

Open-Channel Blockers at the Human $\alpha 4\beta 2$ Neuronal Nicotinic Acetylcholine Receptor

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ABSTRACT

To extend our knowledge of the pharmacological profile of human $\alpha 4\beta 2$ neuronal nicotinic receptors, we investigated the action of hexamethonium on the major brain human nicotinic acetylcholine receptor (nAChR) stably expressed in human embryonic kidney 293 cells. This compound displays all of the characteristics of an open-channel blocker at the human $\alpha 4\beta 2$ nAChR: a voltage-dependent inhibition (more pronounced at hyperpolarized potentials), absence of competition, and use dependence. Moreover, we observed that classic *N*-methyl-D-aspartate open-channel blockers amantadine, 3,5-dimethyl-1-adamantanamine (memantine), and dizocilpine [(+)-MK-801] and the calcium channel antagonist 8-(diethylamino)octyl-

3,4,5-trimethoxybenzoate are powerful inhibitors of the human $\alpha 4\beta 2$ nAChR. Dose-inhibition curves yield, at -100 mV, IC_{50} values in the micromolar range for all of compounds and Hill coefficients below unity. Whole-cell current-voltage relationships display a strong rectification profile at hyperpolarized potentials, and current blockades are fitted adequately by a mathematical model that describes the mechanism of an ion channel block. We conclude that these molecules are powerful human $\alpha 4\beta 2$ open-channel blockers ranking in the following order of potency: amantadine > memantine = hexamethonium > 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate \sim (+)-MK-801.

Neuronal nAChRs belong to the superfamily of ionotropic LGCs (Bertrand and Changeux, 1995). Using the crayfish muscle preparation, it has been shown that muscle nAChRs are activated within a microsecond time scale on binding of the agonist (Franke *et al.*, 1987). Very fast opening of the ionic pore results from the particular structure of these proteins that form both the ligand binding site and transmembrane channel. Given its physical dimensions, this aqueous pore is readily blocked by small molecules. Hexamethonium is a compound initially identified for its ability to block the ACh transmission in autonomic ganglia while leaving muscle nAChRs unaffected (Paton and Zaimis, 1951). The work of Blackman *et al.* (1963) and Ascher *et al.* (1979) suggested that hexamethonium inhibited the ACh-evoked currents in ganglionic neurons by sterically blocking the ionic pore of the nAChR. Extensive investigations have indicated that hexamethonium can be considered as a prototype OCB of the ganglionic nAChRs (Gurney and Rang, 1984) or of the reconstituted chick (Bertrand *et al.*, 1990) and rat (Charnet *et al.*, 1992) $\alpha 4\beta 2$ nAChRs because this small molecule fulfilled the following characteristics: (1) its blocking effect is voltage

dependent (Ascher *et al.*, 1979; Gurney and Rang, 1984; Bertrand *et al.*, 1990; Charnet *et al.*, 1992), (2) it displays a use-dependent mode of action (Gurney and Rang, 1984), and (3) its blocking effect is more pronounced at higher agonist concentrations (Ascher *et al.*, 1979). We therefore apply the term OCB to any compound that complies with these criteria.

Some of the physiological and pharmacological properties of the human $\alpha 4\beta 2$ neuronal nicotinic receptor have been investigated using the patch-clamp technique (Buisson *et al.*, 1996) and indicate that human nAChRs present a distinct profile compared with other vertebrate nAChRs. To further establish the pharmacological signature of the human $\alpha 4\beta 2$ nAChR, we investigated the effect of hexamethonium and TMB-8, a calcium channel antagonist that has been identified as a noncompetitive nicotinic antagonist (Bencherif *et al.*, 1995) (Fig. 1). In addition, we examined the properties of (+)-MK-801 (dizocilpine) at the human $\alpha 4\beta 2$ nAChR and of two other classic NMDA OCBs: amantadine (1-amino-adamantane) and memantine (3,5-dimethyl-1-adamantanamine) (Fig. 1).

Materials and Methods

Stably transfected cells (K177) were grown as described previously (Buisson *et al.*, 1996; Gopalakrishnan *et al.*, 1996) and used at

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; NMDA, *N*-methyl-D-aspartate; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; LGC, ligand-gated channel; OCB, open-channel blocker; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

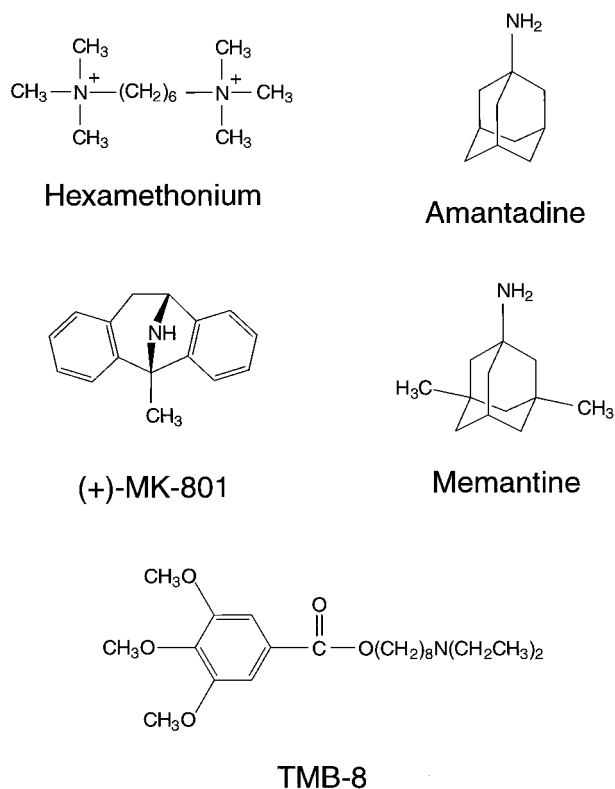


Fig. 1. Chemical formulas of the compounds tested at the human $\alpha 4\beta 2$ neuronal nAChR. Hexamethonium and (+)-MK-801 represent two prototypes of OCBs for central nAChRs and NMDA glutamate receptors, respectively. Amantadine and memantine inhibit NMDA receptors through an open-channel block mechanism. TMB-8 is a voltage-gated calcium channel antagonist displaying a noncompetitive mode of action at several nicotinic receptors (Bencherif *et al.*, 1995). The net charge is 2 for hexamethonium and 1 for amantadine, (+)-MK-801, memantine, and TMB-8 at a physiological pH (7.4).

passages 40–72. Cells were seeded onto 35-mm Petri dishes at low density and recorded 3–5 days later.

