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Can Nicotine Self-Inhibition Account for Its Low Efficacy at the Nicotinic Acetylcholine Receptor from *Torpedo*?

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SUMMARY

Nicotine, a partial agonist, has a very low efficacy at the nicotinic acetylcholine receptor from *Torpedo*, but it is not clear whether this is because it is intrinsically poor at opening the ion channel or because, at concentrations that open the channel, it is also capable of blocking it. In this study, we exploited the action of ethanol, which increases the apparent affinity of cholinergic agonists for channel activation, and demonstrated that the weak action of nicotine is consistent with simultaneous activation and inhibition of the receptor. The presence of ethanol increased the efficacy of nicotine, producing an increase in the initial rate of cation efflux from acetylcholine receptor-rich membrane vesicles, as measured by a rapid quench-flow tracer ion assay. The initial rate of efflux increased with ethanol concentration until, in the presence of 1.5 M ethanol, the response to nicotine was indistinguishable from that of the full agonist carbamylcholine. The concentration-response curves for nicotine were bell-shaped, showing activation at low concentrations and inhibition at higher concentrations. Increasing concentrations of ethanol increased the apparent affinity of nicotine for channel activation and decreased its apparent affinity for channel inhibition. These actions broadened the bell-shaped curve, increasing the maximum response until it was equivalent to that of a full agonist. The apparent affinity of nicotine for its inhibitory site, derived from the aforementioned data, agreed with that determined independently by measuring the inhibition by nicotine of initial rates of ion efflux in response to acetylcholine. A value for the apparent affinity of nicotine for channel opening was estimated from the dependence of this parameter on ethanol concentration. When combined, these two parameters predicted the bell-shaped concentration-response curve for the action of nicotine. The results presented in this study are consistent with the notion that the efficacy of nicotine is determined by its relative affinities for channel activation and channel inhibition, but they do not rule out other contributions.

It is a well known fact that for any biological system various agonists have different intrinsic activities, or efficacies (1, 2). A compound with very low efficacy fails to elicit the maximal response of which a system is capable, even when occupying all available receptors, and is known as a partial agonist.

The nAcChoR is one of the most thoroughly studied receptor systems. The neuromuscular junction has provided a convenient and readily accessible experimental model for electrophysiological experiments (3), and the abundant receptor present in the electroplaque membranes of *Torpedo* and *Electrophorus* has been used in biochemical investigations of drug-receptor relationships (4–6). The structure and function of this ligand-gated ion channel are widely documented (for an excellent review, see the work of Stroud (7)].

However, even in this well characterized system, the molecular mechanisms of partial agonist action have not been unequivocally elucidated. Originally, it was suggested that partial agonists were intrinsically poor at opening the ion channel (8). More recently, the observation of cholinergic self-inhibition, i.e., that high concentrations of full cholinergic agonists inhibit nAcChoR-mediated cation flux (6, 9–11), prompted the suggestion that it is the difference in affinity of an agonist for its activation and inhibition sites that defines its efficacy (12, 13). Although good evidence has been obtained that partial agonists simultaneously have agonist and local anesthetic-like actions on nAcChoR (13, 14), it is not possible to tell whether this is the sole reason for their weak cholinergic action or whether they also exhibit a genuinely poor ability to open the ion channel (15, 16).

Ethanol enhances the apparent affinity of cholinergic agonists for channel activation without changing the maximum attainable flux (17), and this action occurs without an alteration of single-channel conductance (18, 19). If partial agonists are affected in the same way, this action can be exploited to test the hypothesis that the efficacy of an agonist increases as the difference in its affinity for its activating and inhibiting sites

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ABBREVIATIONS: nAcChoR, nicotinic acetylcholine receptor(s); α-BTX, α-bungarotoxin.

on the receptor increases. The results presented in this study are consistent with this model for the partial agonism of nicotine at nAcChoR.

Materials and Methods

Preparation of *Torpedo* postsynaptic membranes. Postsynaptic membranes from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared using sucrose density gradient centrifugation at 4°, essentially as described by Braswell *et al.* (20). Membrane suspensions [5–10 mg of protein/ml, as measured by a modified Lowry method (21), and 7–15 μM in [^{3}H] acetylcholine binding sites, assayed as described (20)] were kept frozen at −80° for up to 6 months and were thawed within 48 hr of use. Before flux assays using acetylcholine, vesicles were incubated for 20 min with 0.1 mM diisopropylfluorophosphate at 4° to inhibit acetylcholinesterase. Flux responses were not altered by this treatment (22).

Measurement of nAcChoR cation channel function. Cation channel function was assayed by measuring agonist-induced $^{86}Rb^+$ efflux from sealed native *Torpedo* electroplaque vesicles at 4°, with either a manual 10-sec ion flux measurement or a rapid quenched-flow technique as described in detail by Forman *et al.* (6). Vesicles were loaded with $^{86}Rb^+$ overnight and extravesicular radioactivity was separated by exclusion and ion exchange chromatography. When necessary, the number of active receptor-channel complexes was reduced by blocking with the irreversible inhibitor α-BTX to prevent full equilibration of tracer within the experimental assay time (6).

Estimations of the experimental initial $^{86}Rb^+$ efflux rates. Measurements of $^{86}Rb^+$ efflux from α-BTX-treated vesicles at 4° were made after different periods, starting from the shortest time obtainable with the rapid mixing apparatus (2–3 msec). Flux times were calculated from measurement of flow rate through the rapid mixing device and the volume of the reaction tube used (that is, the volume between the first mixer, where flux is initiated, and the second mixer, where flux is quenched) (see the report by Forman *et al.* (6)].

