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ACCELERATED COMMUNICATION

Varenicline Is a Partial Agonist at $\alpha4\beta2$ and a Full Agonist at $\alpha7$ Neuronal Nicotinic Receptors

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ABSTRACT

Varenicline, a new nicotinic ligand based on the structure of cytisine, has recently been approved by the U.S. Food and Drug Administration for use as a smoking cessation aid. Varenicline has been shown to be a partial agonist of $\alpha4\beta2$ receptors, and in equilibrium binding assays, it is highly selective for the $\alpha4\beta2$ receptor. In this study, we have examined the functional activity of varenicline at a variety of rat neuronal nicotinic receptors expressed in *Xenopus laevis* oocytes and assayed under two-electrode voltage clamp. We also find that varenicline is a potent, partial agonist at $\alpha4\beta2$ receptors, with an EC_{50} of 2.3 $\pm$ 0.3 μM and an efficacy (relative to acetylcholine) of 13.4 $\pm$ 0.4%. Varenicline has lower potency and higher efficacy at $\alpha3\beta4$ receptors, with an EC_{50} of 55 $\pm$ 8 μM and an efficacy of 75 $\pm$ 6%. Varenicline also seems to be a weak partial agonist at $\alpha3\beta2$ and $\alpha6$-containing receptors, with an efficacy $<$10%. It is remarkable that varenicline is a potent, full agonist at $\alpha7$ receptors with an EC_{50} of 18 $\pm$ 6 μM and an efficacy of 93 $\pm$ 7% (relative to acetylcholine). Thus, whereas varenicline is a partial agonist at some heteromeric neuronal nicotinic receptors, it is a full agonist at the homomeric $\alpha7$ receptor. Some combination of these actions may be involved in the mechanism of varenicline as a smoking cessation aid.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels composed of α and β subunits that assemble to form pentamers with a variety of pharmacological and biophysical properties (Corringer et al., 2000). These receptors are widely distributed in the CNS and have been proposed as potential therapeutic targets for a variety of conditions and disorders, such as Alzheimer's disease, anxiety, depression, drug addiction, epilepsy, pain, Parkinson's disease, schizophrenia and Tourette's syndrome (Jensen et al., 2005). The development of subtype selective ligands is an essential part of attempts to make progress in these areas. Therefore, much effort is currently directed toward synthesizing and screening analogs of various nicotinic compounds (recently reviewed in Jensen et al., 2005). The $\alpha4\beta2$ nAChR (and more complex $\alpha4\beta2$-containing receptors) is a target of particular interest for the development of smoking cessation therapies because of the location of this receptor on presynaptic terminals in the striatum and the role of this receptor in modulating dopamine release (Salminen et al., 2004).

Cytisine is a plant alkaloid with a relatively rigid conformation. In equilibrium binding assays, cytisine is selective for the $\alpha4\beta2$ subunit combination, compared with other important nAChR subtypes such as $\alpha3\beta4$ and $\alpha7$ (Parker et al., 1998; Stauderman et al., 1998; Chavez-Noriega et al., 2000; Carbonnelle et al., 2003; Slater et al., 2003; Xiao et al., 2004). In functional assays, cytisine shows greater potency at $\alpha4\beta2$ receptors than at many other subunit combinations. Cytisine also displays a wide variation in efficacy at various subunit combinations. Although cytisine is a high-efficacy agonist at $\alpha7$ receptors and at various $\beta4$-containing receptors, such as $\alpha3\beta4$, cytisine is a low-efficacy partial agonist at $\alpha4\beta2$ and other $\beta2$ containing receptors (Luetje and Patrick, 1991; Papke and Heinemann, 1994; Chavez-Noriega et al., 1997; Stauderman et al., 1998; Chavez-Noriega et al., 2000; Papke and Porter Papke, 2002; Carbonnelle et al., 2003; Slater et al., 2003). Manipulation of the cytisine structure results in changes in the efficacy at various neuronal nAChRs. For

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine.

example, bromination or iodination of the 3-position in the pyridone ring of cytisine preserves the full efficacy at $\alpha7$ receptors and increases the efficacy at $\alpha4\beta2$ receptors (Slater et al., 2003), whereas the addition of side groups at the basic nitrogen of cytisine can reduce or eliminate efficacy for various receptors (Carbonnelle et al., 2003).