Electrophysiological recordings. All experiments were performed at room temperature (20°) in salt solution (containing 120 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 25 mM glucose, 10 mM HEPES, and 1 μM atropine (for blocking possible endogenous muscarinic receptors); pH 7.4 with NaOH. Patch pipettes (2–5 M Ω) were pulled from glass borosilicate (1.2-mm outer diameter) and filled with 5 mM NaCl, 10 mM CsCl, 120 mM CsF, 2 mM MgCl_2 , 10 mM HEPES, and 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, pH 7.4 with CsOH. Currents, which were recorded in isolated cells using an Axopatch 200A or 200B amplifier (Axon Instruments, Foster City, CA), were filtered at 1 kHz, digitized at 2–5 kHz, and stored on a personal computer equipped with an analog-to-digital converter (ATMIO-16D; National Instrument, Austin, TX) and the DATAC package (Bertrand and Bader, 1986). Data were analyzed on a Macintosh Performa 5200 using the MacDATAC program. Fast superfusion of the cells was performed with a custom-made multibarrel (Buisson *et al.*, 1996; Bertrand *et al.*, 1997) or a 300- μm glass theta-tube actuated by a piezoquartz device (Physik Instrument, Berlin, Germany). Both systems allow solution exchanges in the millisecond range (Franke *et al.*, 1987; Buisson *et al.*, 1996). Chemicals were purchased from Sigma Chemical (St. Louis, MO), Fluka Chemical (Ronkonkoma, NY), and Research Biochemicals (Natick, MA).

Voltage-ramp protocols were performed as follows: the membrane was held at 40 mV, and a first ramp (from 40 to –140 mV in 2 sec) was applied in the standard saline medium (without agonist) for determination of the leak current. Three seconds later, 1 μM ACh

was delivered for 3 sec, and the voltage-ramp was applied 400 msec after the onset of delivery. Data presented herein were obtained through subtraction from the leak current. For ramps starting at negative potential, the same protocol was used, but voltage command ranged from –140 to 40 mV.

Dose-inhibition curves. Every 10 sec, a voltage-ramp protocol (see above) was performed first with 1 μM ACh and then with increasing concentrations of the inhibitor. The ACh-evoked currents were measured at –100 mV and normalized to the amplitude of the current elicited by ACh alone. Values were plotted against the concentrations of the inhibitor (on a logarithm scale) and fitted with the empirical Hill equation:

$$y = \frac{1}{1 + \left[\frac{[\text{X}]}{\text{IC}_{50}} \right]^{n_H}} \quad (1)$$

where [X] is the inhibitor concentration, and IC_{50} and n_H represent the half-inhibitory concentration and Hill coefficient, respectively.

Fit of current ratios. Analysis of the current blockade was done according to the model proposed by Zarek and Dani (1995); that is, current-voltage relationships were measured first under control conditions and then in presence of a given concentration of blocking agent, and the ratio of these currents was plotted as a function of the holding voltage. Data were fitted with the equation:

$$\frac{I_b}{I_c} = \frac{1}{1 + \frac{[\text{B}]}{K_d \cdot e^{[\delta z F V / (R \cdot T)]}}} \quad (2)$$

where I_b and I_c are currents recorded during the blockade and control, respectively; [B] is the concentration of the blocking agent; K_d is the dissociation constant of the blocker at 0 mV; δ is the fraction of the membrane field sensed by the blocking particle; V is the voltage command; R is the gas constant; T is the absolute temperature; F is Faraday's number; z is the charge. For clarity in the figures, current ratios (I_b/I_c) are plotted once every 10 recorded points, corresponding to approximately one measurement every 3.75 mV. Unless specified, the holding potential was –100 mV. Values are given as mean \pm standard error.

EC_{50} corresponds to the concentration of agonist evoking a current of half-maximal amplitude. IC_{50} corresponds to the concentration of blocking agent causing a 50% reduction in the current evoked by a pulse of agonist near the EC_{50} value (1 μM ACh unless otherwise indicated).

Results

We first examined the action of the well known ganglionic inhibitor hexamethonium (Fig. 1) on the human $\alpha 4\beta 2$ nAChR (Fig. 2). Previous work has indicated that hexamethonium is a potent inhibitor of the chick (Bertrand *et al.*, 1990) and rat (Charnet *et al.*, 1992) $\alpha 4\beta 2$ nAChRs reconstituted in the oocyte system, with a blocking effect strongly dependent of the membrane potential. As illustrated in Fig. 2A, coapplication of 3 μM hexamethonium efficiently inhibits ACh-evoked currents. The application of hexamethonium to a steady concentration of ACh causes a fast decrease in the ACh-evoked current that reverses readily when the drug is removed (Fig. 2B). Blockade and recovery are both voltage and time dependent, as shown by recordings obtained at –100 and –60 mV, respectively. Although slowly reversible, full recovery from hexamethonium blockade typically is observed after a wash for a few minutes (Fig. 2C). A plot of the percentage of the current inhibition (determined with the voltage-ramp protocol; see Materials and Methods) as a function of the hexame-