Initial $^{86}Rb^+$ efflux rates (k_f) were estimated from eq. 1, which corrects the response for the decline in the $^{86}Rb^+$ concentration gradient as the assay progresses (4, 23):

$$-\ln\,(1 - F_{At}/F_{eq}) = k_f \cdot t \qquad (1)$$

where F_{At} is the flux signal integrated over time t and F_{eq} is the maximal flux signal possible in this system in the absence of α-BTX. For single time points within the linear kinetics period, initial rates (k_f) were also estimated using this equation.

When flux was integrated beyond the linear range, results were expressed as the fraction of maximal flux, $F_A/F_A(\max)$, where $F_A(\max)$ is the maximal response of the membrane preparation with the level of α-BTX used and is estimated from a 10-sec exposure to carbamylcholine. Note that some $^{86}Rb^+$ is trapped in vesicles that do not respond to agonists. Therefore, $F_A(\max)$ is always less than 100% of the trapped counts by an amount that varies somewhat from one vesicle preparation to the next. Typically 70–80% of vesicle-associated $^{86}Rb^+$ is the maximum amount released by carbamylcholine.

Analysis of nicotine concentration-response curves. Concentration-response curves for nicotine derived from flux measurements integrated over longer times (F_A) in *Torpedo* vesicles are bell shaped (6), and individual flux responses can be fitted by nonlinear least squares regression to the biphasic logistic equation:

$$F_A = F_A(\max)\cdot\frac{[A]^{n_1}}{[A]^{n_1} + K_A^{n_1}}\cdot\left(1 - \frac{[A]^{n_2}}{[A]^{n_2} + K_B^{n_2}}\right) \qquad (2)$$

where K_A is the agonist concentration producing 50% of the maximum response, n_1 is the Hill coefficient for activation, K_B is the agonist concentration that inhibits 50% of maximum flux, and n_2 is the Hill coefficient for self-inhibition.

Initial rate concentration-response curves for nicotine were fitted to a similar biphasic logistic equation:

$$k_f = k_f(\max)\cdot\frac{[A]^{n_1}}{[A]^{n_1} + K_A^{n_1}}\cdot\left(1 - \frac{[A]^{n_2}}{[A]^{n_2} + K_B^{n_2}}\right) \qquad (3)$$

where k_f is the initial rate of ion efflux in response to a nicotine concentration $[A]$ and $k_f(\max)$ is the initial rate of ion efflux in the presence of a maximal activating concentration of acetylcholine.

Concentration-response curves for carbamylcholine were fitted to the logistic equation:

$$F_A = F_A(\max)\cdot\frac{[A]^{n_1}}{[A]^{n_1} + K_A^{n_1}} \qquad (4)$$

Inhibitory concentration-response curves for the inhibition by nicotine of the initial rate in response to 1 mM acetylcholine were fitted to the logistic equation:

$$k_f = k_f(\max)\cdot\left(1 - \frac{[A]^{n_2}}{[A]^{n_2} + K_B^{n_2}}\right) \qquad (5)$$

Chemicals. Diisopropylfluorophosphate was from Aldrich Chemical Co. (Milwaukee, WI). α-BTX was from Miami Serpentarium (Miami, FL). Acetylcholine chloride, carbamylcholine chloride, (−)-nicotine di-(+)-tartrate, procaine hydrochloride, ethanol, and buffer reagents were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Acetylcholine and $^{86}Rb^+$ were from New England Nuclear (Boston, MA).

Results

Initial rate of ion efflux in response to nicotine. The initial rate of ion efflux from $^{86}Rb^+$-loaded vesicles was measured at 4° in response to a maximally activating concentration of nicotine (0.3 mM). Spare response was removed from the system by irreversibly blocking 40% of [^{3}H]acetylcholine sites with α-BTX. Fig. 1 shows the initial rate of nicotine-mediated ion efflux over 10 sec, compared with that elicited by a saturating concentration of the full agonist carbamylcholine, at nAcChoR from *Torpedo*. The initial rate of nicotine-mediated efflux was very low and appeared linear for up to 2 sec. After 10 sec both agonists reached an equilibrium maximal flux

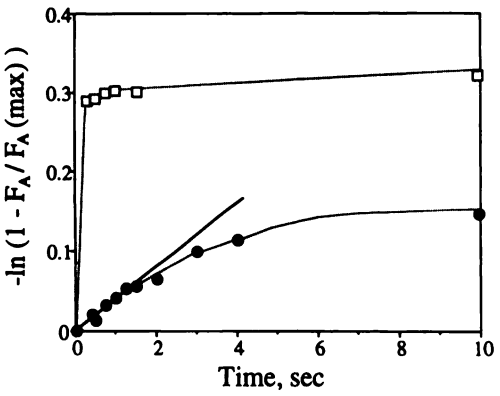


Fig. 1. Initial rates of $^{86}Rb^+$ efflux measured by pulsed quench flow. F_A values were calculated from quenched-flow $^{86}Rb^+$ efflux measurements at 4°, using a single *Torpedo* vesicle preparation that was incubated with α-BTX to block 40% of [^{3}H]acetylcholine sites. Measurements were made in triplicate and flux times were calculated as described in Materials and Methods. Average F_{At} values were analyzed by eq. 1, given in Materials and Methods, fixing the *y*-intercept through zero and ignoring points at $t \geq 2$ sec. The rate coefficient for ion efflux, k_f, is given as the initial slope of this plot (——). The curve was drawn by eye. ●, 0.3 mM nicotine [$F_A(\max) = 15\%$], $k_f = 0.04 \pm 0.0021$ sec^{-1}; □, 5 mM carbamylcholine [$F_A(\max) = 50\%$], k_f cannot be resolved at this time scale.

response [F_A(max) carbamylcholine = 50%; F_A(max) nicotine = 15%]. The rate of ion efflux in response to 5 mM carbamylcholine was too high to be resolved over this time scale. Thus, the reason that nicotine acts as a partial agonist at nAcChoR from *Torpedo* can be explained, at least in part, by the low initial rate of ion efflux in response to this drug, compared with that of a full agonist.

