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Dimensions of the Ion Channel in Neuronal Nicotinic Acetylcholine Receptor as Estimated from Analysis of Conformation-Activity Relationships of Open-Channel Blocking Drugs

Boris S. Zhorov†, Nina B. Brovtsyna‡, Valeriy E. Gmiro§, Nera Ya. Lukomskaya‡, Sergei E. Serdyuk‖, Natalya N. Potapyeva‡, Lev G. Magazanik‡, Dmitriy E. Kurenniy#, and Vladimir I. Skok#

†Pavlov Institute of Physiology, Leningrad B-034, USSR; ‡Sechenov Institute of Evolutionary Physiology and Biochemistry, Leningrad K-223, USSR; §Institute of Experimental Medicine, Leningrad A-22, USSR; Gorki Medical Institute, Donetsk 98, USSR; and #Bogomoletz Institute of Physiology, Kiev 24, USSR

Summary. Relationship between the size of the molecule in the series of organic ions $Et_3\overset{+}{N}—(CH_2)_5—\overset{+}{N}R^1R^2R^3$ (R^i – alkyl or cycloalkyl substituents) and their abilities to block nicotinic acetylcholine receptors (AChRs) due to their open-channel blockade in the neurons of autonomic ganglia and in frog end-plate was analyzed.

All low-energy equilibrium conformations of the drugs were calculated by the molecular mechanics method. A unique rectangular channel profile 6.1 × 8.3 Å, for which the best correlation between blocking activity of the drugs and total population of their conformations being able to penetrate into the channel, was deduced from all those tested.

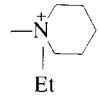
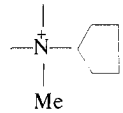
Key Words acetylcholine receptor · ion channel · mechanism of blockade · conformation · structure-activity relationship

Introduction

Although a number of attempts have been made to identify the site in nicotinic acetylcholine receptor (AChR) that binds the open-channel blocking drugs, its localization still remains unclear (Adams, 1976, 1977; Changeux, Giraudt & Dennis, 1987; Gui & Hucho, 1987). Most possibly those intrapore groups which have been suggested to interact with sodium and other penetrating inorganic cations can likewise serve as binding sites for some organic-blocking drugs. Therefore, the activity of these drugs should depend, in particular, on whether their molecules can penetrate deeply enough into the channel to reach their binding site. Dwyer, Adams and Hille (1980) found a correlation between the dimensions of organic ions and their ability to penetrate through the ion channel of the end-plate AChR. The majority of organic drugs are flexible molecules, i.e., they exist as a mixture of different conformers. The specific population of each particular conformer depends on its conformational energy which can be calculated using molecular mechanics. A possibility thus appears, through the analysis of conformation-activity relationship in a series of compounds, to estimate the size of the channel at the level where the binding site is localized. It has been assumed earlier that the binding site for the *bis*-cationic open-channel blocking drugs in AChR of sympathetic ganglion neuron includes two anionic sites separated by a distance corresponding to the interchange distance in penta- and hexamethonium, the most potent open-channel blockers among symmetrical *bis*-ammonium ganglion-blocking agents (*see* Skok, Selyanko & Derkach, 1989). Therefore, compounds used in the present work were pentamethonium derivatives with one variable trialkylammonium cationic group and an unchanged triethylammonium cationic group (Gmiro, Lukomskaya & Serdyuk, 1987). For simplicity, the variable cationic group will further be called the first group, while the constant one will be called the second group. Conformations of the first cationic groups in all substances tested were calculated using molecular mechanics. Earlier this method was applied for the purposes of three-dimensional modeling of recognition sites in adrenergic receptors (Zhorov & Govyrin, 1981; Govyrin & Zhorov, 1984).

According to recent studies, selective blockade of nicotinic synaptic transmission through sympathetic ganglia produced by *bis*-ammonium compounds, pentamethonium derivatives, is entirely due to open-channel blockade of AChRs in ganglion neurons. This conclusion was drawn from strict correlation between the open-channel blocking potencies

Table 1. AChR-blocking activities of the compounds $Et_3—\overset{+}{N}—(CH_2)_5—R$ relative to that of compound (I)

Compound	—R	Blockade of sympathetic ganglia	Blockade of enteric ganglia	Blockade of ACh-induced current in sympathetic neuron
I	$—\overset{+}{N}Me_3$	1.00	1.00	1.00
II	$—\overset{+}{N}Et_2Me$	0.55	1.02	1.06
III	$—\overset{+}{N}Et_3$	0.02	0.01	0.03
IV	$—\overset{+}{N}Me_2n\text{-}Pr$	0.04	0.02	—
V	$—\overset{+}{N}Me_2i\text{-}Pr$	0.13	0.66	
VI		0.27	1.40	0.76
VII		0.01	0.01	—
VIII		0.03	0.03	0.02
IX		0.01	0.02	
X		0.03	0.07	
XI		0.01	0.01	

Compound (I) activity (ED_{50}) is equal to 0.20 μmol/liter for cat sympathetic ganglion, 74.7 μmol/liter for guinea-pig enteric ganglia, and 1.70 μmol/liter for rat sympathetic neuron (single applications of acetylcholine).

of the drugs and their abilities to block ganglionic transmission (Skok, Selyanko & Derkach, 1983; Skok, 1987). This finding allowed us to use, besides the direct open-channel blockade, the drug-induced blockade of the responses recorded from target organs as an indirect estimate of the channel-blocking activities of the drugs.

It is well known that neuronal and end-plate AChR exhibit quite different functional and pharmacological properties. In particular, there are differences between the abilities of many mono- and *bis*-ammonium compounds to block the open channel of end-plate and neuronal AChR (*see* Skok et al., 1989). At the first site, it would be possible to correlate these differences with the differences in the amino acid sequence of neuronal and end-plate AChR which now are evident. Also several genes that encode a number of types of neuronal and muscle nicotinic receptor subunits have now been identified (*see* Steinbach & Ifune, 1989). But, unfortunately, data on chemical structure of receptors are insufficient yet for predicting the fine details of the channel geometry which evidently is of great importance for elucidation of blockade mechanisms. That is why it is the analysis of conformation-activity relationships of the ligands which could help to estimate this geometry.