Varenicline is a recently developed nicotinic ligand that has recently been approved by the U.S. Food and Drug Administration for use as a smoking cessation therapy (Coe et al., 2005a,b,c; Obach et al., 2006). The structure of varenicline (Fig. 1) is loosely based on that of cytisine and it was the partial agonist activity of cytisine that was one of the initial characteristics that led to the development of varenicline (Coe et al., 2005a,b,c). In equilibrium binding assays, varenicline is selective for the $\alpha4\beta2$ receptor compared with $\alpha3\beta4$, $\alpha7$, and muscle-like nAChRs, whereas in a functional assay, varenicline is a partial agonist at $\alpha4\beta2$ receptors (Coe et al., 2005a). In this study, we examined the functional potency and efficacy of varenicline at several neuronal nAChR subunit combinations. We found that varenicline displayed only moderate differences in functional potency but showed wide variation in efficacy at these receptors.

Materials and Methods

Materials. *Xenopus laevis* frogs were purchased from Nasco (Ft. Atkinson, WI). Care and use of *X. laevis* frogs in this study were approved by the University of Miami Animal Research Committee and meet the guidelines of the National Institutes of Health. RNA transcription kits were from Ambion (Austin, TX). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Varenicline. 6,7,8,9-Tetrahydro-6,10-methano-6*H* pyrazino[2,3-*h*][3]benzazepine (varenicline) was synthesized as the dihydrochloride salt using reported methods (Coe and Brooks, 2002; Brooks et al., 2004). We thank Dr. Jotham Coe for helpful suggestions. The structure of varenicline is shown in Fig. 1.

Expression of Neuronal nAChRs in *X. laevis* Oocytes. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester. Oocytes were surgically removed, and follicle cells were removed by treatment with collagenase B for 2 h at room temperature. cRNA encoding the rat $\alpha3$, $\alpha4$, $\alpha6/\alpha3$ (see below), $\alpha7$, $\beta2$, $\beta3$, and $\beta4$ neuronal nAChR subunits was synthesized using mMessage mMachine kits (Ambion). Oocytes were injected with 10 to 40 ng of cRNA in 13 to 50 nl of water and incubated at 19°C in modified Barth's saline [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$ 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/ml gentamicin, 15 mM HEPES, pH 7.6] for 2 to 6 days. For heteromeric nAChRs, cRNA transcripts encoding each subunit were injected into oocytes at a molar ratio of 1:1.

Neuronal nAChRs containing the $\alpha6$ subunit are narrowly distributed, but functionally important, in the mammalian central nervous system (Salminen et al., 2004). Unfortunately, receptors containing this subunit are difficult to functionally express. However a successful strategy has been to use a chimeric subunit consisting of the N-terminal extracellular domain of $\alpha6$ joined to the transmembrane and intracellular domains of $\alpha3$ (Kuryatov et al., 2000; McIntosh et al., 2004; Azam et al., 2005). This chimeric $\alpha6/\alpha3$ subunit was kindly provided by Dr. Michael McIntosh (University of Utah). In accordance with previous reports (McIntosh et al., 2004), we found that whereas functional expression of the $\alpha6/\alpha3$ chimera with the $\beta2$ subunit was very weak, useful levels of functional expression could be achieved with the inclusion of the $\beta3$ subunit. Indeed, $\alpha6\beta2\beta3$ is a functional nAChR subtype in the mammalian central nervous system (Salminen et al., 2004).

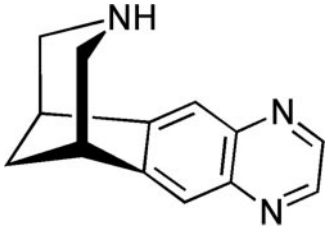

Fig. 1. Structure of varenicline.