Initial efflux kinetics at 4° in response to 0.3 mM nicotine in the absence and presence of ethanol are presented in Fig. 2. Data were collected over 3–16 msec to ensure linear kinetics and were compared with initial rate kinetics in response to the agonist carbamylcholine (5 mM). The maximum flux response to carbamylcholine during this period of time never exceeded 30%. During this short time-frame, the initial rate of carbamylcholine-induced efflux could be determined but that induced by nicotine alone was difficult to resolve because its initial rate was so much lower. However, simultaneous addition of ethanol, which does not affect the response to saturating concentrations of a full agonist like acetylcholine (17), enhanced the rate of ion efflux in response to 0.3 mM nicotine in a concentration-dependent manner. This increase in initial rate was significant at 1.0 M ethanol, and at 1.5 M ethanol the rate was indistinguishable from that of carbamylcholine. It was not possible to use concentrations higher than 2.0 M ethanol in this study because increased nonspecific $^{86}Rb^+$ leak from vesicles overwhelmed the flux responses (24).

Effect of ethanol on nicotine initial rate concentration-response curves. The initial rates of ion efflux in response to a 10^4-fold concentration range of nicotine were measured using single-time point kinetics. A flux integration time of 11 msec was chosen because this is the longest time securely within the region of linear kinetics for all ethanol concentrations (Fig. 2).

Fig. 3 shows flux responses to nicotine, integrated over 11 msec. Nicotine is such a weak agonist that it was only necessary to treat vesicles with α-BTX when the ethanol concentration was ≥1.5 M. Initial rates of ion efflux (k_f) were estimated from eq. 1 and, if the maximum signal was adequate, fitted to eq. 3, fixing k_f(max) to the value established by the measurement of F_A in triplicate at 11 msec in response to 5 mM carbamylcholine during each experimental run. All fitted parameters are given in Table 1, where it can be seen that increasing the ethanol concentration from 1.5 to 2.0 M produced a 2-fold decrease in the apparent dissociation constant for the activating site (K_A) and an insignificant decrease in the affinity of nicotine for its inhibition site. The increasing gap between K_A and K_B resulted in an overall increase in the initial rate of ion efflux at the maximally activating concentration of nicotine, until with 2.0 M ethanol the maximal rate was equivalent to that obtained with 5 mM carbamylcholine. This is consistent with the measurements of initial rates reported above. At lower ethanol concentrations, even maximally activating concentrations of nicotine in the absence of α-BTX elicited such low flux responses at 11 msec [F_A(max) for nicotine plus 1.0 M ethanol, ≤20%; nicotine alone, <5%] that the data could not be fitted to eq. 3.

Although it is known that at millisecond times the maximum flux elicited by acetylcholine is unaffected by ethanol (17) (a

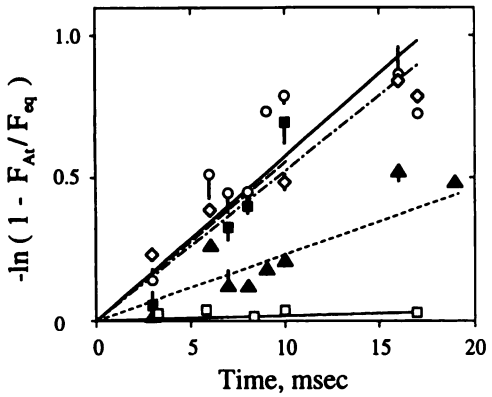


Fig. 2. Effect of ethanol on the initial rate of ion efflux in response to nicotine. Vesicles were pretreated with α-BTX to block approximately 40% of [^{3}H]acetylcholine sites. Measurements of flux responses to a maximally activating concentration of nicotine (0.3 mM) were made at 4° using rapid quenched flow in the presence of varying amounts of ethanol. Data for channel activation up to 20 msec were fitted to eq. 1 through an intercept of zero [——, carbamylcholine (*upper line*) and nicotine (*lower line*); – – –, 1 M ethanol; —·—·—, 1.5 M ethanol; — — — —, 2 M ethanol]. For the purpose of clarity, data points were averaged in the graph. The slopes of the plots, estimates of the initial rate of ion efflux, k_f, are compared with that of a maximally activating concentration of carbamylcholine (5 mM), k_f(nor). Fitted parameters and their standard deviations are as follows [k_f (sec^{-1}), k_f(nor)]: ○, no ethanol, 5 mM carbamylcholine, 0.06 ± 0.002, 1; □, no ethanol, 0.3 mM nicotine, 0, not determined; ▲, 1.0 M ethanol, 0.02 ± 0.001, 0.404; ◇, 1.5 M ethanol, 0.05 ± 0.002, 0.905; ■, 2.0 M ethanol, 0.06 ± 0.004, 0.960.