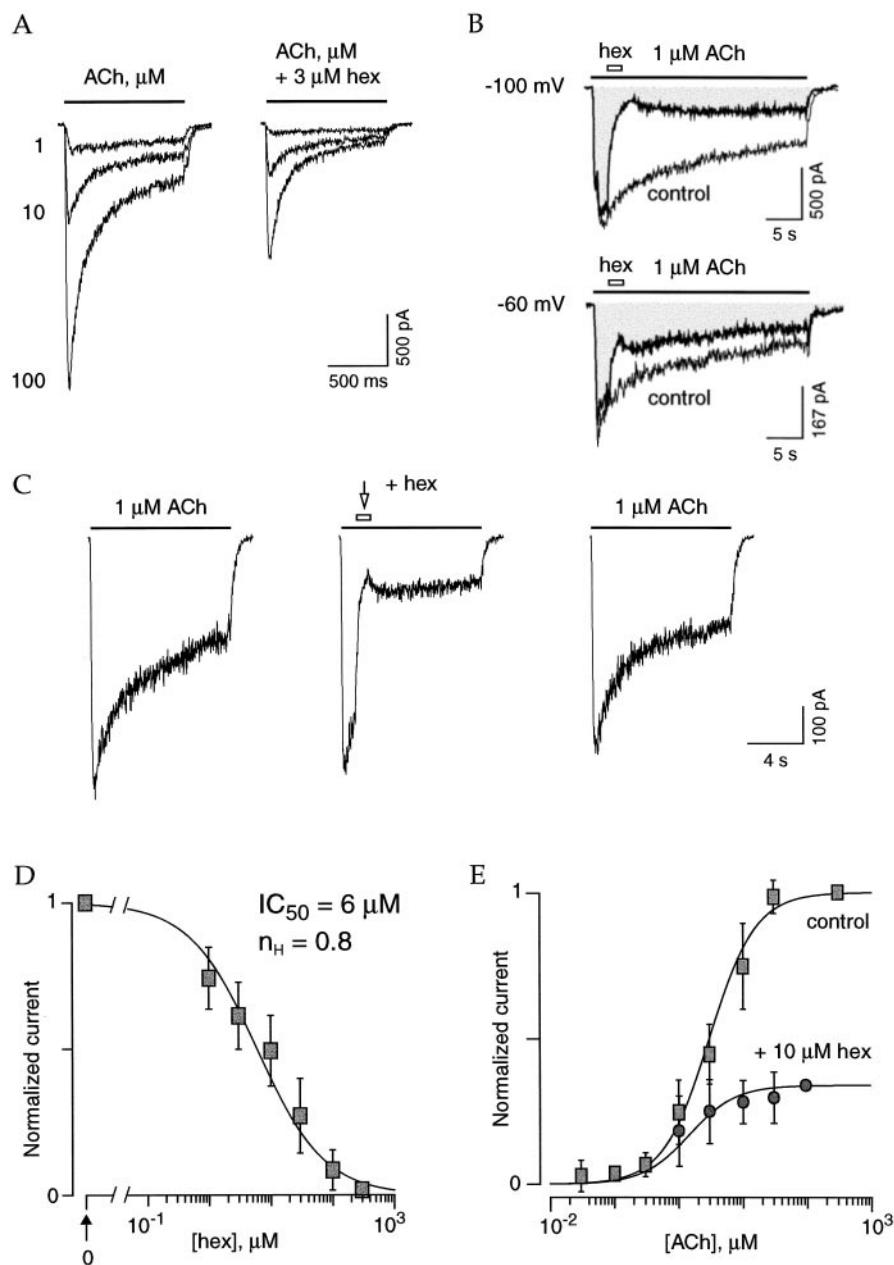


Fig. 2. Hexamethonium, a typical OCB of the human $\alpha 4\beta 2$ nAChR. **A**, Inhibition by hexamethonium (*hex*) of currents evoked by three ACh concentrations. The cell was held at -100 mV. Bars, drug applications. **B**, Time course of hexamethonium blockade and recovery. The application of 30 μM hexamethonium for 2 sec (*open bar*) during a steady exposure to a low ACh concentration (1 μM , 30 sec) causes a fast reduction in the ACh-evoked current. Amplitude of the blockade is voltage dependent [see *top* (-100 mV) and *bottom* (-60 mV) traces]. **C**, Hexamethonium blockade and recovery of ACh-evoked currents. Currents were recorded before and after a 1 -sec hexamethonium blockade (30 μM , *open bar*). ACh pulses (*horizontal lines*) were applied once every minute. Full recovery (*right trace*) was obtained after a ~ 4 -min wash. **D**, Dose-response inhibition curve of hexamethonium at -100 mV. ACh-evoked currents (1 μM ACh) measured in presence of several concentration of hexamethonium are plotted as a function of the antagonist concentration (nine cells; see Materials and Methods). Line through data points, best fit of the empirical Hill equation. **E**, Hexamethonium does not modify the EC_{50} value of ACh for the human $\alpha 4\beta 2$ nAChR. Dose-response curve to ACh obtained in control (*squares*) or presence of 10 μM hexamethonium (*circles*). *Top continuous line*, best fit of empirical Hill equation ($\text{EC}_{50} = 3$ μM , $n_H = 1.2$; see Buisson *et al.*, 1996). Calibrated to the mean current recorded with 1 μM ACh alone, data recorded in presence of 10 μM hexamethonium are fitted (*bottom continuous line*) by the empirical Hill equation, yielding an EC_{50} of 1.5 μM and Hill coefficient of 1.2 (six cells) (Hill equation computed for the standard ACh dose-response curve multiplied by a scaling factor of 0.34).

thionium concentration yields an IC_{50} value of 6 μM and a Hill coefficient of 0.8 (at -100 mV; Fig. 2D). The mean values are summarized in Table 1. Hexamethonium does not significantly modify the EC_{50} value of ACh for the human $\alpha 4\beta 2$ nAChR, as illustrated in Fig. 2E; the ACh dose-response curve performed in the presence of 10 μM hexamethonium gives an EC_{50} value of 1.5 μM with a Hill coefficient of 1.2 (six cells), which is close to the values determined in the absence of hexamethonium. In the presence of 1 μM ACh, voltage-

ramps yield overlaying current-voltage relationships independent of the ramp polarity (Fig. 3A). In contrast, when the same protocols are repeated in the presence of 30 μM hexamethonium, a marked difference in the blockade is observed, with ramps starting at a positive voltage displaying a stronger voltage-dependent blockade (Fig. 3A). Therefore, all the current-voltage relationships presented below were recorded in this configuration. Voltage-ramps performed at 1 μM ACh with increasing concentrations of hexamethonium (1 – 300

TABLE 1
Sensitivity of OCBs

Mean values for IC_{50} and n_H were determined in individual cells by fitting the dose-inhibition curve measured at -100 mV with the empirical Hill equation (see Materials and Methods).

