0026-895X/00/040778-10$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:778–787, 2000

Vol. 58, No. 4
99/857333
Printed in U.S.A.

Rapid Relief of Block by Mecamylamine of Neuronal Nicotinic Acetylcholine Receptors of Rat Chromaffin Cells In Vitro: An Electrophysiological and Modeling Study

RASHID A. GINIATULLIN, ELENA M. SOKOLOVA, SILVIA DI ANGELANTONIO, ANDREI SKORINKIN, MARIA V. TALANTOVA, and ANDREA NISTRI

Biophysics Sector (R.A.G., E.M.S., S.D.A., A.S., M.V.T., A.N.) and INFM Unit (E.M.S., S.D.A., A.N.), International School for Advanced Studies (SISSA), Trieste, Italy; Department of Physiology, Kazan Medical University, Kazan, Tatarstan, Russia (R.A.G., M.V.T.); and Department of Biophysics, Kazan State University, Kazan, Tatarstan, Russia (A.S.)

Received February 23, 2000; accepted August 9, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The mechanism responsible for the blocking action of mecamylamine on neuronal nicotinic acetylcholine receptors (nAChRs) was studied on rat isolated chromaffin cells recorded under whole-cell patch clamp. Mecamylamine strongly depressed (IC_{50} = 0.34 μM) inward currents elicited by short pulses of nicotine, an effect slowly reversible on wash. The mecamylamine block was voltage-dependent and promptly relieved by a protocol combining membrane depolarization with a nicotine pulse. Either depolarization or nicotine pulses were insufficient per se to elicit block relief. Block relief was transient; response depression returned in a use-dependent manner. Exposure to mecamylamine failed to block nAChRs if they were not activated by nicotine or if they were activated at positive membrane potentials. These data suggest that mecamylamine could not interact with receptors either at rest or at depolarized level. Other nicotinic antagonists like dihydro-β-erythroidine or tubocurarine did not share this action of mecamylamine although proadifen partly mimicked it. Mecamylamine is suggested to penetrate and block open nAChRs that would subsequently close and trap this antagonist. Computer modeling indicated that the mechanism of mecamylamine blocking action could be described by assuming that 1) mecamylamine-blocked receptors possessed a much slower, voltage-dependent isomerization rate, 2) the rate constant for mecamylamine unbinding was large and poorly voltage dependent. Hence, channel reopening plus depolarization allowed mecamylamine escape and block relief. In the presence of mecamylamine, therefore, nAChRs acquire the new property of operating as coincidence detectors for concomitant changes in membrane potential and receptor occupancy.

Mecamylamine is a secondary amine acting as an antagonist on neuronal nicotinic acetylcholine receptors (nAChRs) (Ascher et al., 1979; Fieber and Adams, 1991; Nooney et al., 1992). The blocking action of mecamylamine is exerted on all native subtypes of nAChRs despite their different subunit composition (Connolly et al., 1992). In vivo mecamylamine lowers blood pressure and can prevent seizures induced experimentally with the use of nicotine (Gyermek, 1980). In view of this widespread action, which remains, however, highly selective against nAChRs, mecamylamine is commonly used to probe the role of such receptors in central and peripheral synaptic transmission processes.

On the basis of measurements of ACh-evoked currents, mecamylamine has been suggested to be a competitive antagonist on submandibular ganglion cells (Ascher et al., 1979; Gurney and Rang, 1984). Biochemical work on recombinant $\alpha_3\beta_4$ subunit nAChRs (the predominant type expressed in chromaffin cells; Campos-Caro et al., 1997) has shown mecamylamine to block them in a nonsurmountable fashion (Xiao et al., 1998). This type of action might indicate "uncompetitive" antagonism, a term which has been recently used to define the block by memantidine and amantadine of *N*-methyl-D-aspartate (NMDA) receptors (Blanpied et al., 1997; Chen and Lipton, 1997). Uncompetitive antagonism may be caused by two distinct mechanisms: simple open channel block or trapping of the blocker inside the closed channel (for a review, see Dingledine et al., 1999). Lingle (1983) first proposed the trapping mechanism based on experiments on ACh receptors of lobster muscle. On submandibular ganglion neurons, hexamethonium and some closely related derivatives could be trapped by nAChRs and subsequently released by combining depolarization with agonist

This work is supported by grants from Istituto Nazionale di Fisica della Materia (PRA CADY) and from Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica (cofinanziamento ricerca) to A.N. The financial support by the Russian Foundation for Basic Research is gratefully acknowledged by R.A.G., E.M.S., and M.V.T.

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; NMDA, *N*-methyl-D-aspartate; F3, *N*,*N*,*N*-trimethyl-1-(4-*trans*-stilbenoxy)-2-propylammonium iodide; I/V, current/voltage; DHBE, dihydro-β -erythroidine.

application (Gurney and Rang, 1984). Nevertheless, mecamylamine did not share this effect on such ganglion cells. Conversely, Nooney et al. (1992) briefly reported that on bovine chromaffin cells, the antagonism by mecamylamine was voltage dependent. The antagonism by mecamylamine of native nAChRs of autonomic ganglia (Shen and Horn, 1998) and of recombinant $\alpha_3\beta_4$ nAChRs (Nelson and Lindstrom, 1999) was suggested to be caused by simple block of open channels.

The present study provides a quantitative description of the blocking properties of mecamylamine on nAChRs of rat chromaffin cells and its rapid relief when the membrane potential is depolarized in the presence of nicotine. On the basis of these results, we applied a kinetic model to simulate the behavior of nicotine induced currents after exposure to this antagonist to understand the rate limiting steps of this phenomenon. This approach enabled us to propose a scheme to account for the blocking action of mecamylamine.

Experimental Procedures

Patch Clamp Recording. Experiments were carried out on rat adrenal chromaffin cells in vitro as reported previously (Giniatullin et al., 1999). Rats were anesthetized by slowly rising levels of CO_2 and sacrificed by severing the heart vessels. This procedure is in accordance with Italian Animal Welfare Act and with Guide for the Care and Use of Laboratory Animals as adapted and promulgated by the National Institutes of Health.

Chromaffin cells, used within 24 to 48 h from plating, were continuously superfused (3–5 ml/min) with physiological solution containing 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 15 mM glucose, and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. Patch pipettes were pulled from thin glass capillary tubing and had resistance of about 5 MΩ when filled with 120 mM CsCl, 20 mM HEPES, 3 mM Mg_2ATP_3, and 10 mM BAPTA; pH was adjusted to 7.2 with CsOH. Cells were voltage-clamped at −70 mV (unless otherwise indicated) in the whole-cell configuration after obtaining GΩ seals (usually not less than 2 GΩ). Series resistance was compensated by 60%. Nicotine was diluted in physiological solution and delivered by pressure application (10–20 psi) from glass micropipettes (located about 15 μm from the recorded cell). Mecamylamine or other nicotinic antagonists were applied by rapid superfusion via the Rapid Solution Changer RSC-200 (Bio Logic Science Instruments, Grenoble, France). Because the observed antagonist potency of mecamylamine (see *Results*) was similar to that reported previously with other methods of agonist application (Zhang et al., 1999), it is unlikely that pressure-applied nicotine had significantly diluted the mecamylamine concentration at the level of the cell membrane. All test substances were purchased from Sigma (Milan, Italy) with the exception of *N*,*N*,*N*-trimethyl-1-(4-*trans*-stilbenoxy)-2-propylammonium iodide (F3), which was kindly donated by Dr. C. Gotti (Department of Medical Pharmacology, University of Milan, Milan, Italy).

Data Analysis. Data are expressed as mean ± S.E. Statistical significance was assessed with Student *t*-test for parametric data and Wilcoxon test for nonparametric data. At various holding potentials (−120 to −30 mV range) the IC_{50} values (concentration producing 50% reduction in nicotine current amplitude) for mecamylamine block were calculated with the following equation

$$I_c - I_b = \frac{I_c}{1 + \left[\frac{IC_{50}}{[B]}\right]^{n_H}} \quad (1)$$

where I_b and I_c are amplitudes of blocked and control currents, [B] is the mecamylamine concentration, and n_H is the Hill coefficient. These values were plotted against membrane potentials to estimate the IC_{50} value at 0 mV. The latter value was used for the Woodhull's standard equation (Woodhull, 1973).

$$I_b = \frac{I_c}{1 + ([B]/K_d(0\ \text{mV}) \cdot e^{\delta z F V/RT})} \quad (2)$$

assuming that the mecamylamine IC_{50} value could be used instead of its K_d (equilibrium dissociation constant) value. The Woodhull equation enabled us to estimate the portion of membrane electric field sensed by the mecamylamine binding site (expressed as δ) inside the nAChR. For this, we considered that at pH 7.4, this agent is almost fully charged (pK_a = 11.2; Goldstein et al., 1979).