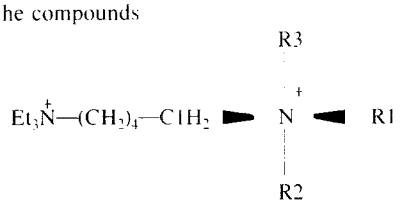
In addition to using the autonomic ganglia, our experiments were also performed on the end-plate to compare the channel-blocking activities of the drugs tested in the ganglia and in the muscle. Preliminary reports on the results were published elsewhere (Zhorov et al., 1989*a*,*b*).

Materials and Methods

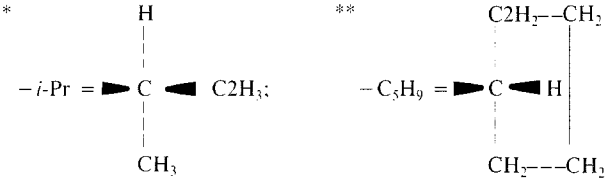
Calculations and Experimental Procedures

According to molecular mechanics calculations, at 25°C 70% of pentamethonium molecules $[(CH_3)_3—\overset{+}{N}—(CH_2)_5—\overset{+}{N}(CH_3)_3]$ are in the fully extended, all-*trans* conformation where electrostatic repulsion between cationic heads is minimal (Rozengart & Zhorov, 1983). For the same reason, in the compounds we worked with the preferred conformation is the extended all-*trans* conformation of pentamethylene chain. Therefore, the electrostatic and Van-der-Waals interactions between cationic heads should only slightly depend on their structure. Based on these assumptions, for the purposes of molecular mechanics calculations, we replaced compounds listed in Table 1 by their diminished models

Table 2. Designation of torsional angles of the compounds



Compound	R1	R2	R3	τ_1	τ_2	τ_3
II	Et	Et	Me	C—C1—N—R1	C1—N—(R2)—C	C1—N—C(R1)—C
III	Et	Et	Et	C1—N—C(R2)—C	C1—N—C(R4)—C	C1—N—C(R1)—C
IV	*n*-Pr	Me	Me	C—C1—N—R1	C1—N—C(R1)—C	N—C(R1)—C—C
V*	*i*-Pr	Me	Me	C—C1—N—R1	C1—N—C(R1)—C2	
VI	Et	$(CH_2)_4$		C—C1—N—R1	C1—N—C(R1)—C	
VII	*n*-Pr	$(CH_2)_4$		C1—N—C(R1)—C	N—C(R1)—C—C	C—C1—N—R1
VIII*	*i*-Pr	$(CH_2)_4$		C—C1—N—R1	C1—N—C(R1)—C2	
IX		$(CH_2)_3CH(CH_2)_3$		C—C1—N—R2		
X	Et	$(CH_2)_5$		C—C1—N—R1	C1—N—C(R1)—C	
XI**	C_5H_9	Me	Me	C—C1—N—R1	C1—N—C(R1)—C2	



where three ethyl groups of the second cationic head were substituted by hydrogen atoms.

Equilibrium conformations of each compound were calculated using the energy minimization method. The starting points were the combinations of the staggered conformations of single bonds in trialkylammonium heads including the bond between pentamethylene chain and the nitrogen atom in nonsymmetrical cationic heads. For pentamethylene chain the all-*trans* conformation was used as initial approximation. Using the molecular mechanics method, we presented conformational energy as a sum of nonbonded interactions, torsional energy and bond-angle deformation energy for nitrogen, carbon and oxygen atoms, with the exception of atoms in methyl groups and butylamine chain. Bond lengths and parameters of the potential functions were chosen as in the work by Zhorov et al. (1975). Nonbonded interactions were calculated using 6-exp potential functions with the parameters developed by Dashevskiy (1974). The merits of this set of parameters are their consistency with a simple set of force constants for bond-angle deformations. Our early calculations revealed a good agreement between optimized conformational maps of acetylcholine calculated with potentials of Dashevskiy and with force field developed by Gelin and Karplus (1975). A universal program for molecular mechanics calculations (Zhorov, 1975) was used. This program minimizes conformational energy in the space of an arbitrarily chosen set of torsional and bond angles with fixed bond lengths. Loop closing potential (Zhorov, 1982) was used for calculation of cyclic molecules. To calculate the dimensions of equilibrium conformations, the Van-der-Waals radius of hydrogen atom was taken as equal to 1.2 Å. The conformational energy minimization was performed with the use of the Davidon method (Fletcher & Powell, 1963) in the space of torsional and bond angles. Minimization procedure was stopped when the norm of gradient became less than 0.1 kcal mol^{-1} rad^{-1}. Derivatives of energy were calculated by the analytical vector method (Zhorov, 1981, 1982).

Torsional angle designations are given in Table 2. In the names of conformers, symbols from left to right correspond to the torsional angles τ_1, τ_2, τ_3 (Table 2). Symbols *g*, *t*, and g^- denote *gauche* ($\tau = 60 \pm 60°$), *trans* ($\tau = 180 \pm 60°$), and (–)-*gauche* ($\tau = -60 \pm 60°$) conformations, respectively. Prefixes e^- and a^- denote equatorial or axial orientation of the N-C1 bond relative to the plane of the piperidine or pyrrolidine ring.

Estimations of Blocking Activities of the Compounds Used

The blocking effects of the compounds upon ACh-induced membrane currents were studied in the neurons of the rat superior cervical ganglion. The isolated ganglion was placed in physiological solution, desheathed and then incubated with collagenase (0.4% Sigma type 1) to clean the cell surface. The ganglion was then continuously perfused with the saline solution throughout the experiment. Membrane currents induced in the ganglion neurons by ACh iontophoretically applied through the extracellular micropipette were recorded at room temperature with the use of the patch-clamp method in the whole-cell recording mode. The recording pipette contained a solution of the following composition (in mM): NaCl 140; $CaCl_2$ 1; EGTA 11; HEPES 10; pH 7.2. A potassium-free solution was used to prevent the appearance of membrane current evoked by activation of muscarinic acetylcholine receptors. The recording technique used was published in more detail elsewhere (Derkach et al., 1987; Selyanko et al., 1988). The blocking compounds were applied with the perfusing