	Hexamethonium	Amantadine	Memantine	(+)-MK-801	TMB-8
IC_{50} (μM)	6.83 ± 2.18	3.44 ± 0.67	6.60 ± 0.92	18.71 ± 3.38	17.20 ± 2.88
n_H	0.76 ± 0.03	0.73 ± 0.02	0.89 ± 0.04	0.70 ± 0.05	0.74 ± 0.05
Cells (<i>n</i>)	6	9	5	7	5

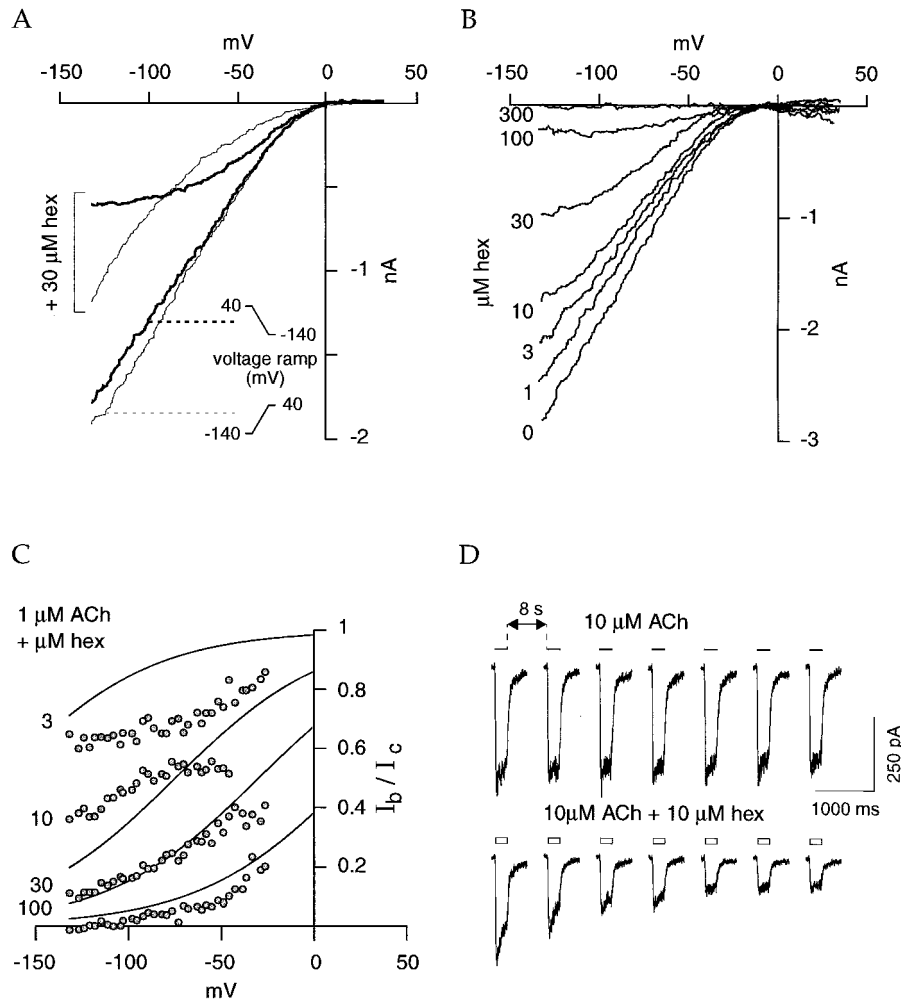


Fig. 3. Hexamethonium blockade is voltage and use dependent. **A**, Hysteresis of the current-voltage relationship under hexamethonium (*hex*). A voltage-ramp (see Materials and Methods) was performed during a 1 μM ACh application first in control and then in the presence of hexamethonium. Although no significant differences were observed in ramp polarities for the current-voltage relationships under the control condition, an important difference is observed when hexamethonium is present with a stronger blockade for the ramp starting at a positive voltage. *Thin lines*, ramp starting from a negative voltage. *Thick lines*, ramp starting from a positive value. **B**, Blockade of hexamethonium is concentration and voltage dependent as illustrated by currents evoked by 1 μM ACh during a voltage-ramp protocol from 40 to -140 mV (in 2 sec). A first ramp was performed during exposure to ACh alone and then during coapplication of increasing hexamethonium concentrations (*left*). **C**, Current ratios (I_b/I_c , as described in Materials and Methods) are plotted as a function of the holding voltage. *Lines through data points*, fit obtained with eq. 2 with a K_d value of 62 μM and a δ value of 0.62. *Left*, values correspond to the concentration of hexamethonium (*hex*) coapplied with ACh. **D**, Hexamethonium (*hex*) blockade is use dependent. Short pulses of ACh (10 μM , 200 msec) were delivered every 8 sec for a control period (*top*); 10 μM hexamethonium then was coapplied with ACh (*bottom*).

μM) reveal a marked voltage-dependent mechanism of block: the fraction of the current inhibited is larger at hyperpolarized membrane potential values (Fig. 3B). This effect can be interpreted in the frame of an open-channel blocking mechanism (Ascher *et al.*, 1979; Bertrand *et al.*, 1990; Charnet *et al.*, 1992). To examine further this hypothesis, we used the single-site model of an ion channel block (Woodhull, 1973). Widely used, this model was adapted later for many LGCs and can be used to fit the current ratio I_b/I_c , where I_b is the current recorded during the blockade, and I_c is the value measured in control. As shown for NMDA, this ratio is described adequately by eq. 2 (Zarek and Dani, 1995). Similar measures performed for several hexamethonium concentrations allowed determination of the mean values of δ and K_d (Fig. 3C, Table 2). Other features of the OCBs are the use-dependent effects (i.e., at a fixed OCB concentration, the fraction of current blockade increases with repetitive agonist stimulations) (Neher and Steinbach, 1978; Gurney and Rang, 1984). As illustrated in Fig. 3D, coapplication of 10 μM hexa-

methonium induces a progressive inhibition of the currents elicited by repetitive pulses of ACh (10 μM , 200 msec). Because hexamethonium enters the nAChR ionic pore, it induces a reduction in the open time of these channels that could be quantified in burst analysis (Colquhoun and Hawkes, 1995). However, given the fast run-down of the $\alpha 4\beta 2$ nAChRs in outside-out patches (Buisson *et al.*, 1996), we could not record under steady state conditions that allow computation of mean open-time histograms to investigate the effect of hexamethonium at the single-channel level. Despite this lack of single-channel measurement, all other evidence indicates hexamethonium behaves as a potent OCB of the human $\alpha 4\beta 2$ nAChR.

The voltage-gated channel antagonist TMB-8 (Fig. 1) has a noncompetitive mode of inhibition at muscle and ganglionic nAChRs, and it was proposed to act via an open-channel block mechanism (Bencherif *et al.*, 1995). We therefore investigated its action on the human $\alpha 4\beta 2$ nAChR. Coapplication of TMB-8 at micromolar concentrations induces a marked

TABLE 2

Parameters of eq. 2

The highest δ values correspond to the deepest sites (from extracellular to intracellular side of the membrane) (see Materials and Methods).