Computer Simulation Method. Simulations were based on the standard theory for receptor activation kinetics in response to agonist binding (Colquhoun and Hawkes, 1977). The same theory was assumed to be applicable to the kinetics of antagonist binding in the presence of the agonist. The nicotinic receptor currents were calculated according to

$$I(t) = V \cdot N \cdot P_{open}(t) \cdot \sigma, \quad (3)$$

where V is the test voltage (mV), N is number of nicotinic channels, P_{open} is the probability of open channel state, and σ is the channel conductance. We assumed σ to be constant at negative holding potentials and to become zero at positive voltage because of strong membrane rectification. Although during simulations we reproduced the full time course of the nicotine evoked responses, for sake of simplicity these were modeled for −70 mV holding potential only.

The voltage dependence (H_i) of a rate constant k_i governing the transition of receptors between distinct kinetic states was obtained from

$$k_i(V) = k_i(0\ \text{mV}) \cdot e^{\frac{V}{H_i}}, \quad (4)$$

where V is the test voltage (mV). During computer simulations, the values for k_i constants were calculated after setting the potential to 0 mV.

In general, based on the mass action law, we formulated a set of differential equations for each kinetic state in analogy with the approach used by Chretien and Chauvet (1998), whereby:

$$\frac{d\bar{P}(t)}{dt} = \bar{P}(t) \cdot Q, \quad (5)$$

where $\bar{P}$ is a vector composed of probabilities of the receptor occupying each kinetic state at time t, and Q is the matrix of transitions between the states. Our in-house-developed program was written in Pascal and used on an IBM-compatible PC to solve numerically this set of differential equations using the eight-order Runge-Kutta method (Baker et al., 1996). The adequacy of the simulated responses to reproduce experimental records was judged by eye.

Results

Characteristics of the Depression of Nicotinic Currents by Mecamylamine

Records in Fig. 1A, taken from a chromaffin cell held at −70 mV, are submaximal inward currents (−320 pA) induced by brief (30 ms) pressure applications of nicotine (0.1 mM pipette concentration). These responses were very reproducible as long as nicotine was applied at intervals of at least 15 s. In the presence of 1 μM mecamylamine (applied via the fast superfusion system), the first response to nicotine (about 5-s exposure to mecamylamine) was only slightly reduced; the extent of the current block, however, was increased with successive applications until it reached steady state (−78 pA current amplitude) approximately 2 min later. On a random sample of 12 cells, the steady state depression was 80 ± 6%.

Once the block was at steady state, its extent was relatively insensitive to the rate of nicotine application within the 0.3 to 0.016 Hz range. Further tests were mainly carried out with 30-ms pressure application of nicotine (0.1 mM) at 0.066 Hz while mecamylamine was administered by fast superfusion. Figure 1B shows average data (n = 12 cells) for the time course of the mecamylamine-induced block of nicotine current amplitude with slow onset (τ = 40 ± 5 s) and minimal recovery on washout. After 10 min, response recovery was 32 ± 9% of control amplitude (n = 4). The reduction in current peak amplitude by mecamylamine was also accompanied by shortening of the monoexponential nicotine current decay from 89 ± 14 to 68 ± 8 ms (n = 9; $P < .05$).

When 1 μM mecamylamine was bath-applied (n = 3), the depression (81 ± 2%) of nicotine current amplitude had also slow onset and recovery. Likewise, when mecamylamine was applied for 1 to 3 min via a separate pressure pipette (1 μM pipette concentration; n = 5), current depression at steady state level was 76 ± 5%.

Figure 1C shows that, on chromaffin cells held at −70 mV, mecamylamine was a rather potent nAChR blocker because, under steady state conditions, the IC_{50} value was 0.34 μM. The Hill coefficient (n_H) value was 1.08, indicating that the stoichiometry for nAChR block apparently required only one mecamylamine molecule.

Voltage Sensitivity of Mecamylamine Block

Several nAChR blockers are known to display a variable degree of voltage dependence (Ascher et al., 1979; Gurney and Rang, 1984; Buisson and Bertrand, 1998). The present experiments were performed to examine if the effect of mecamylamine on chromaffin cells was also voltage dependent. Figure 2A shows the extent and time course of mecamylamine block for two holding potentials. If mecamylamine was tested on cells held at −30 mV, the depression of nicotine (30 ms) currents was relatively weak and readily reversible. Conversely, a much stronger (and longer) block was observed when cells were held at −120 mV (Fig. 2A). Figure 2B shows the plot of percentage depression in nicotine current by various concentrations of mecamylamine (at two different holding potentials). This plot enabled us to calculate H (coefficient for e-fold variation in percentage current depression with membrane potential), which was 50 mV. From the experimentally obtained IC_{50} values of mecamylamine (see Figs. 1C and 2B), it was possible to estimate the IC_{50} value (1.6 μM) at 0 mV. This value was used for Woodhull's equation (Woodhull, 1973) to infer the portion of membrane electric field (expressed as δ) that was sensed by the mecamylamine binding site. The calculated δ of 0.72 suggests that mecamylamine reached a relatively deep site inside the nAChR channels.

The voltage dependence of the block was also explored by constructing full current/voltage (I/V) curves after clamping cells at several membrane potentials within the −120 to +30 mV range as indicated in Fig. 2C. Mecamylamine reduced membrane currents at all test potentials with no change in the apparent reversal potential or in the strong rectifying properties of nAChRs (which allow minimal current flow at positive potentials; Nooney et al., 1992). The mecamylamine-induced depression was proportionally much larger at negative values as shown in Fig. 2D, in which the nicotine current (expressed as ratio of the response in mecamylamine solution over the control one) was plotted versus membrane potential.

Rapid Rescue of Nicotine Currents from Mecamylamine Block Despite Continuous Antagonist Presence

Could the strong voltage dependence of mecamylamine block confer special blocking properties to this antagonist? This issue was tested in experiments like the one shown in Fig. 3A: in the continuous presence of 1 μM mecamylamine, after achieving 89% block of nicotine current amplitude, the

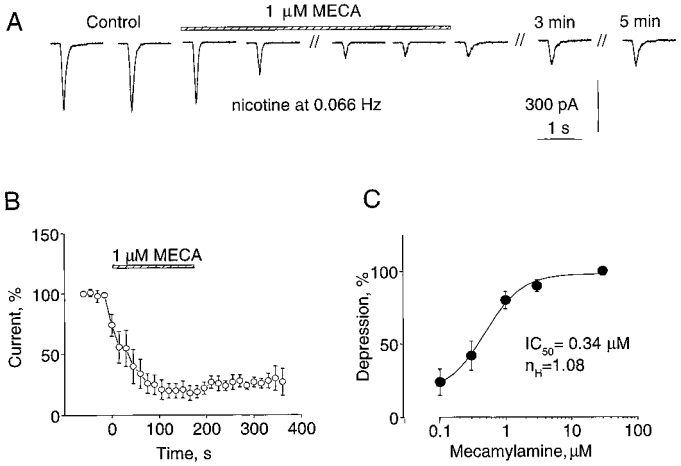

Fig. 1. Effect of mecamylamine on membrane currents evoked by pressure-applied 0.1 mM nicotine. A, control currents elicited by pulsing nicotine at 0.066 Hz are depressed by mecamylamine with steady state block attained at 2 min. Washout is associated with minimal recovery in current amplitude. B, plot of nicotine current amplitude (%) versus elapsed time from start of mecamylamine superfusion. Data are from 12 cells. C, plot of % depression in nicotine current amplitude against log concentration of mecamylamine. The concentration producing 50% reduction was taken as IC_{50} (0.34 μM). The curve had a Hill coefficient (n_H) of 1.1. Data points are from 5 to 12 cells.