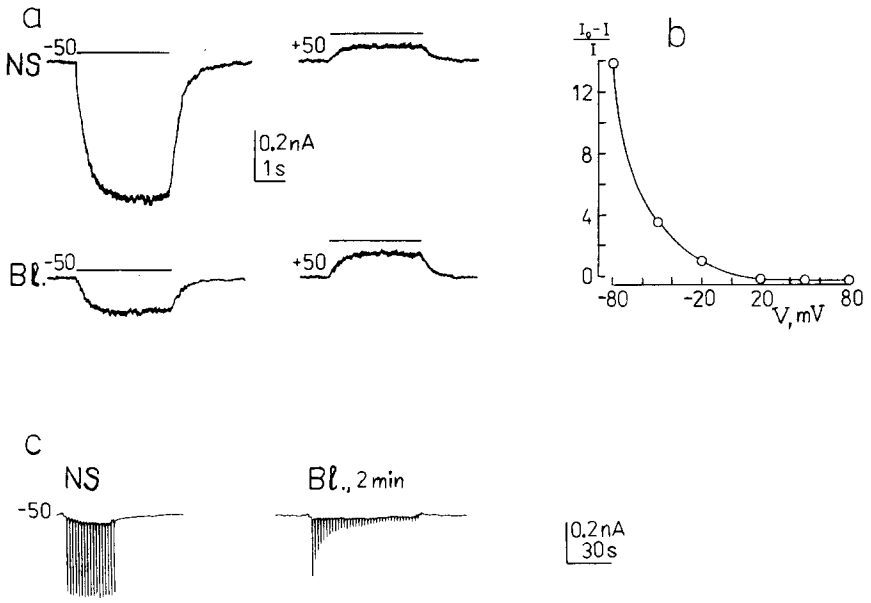


Fig. 1. Blocking effects of a compound (VI) (*see* Table 1) on the ACh-induced membrane current in the neurons of rat superior cervical ganglion. (*a*) The ACh-induced currents recorded in normal solution (upper records) and 5 min after the onset of blocker application (lower records) at membrane potential levels indicated near each record. (*b*) Voltage dependence of blocking effect. The blocking effect, expressed as $(I_0 - I)/I$, where I_0 and I are current amplitudes in normal solution and in the presence of a blocker, correspondingly, is plotted against membrane potential level. (*c*) Frequency dependence of a blocking effect. The currents were evoked by repetitive ACh applications in normal solution (left records) and in the presence of a blocker (right records). Concentration of a blocker was 5 μmol/liter (*a* and *b*) and 20 μmol/liter (*c*). Frequency of ACh application was < 0.01 Hz (*a* and *b*), and 0.5 Hz (*c*). Records *a* and *b* and records *c* were obtained from two different neurons, correspondingly

solution. To estimate blocking activities of the compounds used, the equilibrium dissociation constant K_D was found by interpolation as being equal to a concentration of a compound producing 50% decrease in the ACh-induced current amplitude. The blocking activity was calculated as the inverse of K_D and relative to that of the compound II (*see* Table 1, the last column).

The blocking effects of the compounds on ganglionic transmission were studied in cat superior cervical ganglion *in situ*. Contractions of nictitating membrane evoked by repetitive (5 stim/sec) stimulation of cervical sympathetic nerve were recorded from the animal anaesthetized by the mixture of chloralose and urethan (50 + 500 mg/kg i.v.). The ED_{50} of the blocking compounds injected into lingual artery was estimated, and their blocking activities were calculated as inverses of the ED_{50} values. The method, in more detail, was published elsewhere (Lukomskaya & Gmiro, 1982).

The blockade of suberyldicholine-induced responses of gut muscle was studied in pieces of the guinea-pig small intestine that were isolated and perfused with physiological saline solution at 37°C. Suberyldicholine was used because it selectively activated nicotinic acetylcholine receptors without affecting the muscarinic ones. Both suberyldicholine and the blocking compound tested were applied in perfusion saline, and the gut contractions were recorded. The K_D values were obtained from the shift of the dose-response curve.

To study blocking effects of the compounds upon the end-plate AChRs, the end-plate currents (EPCs) were evoked in frog sartorius muscle by motor nerve stimulation (0.2 stim/sec), and were recorded with the use of the conventional two-electrode voltage-clamp method at room temperature. Concentrations of Ca^{2+} and Mg^{2+} ions in the medium were decreased to 0.9 mM and increased to 6.0 mM, correspondingly, to prevent muscle contraction. The effects of blocking compounds on the EPC amplitude and on the EPC decay time course were studied at membrane potential levels from −60 to −140 mV. Most compounds tested induced biexponential EPC decay, with two time constants proportional to inverses of the rate constants for association and dissociation of a blocking drug with the open channel. The equilibrium dissociation constant value (K_D) for blocking effect of each compound was calculated as a ratio of two corresponding rate constants.

Results and Discussion

Blocking Effects of the Compounds Tested

Blocking effects of compounds (I), (II), (III), (VI) and (VIII) on ACh-induced current were studied in rat superior cervical ganglion neurons (Table 1). All compounds decreased the current in a voltage-dependent manner, their blocking effects being increased by membrane hyperpolarization and decreased by membrane depolarization; the blocking effects disappeared at membrane potentials more positive than 20–50 mV. An example of the voltage-dependent blocking effect produced by compound (VI) is illustrated by Fig. 1*a* and *b*. In a series of ACh applications in the presence of a blocker each

Table 3. The effect of the blocking drugs $Et_3\overset{+}{N}(CH_2)_5R$ on the muscle nicotinic AChR

Compound	R	Membrane potential, (mV)	$k_f \cdot 10^6$ ($M^{-1}S^{-1}$)	k_b (S^{-1})	K_D (mM)
I	$-\overset{+}{N}Me_3$	−80	1.03 ± 0.22 (n = 8)	<50	<0.05
		−120	2.72 ± 0.6 (n = 8)	<50	0.02
II	$-\overset{+}{N}Et_2Me$	−80	0.43 ± 0.1 (n = 3)	784 ± 39 (n = 3)	1.8
		−120	0.77 ± 0.18 (n = 3)	772 ± 91 (n = 3)	1.0
III	$-\overset{+}{N}Et_3$	−80	0.10 ± 0.03 (n = 6)	987 ± 138 (n = 6)	9.9
		−120	1.52 ± 0.3 (n = 5)	1064 ± 204 (n = 5)	0.7
VI	 Et	−80	0.23 ± 0.05 (n = 4)	467 ± 71 (n = 4)	4.4
		−120	0.12 ± 0.03 (n = 4)	494 ± 13 (n = 4)	4.1
VIII	 *i*-Pr	−80	1.10 ± 0.01 (n = 9)	1287 ± 135 (n = 9)	1.7
		−120	0.36 ± 0.1 (n = 9)	967 ± 138 (n = 8)	2.7
IX		−80	0.60 ± 0.1 (n = 4)	1044 ± 23 (n = 4)	1.7
		−120	1.65 ± 0.67 (n = 4)	713 ± 24 (n = 4)	0.4

k_f: association rate constant for the drugs with the open channel; k_b: dissociation rate constant K_D: dissociation constant (determined at two membrane potential levels −80 and −120 mV).