Electrophysiological Methods. Agonist-induced current responses were measured 2 to 6 days after cRNA injection using two-electrode voltage clamp in an automated parallel electrophysiology system (OpusExpress 6000A; Molecular Devices, Sunnyvale, CA). Micropipettes were filled with 3 M KCl and had resistances of 0.2 to 2.0 MΩ. Current responses were recorded at a holding potential of -40 mV to minimize the contribution of calcium activated chloride channels. For all receptors except $\alpha7$, current responses were filtered (four-pole, Bessel, low-pass) at 20 Hz (-3 db) and sampled at 100 Hz. For $\alpha7$ receptors, current responses were filtered (four-pole, Bessel, low-pass) at 100 Hz (-3 db) and sampled at 500 Hz. Current responses were captured and stored using OpusXpress 1.1 software (Molecular Devices). Initial analysis was done using Clampfit 9.1 software (Molecular Devices). Oocytes were perfused at room temperature (20–25°C) with Ringer's saline (115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 0.0001 mM atropine, and 10 mM HEPES, pH 7.2). For all receptors except $\alpha7$, acetylcholine and varenicline were applied for 20 s at a flow rate of 4 ml/min, with 3-min washes between applications. For $\alpha7$ receptors, acetylcholine and varenicline were applied for 5 s at a flow rate of 4 ml/min, with 3-min washes between applications. The acetylcholine concentration, applied before each varenicline application, was 20 μM for $\alpha4\beta2$ (EC_{30}), 110 μM for $\alpha3\beta4$ (EC_{20}), 10 μM for $\alpha3\beta2$ (EC_{20}), 30 μM for $\alpha6/\alpha3\beta2\beta3$ ($\sim EC_{50}$), and 300 μM for $\alpha7$ (EC_{50}). Each current response to varenicline was normalized to the preceding acetylcholine-induced response. Dose response data were fit to the equation $I = I_{max}/[1 + (EC_{50}/X)^{n_H}]$, where I is the current response at agonist concentration (X), I_{max} is the maximum current, EC_{50} is the ligand concentration producing the half-maximal current response, and n_H is the Hill coefficient. Dose response data for inhibition curves were fit to the equation $I = I_{max}/[1 + (X/IC_{50})^{n_H}]$, where I is the current response at agonist concentration (X), I_{max} is the maximum current, IC_{50} is the ligand concentration producing half-maximal inhibition of the current response, and n_H is the Hill coefficient.

Results and Discussion

We first examined the effect of varenicline on rat $\alpha4\beta2$ receptors expressed in *X. laevis* oocytes. Application of 100 μM varenicline yielded a peak current response that was smaller in amplitude than the response evoked by 20 μM ACh (the EC_{30} for ACh), suggesting that varenicline might have a low efficacy at this receptor (Fig. 2B). The response to this high concentration of varenicline desensitized rapidly compared with the response to a low concentration of varenicline (1 μM, Fig. 2A). The small deflection seen when the varenicline is washed away suggests that in addition to the desensitizing effect, varenicline may cause a small amount of channel block. When the $\alpha4\beta2$ receptor was challenged with a range of varenicline concentrations and the current responses were normalized to the maximal response to ACh (see *Materials and Methods*), we found that varenicline was a potent (EC_{50} = 2.3 $\pm$ 0.3 μM) partial agonist with an efficacy 13.3 $\pm$ 0.4% that of ACh (Fig. 2C). These results are similar to what has been previously reported for human $\alpha4\beta2$ (EC_{50} = 2.3 μM, efficacy = 24% relative to nicotine; Coe et al., 2005a). When a range of varenicline concentrations were

coapplied with ACh (40 μM, the EC_{60}), we found that varenicline can antagonize the ACh response with an IC_{50} of 0.2 $\pm$ 0.03 μM (Fig. 2D), consistent with the idea that varenicline is a potent partial agonist of $\alpha4\beta2$ receptors.

Next, we examined the action of varenicline on other neuronal nAChR subtypes. Application of 200 μM varenicline to an oocyte expressing $\alpha3\beta4$ receptors yielded a peak current response with an amplitude substantially larger than the response evoked by 110 μM ACh (the EC_{20} for ACh), suggesting that varenicline might have a high efficacy at this receptor (Fig. 3B). The response to this high concentration of varenicline desensitized rapidly, compared with the response to a lower concentration of varenicline (30 μM, Fig. 3A). The small deflection seen when the varenicline is washed away suggests that in addition to the desensitizing effect, varenicline may cause a small amount of channel block. When the $\alpha3\beta4$ receptor was challenged with a range of varenicline concentrations and the current responses normalized to the maximal response to ACh (see *Materials and Methods*), we found that varenicline had an efficacy of 75 $\pm$ 6% at these receptors (Fig. 3C). Varenicline was 24-fold less potent at $\alpha3\beta4$ receptors (EC_{50} = 55 $\pm$ 8 μM) than at $\alpha4\beta2$ receptors.