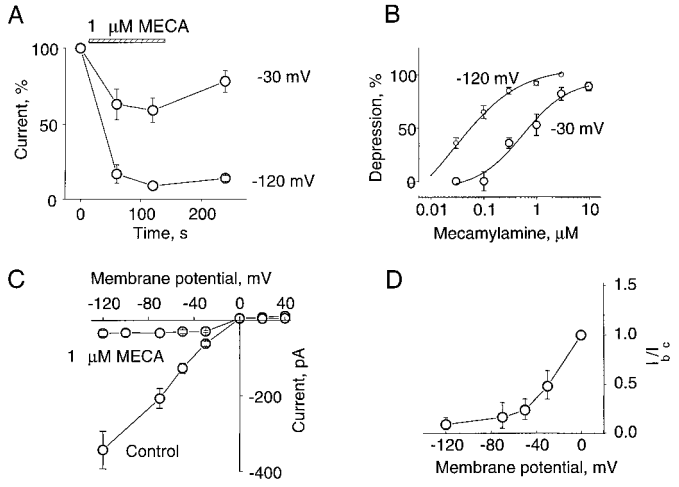

Fig. 2. Voltage dependence of mecamylamine block. A, time course of changes in nicotine current amplitude (%) in the presence of 1 μM mecamylamine (applied as indicated by the horizontal bar) at −30 or −120 mV holding potential. Compare stronger block at −120 mV with less recovery after washout. Data are from five to eight cells. B, plot of the depression of nicotine induced currents by increasing log concentrations of mecamylamine at −120 or −30 mV holding potential. Data are from five cells. C, I/V curve obtained by clamping cells (n = 5) at various holding potentials in control solution or in 1 μM mecamylamine solution. D, plot of nicotine current block (expressed as I_b/I_c; i.e., the ratio of blocked current over control) versus different membrane potentials (n = 5). Note stronger block at more negative membrane potentials (1 μM mecamylamine solution).

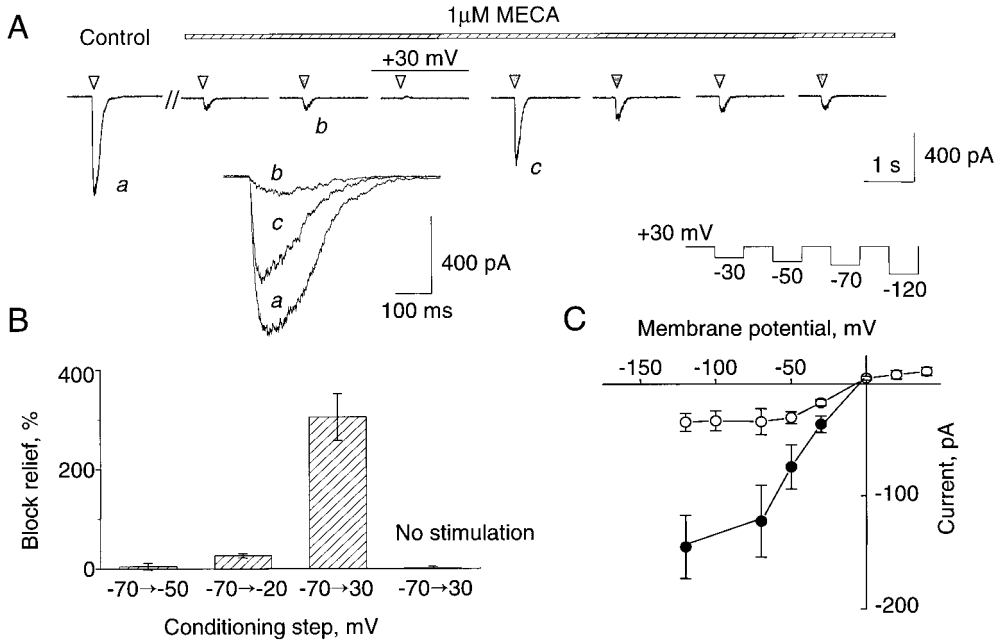

Fig. 3. Rapid relief from 1 μM mecamylamine block. A, control current evoked by nicotine pulses (arrowheads) is reduced at steady state by mecamylamine. After combining depolarization to +30 mV (horizontal line) with nicotine pulse (note slight outward current), the subsequent application of nicotine transiently generated a strong inward current, which was then blocked again. Inset shows samples (a–c) of nicotine currents on faster time base and at higher gain taken from likewise labeled traces. B, histograms indicating block relief (as % of the current amplitude before the conditioning protocol) induced by depolarization from −70 mV to −50, −20 or +30 mV in the presence of nicotine or to +30 mV without nicotine (no stimulation). Data are from six cells. C, I/V relation for the nicotine current during mecamylamine steady state block (○) and for the response (●) immediately after the depolarization sojourn at +30 mV; $n = 5$.

membrane potential was shifted to +30 mV for 15 s during which a single nicotine pulse was applied to elicit a very small response only (16 pA). Nevertheless, on return to standard holding potential (−70 mV), nicotine evoked a substantial current that was only 38% smaller than initial responses in control solution. The protocol of nicotine pulse application was continued to reveal rapid re-establishment of current block (88%) analogous to the one observed before the transient depolarization test. The inset to Fig. 3A shows (at faster speed and higher gain) superimposed records of nicotine currents in control solution, in the presence of mecamylamine and immediately after membrane depolarization with associated block relief. Block relief expressed as ratio of currents before and after the combined application of a depolarizing step and nicotine was 406 ± 83% ($n = 9$). Thus, the relieved current corresponds to 64 ± 7% of the control one.

Using a protocol similar to the one of Fig. 3A with membrane depolarization to variable levels, it was apparent that relief of mecamylamine block was related to the value of membrane depolarization and that depolarization alone (without concomitant application of nicotine) could not temporarily restore current amplitude (Fig. 3B). From data like those of Fig. 3B it was possible to calculate the value of H as 22.5 mV (that is, the coefficient for e-fold change in block relief intensity). Depolarization duration was less critical than amplitude to determine relief of block because essentially the same results were obtained when the pulse (100 mV from −70 to +30 mV) was varied from 0.5 to 15 s.

Figure 3C shows the I/V relation for nicotine currents during mecamylamine steady-state block (Fig. 3C, ○) and for responses (Fig. 3C, ●) immediately after the depolarization sojourn at +30 mV. Thus, at least within the −70 to 0 mV range, the characteristics of the nicotine current during block relief were relatively similar to those observed in control solution.

Voltage-dependent interaction at the level of the mecamylamine-blocked receptors might also have been caused by intracellular Mg^{2+}, which is known to block nAChR currents by deep penetration through the nicotinic channels (Ifune and Steinbach, 1991). If Mg^{2+} exerted this action on chromaffin cell receptors as well, it might have knocked off mecamylamine from the channel and thus contributed to block relief at positive potential. This possibility was tested in the present experiments by using an intracellular patch solution from which Mg^{2+} was omitted and replaced in an equimolar fashion by Na^{+}. Under these conditions, the steady state block of 20-ms nicotine currents produced by mecamylamine (1 μM) was 87 ± 2% (5 cells). On the same cells, membrane depolarization to +30 mV in conjunction with a nicotine pulse brought about 362 ± 42% block relief, which amounted to an average nicotine current amplitude of 61 ± 4% of the control response before mecamylamine. Thus, these data make it unlikely that intracellular Mg^{2+} indirectly generated block relief.