subsequent response was smaller than the previous one (frequency-dependent blocking effect, Fig. 1*c*). Both these results are consistent with the open-channel blocking mechanism (Adams, 1976; Hille, 1984). The activities of the compounds in blocking ACh-induced current could differ in various compounds more than 50-fold (e.g., compounds I and VIII, the last column of Table 1).

Blocking activities of the compounds in their effects on synaptic transmission through the cat superior cervical ganglion and on the suberyldicholine-induced contractions of the guinea-pig small intestine are also given in Table 1. Relative ganglionic-blocking activities of the compounds tested were very similar regardless of what object they were estimated. This similarity could be interpreted in favor of the above assumption that the ganglionic-blocking effect produced by *bis*-ammonium compounds in different ganglia is due to a similar mechanism, the open-channel blockade of ganglionic AChRs.

The above compounds were also shown to block, although markedly less strongly, the postsynaptic current in the frog end plate (Table 3). The drugs induced biexponential decay of postsynaptic current, their effect being voltage dependent. This provides evidence supporting the open-channel mechanism of blockade (Peper, Bradley & Dreyer, 1982). We thus could measure directly the association and dissociation rate constants of the interaction of the drugs with the open channel.

The simplest sequential model (Adams, 1976) suggests that a blocking agent interacts with the open channel with the forward (k_f) and backward (k_b) rate constants:

$$2A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* + B \underset{k_b}{\overset{k_f}{\rightleftharpoons}} A_2R^*B$$

where A is agonist, B is blocking agent, R is the acetylcholine receptor, A_2R, A_2R^* and A_2R^*B are the agonist-receptor complexes with closed, open and blocked ion channel, correspondingly, and k_{+1}, k_{-1}, α, β, k_f and k_b are the rate constants.

The k_f and k_b values were estimated as the inverses to the time constants that characterize the blocker-induced biexponential decay of the EPC (Peper et al., 1982). The values of k_f, k_b and $K_D = k_b/k_f$ thus obtained are shown in Table 3. One can see from the comparison of Table 3 with Table 1 that a sequence in relative activities of the compounds

found in the end plate reveals the same tendency as that observed in the ganglia. In both cases essential decrease of activity was correlated with the enlargement of trialkylammonium head (e.g., the compounds (II) and (III)). These data indicate some similarity in the mechanisms of action of *bis*-cationic blocking drugs on muscle and neuronal AChRs. It should be noted, however, that the absolute values of k_b and K_D differ markedly in these two objects (*see below*).

Considering ganglion-blocking activities of the compounds tested, one should emphasize that compounds (III) and (VI), although being equal in total number of carbon atoms and only slightly different in the chemical structure of their first cationic head, exhibit essentially different activity. The same is observed in compounds (II), (IV) and (V) which possess the identical brutto-formula of the first cationic head (NC_5H_{13}). Such variations in the activity of the compounds with related structure cannot be accounted for by their different hydrophobic properties. However, these data seem surprising only if two-dimensional structural formulae of the molecules are considered, while they could be accounted for by using the three-dimensional models of the blocking molecules. For this purpose, all equilibrium conformations of the first cationic heads in compounds (I)–(XI) were calculated. The results are now described.

Conformational Characteristics of the Blocking Compounds

Energies of all low-energy equilibrium conformations of the compounds (I)–(XI) are listed in Table 4. The number of equilibrium conformations varies from 1 in compound (I) up to 54 in compound (VII). Due to nonbonded repulsion between substituents, many equilibrium conformations are the high-energy ones. For example, among 27 equilibrium conformations of the compound (III), only 3 conformations are of the energy lower than 3 kcal/mol. In compounds (VI), (VII), (VIII) the pyrrolidine ring is in the envelope conformation with the bond N—C^{α} being in equatorial or in axial orientation with respect to the plane of four carbon atoms of the ring. In compounds (VI) and (VII) that have two unbranched substituents at pyrrolidine nitrogen, both axial and equatorial orientations of the bond N—C^{α} are equal in energy. In contrast, in compounds (VIII) and (X), most preferable is the equatorial orientation of the branched substituent. The chair conformation of the piperidine cycle is the most preferable in compound (X), with axial and equatorial orientations of the bond N—C having the same probability due to comparable volume of two unbranched substituents at nitrogen atom.

In equilibrium conformations optimal bond angles of some atoms essentially deviate from those in the ideal tetrahedral models (109.5°). For example, in all compounds tested their bond angle A(C^{β}-C^{α}-N) is within the range 117–119°, which is characteristic for the angle A(N-C^{α}-C^{β}) in the crystals of acetylcholine (Canepa, Pauling & Sörum, 1966; Herdklotz & Sass, 1970).

Relationship between Geometry and Activity of the Blocking Drugs

As only one of the cationic groups (the first) is variable in the series of compounds tested, while another group (the second) remains constant, the observed differences in blocking activities should be due to differences in the structure of only the variable cationic group. According to existing models (Kistler et al., 1982; Unwin, Toyoshima & Kubalek, 1988), the channel of AChR has a wide entrance (mouth) facing extracellular space and more deeply located narrow part. The simplest explanation for the observed differences in blocking activities of the compounds would be that the second (in most cases the largest) group binds to the mouth, while the first group binds more deeply in the narrow part of the channel. On this assumption, the increase in the size of the first group to a size greater than that of the channel, should result in a decrease of blocking activity. All equilibrium conformations of each blocking compound could thus be divided into two categories, the compact and noncompact ones, depending on whether their first cationic group can penetrate into the narrow part of the channel. Therefore, the possibility appears to estimate the profile of the narrow part of the channel as that corresponding to the best correlation between the blocking activity and the population of the compact conformers in each compound tested.

