 0.06 msec (54 spectra).

DISCUSSION

We have evaluated several semirigid nicotinic agonists with the goal of relating structure to action. The three actions measured were over-all potency as assayed by contracture of the rectus abdominis muscle of the frog, and average lifetime and conductance of the ion channels associated with the AChR. The channel properties estimated by noise analysis are thought to be elemental units of AChR action, of which potency is a function. The agonists were conformationally constrained to remove some of the uncertainty in relating structure to action. Three of the agonists were modeled on AnTX-a, a potent agonist that closely resembles ACh in its action (5).

Potency ratios. Natural, (+)-AnTX-a was the most potent of the agonists, being about 30 times more potent than Carb. This enantiomer was at least twice as potent as the racemic mixture, which shows that (−)-AnTX-a is a very weak agonist or perhaps is an antagonist. This finding contrasts with that of Carmichael *et al.* (4), who found no difference between (+)- and (±)-AnTX-a. The stereoselectivity of the AChR for AnTX-a supports the view that the AChR does recognize chirality and may recognize more than two points on the agonist molecule. The high (>1000:1) stereoselectivity of the AChR for the moderately potent agonist, *trans*-3-acetoxy-1-methylthiane (15), studied on the rectus abdominis muscle of the frog *R. temporaria* also supports this view. Stereoselectivity by the nicotinic AChR has not been widely appreciated, partly because few optically active nicotinic agonists are known, and fewer of their enantiomeric pairs have been tested. The stereoselectivity of nicotinic receptors for the enantiomers of one agonist, nicotine, was found to vary considerably between various preparations (16). Another pair, (+)- and (−)-muscarone, does show little difference in nicotinic activity between enantiomers (17–19), but this result can be explained by the following hypothesis. We propose that the AChR donates a hydrogen bond to the agonist [as Beers and Reich (20) suggested] and recognizes a plane defined by the hydrogen bond acceptor (i.e., the carbonyl group and its two substituents). The anionic site of the AChR is positioned out of this plane, off of the C=O axis. These conditions suffice to establish chirality at the recognition site, and furthermore help to explain the effect that quaternization has on agonist potency. The muscarone enantiomers are nearly equipotent because the quaternary nitrogen lies *on* the carbonyl plane (data from ref. 21) so that the methyl groups, which, like ACh probably bear the positive charge (22), can project on either side of this plane, regardless of the enantiomer. This hypothesis explains the function of quaternization as a forced displacement of a methyl group out of the carbonyl plane. Although 3-acetylpyridine methiodide is a quaternary amine, the *N*-methyl group of this feeble agonist lies on the carbonyl plane. Arecoline (23, 24) and arecolone (our results) are weaker than their *N*-methyl derivatives because steric crowding enforces the equitorial conformation, in which the methyl group is approximately on the carbonyl plane. In polycyclic agonists, such as AnTX-a, cytisine and nicotine, quaternization is unnecessary because the ring systems themselves separate the cationic regions from the carbonyl plane. *N*-methylation of nicotine, for example, enhances potency by only 1.8-fold (25), and successive methylation of (−)-cytisine leads to successive *decreases* in potency (26). However, the activity of (−)-ferruginine did not fit this hypothesis. It is not clear why *N*-methylation so markedly enhanced its activity.

Two of the agonists, arecolone methiodide and (−)-ferruginine methiodide, have not been tested previously for nicotinic activity. Arecolone methiodide in particular may have future usefulness because it is very potent (Table 1), while being chemically stable and simpler than bicyclic agonists. Our finding of a potency ratio of 1.3 for arecoline methiodide to Carb agrees with the ratio of 1.4 found previously (24). From our results one can calculate a potency ratio of cytisine to TMA of 5.5. By multiplying previous values for equipotent molar ratios involving cytisine, β-pyridylmethyltrimethylammonium, *m*-hydroxyphenylpropyltrimethylammonium, and TMA (25–27), one can compute a similar potency ratio of 5.3 as obtained by Barlow and co-workers. We found muscarone to be relatively weak, 0.8 times the potency of Carb. This contrasts with the reports that muscarone is twice as potent as ACh (17, 18, 28). In the previous studies, however, no anticholinesterase agent was used, which can easily account for the discrepancy (we find ACh + 10 μM neostigmine to be about equipotent with (+)-AnTX-a in contracture experiments).

Channel properties. Channel properties did vary among the agonists (Figs. 3 and 4; Table 2) but by less than 2-fold (at −90 mV). Differences in τ were complicated by the finding of differences in the voltage sensitivity in τ among the agonists (discussed below). Can differences in channel properties (τ and γ) account for variations in potency? To find out, we calculated average charge admitted per channel at −90 mV [τ (at −90 mV) × γ × 0.09 V] for each agonist and plotted this value and relative potency as a scattergram (Fig. 5). Since no correlation is seen, we conclude that channel opening frequency dominates γ and τ in deciding potency. Opening frequency, in turn, may be controlled by desensitization or other types of concomitant blockade, which may vary among the agonists.