	Hexamethonium	Amantadine	Memantine	(+)-MK-801	TMB-8
δ	0.52 ± 0.16	0.65 ± 0.14	0.52 ± 0.08	0.29 ± 0.14	0.14 ± 0.05
K_d (μM)	67 ± 16	63 ± 15	57 ± 14	75 ± 50	126 ± 75
Cells (<i>n</i>)	5	3	3	3	3

reduction in ACh-evoked currents comparable to that obtained with hexamethonium (data not shown). A dose-inhibition curve (Fig. 4A) at -100 mV yields an IC_{50} value of $15 \mu M$ and a Hill coefficient of 0.7 (mean values given in Table 1). Moreover, as illustrated in Fig. 4B, the effect of TMB-8 is voltage dependent, and the current ratio I_b/I_c can be described with the use of eq. 2. These results indicate that as proposed initially (Bencherif *et al.*, 1995), TMB-8 should bind within the ionic pore of $\alpha 4\beta 2$ nAChRs.

Dose-inhibition protocols performed with increasing concentrations of (+)-MK-801 yielded an IC_{50} value of $15 \mu M$ and a Hill coefficient of 0.7 at -100 mV for the human $\alpha 4\beta 2$ nAChR (Fig. 5A; mean values given in Table 1). The voltage-dependence of the (+)-MK-801 effect (see Fig. 5B) is illustrated by the correlation observed between the current ratio I_b/I_c and predictions made on the basis of eq. 2 (see Materials and Methods and Table 2). Together with the results of others (Ramoa *et al.*, 1990; Amador and Dani, 1991; Briggs

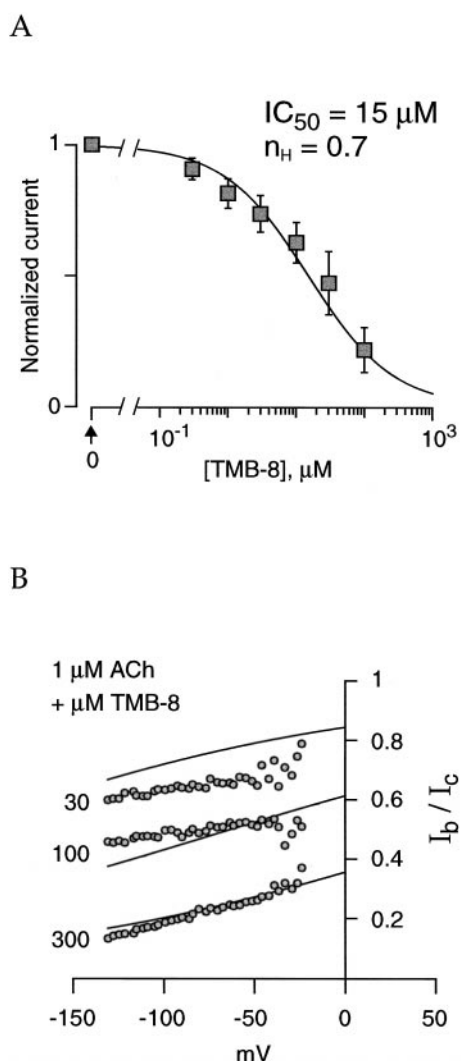


Fig. 4. The calcium channel antagonist TMB-8 inhibits the human $\alpha 4\beta 2$ nAChR. A, Dose-inhibition curve determined at -100 mV (squares, five cells; see Materials and Methods). Data are fitted with the empirical Hill equation (continuous line). B, Plot of the current ratios (I_b/I_c) as a function of cell voltage. Lines through data points, fit obtained with eq. 2 with a K_d value of $160 \mu M$ and a δ value of 0.19. Left, values correspond to the concentration of TMB-8 coapplied with ACh.

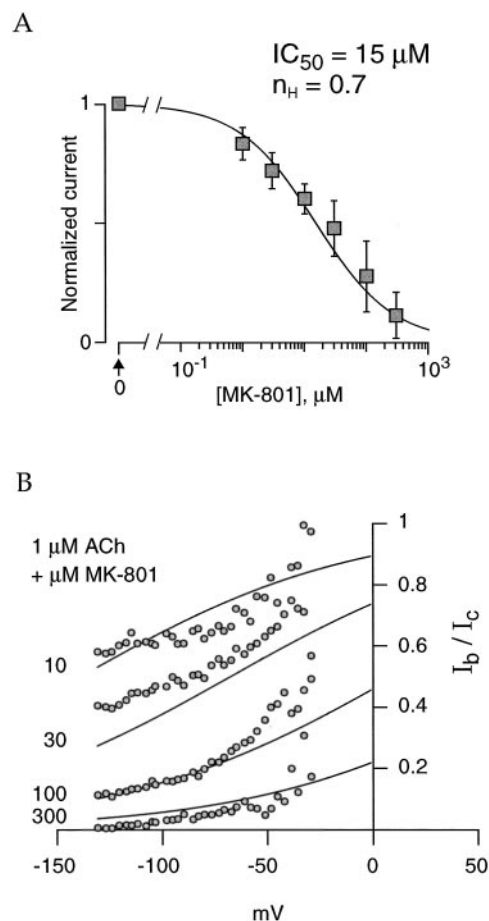


Fig. 5. (+)-MK-801 inhibits $\alpha 4\beta 2$ nAChRs. A, Dose-inhibition curve (squares) measured at -100 mV (five cells; see Materials and Methods). Continuous line, data are fitted with the empirical Hill equation (see Materials and Methods). B, (+)-MK-801 blockade is voltage dependent. Plot of the current ratios (I_b/I_c) as a function of the holding voltage. Lines through data points, fit obtained with eq. 2 with a K_d value of $7.5 \mu M$ and a δ value of 0.39. Left, values correspond to the concentration of MK-801 coapplied with ACh.

and Mckenna, 1996), our data confirm that (+)-MK-801 is a powerful OCB of central nAChRs.