Let us approximate the profile of the narrow part of the channel (the clear space inside the channel crossectioned by the plane normal to its longitudinal axis) by a rectangle with the sides a and b ($a < b$). A molecule M_i ($i = 1,2 \ldots 11$) has N_i conformers C_{ij} ($j = 1,2 \ldots N_i$) with the energies E_{ij} (Table 4). Relative concentration (population) of the conformer C_{ij}

$$p_{ij} = [\exp(-E_{ij}/RT)] / \sum_{k=1}^{N_i} \exp(-E_{ik}/RT) \qquad (1)$$

Where $R = 0{,}001987$ kcal/mol is the universal gas constant and T is temperature taken equal to 300°K.

Table 4. Energy (kcal/mol) of equilibrium conformations of the compounds (II)–(XI)

Compound	N—Cl *	τ_3	τ_1, τ_2								
			gg	gt	gg^-	tg	tt	tg^-	g^-g	g^-t	g^-g^-
II		g	0.2	0.1	2.6	2.9	0.3	2.8	2.8	3.0	
		t	0.8		0.3			0.0	0.2	2.9	2.8
		g^-			3.0		0.8	0.2	0.0	0.2	2.6
III		g	3.0			0.3		3.0	3.0		
		t							0.0		
		g^-		3.0					3.0	0.3	3.0
IV		g	2.1	0.0	1.6	2.4	0.6			2.9	
		t	2.6	0.6	2.9		0.9		2.6	0.6	2.5
		g^-		2.9		2.6	0.6	2.4	1.8	0.0	2.1
V		–		0.0	2.0	0.0		2.1	1.0	1.1	0.5
VI	e	–	1.8	1.8		0.0		1.8		0.0	1.8
	a	–	1.7	1.7		0.0		1.8		0.0	1.8
VII	e	g	1.6	0.0	1.9		1.9				
	e	t		1.9						1.9	
	e	g^-			2.9		1.9		1.9	0.0	1.6
	a	g	1.0	0.0	1.8		1.9			3.0	
	a	t	2.6	1.8						1.8	2.6
	a	g^-		3.0			1.9		1.8	0.0	1.0
VIII	e	–	1.2			1.0		0.6	1.5	0.7	
	a	–	0.0	2.4	2.5	0.4	0.1	0.1	0.8	0.1	
X	e	–	0.1			1.1		1.1			0.1
	a	–	0.0	1.1	3.0				3.0	1.0	0.0
XI	e	–	0.0		2.8	0.9	0.9	0.2		0.0	2.8
	a	–	0.9			2.3	2.3	0.8		0.9	

In the compound (IX) energies of g, t and g^- conformers are equal to 0.0, 2.74, and 2.74 kcal/mol, respectively. Spaced positions correspond to conformers with energy > 3 kcal/mol.
*Orientation of N—Cl bond relative to the ring plane: e, equatorially; a, axially.

Let us designate as l_{ij} and m_{ij} ($l_{ij} < m_{ij}$) the dimensions of the minimal rectangle that accommodates the projection of trialkylammonium head of conformer C_{ij} with the straight line between its two nitrogen atoms normal to the plane of the rectangle. To find dimensions l_{ij} and m_{ij}, conformer C_{ij} was rotated around the above straight line in 1° increments. Small cationic head of conformer C_{ij} would penetrate the narrow part of the channel and bind to the deep anionic site if

$$l_{ij} \leqslant a \text{ and } m_{ij} \leqslant b. \tag{2}$$

The conformers which satisfy condition (2) are considered compact. The total population of compact conformers of compound M_i, P_i, is determined as

$$P_i = \sum_{j=1}^{N_i} \sigma_{ij}\mu_{ij}p_{ij} \tag{3}$$

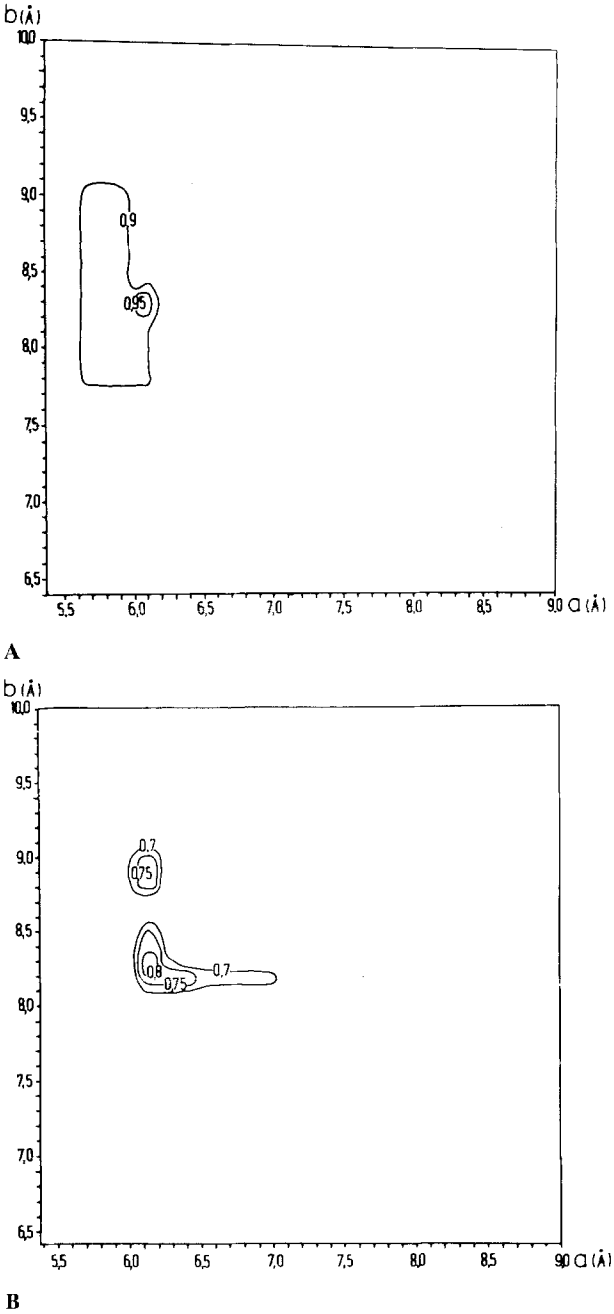
where

$$\sigma_{ij} = \begin{cases} 1, \text{ if } l_{ij} \leqslant a \\ 0, \text{ if } l_{ij} > a, \end{cases} \tag{4}$$

and

$$\mu_{ij} = \begin{cases} 1, \text{ if } m_{ij} \leqslant b \\ 0, \text{ if } m_{ij} > b. \end{cases} \tag{5}$$