Many compounds are known to block the AChR at

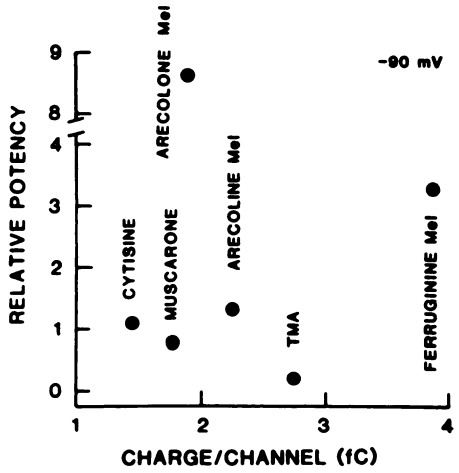

FIG. 5. *Scattergram of relative (to Carb) agonist potency and average charge traversing an open channel*

Relative potency was determined by muscle contracture experiments. Average charge per channel is the product of τ (at −90 mV), × γ × 0.09 V. No correlation between potency and charge/channel is evident.

sites apart from the recognition site for ACh (2). Although blockade may be characterized as of an open- or closed-channel type or may be described as an acceleration of desensitization, the relationship among these actions is obscure. Certainly with perhydrohistrionicotoxin (29) and probably with most other antagonists of this type, stereochemistry is of minor importance; hydrophobic forces seem dominant. One expects that agonists, too, may produce similar sorts of blockades. This nonstereospecific action may be responsible for the potency ratio, which slightly exceeds 2:1, for (+)- to (±)-AnTX-a. It is possible that variations in γ and τ seen by various agonists have less to do with differences in activated states than they do with blockade. The agonist could act as if it occludes the open-ion channel, as decamethonium is thought to do (30), thereby shortening channel lifetime or chopping the open phase as does the local anesthetic QX222 (31). The latter mechanism would lead to double Lorentzian spectra unless chopping frequency exceeded the bandpass used for the Fourier analysis. No double Lorentzian spectra were evident, however. Generally, noncompetitive antagonists show voltage sensitivity such that their rates or equilibrium levels of blockade increase with hyperpolarization (e.g., refs. 2, 30, 31). If the agonists were blocking open channels, those agonists that produced the shortest channel lifetimes would be expected to have the shallowest ln τ versus membrane potential plots and conversely. This result was not seen. Of the six semirigid agonists tested here, (−)- cytisine and (−)-ferruginine methiodide induced opposite extremes in channel lifetime (for any given membrane potential) but had plots of ln τ versus membrane potential with indistinguishable slopes (Table 2; Fig. 4). Thus, whereas concomitant blockade is still a possible action of these agonists, no clear evidence in its favor emerged from these studies.

As seen in Table 2, the slopes of the regression lines for ln τ versus membrane potential did seem to vary among some of the agonists. The slopes were found to be significantly heterogeneous by an analysis of variance test. This new finding is now under study using patch-clamp experiments. If confirmed, it would show that this voltage sensitivity is *not* an intrinsic property of the AChR at a given temperature. If the voltage sensitivity is due to a change in dipole moment in the AChR that accompanies channel closure (32), our evidence would suggest that the agonists can influence this change in different ways.

There is no clear relationship between γ or τ and agonist structure. Small alterations in the molecular structure of the agonist alter γ and τ in unpredictable ways. This could be because γ and τ, estimated by noise analysis, do not accurately reflect elementary molecular properties or because geometric structure gives little clue into subtle interactions involving hydrophobic effects, polarizability, hydrogen bonding, charge distribution, etc. These more subtle molecular properties are being investigated by computer modeling methods.

Variations in channel properties (both γ and τ) in response to different agonists, as determined by noise analysis, have been reported by numerous authors (see a tabulation of these results in ref. 1). The method has well-known, inherent limitations. Noise analysis condenses details of channel currents into only few (two here) parameters. Fine structure of channel currents and infrequent events, seen in single-channel (patch-clamp) recordings, are usually unresolved by noise analysis. High-frequency events are necessarily attenuated and smoothed by the filtering that is required by the sampling procedure. In addition, a discrepancy between the results of noise analysis and patch clamp may exist. Variations in channel conductance among agonists, as determined by noise analysis, reported previously (e.g., ref. 1) were not evident in the short patch-clamp records shown by Neher and Sakmann (33). This discrepancy may be a consequence of sampling error, the preparation (innervated versus denervated frog muscle), or other factors that await clarification. Although the molecular interpretation may be considered tentative, the method of noise analysis does reveal changes in channel response due to the agonists we investigated.

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