As presented in Fig. 6A, we observed that coapplication of $10 \mu M$ memantine with $10 \mu M$ ACh induced a progressive and use-dependent inhibition of the $\alpha 4\beta 2$ -evoked currents. Partial to full recovery can be observed after an extensive wash-out and is dependent on the holding voltage, as illustrated in Fig. 6B. Similar results were obtained with amantadine (data not shown). Dose-inhibition curves determined at -100 mV reveal the very high potency of both compounds to inhibit the human $\alpha 4\beta 2$ nAChRs. Amantadine displays the lowest IC_{50} value of all the compounds investigated in the current study (Fig. 6C, Table 1), whereas memantine potency is comparable to that of hexamethonium (Fig. 6D, Table 1). Non-competitive antagonists, such as hexamethonium, decrease the ACh maximal amplitude but do not modify its EC_{50} value for the nAChR (see above and Fig. 2D). We then computed the ratio of the current evoked by ACh in the presence of $10 \mu M$ memantine to the current recorded without this inhibitor. The mean ratio is 0.42 ± 0.01 , 0.48 ± 0.05 , and 0.42 ± 0.02 for 1, 10, and $100 \mu M$ ACh, respectively (three cells). Thus, the EC_{50} value of ACh for the human $\alpha 4\beta 2$ nAChR seems to not be modified by the presence of $10 \mu M$ memantine and

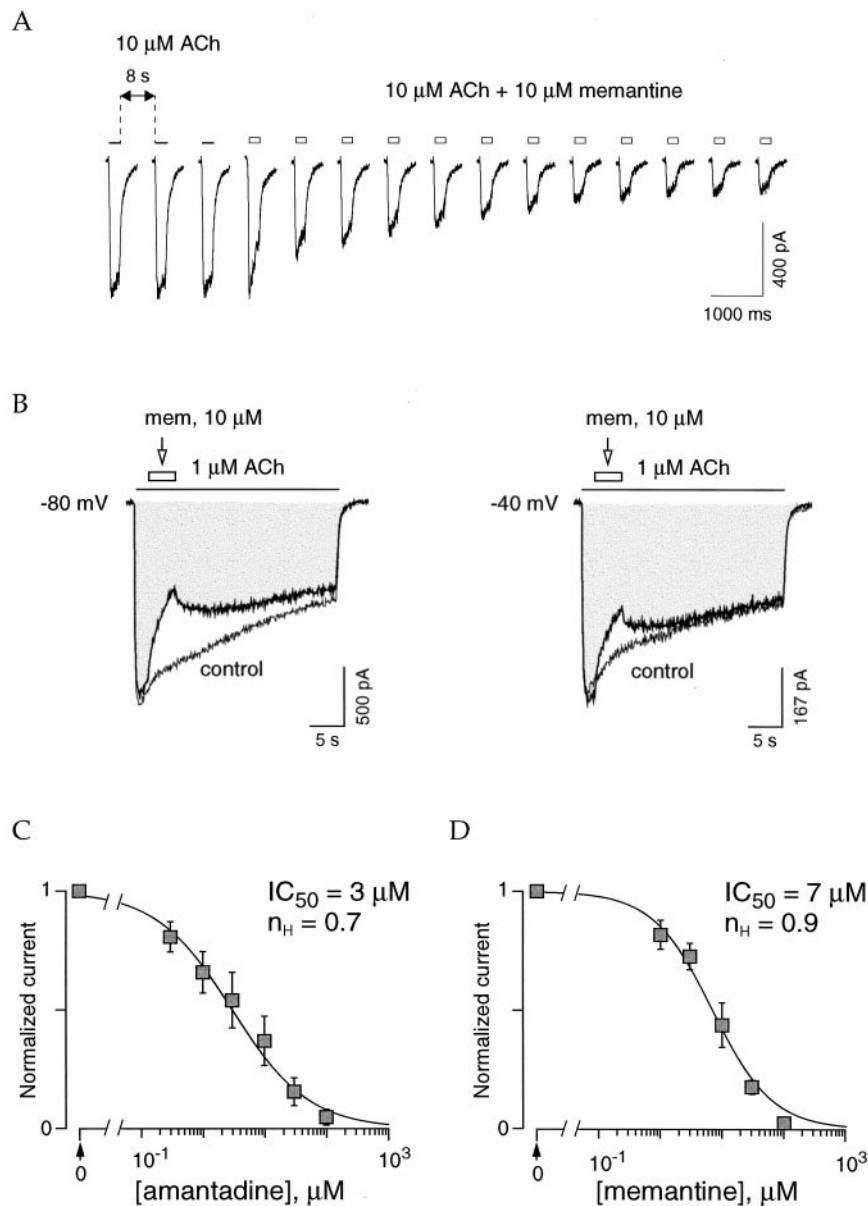


Fig. 6. Amantadine and memantine are potent OCBs of the human $\alpha 4\beta 2$ nAChR. A, Memantine inhibits human $\alpha 4\beta 2$ nAChRs in a use-dependent manner. Short pulses of ACh ($10 \mu\text{M}$; 200 msec) were delivered every 8 sec for a control period (*left*). Memantine ($10 \mu\text{M}$) then was coapplied with ACh (*right*). Even with a 8-sec interval of washout between the applications, memantine induces a progressive block of the ACh-evoked current until it reaches a steady state of inhibition (representative of four experiments). B, Memantine blockade is easily reversible. The application of a short pulse of memantine (*mem*) ($10 \mu\text{M}$, 2 sec) during a prolonged exposure to a low ($1 \mu\text{M}$) ACh pulse causes a fast and reversible reduction of the evoked current. Although incomplete recovery is observed after a 26-sec washout at -80 mV , full recovery was readily obtained when the same experimental paradigm was repeated at a holding potential of -40 mV . C and D, Dose-inhibition curves (*squares*) of amantadine and memantine at -100 mV (nine cells for amantadine and five cells for memantine). *Continuous lines*, values corresponding to the empirical Hill equation (see Materials and Methods).

suggests a noncompetitive mechanism of blockade for this compound. As observed previously with hexamethonium (Fig. 3A), voltage-ramps recorded in the presence of memantine (or amantadine) show hysteresis depending on the ramp polarity (Fig. 7A). In addition, a marked voltage-dependent mechanism of inhibition is illustrated by the current-voltage relationships recorded under increasing concentrations of memantine (Fig. 7B). Similar data were obtained with amantadine (data not shown). The voltage dependence of I_b/I_c for memantine and amantadine is presented in Fig. 7, C and D. Theoretical values are in good agreement with experimental data (see Materials and Methods and Table 2).

Discussion

We investigated the action at the human $\alpha 4\beta 2$ nAChR of compounds that are known to penetrate and block by steric hindrance the ionic pore of LGCs as well as voltage-gated channels.