Prevention of Mecamylamine-Blocking Action

The present experiments were carried out to find out, despite continuous, standard application of mecamylamine, whether it was actually possible to prevent its antagonist effect. For this purpose, tests analogous to those performed by Gurney and Rang (1984) with methonium compounds were employed. Figure 4a shows that, on a cell held at −70 mV, 3-min application of mecamylamine (without applying nicotine) had no effect on the response to nicotine applied 2 min later in control solution. When repeated pulses of nicotine (0.066 Hz) were applied to the same cell held at +30 mV during mecamylamine application, there was no nicotine response in the presence of the antagonist, but a full response appeared after 2 min washout (Fig. 4b). In contrast with this observation, when the same cell was then subjected to the usual protocol of applying mecamylamine at −70 mV during repeated pulses of nicotine, there was strong depression of current amplitude (by 95% at 3 min), which recovered minimally (20% of control) after 2 min washout (Fig. 4c). Similar data were obtained from five cells. In accordance with the use-dependent properties of mecamylamine block, these results indicate that the mere presence of this drug was insuf-

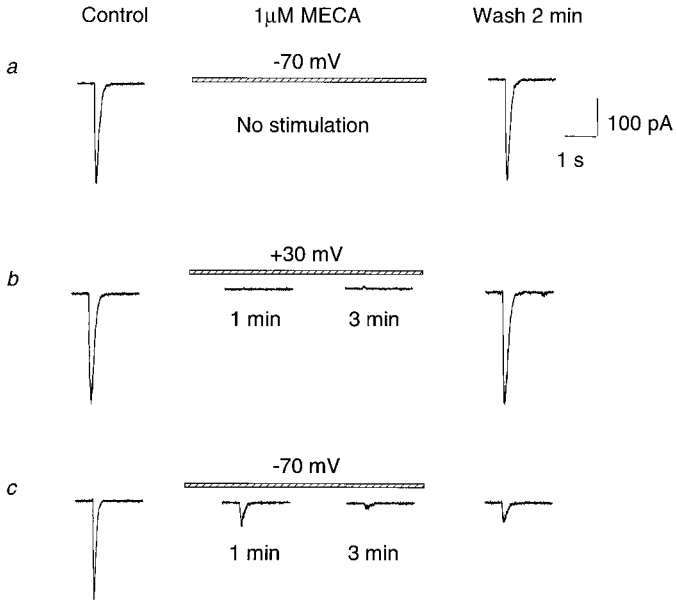

Fig. 4. Prevention of mecamylamine block. a, 3-min application of mecamylamine (hatched bar) to a cell held at −70 mV without pulse application of nicotine fails to affect responses to nicotine after mecamylamine wash. b, if the cell is depolarized to +30 mV and nicotine pulses are applied (0.066 Hz) throughout the 3-min mecamylamine application, subsequent response to nicotine after mecamylamine wash is unaffected. c, nicotine response is readily and strongly blocked when applied in the presence of mecamylamine at −70 mV and, despite 2-min wash of mecamylamine, the nicotine current remains largely reduced. All records are from the same cell.

ficient to block nAChRs unless they were also activated to generate inward currents.

Protocols for Rescuing AChRs from Mecamylamine Block Remain Effective over an Extended Period

As the combined depolarization and single agonist application were so effective in producing block relief, one interesting question was for how long after this test the block reversal could still be observed despite the continuous presence of mecamylamine. To explore this aspect nicotine currents were blocked by 1 μM mecamylamine (87% at steady state at −70 mV; see Fig. 5A). The relief protocol consisted in the standard depolarization to +30 mV associated with a pulse of nicotine, which elicited a small outward current (30 pA). Fifteen seconds later at −70 mV and still in the presence of mecamylamine, the inward current was very large (Fig. 5A, a; 330% of the response before depolarization). Interestingly, an equally strong current rescue was observed when the nicotine pulse was applied after 1 min rest (Fig. 5A, b; 391%). Even a longer resting period (5 min) in the presence of mecamylamine preserved the block relief (Fig. 5A, c) as the current amplitude was 378% of the one before depolarization. On average, for five cells tested at 15 s or 5 min, block relief was essentially the same (Fig. 5B).

As the association of nicotine application plus strong membrane depolarization relieved AChR block, we explored whether this combination could speed up recovery from this block during mecamylamine washout. Figure 6A shows that, after establishing strong block of nicotine currents by mecamylamine, 150-s washout was accompanied by weak recovery in current amplitude (20%) to test pulses; nevertheless, depolarization to +30 mV plus a single nicotine pulse were able to elicit a very large recovery (249% with respect to amplitude before depolarization). Fig. 6B shows that on a sample of six cells the restored current amplitude remained relatively stable in control solution after the depolarizing pulse (●, 84 ± 8% of relieved current at 60 s), unlike the rapid block return observed in the continuous presence of mecamylamine (○, see also data reported in Fig. 1B). Note that the nicotine current amplitude 3 min after wash and after conditioning depolarization was 68 ± 14% of control current, whereas in the absence of depolarization the current was 27 ± 11% of control (see Fig. 1B).

Comparison between Mecamylamine and Other Nicotinic Receptor Antagonists

The complex properties of nAChR block by (and recovery from) mecamylamine raised the issue of whether other cholinergic blockers also shared similar properties on rat chromaffin cells in vitro. Figure 7A shows that dihydro-β-erythroidine (DHβE; 400 μM) rapidly, extensively (by 91 ± 1%;

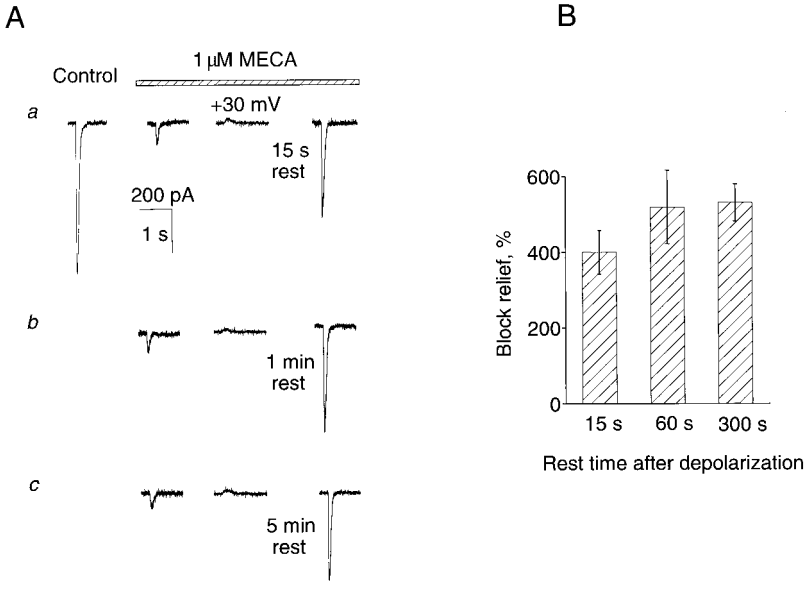

Fig. 5. Relief from mecamylamine block can still be observed after long rest. A, after achieving steady state block of nicotine current (8–10% in a–c), membrane depolarization to +30 mV plus application of nicotine pulse generates substantial block relief (54–77% of control current in a–c) observed after 15 s (a), 1 min (b), or 5 min (c) rest. All records are from the same cell held at −70 mV. B, bar chart showing equivalent degree of block relief (expressed as % of steady-state blocked current before test protocol) after 15 to 300 s of rest; $n = 5$.

$n = 4$) and reversibly blocked responses to nicotine. The introduction of the protocol of strong depolarization plus nicotine application made no difference to the DHβE-evoked block. Similar data were observed with *d*-tubocurarine (10 μM; block by 46 ± 10%; $n = 3$), or the oxystilbene derivative F3 (100 nM; block by 44 ± 3%; $n = 30$). Proadifen (10 μM) blocked nicotine currents by 83 ± 1% ($n = 5$), a phenomenon partly relieved by membrane depolarization as indicated in Fig. 7B. Data summarizing observations based on depolarization plus nicotine application are shown in Fig. 7B.

Computational Modeling of Mecamylamine Action on nAChRs

The mechanism of mecamylamine action on nAChRs was further investigated using computer-assisted simulation of whole-cell currents induced by nicotine in the presence of this antagonist and during washout. Data modeling should indicate whether the experimental data could be adequately fitted by assuming either simple open channel block by mecamylamine or a more complex process involving changes in receptor kinetics.

A model obtained by computer simulations was considered useful only if it reproduced the action of mecamylamine in accordance with four stringent criteria, namely: 1), slow, use-dependent onset of block; 2), attainment of steady state block; 3), very rapid block relief by depolarization plus nicotine application; 4), slow recovery on washout. In Fig. 8A, these four characteristics are clearly demonstrated by typical traces of experimentally recorded inward currents from a chromaffin cell (clamped at −70 mV) exposed to brief (30 ms) pulses of nicotine and are labeled a–d accordingly. The dashed horizontal bar indicates the application of mecamylamine; membrane depolarization to +30 mV is represented by a filled bar.

In general, we assumed that nAChR activation and deactivation could be normally described by a simplified scheme (reviewed by Colquhoun, 1998) in which:

$$R + A \underset{k_1^-}{\overset{k_1^+}{\longleftrightarrow}} AR + A \underset{k_2^-}{\overset{k_2^+}{\longleftrightarrow}} A_2R \underset{\alpha}{\overset{\beta}{\longleftrightarrow}} A_2R^*$$

where k_1^+, k_1^-, k_2^+, k_2^-, β, and α are rate constants for agonist binding/unbinding and isomerization, A is agonist, and R and R^* represent the closed and open states of the channel, respectively. Note that although β has minimal voltage dependence, α has a moderate degree of voltage dependence ($H = 156$ mV; Mathie et al., 1990; Maconochie and Knight, 1992). In view of the short pulse application of nicotine, which elicited closely reproducible responses, we implied that there was no significant receptor desensitization.