Let us assume that (*i*) there is a correlation between the population of compact conformers of the compounds and relative affinities of these compounds for the receptor, and (*ii*) the correlation is described by a linear regression equation. It is obvious that the correlation coefficient for the above model should depend only upon the chosen dimensions a and b of the narrow part of the channel. Considering a and b as the variables, it is possible to calculate optimal values which correspond to a maximum of correlation coefficient (r_{max}). The value of r_{max} could at the same time serve as a criterion for



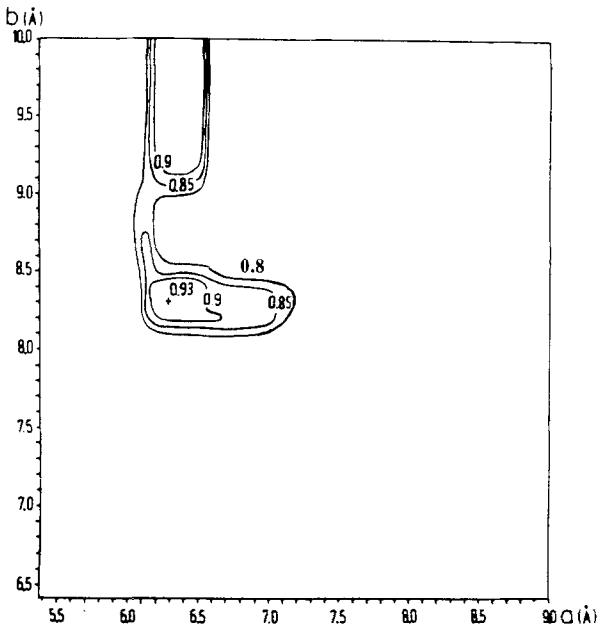


Fig. 2. The lines indicating high equal values of correlation coefficient between relative blocking activities of the compounds tested and population of their compact conformations, as corresponding to different values of a and b (the size of the channel, *see* text). Blocking activities were estimated from (*a*) blockade of synaptic transmission through cat superior cervical ganglion, (*b*) blockade of suberyldicholine-induced contraction of guinea-pig intestine, and (*c*) blockade of ACh-induced membrane currents in rat superior cervical ganglion. The maps were calculated in 0.1-Å increments for a and b

the model reliability, in particular, for the validity of the assumption that the dimensions of the first cationic head are crucial to the relative affinity of a ligand.

Figure 2 illustrates how the correlation coefficient between the blocking activities of the compounds and the population of their compact conformers depends on the channel dimensions a and b. The maps were calculated, with a and b values varied in 0.1-Å increments. As a result, the set of equilibrium conformations of each compound appeared divided into compact and noncompact subsets, in accordance with the criterion (2). For each combination of a and b, the correlation coefficient between known activity of a drug and the total population of subset of its compact conformations was calculated. In the experiments with synaptic transmission through sympathetic ganglion (Fig. 2*a*) and responses of enteric ganglia to agonist (Fig. 2*b*) maximal correlation coefficient correspons to the same channel dimensions, namely, $a = 6.1$ Å and $b = 8.3$ Å. In the experiments with ACh-induced membrane current in sympathetic neurons (Fig. 2*c*) maximal correlation coefficient correspond to dimensions $a = 6.3$ Å, $b = 8.3$ Å, and; besides, an additional region ($a \approx 6.3$ Å, $b > 9$ Å) of high correlation coefficients exists. These peculiarities are apparently due to low number of observations in the latter case.

Therefore, one can conclude that the region of the channel responsible for binding of the first cationic head is characterized by the following dimensions: $a = 6.1$ Å and $b = 8.3$ Å. Those conformers that can penetrate this narrow spot should be, according to the above hypothesis, considered as biologically active (productive) ones.

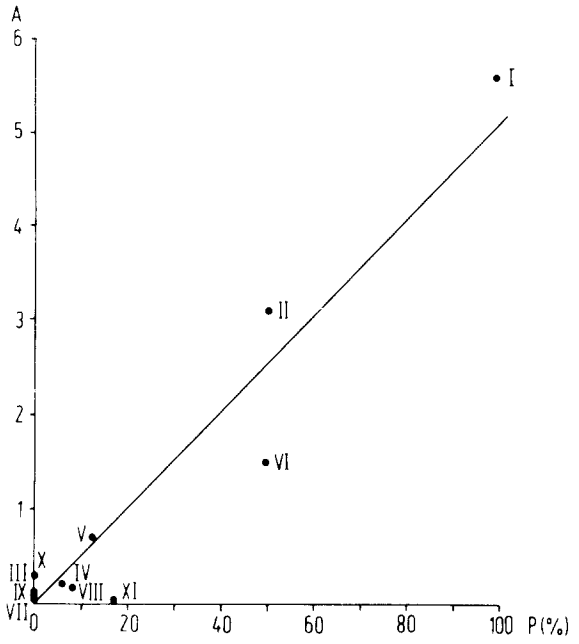


Fig. 3. The correlation field between the drug activities (*A*, relative units) and the total population of the corresponding productive conformations (*P*, %)

The correlation between the drug activities and population of the corresponding productive conformations is shown in Fig. 3. The experimental points in this plot are distributed rather nonuniformly, as could be expected from the blocking activities of the compounds (Table 1). The course of the regression line is determined mainly by the active compounds (I), (II) and (VI), on one hand, and by the group of less active compounds with low population of productive conformations, on the other hand.

The correlation observed between the population of productive conformations of the blockers and their biological activity agree with a concept that the energy of intramolecular interactions in a ligand (ΔE^L) is an independent contribution into the free energy (ΔG) of the ligand-receptor complex (Zhorov & Govyrin, 1981). Consequently, $1/K_D = \exp(-\Delta G/RT)$ should increase directly with $\exp(-\Delta E^L/RT)$. In other words, receptor binds not only low-energy, but also high-energy productive conformers; but in the latter case, binding is paid by the increase of free energy of ligand-receptor complex, and, consequently, by the loss of "biological activity" of the ligand.