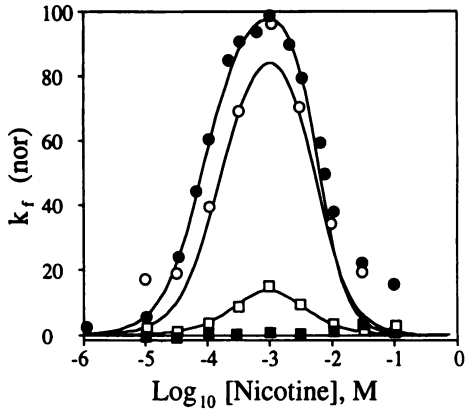


Fig. 3. Nicotine concentration-response curves for efflux rate in various concentrations of ethanol. Initial efflux rates were calculated from 11-msec $^{86}Rb^+$ efflux measurements, in response to a range of nicotine concentrations, from two different *Torpedo* vesicle preparations that had been incubated with α-BTX to block 40% of [^{3}H]acetylcholine sites (1.5 and 2.0 M ethanol) or that had not been incubated with α-BTX. Values of the efflux were normalized to the maximal flux obtained with 5 mM carbamylcholine (integrated for 10 sec and were fitted by nonlinear least squares to the biphasic equation given in the text (eq. 3). Data are shown for nicotine in the presence of 0.5 M ethanol (■), 1.0 M ethanol (□), 1.5 M ethanol (○), and 2.0 M ethanol (●). Fitted parameters for the 1.5 M ethanol and 2.0 M ethanol data are given in Table 1; for 0.5 M ethanol and 1.0 M ethanol the lines are drawn by eye.

TABLE 1

Parameters for 11-msec nicotine concentration-response curves

Apparent dissociation constants and Hill coefficients (mean ± standard deviation) for activation (K_A and n_1, respectively) and inhibition (K_B and n_2, respectively) were derived from nonlinear least squares analysis of 11-msec flux measurements in Fig. 3, using eq. 3 with the normalized k_f(max) fixed at 1. Data for ethanol concentrations of ≤1.0 M could not be fitted.

[Ethanol]	K_A	n_1	K_B	n_2	K_B/K_A
M	μM		mM		
1.5	170 ± 46	1.3 ± 0.45	5.3 ± 1.40	1.5 ± 0.54	31
2.0	89 ± 7.5	1.3 ± 0.14	6.2 ± 0.39	1.9 ± 0.24	70

result that we confirmed), carbamylcholine is a slightly weaker agonist than acetylcholine (6). Therefore, in experiments in which flux was integrated for 11 msec with vesicles that were blocked with α-BTX, we examined whether 1.5 M ethanol could enhance the maximum flux elicited by carbamylcholine. Addition of ethanol to the maximum stimulating concentration of carbachol (10 mM) increased the maximum flux to that of acetylcholine. Similarly, nicotine (0.3 mM) with ethanol achieved the response of acetylcholine. This effect was also observed with maximum stimulating concentrations of suberyldicholine (1 mM). Thus, the efficacy of all three agonists in the presence of sufficient amounts of ethanol equaled that of acetylcholine.

Integrated nicotine concentration-response curves. Because of the low ratio of nicotine-stimulated efflux to nonspecific $^{86}Rb^+$ leak in millisecond experiments, nicotine concentration-response relationships were further investigated using a 10-sec manual ion efflux assay. Although the response is not linear with time for 10 sec, effectively decreasing the experimentally observed K_A below the value expected from kinetic studies (6), this decrease provides the practical advantage of further separating the activation and inhibition curves. The theoretical disadvantage of nonlinear kinetics is offset somewhat by the empirical observation that the ethanol-induced increase in apparent agonist affinity is essentially independent of flux integration time (17). Thus, the relative ethanol-induced shifts in K_A obtained from millisecond and 10-sec flux measurements are self-consistent, even though the absolute values are different. In addition, the inhibition constants of noncompetitive inhibitors are insensitive to the time over which flux is integrated (22).

For quantitative analysis of agonist concentration-response curves at 10 sec, it is customary to remove spare response from the system by irreversibly blocking approximately 85% of the receptors with α-BTX (6). However, the maximal flux response to nicotine is so low that nicotine concentration-response curves could be measured in untreated membrane preparations without emptying the vesicles of tracer ions over the time course of the assay. In order to fit the biphasic nicotine concentration-response curve to eq. 2 while making the assumption that nicotine is a full agonist, the maximal response to a full agonist, F_A(max), must be known; it may be approximated experimentally as the response to 5 mM carbamylcholine determined in the same vesicles and over the same integration time. Results of this analysis are given in Table 2. However, in a membrane preparation not treated with α-BTX the measured F_A(max) elicited by full agonists will always be underestimated by this procedure because "spare receptors" cause complete emptying of the vesicles well within the assay time. This was the case for concentration-response curves determined at 0 and 0.5 mM ethanol in the absence of any α-BTX. As described in the Appendix, a theoretical maximal response assuming 100% activated receptors and a limitless supply of $^{86}Rb^+$, F_A(max)100, was, therefore, projected for each of these batches of vesicles. This value was then used in the fitting of nicotine flux response data from untreated membrane vesicles. The fitted parameters from this analysis are also given in Table 2. For example, 0, 40, and 75% of receptors were blocked with α-BTX for experiments with ethanol concentrations of 0, 0.5, and >0.5 M, respectively. At the two lowest α-BTX concentrations, 5 mM carbamylcholine emptied the vesicles within the flux assay time and the procedure in the Appendix was applied to estimate F_A(max)100, yielding values of 275% (0% α-BTX block) and 89% (40% α-BTX block). At 75% α-BTX block, all spare response was removed from these vesicles and the maximum response with nicotine in the presence of 0.75–2.0 M ethanol was the same as that with carbamylcholine.