Model 1. The simplest case would be that mecamylamine action is explained on the basis of simple open channel block as assumed recently, for instance, by Shen and Horn (1998).

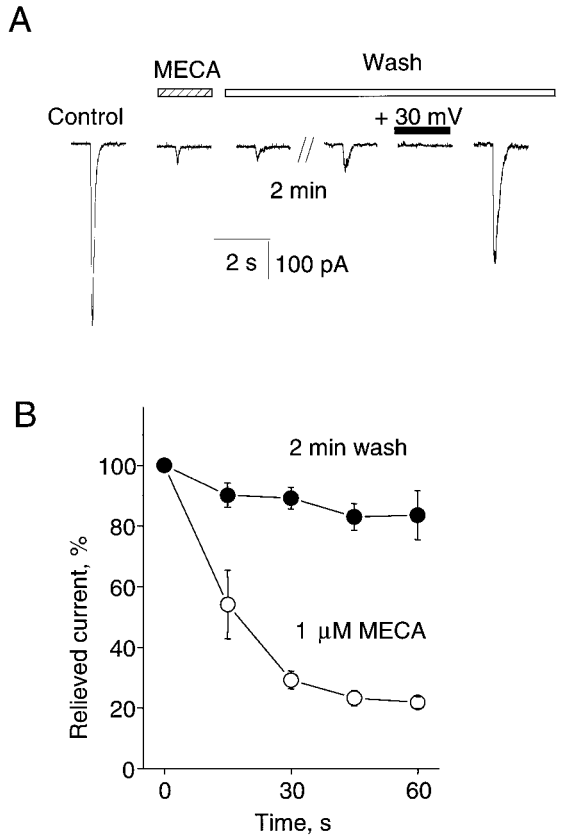

Fig. 6. Accelerated recovery from mecamylamine block by depolarization plus nicotine application. A, after attaining steady state block (20%) by mecamylamine of nicotine current (see horizontal hatched bar), responses to repeated nicotine pulses (0.066 Hz) are monitored in standard Krebs' solution (horizontal open bar). Recovery is slight after 2 min, but it is greatly enhanced after +30 mV depolarization (filled bar) plus nicotine pulse (70% of control response). B, time course of nicotine current amplitude (as % of amplitude of relieved current) either in the continuous presence of mecamylamine (○; $n = 6$) or during washout of mecamylamine (●; $n = 6$). Note that, during washout, the current amplitude remained sustained with only a slight decline (84 ± 8% of relieved current at 60 s), perhaps due to rebinding of mecamylamine to nAChRs.

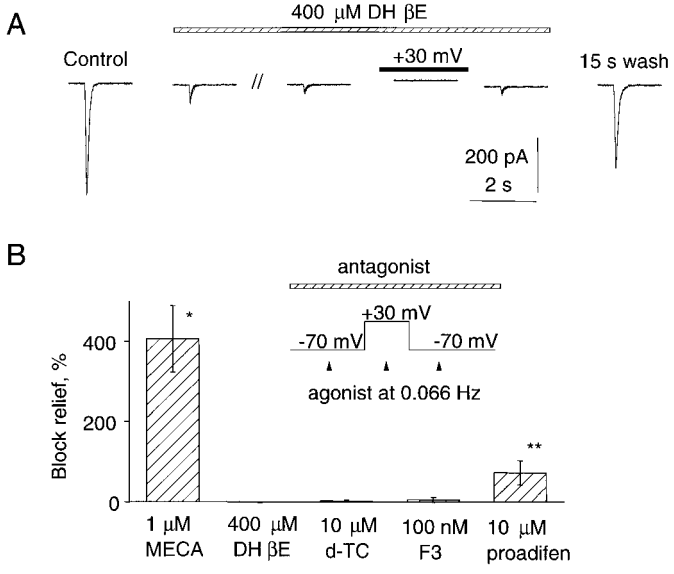

Fig. 7. Lack or minimal relief of nAChR block by various cholinergic antagonists. A, nicotine current (0.066 Hz pulses) is rapidly (<5 s) blocked by DHβE. Apparent steady-state antagonism is obtained in 30 s. Combined depolarization to +30 mV (filled bar) plus nicotine pulse does not evoke block relief. Prompt current recovery (15 s) is observed after washout of antagonist. B, bar chart showing block relief (as % of blocked current before test protocol; see scheme in inset) in the presence of mecamylamine (MECA; $n = 9$), DHβE ($n = 4$), *d*-tubocurarine (*d*-TC; $n = 3$), F3 ($n = 4$), or proadifen ($n = 5$). *, $P < .01$, **, $P < .05$.

This can be represented as

$$R + A \underset{k_1^-}{\overset{k_1^+}{\longleftrightarrow}} AR + A \underset{k_2^-}{\overset{k_2^+}{\longleftrightarrow}} A_2R \underset{\alpha}{\overset{\beta}{\longleftrightarrow}} A_2R^* + B \underset{k_b^-}{\overset{k_b^+}{\longleftrightarrow}} A_2RB$$

where B is mecamylamine, k_b^+ is the blocking rate constant, and k_b^- is the unblocking rate constant. Because nAChRs of rat chromaffin cells are very largely made up by $\alpha_3\beta_4$ subunits (Campos-Caro et al., 1997) and the efficacy of ACh and nicotine on $\alpha_3\beta_4$ is very similar (Luetje and Patrick, 1991; Sivilotti et al., 1997), the activation/deactivation kinetics of nAChRs were taken from Mathie et al. (1991), Maconochie and Knight (1992), and Bennett et al. (1997). The values of these reaction rate constants (k_1^+, k_1^-, k_2^+, k_2^-, β, α) and the voltage sensitivity of α are listed in Table 1 (column 1),

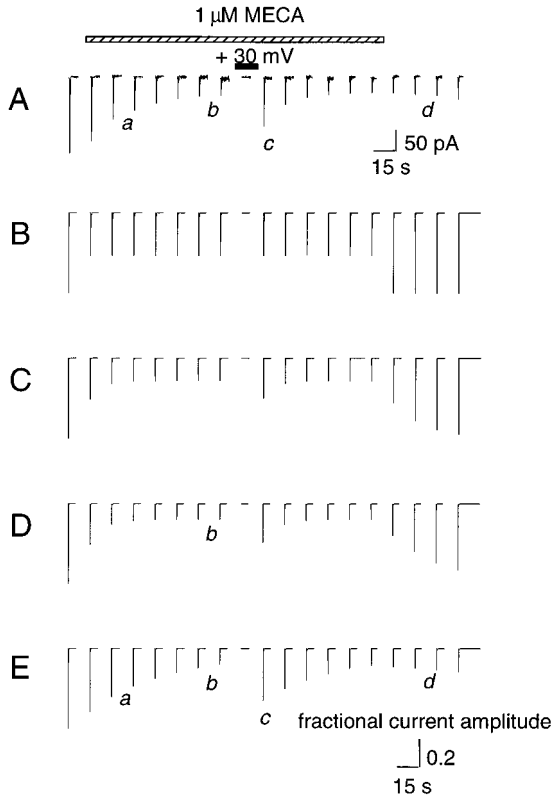

Fig. 8. Comparison of experimentally observed nicotine currents with computer simulated responses. A, experimental current traces recorded from a chromaffin cell after pulse application (30 ms) of nicotine in control solution or in the presence of mecamylamine (dashed horizontal bar). Membrane depolarization to +30 mV is indicated by the filled horizontal bar. Labels a–d indicate distinctive characteristics of the mecamylamine action; namely its slow, use-dependent onset (a), the steady-state block (b), the rapid relief by combining depolarization with nicotine application (c), and the slow recovery on wash (d). A model of receptor block is acceptable only if it reproduces all these features. B, simulated responses obtained using the model 1 (see text) and the conditions indicated in Table 1 indicate inadequacy of this model. C, simulated responses using model 2 without alterations in rate constants: poor correspondence indicates inadequate model conditions. D, simulated responses obtained using model 2 when the rate constant β' for isomerization of the agonist bound blocked receptor is halved: the simulation partly reproduces conditions experimentally observed (level of steady state block indicated by b). E, simulated responses obtained by reducing β' together with increasing the rate constant k_b^- for mecamylamine unbinding from receptors (see text). These responses can simulate experimental data, thus suggesting it to be a model adequate to fit the present findings. Amplitude of simulated responses was expressed as the probability to observe a certain channel state after a pulse of agonist (see eq. 3).