In contrast to the above findings observed in neuronal AChRs, the analysis of the relationship between conformational properties of the drugs and their abilities to block muscle AChRs failed to give a strict maximum of the correlation coefficient as a function of *a* and *b*. The reason for this difference is that compound (I) is much more active than other compounds which are similar in their low blocking activities.

The dimensions of equilibrium conformations of some compounds and the indications of their productivities relative to AChRs of sympathetic and parasympathetic ganglia are summarized in Table 5. None of three low-energy conformers of the low-active compound (III) is able to penetrate into the narrow spot of the channel (Fig. 4); i.e., none is a productive one. At the same time, the majority of 11 optimal conformations of the active compound (II) are productive ones (Fig. 5).

Closing two ethyl substituents of low-active compound (III) in a five-member ring in compound (VI) yields 49% population of productive conformations (Fig. 6).

The distinguishing features of compounds (IV) and (V) exhibiting essentially different activities are *n*-propyl and isopropyl substituents in their trialkylammonium "heads." At first sight, the branched isopropyl substituent should prevent interaction of the compound with the intrapore anionic site to a greater extent than *n*-propyl substituent would do. However, compound (V) is more active than compound (IV). Table 5 shows that the population of productive conformers in compound (V) is twice as high as that in compound (IV). Comparatively low activity of compound (IV) is due to conformation *ttt* which is the only productive one among nine low-energy conformations.

According to the model presented the invariable triethylammonium group of the blockers, which is the largest one in the majority of the molecules studied, cannot penetrate the narrow part of the channel. So the binding site for this group (the second anionic site) should be located somewhere between the narrow part and outer mouth of the channel. So far as the first anionic site is concerned it has either to correspond to the narrow part of the channel or to be located deeper than it. The kinetic data on binding of *bis*-cationic blockers helps to choose between these two possibilities. Indeed, if the narrow spot were located between two anionic sites, one should expect that an increase in the size of trialkylammonium head would diminish not only the association but also the dissociation rate of drug binding. The millisecond range of ligand-receptor complex lifetime is long enough for multiple conformational changes in trialkylammonium head. So this head would easily "forget" the conformation in which it has penetrated the narrow spot, and therefore, one should expect a decrease of the dissociation rate for large blocking molecules. The rate constants of association for compounds (II, VI, and IX) with muscle AChR varies to a greater extent than dissoci-

Table 5. Characteristics of the optimal conformations of the compounds (I)–(V) $Et_3\overset{+}{N}—(CH_2)_5—R$

Compound	R	Conformer	Energy, kcal/mol	Population, %	Dimensions, Å		Productivity*
					l	*m*	
I	$—\overset{+}{N}Me_3$	—	0.0	100	5.6	6.5	+
II	$—\overset{+}{N}MeEt_2$	tg^-t	0.0	13.1	5.7	7.8	+
		g^-gg^-	0.0	13.1	5.7	9.1	−
		gtg	0.1	11.1	5.7	7.8	+
		tg^-g^-	0.2	9.5	6.2	8.2	−
		g^-gt	0.2	9.5	6.1	8.2	+
		g^-tg^-	0.2	9.5	6.1	8.2	+
		ggg	0.2	9.5	6.2	8.2	−
		gg^-t	0.3	8.1	6.6	7.3	−
		ttg	0.3	8.1	6.6	7.3	−
		ttg^-	0.8	3.6	5.9	8.3	+
		ggt	0.8	3.6	5.9	8.3	+
III	$—\overset{+}{N}Et_3$	g^-gt	0.0	43.0	5.7	9.1	−
		tgg	0.3	26.4	7.1	8.1	−
		g^-tg^-	0.3	26.4	7.1	8.2	−
IV	$—\overset{+}{N}Me_2n$-Pr	g^-tg^-	0.0	25.8	5.8	9.1	−
		gtg	0.0	25.8	5.8	9.1	−
		ttg	0.6	9.8	6.0	8.4	−
		ttg^-	0.6	9.8	6.0	8.4	−
		g^-tt	0.6	9.8	6.0	8.4	−
		ggt	0.6	9.8	6.0	8.4	−
		ttt	0.9	6.0	6.1	6.9	+
		g^-gg	1.6	1.9	5.7	8.6	−
		g^-gg^-	1.8	1.4	5.7	8.6	−
V	$—\overset{+}{N}Me_2i$-Pr	tg	0.0	34.6	6.2	7.6	−
		gt	0.0	34.6	6.2	7.7	−
		g^-g^-	0.5	15.4	6.5	6.6	−
		g^-g	1.0	6.8	5.9	7.1	+
		g^-t	1.1	5.8	5.9	7.2	+
		gg^-	2.0	1.3	6.6	7.5	−
		tg^-	2.1	1.1	6.6	7.5	−

* +: productive conformation; −: nonproductive conformation.

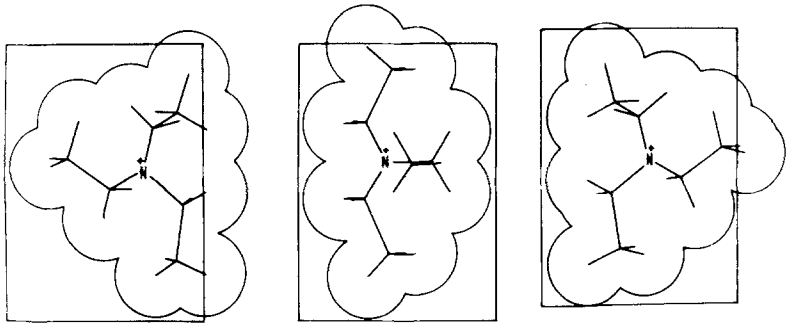


Fig. 4. Projections of triethylammonium head of the compounds (III) on the plane normal to the axis passing through nitrogen atoms. Rectangle designates the dimensions (6.1 × 8.3 Å) of the narrow spot of the channel. Low-energy conformations tgg, g^-gt and g^-tg^- are nonproductive

ation rate constants (Table 3). Recently the values of rate constants k_f and k_b were also estimated for blockade of AChR open channel in rabbit superior cervical ganglion with a series of symmetrical compounds $[(CH_3)_3\overset{+}{N}—(CH_2)_n—\overset{+}{N}(CH_3)_3$, $n = 4$–$7]$ (XII)–(XV) (Skok et al., 1983, 1984; Gurney & Rang, 1984) and with a nonsymmetrical compound $[(CH_3)_3N$-$(CH_2)_5$-$NH_2\ ^iPr]$ (XVI) (Skok et al., 1983, 1984). Blocking activities of these compounds correlated with k_f rather than with k_b values. All these data suggest that the narrow part of the AChR is located in the vicinity of the first anionic site.