Fig. 4 shows the series of biphasic nicotine concentration-response curves obtained in the presence of various concentrations of ethanol, when the data are analyzed as described above. The data points are shown normalized to the maximal response projected for each membrane preparation [F_A(max)100]. For curves determined in the presence of >0.5 M ethanol, the maximum nicotine response is the same as that of a full agonist, and the nonlinear least squares regression was fitted to eq. 2 and constrained to pass through this maximal value [F_A(max)100]. The parameters are given in Table 2. For data with ≤0.5 M ethanol, which does not achieve the maximum possible flux, two analyses are possible. First, the curves shown are unconstrained analyses of the data. Second, one may assume that nicotine behaves as a full agonist and constrain the fit to the maximum flux exhibited by full agonists; the parameters for this case are given in Table 2.

Ethanol, in the concentrations used in this study, significantly increased the apparent affinity of nicotine for its activation site, while producing a smaller decrease in the apparent affinity of the nicotine inhibitory site (Table 2). As the ethanol

TABLE 2

Parameters for 10-sec nicotine concentration-response curves

Parameters were determined as in Table 1, but these 10-sec flux data (Fig. 4) were fitted to eq. 2 with F_A(max) fixed at the value determined for 100% active receptors [F_A(max)100].

[Ethanol]	F_A(max)100	K_A	n_1	K_B	n_2	K_B/K_A
M		μM		mM		
0	267[a]	62 ± 6.5	2.2 ± 0.36	1.1 ± 0.10	2.7 ± 0.92	18
0.5	89[a]	58 ± 9.4	1.9 ± 0.22	1.5 ± 0.15	2.8 ± 0.61	26
0.75	50	45 ± 6.8	1.5 ± 0.23	1.7 ± 0.13	2.7 ± 0.36	38
1.0	50	26 ± 2.7	1.5 ± 0.19	2.1 ± 0.16	1.8 ± 0.18	81
1.5	50	11 ± 1.6	1.2 ± 0.29	2.6 ± 0.32	1.7 ± 0.26	236
2.0	50	5.2 ± 0.62	0.8 ± 0.13	3.4 ± 0.30	1.6 ± 0.19	654

[a] Determined by extrapolation, as described in the Appendix.

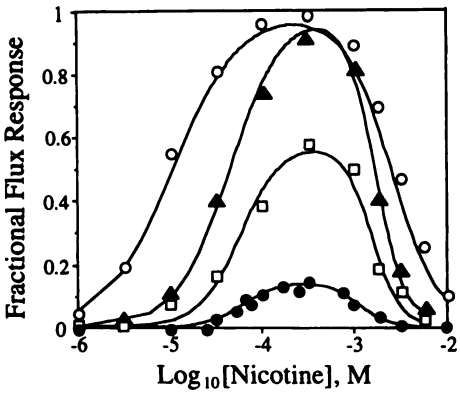


Fig. 4. Effect of ethanol on the integrated nicotine concentration-response curve. Nicotine concentration-response curves were measured using 10-sec $^{86}Rb^+$ efflux measurements from vesicle preparations, differentially blocked with α-BTX, in the presence of various concentrations of ethanol. Ethanol and α-BTX block conditions were as follows: ●, nicotine alone, untreated membranes; □, plus 0.5 M ethanol, 40% block; ▲, plus 1.0 M ethanol, 75% block; ○, plus 1.5 M ethanol, 75% block. Data were fitted to eq. 2 by nonlinear least squares regression (see text).

concentration increased, a leftward shift of the measured activation curves occurred, resulting in a decrease in K_A, and a smaller rightward shift of the inhibitory curve, resulting in an increase in K_B.

Noncompetitive inhibitory concentration-response curves for nicotine. It was not possible to obtain control nicotine concentration-response curves at short time intervals. In order to obtain a value for the inhibitory dissociation constant for nicotine in the absence of desensitization, the ability of nicotine to noncompetitively inhibit acetylcholine-stimulated ion flux was measured over a range of nicotine concentrations at 11 msec. Previous work (6) has shown that at 11 msec a concentration of 1–10 mM acetylcholine should saturate the agonist activation sites on *Torpedo* nAcChoR (K_A = 50 μM), while having no effect on the agonist self-inhibition sites (K_B = 110 mM). Thus, by measuring the effect of nicotine on the flux response to 1–10 mM acetylcholine, it was possible to examine the inhibitory action of nicotine independently of its channel-activating properties.

Noncompetitive inhibition by nicotine of the flux response to acetylcholine was measured in vesicles without spare receptors, using the rapid quench-flow method (three experiments; flux integrated over 10–11 msec; 1–10 mM acetylcholine) and 10-sec integrations (one experiment; 3 mM acetylcholine). In the former set, initial rates (k_f) were estimated by single-time point kinetics from eq. 1. Nicotine inhibition curves could be fitted to the logistic function (eq. 5) by nonlinear least squares analysis. The apparent dissociation constant of nicotine, K_B, for inhibition of nAcChoR activity was independent of the acetylcholine concentration and integration time over the range studied, yielding average values of K_B of 1.2 ± 0.41 mM (n_2 = −2.3 ± 0.096). This value agrees with that obtained from nicotine concentration-response curves integrated over 10 sec (Table 2) and is in agreement with previous observations with local anesthetics that inhibitory concentrations do not vary with the time of integration (12).

The apparent dissociation constant of nicotine, K_B, was found to increase with ethanol concentration, such that 2 M ethanol caused a 5-fold increase (Fig. 5). This compares to a 3-fold shift of K_B deduced from nicotine concentration-response curves integrated over 10 sec (Table 2). The millisecond value of K_B at 1.5 M ethanol was also in good agreement with that in Table 1.