where H is the coefficient for e-fold constant change with membrane potential. Whereas the k_b^+ value was taken from Nelson and Lindstrom (1999), the k_b^- value (42 s^{-1}) and its H coefficient (80 mV) were calculated from $IC_{50}{*}k_b^+$ and $1/H(k_b^-) = 1/H(IC_{50}) + 1/H(k_b^+)$. Figure 8B shows that this model could not fit our experimental data. Note that responses elicited by 20-ms application of nicotine (0.1 mM pipette concentration) corresponded to approximately the half-maximal effect (Giniatullin et al., 1999). Even when we increased β from 460 to 27,000 s^{-1} (Mathie et al., 1991), the model remained unsuitable to describe the data. These observations suggest that simple open channel block was presumably not the mechanism responsible for the action of mecamylamine.

Model 2. The inability of model 1 to describe the present data introduced the need for testing a different kinetic scheme, like, for example, the one originally developed by Blanpied et al. (1997) to describe antagonist trapping inside NMDA channels, whereby

$$A + R \underset{k_1^-}{\overset{k_1^+}{\longleftrightarrow}} AR + A \underset{k_2^-}{\overset{k_2^+}{\longleftrightarrow}} A_2R \underset{\alpha}{\overset{\beta}{\longleftrightarrow}} A_2R^* + B$$

$$k_b^- \updownarrow k_b^+$$

$$RB \underset{k_1^{+'}}{\overset{k_1^{+'}}{\longleftrightarrow}} ARB + A \underset{k_2^{-'}}{\overset{k_2^{+'}}{\longleftrightarrow}} A_2RB \underset{\alpha'}{\overset{\beta'}{\longleftrightarrow}} A_2RB$$

In this format the model predicts that the blocker B becomes trapped inside the receptor channel, whereas the agonist comes off it. Note that the present model is distinct from the one proposed by Lingle (1983); here we assumed agonist unbinding from blocked receptor. In our model, trapping of mecamylamine is represented by the set of reactions in the lower arm of the scheme, whereas untrapping is given by the transition from the lower arm to the upper one. Because the kinetics of agonist association/dissociation for blocked nAChRs are not measurable with conventional electrophysiological methods, it was first assumed that they were analogous to those of unblocked receptors. For this initial simulation, we used the k_b^- (H = 80 mV) and the k_b^+ (H = −100 mV) values employed for model 1. In this way we could not replicate the experimental observations because the onset and washout of the mecamylamine block were too fast and the block relief relatively weak (see Fig. 8C). We next tried to change k_b^- values (which were not experimentally obtained) by either increasing them up to 200 s^{-1} or decreasing them to 5 s^{-1}. In the first case, the simulated responses lacked block relief, whereas, in the second case, they displayed rapid recovery (not shown).

This modeling test seemed to suggest that our initial assumption of identical kinetics for blocked or unblocked receptors was inadequate. Because Dilmore and Johnson (1998) have concluded that the operation of trapped NMDA receptors becomes significantly slower, we extended this concept to the mecamylamine-bound nAChRs by implying a retarded rate of nicotine binding to them and/or their sluggish gating. As shown in Fig. 8D, slowing β' from 460 to 220 s^{-1} was insufficient to improve the correspondence of simulated records to experimental ones (inadequate block relief, fast

onset and washout) although the extent of current depression was comparable with the real responses (see b in Fig. 8D). A more drastic reduction in the value of β' to 2 s^{-1} led to virtually full suppression of simulated currents (data not shown).

We next explored whether other steps in model 2, downstream of blocked/agonist-bound/closed receptors, might have been responsible for the phenomenon. In practice, we considered a possible limiting role for the unbinding/binding of nicotine to the closed/blocked receptors. To test this possibility, we reset the β' value to the same as β (460 s^{-1}), and we assigned values to $k_1^{+\prime}$ (H = 20 mV) and $k_2^{+\prime}$ (H = 20 mV) smaller than k_1^{+} and k_2^{+}. When this difference was 1.25-fold smaller (with a corresponding increase by 1.25-fold in $k_1^{-\prime}$ (H = −20 mV) and $k_2^{-\prime}$ (H = −20 mV) over k_1^{-} and k_2^{-}), we observed results (data not shown) analogous to those found previously (see Fig. 8D) with a mere reduction in β' to 220 s^{-1}. When the ratio for the constants of blocked over unblocked receptors was raised to 3.5, the current block became too strong with virtually no recovery (data not shown). These simulations suggested that strong reductions in the rate constants of the reactions downstream to the complex of agonist and mecamylamine bound, active receptor were insufficient per se to replicate the experimental data. Equally inadequate were the simulations based on wide changes (5–190 s^{-1} range; H = 20/100 mV) in the k_b^{-} values only (data not shown).

It seemed plausible that the mechanism of mecamylamine block was complex and that once nAChRs had been bound by mecamylamine, their kinetics were disrupted at more than just a single reaction step. To test this assumption, we run simulations by changing a series of parameters like k_b^{-}, and $k_1^{+\prime}$, $k_2^{+\prime}$, k_1^{-}, and k_2^{-}, as indicated above. Limited success was obtained when k_b^{-} was 190 s^{-1} and the ratio of the constants for blocked receptors over unblocked receptors was 3.5 (data not shown). However, even in this case, the block relief was too intense and required an additional assumption, namely that the constants for closed/blocked receptors were strongly voltage dependent (H = 20 mV). The latter did not seem a plausible phenomenon and lacked any experimental support. We then turned our attention to the reactions involving open/bound receptors and found the most satisfactory simulation (see Fig. 8E) to match experimental data when β' was 112 s^{-1}, k_b^{-} was 200 s^{-1}, and all the other constants unchanged with respect to the reaction scheme describing the interaction of nicotine with unblocked receptors. Note that for this model we used H = 22.5 mV for β' to achieve a realistic block relief and that this H value corresponds to the experimentally observed voltage dependence of block relief (assigning voltage dependence to k_b^{-} played now a very minor role in the observed simulations). In summary then, although slowing of agonist binding to blocked receptors might contribute to the depression of nicotine-induced responses, slowing of the isomerization reaction together with a larger value for the constant of mecamylamine untrapping was sufficient to reproduce all features of mecamylamine interaction with nAChRs. Of course, these results cannot imply that these conditions are the only realistic ones to generate the experimentally observed responses. Instead, they indicate that if these conditions were met, nAChRs in the presence of mecamylamine can show responses analogous to the experimental data.

Discussion

The present study provides a novel, quantitative description of the strong block of chromaffin cell nAChRs by mecamylamine. Such a block could be rapidly relieved by combining a large membrane depolarization with nicotine application, suggesting that mecamylamine was trapped inside nAChRs from which it could be subsequently released on channel reopening. Activation of nAChRs in the presence of mecamylamine was therefore possible as long as there was coincidence of agonist binding with strong membrane depolarization. Computer modeling enabled us to outline the rate limiting step(s) of this phenomenon.

Block by Mecamylamine of nAChRs. On autonomic nAChRs, the action of mecamylamine is either competitive (Asher et al., 1979; Gurney and Rang, 1984) or noncompetitive (Fieber and Adams, 1991; Nooney et al., 1992), a difference caused, perhaps, by different subunit compositions of these receptors. Even within the same tissue, namely the superior cervical ganglion, mecamylamine produces distinct types of receptor block depending on the cells examined (Shen and Horn, 1998).