We also examined the efficacy of varenicline at $\alpha3\beta2$ receptors and $\alpha6$-containing receptors (as represented by an $\alpha6/\alpha3$ chimera coexpressed with the $\beta2$ and $\beta3$ subunits, see *Materials and Methods*). When 100 μM varenicline was applied to oocytes expressing $\alpha3\beta2$ receptors, current responses were 3.7 $\pm$ 0.4% of the maximal ACh response. This was a saturating response, in that 1 mM varenicline did not yield larger current responses (data not shown). Thus, varenicline is a low-efficacy partial agonist at $\alpha3\beta2$ receptors, with an $EC_{50} < 100$ μM. Varenicline also displayed low efficacy on the $\alpha6/\alpha3\beta2\beta3$ receptor, with 100 μM varenicline yielding responses that were 8.8 $\pm$ 1.4% of the maximal response to ACh. Again, this seems to be a saturating response, because the response to 10 μM varenicline was similar (data not shown).

Although varenicline proved to be a partial agonist at many of the neuronal nAChR subtypes that we tested, the effect of varenicline was remarkably different at $\alpha7$ containing receptors. When 100 μM varenicline was applied to $\alpha7$ expressing oocytes, peak current responses were substantially larger than the responses to 300 μM ACh (the EC_{50}), suggesting that varenicline has high efficacy at these receptors (Fig. 4A). This was borne out when we applied a range of varenicline concentrations to $\alpha7$-expressing oocytes. Varenicline was a potent full agonist, activating $\alpha7$ receptors with an EC_{50} of 18 $\pm$ 6 μM and an efficacy that was 93 $\pm$ 7% of the maximal response to ACh (Fig. 4, A and B). The rapid desensitization kinetics of the $\alpha7$ receptor at all agonist concentrations has raised concerns about the use peak current measurements to study the $\alpha7$ receptor and an alternate method, net charge analysis, has been proposed (Papke and Porter Papke, 2002). When using net charge analysis, the EC_{50} for

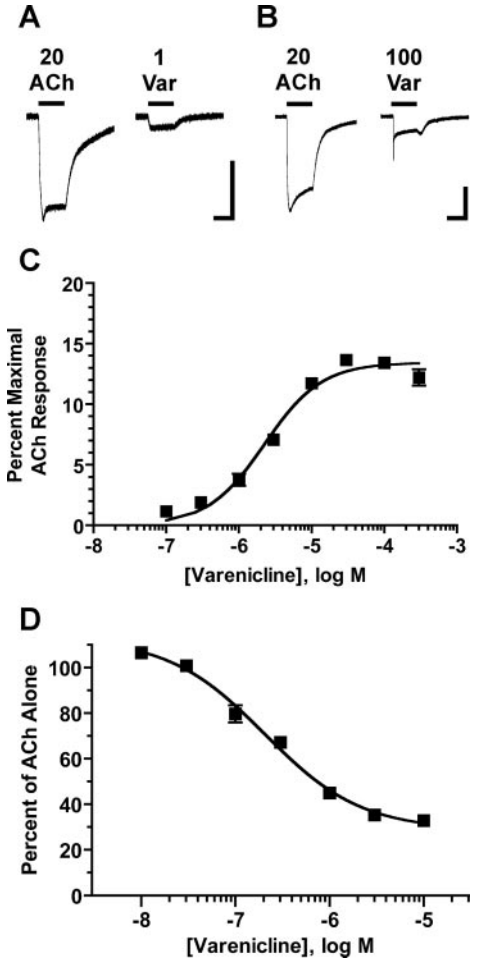

Fig. 2. Action of varenicline at $\alpha4\beta2$ receptors. A, current responses of an oocyte expressing $\alpha4\beta2$ receptors to 20 μM ACh and 1 μM varenicline. Scale: 200 nA, 20 s. B, current responses of an oocyte expressing $\alpha4\beta2$ receptors to 20 μM ACh and 100 μM varenicline. Scale: 200 nA, 20 s. C, responses of $\alpha4\beta2$ expressing oocytes to a range of varenicline concentrations, normalized to the maximal ACh response. Values are the mean $\pm$ S.E.M., n = 5. D, varenicline inhibition of ACh responses. Responses of oocytes to 40 μM ACh (EC_{60}) combined with various concentrations of varenicline are normalized to the response to ACh alone. Values are the mean $\pm$ S.E.M., n = 4 to 5.