Hexamethonium is one of the first compounds used to

discriminate the ganglionic and muscle nAChRs (Paton and Zaimis, 1951) and later was characterized as being a potent OCB of nAChRs of the rat parasympathetic ganglion cells (Ascher *et al.*, 1979) and of the reconstituted chick and rat $\alpha 4\beta 2$ nAChRs (Bertrand *et al.*, 1990; Charnet *et al.*, 1992). A few years ago, Bencherif *et al.* (1995) presented biochemical evidence suggesting that the calcium channel antagonist TMB-8 must be a powerful noncompetitive antagonist of peripheral and central nAChRs, with an IC_{50} value in the low micromolar range, indicating this compound inhibits almost equipotently calcium channels and nAChRs. Although first identified as a specific NMDA noncompetitive antagonist (Wong *et al.*, 1986) with open-channel blockade properties (Huettner and Bean, 1988), (+)-MK-801 later was shown to block nAChRs within the same range of concentrations (Ramoa *et al.*, 1990; Amador and Dani, 1991). Similarly, amantadine and memantine are powerful OCBs of NMDA receptors acting at micromolar concentrations (Chen, 1992; Parsons *et al.*, 1996; Chen and Lipton, 1997) and, as shown

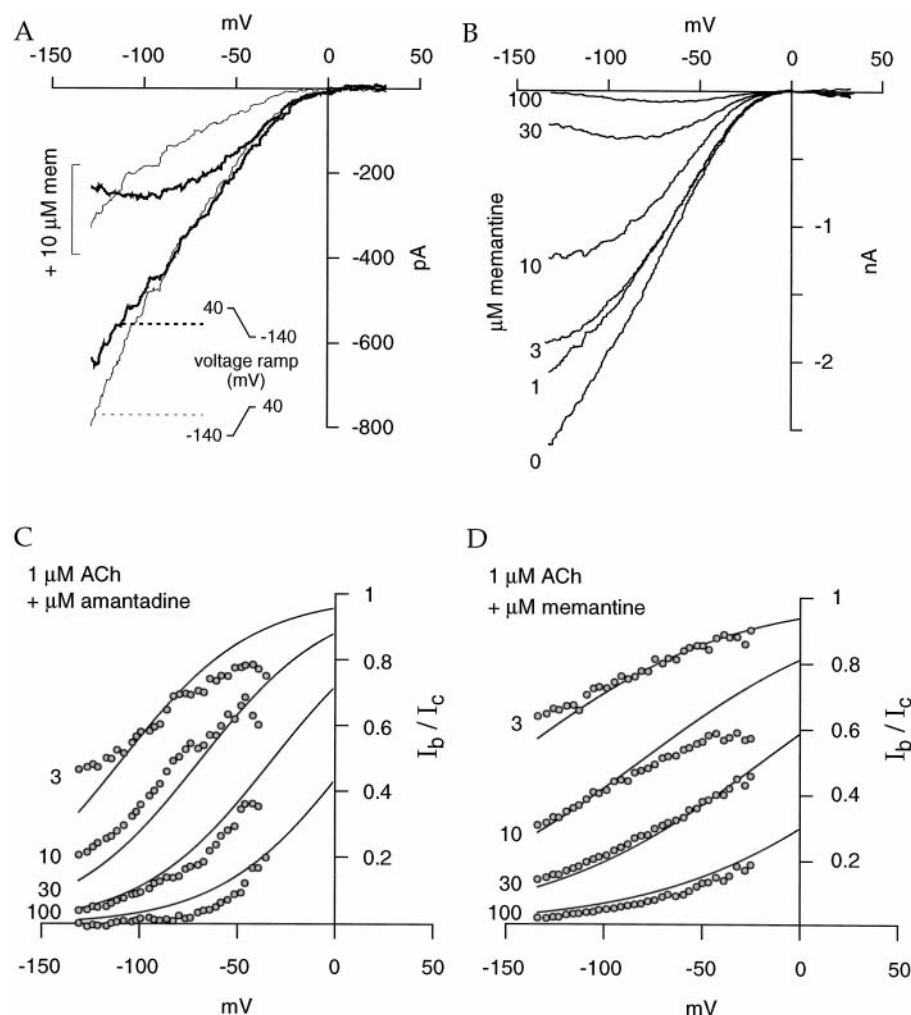


Fig. 7. Blockade of human $\alpha 4\beta 2$ nAChRs by amantadine and memantine is voltage dependent. **A**, Current-voltage relationships recorded for positive or negative voltage protocols in control and the presence of $10 \mu\text{M}$ memantine (*mem*). Hysteresis of memantine blockade is observed clearly in a comparison of the two conditions (*thin line*, ramp starts at -140 mV ; *thick line*, ramp starts at 40 mV). The current-voltage relationship of the ACh-evoked current is independent of the ramp polarity. The slight difference between the two curves may be attributed to a partial desensitization of $\alpha 4\beta 2$ nAChRs. However, when memantine ($10 \mu\text{M}$) is coapplied with ACh, the voltage dependence of its block is observed only under a decreasing voltage-ramp protocol. **B**, Blockade of memantine is concentration and voltage dependent as illustrated by currents evoked by $1 \mu\text{M}$ ACh during a voltage-ramp protocol from 40 to -140 mV (in 2 sec). A first ramp was performed during exposure to ACh alone and then during coapplication of increasing memantine concentrations (*left*). **C** and **D**, Plot of the current ratios (I_b/I_c) as a function of the cell voltage. *Lines through data points*, fit obtained with eq. 2 with a K_d value of $80 \mu\text{M}$ and a δ value of 0.76 for amantadine and a K_d value of $42 \mu\text{M}$ and a δ value of 0.45 for memantine. *Left*, values correspond to the concentration of OCB coapplied with ACh.

more than two decades ago (Albuquerque *et al.*, 1978; Tsai *et al.*, 1978), amantadine has a blocking effect at the muscle nAChR in the range of $100 \mu\text{M}$. Moreover, micromolar concentrations of amantadine inhibit the α -bungarotoxin-sensitive current evoked by ACh in cultured rat hippocampal neurons (Matsubayashi *et al.*, 1997).