The present study carried out on rat chromaffin cells demonstrated the blocking action of mecamylamine to be slow in onset (compare it with DHβE or with F3; Giniatullin et al.,

TABLE 1
Kinetic values used for computer modelling of mecamylamine block of nicotine currents

	Model 1 (Fig. 8B)		Model 2 (Fig. 8C)		Model 2 (Fig. 8D)		Model 2 (Fig. 8E)	
	Values at 0 mV	H	Values at 0 mV	H	Values at 0 mV	H	Values at 0 mV	H
		mV		mV		mV		mV
k_1^{+}	40.8 μM^{-1} s^{-1}		40.8 μM^{-1} s^{-1}		40.8 μM^{-1} s^{-1}		40.8 μM^{-1} s^{-1}	
k_1^{-}	4170 s^{-1}		4170 s^{-1}		4170 s^{-1}		4170 s^{-1}	
k_2^{+}	20.4 μM^{-1} s^{-1}		20.4 μM^{-1} s^{-1}		20.4 μM^{-1} s^{-1}		20.4 μM^{-1} s^{-1}	
k_2^{-}	8340 s^{-1}		8340 s^{-1}		8340 s^{-1}		8340 s^{-1}	
β	460 s^{-1}		460 s^{-1}		460 s^{-1}		460 s^{-1}	
α	45 s^{-1}	156	45 s^{-1}	156	45 s^{-1}	156	45 s^{-1}	156
k_b^{+}	23 μM^{-1} s^{-1}	−100	23 μM^{-1} s^{-1}	−100	23 μM^{-1} s^{-1}	−100	23 μM^{-1} s^{-1}	−100
k_b^{-}	42 s^{-1}	80	42 s^{-1}	80	42 s^{-1}	80	200 s^{-1}	
α'			45 s^{-1}	156	45 s^{-1}	156	45 s^{-1}	156
β'			460 s^{-1}		220 s^{-1}		112 s^{-1}	22.5
$k_2^{-\prime}$			8340 s^{-1}		8340 s^{-1}		8340 s^{-1}	
$k_2^{+\prime}$			20.4 μM^{-1} s^{-1}		20.4 μM^{-1} s^{-1}		20.4 μM^{-1} s^{-1}	
$k_1^{-\prime}$			4170 s^{-1}		4170 s^{-1}		4170 s^{-1}	
$k_1^{+\prime}$			40.8 μM^{-1} s^{-1}		40.8 μM^{-1} s^{-1}		40.8 μM^{-1} s^{-1}	

1999), potent (IC_{50} = 0.34 μM), and persistent on washout. The reduction in current decay during mecamylamine block is similar to that found on recombinant $\alpha_3\beta_4$ receptors (Zhang et al., 1999). The use and voltage dependence of mecamylamine block confirms that the effect of mecamylamine can be classified as "uncompetitive" antagonism. This type of block, which has recently been investigated in relation to glutamate receptors (Blanpied et al., 1997; Chen and Lipton, 1997), implies that the blocker only acts if the receptor has been activated. This form of antagonism is therefore dissimilar from conventional noncompetitive antagonism when the blocker can interacts with resting as well as activated receptors (for a recent review, see Dingledine et al., 1999).

Mechanism of Mecamylamine Action. Because mecamylamine has a pK_a value of 11.2, more than 99% of this compound will be ionized at pH 7.4 (Goldstein et al., 1979), making possible its interaction with the strong negative charges inside the nicotinic channel (Pascual and Karlin, 1998). This property suggests that, at negative membrane potentials, mecamylamine can deeply penetrate into open nAChRs as indicated by our calculations based on the Woodhull (1973) method.

The ability of membrane depolarization to reverse the mecamylamine block was a striking property of the action of this antagonist. Lingle (1983) had briefly reported a similar phenomenon on crustacean muscle nicotinic receptors. Intracellular processes played little, if any, role in rapid recovery from mecamylamine. Thus, potential involvement of Ca^{2+}-dependent second messengers because of depolarization-evoked Ca^{2+} influx was made unlikely by the fact that cells had been dialyzed with a solution containing the fast Ca^{2+} chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid). In addition, simple depolarization (which should have opened voltage dependent Ca^{2+} channels) without activation of nAChRs was ineffective on mecamylamine block. Furthermore, we found no evidence to support a role for intracellular Mg^{2+} (Ifune and Steinbach, 1991) in displacing mecamylamine from blocked receptors. The special voltage dependence of the mecamylamine block and unblock should therefore be explained differently.

In this sense, we might imagine at least two distinct mechanisms underlying uncompetitive antagonism: 1) mecamylamine bound to (and came off from) a site deep inside the channel with strong voltage dependence that was also manifested as depolarization-dependent block relief (similar to positively charged channel blockers; Adams and Feltz, 1980; Chen and Lipton, 1997); 2) mecamylamine entered open nAChR channels, in which it remained trapped after receptor closure; in this case, voltage-dependence resided in the processes that control opening and closure of mecamylamine-bound channels, and agonist sensitivity. The present experimental data based on whole-cell currents could not resolve which steps were voltage dependent. However, even single channel recordings may not help to separate a voltage-dependent, open-channel block from a voltage-dependent trapping mechanism, particularly if the latter is manifested as nonspecific decrease in macroscopic charge transfer or reduction in opening frequency (Neher, 1983; Nelson and Lindstrom, 1999). Indeed, using mecamylamine at relatively high (5–50 μM) concentrations on recombinant nAChRs of excised patches, Nelson and Lindstrom (1999) observed that the persistent, strong reduction in channel open frequency (which might be related to trapping mechanisms) precluded estimation of the channel closed state. Further understanding of the mode of mecamylamine interaction with nAChRs was therefore sought with computer modeling in which we changed the rate constants of one or more reaction steps of distinct kinetic schemes.

Computer Modeling of Mecamylamine Action. By testing the effects of discrete changes in the rate constants of the various reactions underlying mecamylamine/nAChR interaction, it was possible to simulate how cell responses should have been generated if a certain step was rate limiting for the observed phenomenon. This approach had inherent limitations because of the selection of values for reaction constants not directly measured here. Nevertheless, strong reliance on ensuring that simulated responses could match a set of crucial criteria inherent to the action of mecamylamine should have prevented unrealistic conclusions. Modeling results should predict certain receptor properties to be tested in future experiments.

Simple open channel block (even assuming voltage-dependent interaction by mecamylamine with nAChRs) could not match the criteria necessary to simulate closely the experimental data. Further progress was therefore attempted by using a more complex receptor scheme (Blanpied et al., 1997; Dilmore and Johnson, 1998) employed previously to model the action of antagonists trapped inside NMDA receptors. If the kinetics of agonist interaction with mecamylamine-bound receptors were left as in control conditions, modeling failed to meet the prearranged set of criteria. This result indicated that the mere presence of mecamylamine inside the channel could not account for the observed phenomena. A suitable model for the present data was obtained when the rate constant for isomerization of nAChRs bound by nicotine was assumed to become slow and voltage-dependent, whereas mecamylamine unbinding from the channel did not need voltage sensitivity. These observations suggest that, once mecamylamine had bound nAChRs, the latter rapidly converted into an inactive state despite the presence of nicotine. The predictions raised by the present model, however, will need validation through experimental tests.

How General Is the Phenomenon of Block Relief? A trapping block mechanism has first been proposed for tetraethylammonium acting on potassium channels (Armstrong, 1971). The suggestion of an analogous process for cholinergic receptors originated from a report on lobster muscle receptors activated by slowly applied cholinergic agonists (Lingle, 1983). On submandibular ganglia, Gurney and Rang (1984) observed substantial relief of the hexamethonium block but not of the mecamylamine one. On bovine chromaffin cell nAChRs, Nooney et al. (1992) briefly reported mecamylamine block relief by depolarization plus ACh application without further quantitative data. In our study, the characteristics of block analogous to that by mecamylamine were not found with the competitive antagonists DHβE (Xiao et al., 1998) or F3 (Giniatullin et al., 1999). Note that despite the demonstrated channel blocking action of tubocurarine on submandibular ganglia (Ascher et al., 1979), this compound did not share the peculiar voltage dependent block relief observed with mecamylamine in the present study. Weak block relief was observed in the case of proadifen, an agent that facilitates receptor desensitization (SKF-525A; Giniatullin et al.,

1989). In general, one may conclude that the rapid reversal of the action of mecamylamine on chromaffin cells remains an outstanding, although not unique, case. This agent could thus be a tool to identify the molecular structure inside the nAChR channel so sensitive to trapping block.