The Hill coefficient for the inhibitory site for nicotine was always greater than 1 and had a value of 2 in the absence of ethanol. This contrasts with other agonists, whose Hill coefficients we have found to be 1 (6).

Discussion

Models of partial agonism at nAcChoR. It is clear from the results presented in this paper that nicotine has a considerably weaker effect on $^{86}Rb^+$ efflux than does a full agonist at *Torpedo* nAcChoR. This confirms the initial observations of the flux response to nicotine at *Torpedo* nAcChoR by Forman *et al.* (6) and is consistent with electrophysiological studies by Gardner *et al.* (25) and Rozental *et al.* (26), which demonstrate the low efficacy of (−)-nicotine at the frog neuromuscular nAcChoR. Thus, nicotine meets the criterion of a partial agonist, as defined by Stephenson (1), that such a compound even when occupying all receptors fails to bring about the maximal response of which the system is capable.

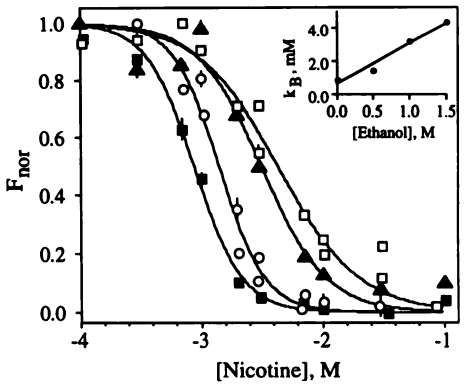
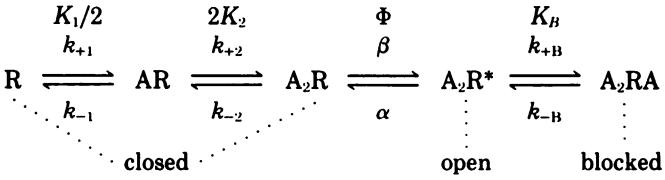


Fig. 5. Ethanol-induced increase of the inhibition constant of nicotine for acetylcholine-induced ion efflux, showing noncompetitive inhibition by nicotine of the flux response to acetylcholine (1 mM) integrated over 11 msec. Nicotine inhibition curves, obtained on a matched set of vesicles in which spare response was removed with α-BTX, were fitted to eq. 5 by nonlinear least square analysis. K_B (mean ± standard deviation; mM) and n_2 (mean ± standard deviation) were as follows: nicotine with 1 mM acetylcholine (■), 0.89 ± 0.073, −2.2 ± 0.42; plus 0.5 M ethanol (○), 1.41 ± 0.075, −2.4 ± 0.25; plus 1 M ethanol (▲), 3.2 ± 0.38, −1.7 ± 0.29; plus 1.5 M ethanol (□), 4.3 ± 0.42, −1.3 ± 0.16. *Inset*, increase of K_B with ethanol concentration (slope, 0.0021 ± 0.0003; intercept, 0.79 ± 0.03 mM). Each curve is normalized to its own k_f (max); see eq. 5.

All cholinergic agonists inhibit the channels they open (6, 10, 11). For example, single-channel studies of the action of nicotine on nAcChoR from frog neuromuscular junction clearly show channel-blocking events (26), and noise analysis of the action of decamethonium at nAcChoR showed two distinct kinetic components and a channel-blocking mechanism similar to that of procaine (14). These observations have led the classical sequential scheme of receptor activation (27) to be modified to include self-inhibition by the agonist itself [see, for example, the work of Marshall *et al.* (15)]:



where $K_1 = k_{-1}/k_{+1}$, $K_2 = k_{-2}/k_{+2}$, $\Phi = \beta/\alpha$, and $K_B = k_{-B}/k_{+B}$. In this scheme, the flux response to an agonist depends on the fraction of receptors in the activated open state (A_2R^*) and the conductance properties of the open ion channel.

An agent might be a partial agonist for several reasons, i.e., its activated state might have a lower conductance than that produced by a full agonist (1), it might be able to convert only a small proportion of closed, fully occupied receptors (A_2R) to the open state (A_2R^*) (8), or a large proportion of open channels may be blocked by the agonist (A_2RA) (6, 13). The first possibility is unlikely, because Gardner *et al.* (25) have demonstrated that a variety of agonists with differing efficacies, including nicotine, all elicited exactly the same single-channel conductances as acetylcholine in nAcChoR at the frog motor endplate and in cultured rat myotubes. Moreover, all the drugs showed the same reversal potential, suggesting that in all cases the activated receptor adopted the same conformation. Therefore, within the framework of the above scheme, the low efficacy of a partial agonist occurs because the channel has either a low

probability of being open (Φ is large) or a high probability of being blocked upon opening ($K_A = K_1 \cdot K_2/\Phi \approx K_B$).

Significantly, in a series of agonists, acetylcholine exhibits the largest difference between K_A and K_B, suggesting that this is the defining characteristic of a good agonist, and there are no partial agonists known where blocking is not observed (6). For very weak agonists such as decamethonium and phenyltrimethylammonium (and nicotine), channel blockade occurs at all agonist concentrations that open ion channels and is very rapid (13). Thus, channel activation events cannot be analyzed in the absence of channel blockade, individual channel blocking events cannot be resolved, and determination of the probability of the channel being open (p_o) is confounded by the presence of self-inhibition. Indeed, in a recent study, although single-channel recordings revealed that decamethonium had a very low probability of activating the ion channel ($p_o \sim 0.01$), it was not possible to tell whether this was due to the concomitant channel blockade or to an intrinsically low probability of opening the ion channel (13). Although preliminary results using rapid perfusion of outside-out patches and measurements of macroscopic currents remove ambiguities attendant on fast desensitization, there remains the fundamental problem that the dissociation coefficients for activation and inhibition are comparable ($K_B \approx K_A$) (16).