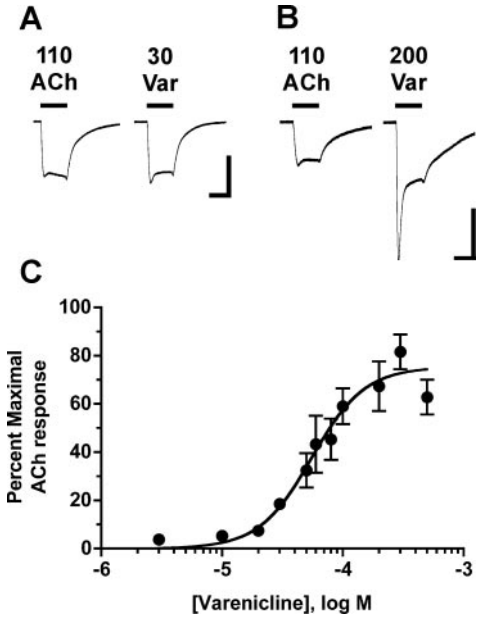

Fig. 3. Action of varenicline at $\alpha3\beta4$ receptors. A, current responses of an oocyte expressing $\alpha3\beta4$ receptors to 110 μM ACh and 30 μM varenicline. Scale: 0.5 μA, 20 s. B, current responses of an oocyte expressing $\alpha3\beta4$ receptors to 110 μM ACh and 200 μM varenicline. Scale: 0.5 μA, 20 s. C, responses of $\alpha3\beta4$ expressing oocytes to a range of varenicline concentrations, normalized to the maximal ACh response. Values are the mean $\pm$ S.E.M., n = 4 to 10.

ACh is greatly reduced and 300 μM ACh becomes a saturating concentration (Papke and Porter Papke, 2002). When we examine the actions of varenicline on α7 receptors using net charge analysis, we again find that varenicline is a potent, high efficacy agonist, with an EC_{50} of 2.3 $\pm$ 0.2 μM and an efficacy of 84 $\pm$ 2%.

We find relatively modest differences in the functional potency of varenicline at several neuronal nAChRs. The EC_{50} for varenicline activation of $\alpha4\beta2$ is 2.3 $\pm$ 0.3 μM, whereas varenicline was 8- and 24-fold less potent at α7 and $\alpha3\beta4$ receptors, respectively. These results contrast with the high degree of selectivity reported for varenicline at $\alpha4\beta2$ compared with α7 and $\alpha3\beta4$ receptors (Coe et al., 2005a). However, in this earlier study, it is the equilibrium binding affinity of varenicline that is being compared. In this context, varenicline is highly selective for $\alpha4\beta2$, displaying an affinity that is 4000- and >5000-fold greater than the affinities for the $\alpha3\beta4$ and α7 subtypes, respectively. The binding affinity derived from this type of assay is primarily the affinity of the ligand for the desensitized state(s) of the receptor. Unfortunately, large differences in affinity observed in equilibrium binding assays are generally not observed in functional assays, and the rank order of affinities do not necessarily correlate with the rank orders of functional potency or efficacy (Avalos et al., 2002; Jensen et al., 2005). Thus, we find that the functional potency of varenicline at $\alpha4\beta2$, $\alpha3\beta4$, and α7 receptors varies by 24-fold or less. More interesting is the variation in efficacy, which ranges from low (<15% for $\alpha4\beta2$, $\alpha3\beta2$, and α6-containing receptors), to high (93% and 75% for α7 and $\alpha3\beta4$ receptors, respectively). This variation in efficacy is not surprising considering that the initial inspiration for the development of varenicline was cytisine (Coe et al., 2005a), an agonist that displays low efficacy at $\alpha4\beta2$ receptors and high efficacy at $\alpha3\beta4$ and α7 receptors (Luetje and Patrick, 1991; Papke and Heinemann, 1994; Chavez-Noriega et al., 1997, 2000; Stauderman et al., 1998; Papke and Porter Papke, 2002; Carbonnelle et al., 2003; Slater et al., 2003). Compared with cytisine, varenicline has a similarly low efficacy at $\alpha4\beta2$ and other $\beta2$-containing receptors and a similarly high efficacy at $\alpha3\beta4$ and α7 receptors.

We have characterized the pharmacological properties of varenicline using rat neuronal nAChRs. Thus, it is important to consider how our results may relate to the actions of varenicline at human receptors. As discussed above, varenicline displays similar potency and efficacy values at both rat and human $\alpha4\beta2$ receptors (Table 1) (Coe et al., 2005a). In addition, cytisine displays similar potencies and efficacies on both rat and human $\alpha4\beta2$, $\alpha3\beta4$, and α7 receptors (Chavez-Noriega et al., 1997; Papke and Porter Papke, 2002; Carbonnelle et al., 2003). Thus, the actions of varenicline on rat neuronal nAChRs that we describe here are likely to be relevant to the actions of varenicline on human receptors.