Functional Implications. One interesting aspect was the requirement for the coincidence of two factors to achieve rapid block relief. One was a selective signal, namely activation of nAChRs, whereas the other was nonspecific cell depolarization. This situation is reminiscent of the Mg^{2+} block (and relief) of NMDA receptors by combining membrane depolarization with glutamate application (reviewed by Dingledine et al., 1999). In the case of chromaffin cells (and perhaps even of brain nicotinic receptors sensitive to mecamylamine that can cross the blood-brain barrier), this antagonist might generate a system detecting the coincidence of pre- and postsynaptic activity (i.e., a Hebbian synapse). In other words, application of a drug like mecamylamine might simply unveil a phenomenon generated in standard conditions by endogenous substances acting together with acetylcholine. Physiological compounds that might possess a mecamylamine like-action are 5-hydroxytryptamine (Grassi, 1999), or substance P (Clapham and Neher, 1984; Boyd and Leeman, 1987), which depress nAChR function by voltage-dependent but incompletely understood mechanisms. Depolarization of the postsynaptic membrane necessary for block relief might be achieved via a distinct synaptic input, which, together with endogenous acetylcholine, might quickly restore cholinergic transmission. ATP (Ralevic and Burnstock, 1998) is one potential candidate for such interplay with blocked nAChRs.

Acknowledgments

We thank Dr. S. Antonov, École Normale Superieure, Paris, for helpful discussions and Dr. Massimo Righi for assistance in the preparation of chromaffin cell cultures.

References

Adams PR and Feltz A (1980) End-plate channel opening and the kinetics of quinacrine (mepacrine) block. *J Physiol (Lond)* **306:**283–306.
Armstrong CM (1971) Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J Gen Physiol* **58:**413–437.
Ascher P, Large WA and Rang HP (1979) Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells. *J Physiol (Lond)* **295:**139–170.
Baker TS, Dormand JR and Prince PJ (1996) Continuous approximation with embedded Runge-Kutta methods. *Appl Numl Math* **22:**51–62.
Bennett MR, Farnell L, Gibson WG and Lavidis NA (1997) Synaptic transmission at visualized sympathetic boutons: Stochastic interaction between acetylcholine and its receptors. *Biophys J* **72:**1595–1606.
Blanpied TA, Boeckman FA, Aizenman E and Johnson JW (1997) Trapping channel block of NMDA-activated responses by amantadine and memantine. *J Neurophysiol* **77:**309–323.
Boyd ND and Leeman SE (1987) Multiple actions of substance P that regulate the functional properties of acetylcholine receptors of clonal rat PC12 cells. *J Physiol (Lond)* **389:**69–97.
Buisson B and Bertrand D (1998) Open-channel blockers at the human $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* **53:**555–563.
Campos-Caro A, Smillie FI, Dominguez del Toro E, Rovira JC, Vicente-Agullo F, Chapuli J, Juiz JM, Sala F, Ballesta JJ and Criado M (1997) Neuronal nicotinic acetylcholine receptors on bovine chromaffin cells: Cloning, expression and genomic organization of receptor subunits. *J Neurochem* **68:**488–497.
Chen HS and Lipton SA (1997) Mechanism of memantine block of NMDA-activated channels in rat retinal ganglion cells: Uncompetitive antagonism. *J Physiol (Lond)* **499:**27–46.
Chretien JM and Chauvet GA (1998) An algorithmic method for determining the kinetic system of receptor-channel complex. *Math Biosci* **147:**227–257.
Clapham DE and Neher E (1984) Substance P reduces acetylcholine-induced currents in isolated bovine chromaffin cells. *J Physiol (Lond)* **347:**255–277.
Colquhoun D (1998) Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.
Colquhoun D and Hawkes AG (1977) Relaxation and fluctuations of membrane currents that flow through drug-operated channels. *Proc R Soc Lond B Biol Sci* **199:**231–262.
Connolly J, Boulter J and Heinemann SF (1992) Alpha 4–2 beta 2 and other nicotinic acetylcholine receptor subtypes as target of psychoactive and addictive drugs. *Br J Pharmacol* **105:**657–666.
Dilmore JG and Johnson JW (1998) Open channel block alteration of *N*-methyl-D-aspartic acid receptor gating by an analog of phencyclidine. *Biophys J* **75:**1801–1816.
Dingledine R, Borges K, Bowie D and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* **51:**7–61.
Fieber LA and Adams DJ (1991) Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J Physiol (Lond)* **431:**215–237.
Giniatullin R, Di Angelantonio S, Marchetti C, Sokolova E, Khiroug L and Nistri A (1999) Calcitonin gene-related peptide rapidly downregulates nicotinic receptor function and slowly raises intracellular Ca^{2+} in rat chromaffin cells in vitro. *J Neurosci* **19:**2945–2953.
Giniatullin RA, Khamitov KhS, Khazipov R, Magazanik LG, Nikolsky EE, Snetkov VA and Vyskocil F (1989) Development of desensitization during repetitive end-plate activity and single end-plate currents in frog muscle. *J Physiol (Lond)* **412:**113–122.
Goldstein A, Aronov L and Kalman SM (1979) *Principles of Drug Action: The Basis of Pharmacology*, p 194, John Wiley & Sons, Inc, New York.
Grassi F (1999) 5-Hydroxytriptamine blocks the fetal more potently than the adult mouse muscle acetylcholine receptor. *Pfluegers Arch* **437:**903–909.
Gurney AM and Rang HP (1984) The channel-blocking action of methonium compounds on rat submandibular ganglion cells. *Br J Pharmacol* **82:**623–642.
Gyermek L (1980) Methods for the examination of ganglion-blocking activity, in *Pharmacology of Ganglionic Transmission* (Kharkevich DD ed) pp 63–122, Springer Verlag, Berlin.
Ifune CK and Steinbach JH (1991) Voltage dependent block by magnesium of neuronal nicotinic acetylcholine receptor channels in rat phaechromocytoma cells. *J Physiol (Lond)* **443:**683–701.
Lingle C (1983) Different types of blockade of crustacean acetylcholine-induced currents. *J Physiol (Lond)* **339:**419–437.
Luetje CW and Patrick J (1991) Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J Neurosci* **11:**837–845.
Maconochie DJ and Knight DE (1992) A study of bovine adrenal chromaffin nicotinic receptor using patch clamp and concentration-jump techniques. *J Physiol (Lond)* **454:**129–153.
Mathie A, Colquhoun D and Cull-Candy SG (1990) Rectification of currents activated by nicotinic acetylcholine receptors in rat sympathetic ganglion neurones. *J Physiol (Lond)* **427:**625–655.
Mathie A, Cull-Candy SG and Colquhoun D (1991) Conductance and kinetic properties of single nicotinic acetylcholine receptor channels in rat sympathetic neurones. *J Physiol (Lond)* **439:**717–750.
Neher E (1983) The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J Physiol (Lond)* **339:**663–678.
Nelson ME and Lindstrom J (1999) Single channel properties of human $\alpha3$ AChRs: Impact of $\beta2$, $\beta4$ and $\alpha5$ subunits. *J Physiol (Lond)* **516:**657–678.
Nooney JM, Peters JA and Lambert JJ (1992) A patch clamp study of the nicotinic acetylcholine receptor of bovine adrenomedullary chromaffin cells in culture. *J Physiol (Lond)* **455:**503–527.
Pascual JM and Karlin A (1998) State-dependent accessibility and electrostatic potential in the channel of the acetylcholine receptor. *J Gen Physiol* **111:**717–739.
Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50:**413–492.
Shen W-X and Horn JP (1998) Mecamylamine selectively blocks nicotinic receptors on vasomotor sympathetic C neurons. *Brain Res* **788:**118–124.
Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R and Colquhoun D (1997) Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour. *J Physiol (Lond)* **500:**123–138.
Woodhull AM (1973) Ionic blockage of sodium channels in nerve. *J Gen Physiol* **61:**678–708.
Xiao Y, Meyer EL, Thompson JM, Surin A, Wroblewski J and Kellar KJ (1998) Rat $\alpha3/\beta4$ subtype of neuronal nicotinic acetylcholine receptor stably expressed in a transfected cell line: Pharmacology of ligand binding and function. *Mol Pharmacol* **54:**322–333.
Zhang J, Xiao Y, Abdrakhmanova G, Wang W, Cleemann L, Kellar KJ and Morad M (1999) Activation and Ca^{2+} permeation of stably transfected $\alpha_3\beta_4$ neuronal nicotinic acetylcholine receptor. *Mol Pharmacol* **55:**970–981.

Send reprint requests to: A. Nistri, SISSA, Via Beirut 4, 34014 Trieste, Italy. E-mail: nistri@sissa.it