In our initial rate studies, maximally activating concentrations of nicotine elicit an initial rate of flux response about 1 order of magnitude lower than that of carbamylcholine (Fig. 2). Thus, if nicotine can open ion channels as efficiently as a full agonist, the resulting flux response must be very rapidly curtailed, suggesting that channel blockade by nicotine is too fast to be resolved as a separate component in the millisecond time frame.

Rapid channel inactivation by nicotine is consistent with that produced by high concentrations of all other cholinergic agonists. High concentrations of acetylcholine, carbamylcholine, and suberyldicholine inhibit nAcChoR-mediated cation flux, resulting in bell-shaped concentration-response curves (6, 9, 10, 12). This self-inhibiting action seems similar to the inhibition obtained with a local anesthetic, with both actions appearing in electrophysiological studies as rapid brief closures of ion channels (10, 11, 28), the inhibitory potencies of both compounds being enhanced by membrane hyperpolarization (9–11), and both compounds appearing to act at the same inhibitory site on nAcChoR (22). Indeed, electrophysiological studies of the action of nicotine at neuromuscular nAcChoR do demonstrate channel flickering events characteristic of rapid, transient, channel blockade (26). However, because inhibition by nicotine in our studies is associated with a higher Hill coefficient than that for other agonists the underlying mechanism may differ.

Nicotine as a full agonist in the presence of ethanol. One object of our study was to test whether the dramatic effect of ethanol on the K_A of agonists was also present with partial agonists and, if so, whether this might help to resolve the dilemma outlined above. Our results show that over any time scale from 3 msec to 10 sec, in the presence of sufficient ethanol, maximal stimulation by nicotine elicits essentially the same response as does a full agonist. Furthermore, integrated flux concentration-response curves at 11 msec and at 10 sec demonstrate unequivocally that ethanol, concomitantly with increasing the maximum response elicited by nicotine, increases the difference between K_A and K_B. Significantly, single-channel studies have shown that ethanol does not alter the single-channel conductance of activated nAcChoR (18) and in tracer ion flux studies the maximal response to a full agonist is not increased by ethanol (17). Therefore, nicotine becomes a full agonist in the presence of ethanol because of a decrease in K_A and a simultaneous increase in K_B. Thus, there is no intrinsic reason why nicotine should not be a full agonist, suggesting that nicotine binds to the cholinergic site in a manner comparable to that of other agonists and fully activates the associated channel. Indeed, recent studies show that nicotine binding is associated with the same amino acids as is that of other cholinergic ligands (29).

A priori, ethanol might decrease K_A by an action on the binding steps (K_1, K_2) or on the open probability (Φ), and it is necessary to resolve this issue in order to establish the mechanism of nicotine as a partial agonist. Independent evidence, summarized by Forman *et al.* (17) and based on both electrophysiological (30) and rapid kinetic (17) studies, suggests, but does not prove, that ethanol acts at the binding step by slowing dissociation of the agonist. More recently, single-channel studies confirm that ethanol increases agonist affinity and does not affect conductance (19),[2] but it may also slow channel closing (19). These studies involve prolonged exposures to ethanol, and the ensuing desensitization is a complicating factor. The advent of rapid-perfusion patch-clamping may offer a solution to this problem (16).

Our present study demonstrates that K_B increases with ethanol concentration. This new observation provides a parsimonious rationale for the observed shifts in both K_A and K_B, one that strengthens the possibility that ethanol increases the open probability. If the only action of ethanol was to stabilize the open channel state, it would simultaneously decrease K_A and increase K_B. Our data are of insufficient accuracy to test this simple hypothesis. Although it remains impossible to assess the contribution of channel opening to partial efficacy, our work suggests that the use of ethanol in electrophysiological studies using rapid perfusion techniques (to avoid desensitization) could lead to a resolution of this intractable problem.

Independent estimation of the K_A of nicotine. Classically, it should be possible to obtain the K_A of a partial agonist by using it as a competitive inhibitor of a full agonist but, because of the ability of nicotine to block the channel in the same concentration range in which it binds to the agonist site, this would be difficult. The only other means of obtaining K_A for such a compound is to measure its full concentration-response curve and thus estimate the values of K_A and K_B by fitting the flux data to a logistic equation (eq. 2). Biphasic partial agonist concentration-response relationships can be fitted by overlapping curves for channel activation and inhibition (6, 13, 15). However, with nicotine the degree of overlap of the two curves is too severe; even maximally activating concentrations of nicotine (0.3 mM) in the absence of α-BTX blockade elicited only a marginal response at *Torpedo* nAcChoR (Fig. 2). Thus, in order to obtain data that could be analyzed accurately, it was necessary to integrate flux response over several seconds and to use vesicles that had not been pretreated with α-BTX. Over this time scale, the kinetics are nonlinear

[2] Dilger, personal communication.

and fast desensitization occurs; consequently, the true K_A cannot obtained.

Forman *et al.* (17) observed that the effect of ethanol on agonist K_A was independent of the time scale of the experiment, provided that the shift in K_A was measured relative to its ethanol-free value, K_A^0, on the same time scale. Thus, the logarithm of the relative decrease in K_A, log (K_A^0/K_A^E), decreased linearly with ethanol concentration (0–1.5 M), independently of both the integration time used in the flux experiment (5 msec to 10 sec) and the agonist (carbamylcholine or acetylcholine).