All of the actions of varenicline should be taken into account when considering a potential mechanism for smoking cessation. Varenicline is a potent partial agonist at $\alpha4\beta2$ receptors, a less potent high efficacy agonist at $\alpha3\beta4$ receptors, a partial agonist at $\alpha3\beta2$ and α6-containing receptors, and a potent full agonist at α7 receptors. The involvement of presynaptic α4- and α6-containing receptors in regulating dopamine release in the striatum (Salminen et al., 2004), makes the partial agonism of varenicline at these receptors an attractive potential mechanism (Coe et al., 2005a). However, the potent high efficacy activation of α7 receptors may also be important. In addition, because therapeutic use of varenicline involves long-term exposure, it may be that it is the greater affinity of varenicline for the $\alpha4\beta2$ receptor in the desensitized state(s) that is important. That is, varenicline may have a selectively greater ability to maintain $\alpha4\beta2$ receptors in a desensitized state. Although it is possible that one of these actions is critical, it is also possible that that some combination of these actions might work in concert to achieve a desirable smoking cessation outcome. Regardless of the exact mechanism of action, it is ultimately the results of clinical trials that determine the usefulness of varenicline as a smoking cessation therapeutic. It should also be noted that the potent, high-efficacy agonism of the α7 receptor suggests that varenicline may have utility as a treatment of such disorders as schizophrenia and Alzheimer's disease (Jensen et al., 2005).

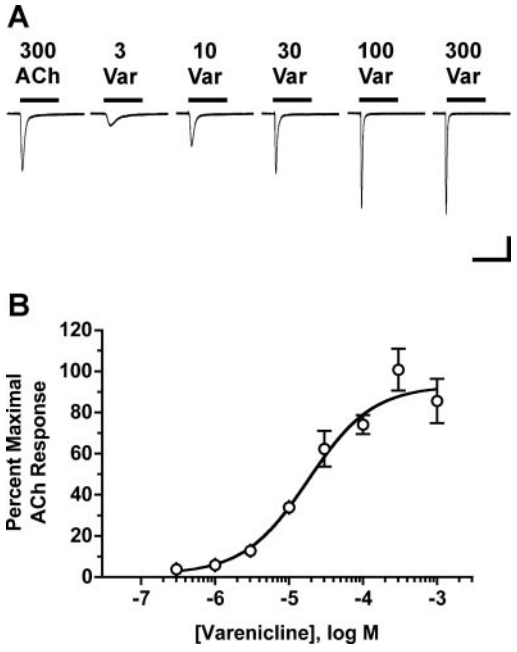

Fig. 4. Action of varenicline at α7 receptors. A, current responses of an oocyte expressing α7 receptors to 300 μM ACh and various concentrations of varenicline (in μM). Scale: 1 μA, 5 s. B, responses of α7 expressing oocytes to a range of varenicline concentrations, normalized to the maximal ACh response. Values are the mean $\pm$ S.E.M., n = 5 to 11.

TABLE 1

Functional potency and efficacy values for varenicline at rat neuronal nAChRs

EC_{50} values were determined from the fit data in Figs. 2C, 3C, and 4B. Efficacy values are presented as the percentage of maximum ACh response (see *Materials and Methods*). Efficacy values for $\alpha4\beta2$, $\alpha3\beta4$, and α7 are taken from the fit data in Figs. 2C, 3C, and 4B, respectively. Efficacy values for $\alpha3\beta2$ and $\alpha6/\alpha3\beta2\beta3$ are derived from a single concentration of varenicline (100 μM). All values are presented as mean $\pm$ S.E.M.

Receptor	EC_{50}	n_H	Efficacy
	μM		%
$\alpha4\beta2$	2.3 $\pm$ 0.3	1.1 $\pm$ 0.1	13.4 $\pm$ 0.4
$\alpha3\beta4$	55 $\pm$ 8	2.0 $\pm$ 0.5	75 $\pm$ 6
$\alpha3\beta2$	N.D.	N.D.	3.7 $\pm$ 0.4
$\alpha6/\alpha3\beta2\beta3$	N.D.	N.D.	8.8 $\pm$ 1.4
$\alpha7$	18 $\pm$ 6	1.0 $\pm$ 0.3	93 $\pm$ 7

N.D., not determined.

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