Our results demonstrate that the five compounds listed above inhibit the human $\alpha 4\beta 2$ nAChR in the low micromolar range (Table 1). A low Hill coefficient was observed for all compounds tested. Usually interpreted as indicative of cooperativity, the low Hill coefficient suggests the absence of cooperativity in the blockade processes of the antagonist tested. The discrepancy between the low Hill coefficients observed herein and a predicted value of unity, however, cannot be explained on the basis of the channel blockade. Moreover, no substantial modification of the EC_{50} value to ACh was observed for the two compounds tested (hexamethonium and memantine) and thus provide further evidence of a noncompetitive mode of blockade. Although surprising at first, the absence of a significant displacement of the EC_{50} , in presence of hexamethonium, which should have been expected based on our knowledge of OCBs (Ascher *et al.*, 1979), can be interpreted as indicating that this compound binds with equal affinity to the resting and active states. Further work is needed to confirm this hypothesis. Full recovery from blockade was observed for the five OCBs tested as illus-

trated, for example, for hexamethonium and memantine in Figs. 2 and 6. Moreover, it should be noted that these two compounds, which display different voltage-dependent properties (see below), exhibit a significant difference in the recovery time course; hexamethonium is more difficult to wash than memantine, a difference that may indicate that hexamethonium binds more tightly in the pore than memantine.

OCBs generally are known to exhibit a marked voltage-dependent inhibition (Bertrand *et al.*, 1997). One illustration of this property is given by the currents, recorded at different potentials, in the presence of a determined concentration of hexamethonium (Fig. 2B) or memantine (Fig. 6B): the fraction of the inhibited ACh-evoked current increased with more negative holding potential values. This observation is reinforced further on examination of current-voltage relationships (Figs. 3A and 7A). An hysteresis common to all the compounds tested was observed when comparing data obtained with ramps of opposite polarities. This phenomenon is best explained by assuming that compounds enter and block the channel more readily when starting from positive voltages than from negative values. To obtain the best revelation of the channel blockade, all experiments were conducted with voltage-ramps starting at positive values.

In the presence of each of the five compounds investigated in this work, current ratios are fitted adequately using a model adapted from that originally proposed for OCBs

(Woodhull, 1973) (eq. 2 and Table 2). The δ parameter represents the fraction of the membrane electrical field that is sensed by the OCB at its binding site. The highest δ values determined for hexamethonium, amantadine, and memantine suggest that the putative channel binding site for these molecules must be located near the middle of the field across the ionic pore (assuming a constant electrical field across the lipid bilayer). According to this model, TMB-8 and (+)-MK-801 should bind to another site located in the upper 10–20% of the pore electrical field (Table 2). Thus, we propose that these molecules inhibit the human $\alpha 4\beta 2$ nAChR via an open-channel block mechanism but may remain trapped at distinct locations within the ionic pore. A comparison of the recovery from open-channel blockade of hexamethonium and memantine reveals that inhibition induced by this second compound recovers more easily than that with hexamethonium. From previous studies performed on chick $\alpha 4\beta 2$ nAChR, it is known that full recovery from hexamethonium blockade is achieved only with a so-called pop-out protocol (Bertrand *et al.*, 1990) that consists of depolarizing the cell in the presence of ACh. Thus, it can be proposed that hexamethonium indeed remains more strongly bound within the ionic pore than memantine. Further evidence for an open-channel block mechanism is given by the use-dependent effect observed for the three compounds tested (hexamethonium, memantine, and amantadine). Compounds termed OCB often can block the receptor even in its closed state, as first illustrated by Adams (1977) for procaine blockade of the ACh receptor at the neuromuscular junction. Currently, however, the complex properties of neuronal nAChRs preclude detailed analysis that would allow discrimination of the possibility of interaction of blocking agents with the closed or desensitized conformations or both.

In the late 1970s, the emergence of the patch-clamp technique led to the investigation of blocking mechanisms of compounds at the single-channel level. Experiments performed with muscle nAChRs confirmed a previous hypothesis that local anesthetics such as QX-222 or QX-314, as well as curare, are entering opened nAChRs and blocking them via a steric hindrance that is revealed through reduction in the mean open time (Neher and Steinbach, 1978). More recently, site-directed mutagenesis and reconstitution experiments allowed the identification of residues lining the ionic pore of the muscle or neuronal $\alpha 7$ nAChRs that interact with QX-222 (Charnet *et al.*, 1990; Revah *et al.*, 1991). These studies confirmed the hypothesis derived from previous macroscopic observations (Beam, 1976; Colquhoun *et al.*, 1979). Although indispensable for the confirmation of the blockade mechanisms at the molecular level, $\alpha 4\beta 2$ single-channel measurements must await study in the outside-out configuration. The substantial run-down observed under these experimental conditions precludes an analysis of single channels under steady state conditions (Buisson *et al.*, 1996).

Because the $\alpha 4\beta 2$ subtype may be the predominant form of the human brain nAChRs that binds (–)-nicotine with high affinity (Gopalakrishnan *et al.*, 1996) and is responsible for nicotine addiction, it follows that the effect induced by this tobacco alkaloid must be related to this type of nAChR. The addictive properties of nicotine are well documented in rodents, and it was shown that nicotine stimulates dopamine transmission in the nucleus accumbens (Pontieri *et al.*, 1996) in a manner similar to that of cocaine (Merlo Pich *et al.*,

1997). Belonging to the mesolimbic system, the nucleus accumbens is of critical importance in the reinforcing properties of addictive drugs (Nisell *et al.*, 1995). The high affinity nicotine binding sites localized in the nucleus accumbens of the rat are suggestive of expression of the $\alpha 4\beta 2$ subunits in this area (Clarke and Pert, 1985). In the view of these data, it follows that $\alpha 4\beta 2$ nAChR antagonists should constitute potent pharmacological tools in the treatment of smoking cessation. Among the compounds tested in this study, amantadine and memantine display the best inhibition properties at the human $\alpha 4\beta 2$ nAChR, with a use-dependent effect. It is of value to recall that both substances have been used clinically for therapy with persons with Parkinson's disease for >25 years (Danielczyk, 1995). In the brain of treated patients with Parkinson's disease, the extracellular concentration of amantadine is estimated to be $\sim 10 \mu\text{M}$ (Kornhuber *et al.*, 1995), a value that is close to the IC_{50} values determined for the human $\alpha 4\beta 2$ nAChR. Thus, in smoking cessation trials, amantadine (or memantine) could be used at concentrations equal to or lower than those used for the treatment of parkinsonism, with good knowledge of the side effects. In conclusion, we propose that hexamethonium, TMB-8, (+)-MK-801, amantadine, and memantine are potent OCBs of the human $\alpha 4\beta 2$ nAChR and that some of the clinical effects observed for amantadine and/or memantine might be related to their action on the neuronal nAChRs.

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