Unfortunately, we cannot determine K_A^0 for nicotine, but the analysis will work just as well if we normalize to a constant ethanol concentration such as 1.5 M. The plot of log $(K_A^{1.5}/K_A^E)$ for acetylcholine is a straight line between 0 and 1.5 M ethanol, with only a slight deviation at 2 M. Our 10-sec nicotine K_A data (Table 2) when reduced similarly fall close to the acetylcholine data between 0.5 and 2.0 M ethanol. The value for K_A in the absence of ethanol is very insecure because of the large difference between the observed maximum response and F_A(max)100 and, not surprisingly, it lies off the line. The K_A values for nicotine at millisecond times (Table 1) have the same ratio as the corresponding values for acetylcholine. Thus, in general the relative K_A values of both acetylcholine and nicotine have the same dependence on ethanol concentration, and an extrapolation of the nicotine data at high ethanol concentration to zero ethanol provides an estimate of the K_A of nicotine. The best estimate of the real K_A, obtained on a millisecond time scale where the kinetics are linear and desensitization is minimal, is ~4 mM. Thus, the affinity of nicotine for its agonist sites in the resting state is less than that for the channel inhibition sites. In Fig. 6 the predicted behavior for this combination of K_A and K_B is shown and compared with the behavior in the presence of 1.5 M ethanol. This figure illustrates how the ethanol-induced changes in fitted parameters for activation and inhibition, which result in an increase in the K_B/K_A ratio for nicotine as the alcohol concentration increases, result in a dramatic increase in the efficacy of nicotine until, in the presence of 2.0 M ethanol, the maximal response to nicotine is indistinguishable from that of a full agonist. Thus, its channel-inhibitory activity prevents nicotine from revealing how good an agonist it is.

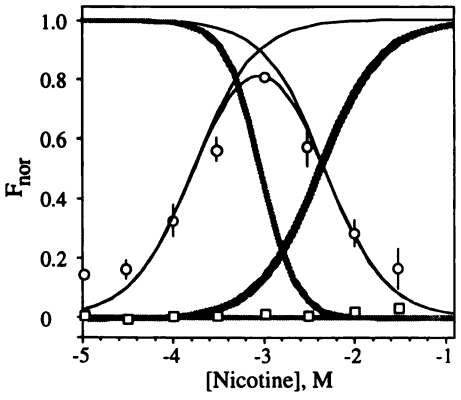


Fig. 6. Overlap of the activation and inhibition curves for nicotine, limiting its response. The sigmoid curves with positive slopes represent occupancy of the agonist sites by nicotine; those with negative slopes represent (1-occupancy of the inhibition sites). Parameters for nicotine alone (K_A, 4.0 mM; n_1, 1.3; K_B, 0.89 mM; n_2, 2.3) (□) were determined as described in the text (see Discussion); parameters for nicotine plus 1.5 M ethanol (——) are from Table 1. The bell-shaped curves are the net response (eq. 3). Data points (□, nicotine alone; ○, nicotine plus 1.5 M ethanol), which are from 11-msec experiments normalized to the response to a saturating concentration of acetylcholine, F_{nor}, are simply superimposed on the simulations. Standard deviations are indicated where they exceed the size of the symbol.

Conclusions. In this study we have demonstrated that the apparent efficacy of nicotine is increased when the relative affinity of nicotine for the activation site on nAcChoR is increased by ethanol, thus separating the channel-activating and -inhibiting actions of this drug. These results are consistent with, but do not prove, the hypothesis that the low efficacy of nicotine at nAcChoR from *Torpedo* is not due to an intrinsic inability to activate the receptor but instead is due to its self-inhibiting action at all activating agonist concentrations and, in the absence of channel blockade, nicotine could produce a flux response comparable to that of a full agonist. The use of ethanol in electrophysiological experiments using rapid perfusion techniques may help to solve the problem of the mechanism of partial agonist action.

Appendix: Calibration of Membrane Preparations

The local anesthetic procaine inhibits agonist-mediated ion efflux from *Torpedo* vesicles with apparent dissociation constant K_P and Hill coefficient of ~1 in the 10-sec manual ion flux assay. Therefore, the fraction of sites (y_P) occupied by a concentration of procaine, P, will be given by a simple hyperbolic equation:

$$y_P = \frac{P}{P + K_P}$$

If inhibition of agonist-mediated efflux is measured in a vesicle preparation containing spare receptors, then the concentration of procaine required to inhibit 50% flux activity will be increased from K_P to K_{P^*}, because procaine binding to spare receptors will not affect the flux response measured. The fraction of receptors (y_{P^*}) occupied by procaine concentration K_{P^*} represents the fraction of spare receptors plus the fraction of receptors required to inhibit 50% of the flux response, and this is given by:

$$y_{P^*} = \frac{K_{P^*}}{K_{P^*} + K_P}$$

The fraction of spare receptors, y_{SP}, is given by:

$$y_{SP} = y_{P^*} - (1 - y_{P^*}) = 2y_{P^*} - 1$$

If only $(1 - y_{SP})$ of the true maximum flux is measured and flux is proportional to fraction of activated receptors, then the theoretical maximum for 100% activation can be projected:

$$F_A(\text{max})100 = \frac{F_A(\text{max})}{(1 - y_{SP})}$$

Procaine inhibitory concentration-response curves were measured for each membrane preparation after spare response had been removed and for the same membrane preparation in the absence of α-BTX. From the above derivation it was possible to quantitate the fraction of spare receptors in each preparation and thus estimate the unblocked F_A(max)100 for each experimental system. This value was fixed as F_A(max) in the nonlinear least squares analysis, fitting control nicotine flux data to the logistic equation given in the text.

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