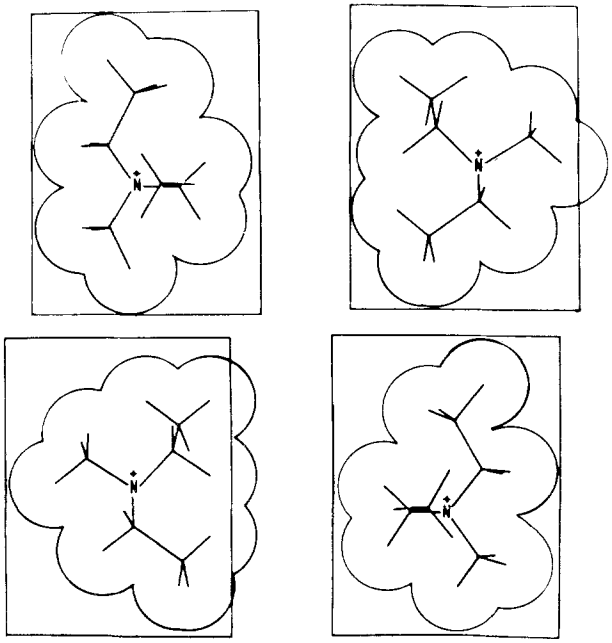


Fig. 5. Projections of methyldiethylammonium head of the compound (II) on the plane normal to the axis passing through nitrogen atoms. Rectangle designates the dimensions (6.1 × 8.3 Å) of the narrow spot of the channel. Low-energy conformations g^-gt and g^-tg^- are productive, while gg^-t and ttg are nonproductive

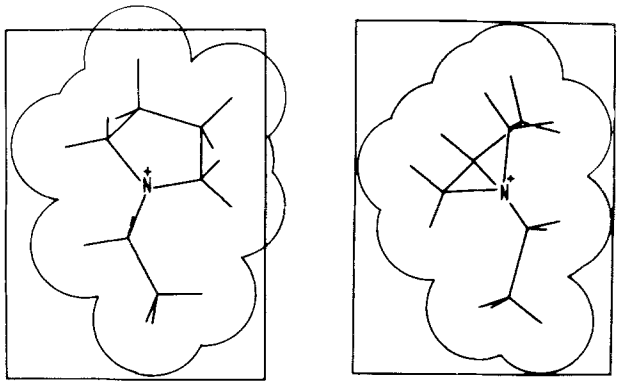


Fig. 6. Projections of ethylpyrrolidine head of the compound (VI) on the plane normal to the axis passing through nitrogen atoms. Rectangle designates the dimensions (6.1 × 8.3 Å) of the narrow spot of the channel. Low-energy conformation egt is productive, while eg^-t is nonproductive

Is the above narrow spot identical to the selectivity filter which prevents passing of anions and large organic cations through the channel of nicotinic AChR? According to the model of Hille (1984), the essential feature of the selectivity filter is a ring of oxygen atoms which diminish free-energy barrier for partial dehydration of passing cations. On the other hand, recent findings made with site-directed mutagenesis suggest that both the permeant ions and the

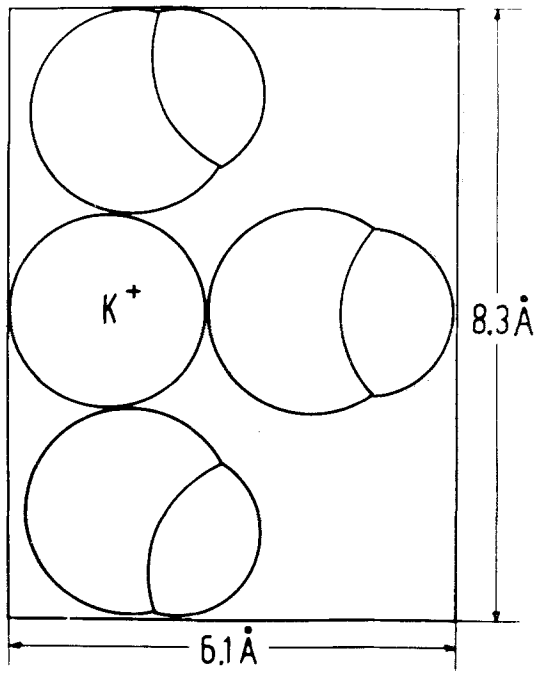


Fig. 7. The ion channel narrow part of the calculated dimensions (6.1 × 8.3 Å) fits an inorganic cation as large as potassium surrounded from all but one side by water molecules of the first hydration shell (axial water molecules are omitted). Taking into account high flexibility of water-ion-water bond angles, the same rectangle would fit partly dehydrated potassium ion with any energetically possible configuration of the shell

open-channel blockers may interact with the polar amino acid residues located either close to M2 membrane-spanning region (Imoto et al., 1988) or within this region (Leonard et al., 1988) in nicotinic AChRs of fish electric organ and mouse end-plate, correspondingly. However, no information of this kind has been obtained so far from neuronal nicotinic AChR. The ion channel of AChR does not exhibit selectivity for any particular inorganic cation (Dwyer et al., 1980). The calculated dimensions of the ion channel narrow part predict that an inorganic cation as large as potassium can pass through the spot losing only one water molecule of the first hydration shell (Fig. 7).

Figure 8 presents the proposed model of drug-receptor complex. To some extent, our model resembles the recent model of Charnet et al. 1990) where a local anesthetic molecule binds into the tapered channel.

The voltage dependence of a drug action can indicate how deep in the pore the binding site for this drug is located. (Adams, 1977; Hille, 1984). Voltage dependence of rate constants for binding of compounds (XII)–(XVI) to the open channel suggests that the binding site is located in the middle one-third of membrane electric field (*see* Skok et al.,

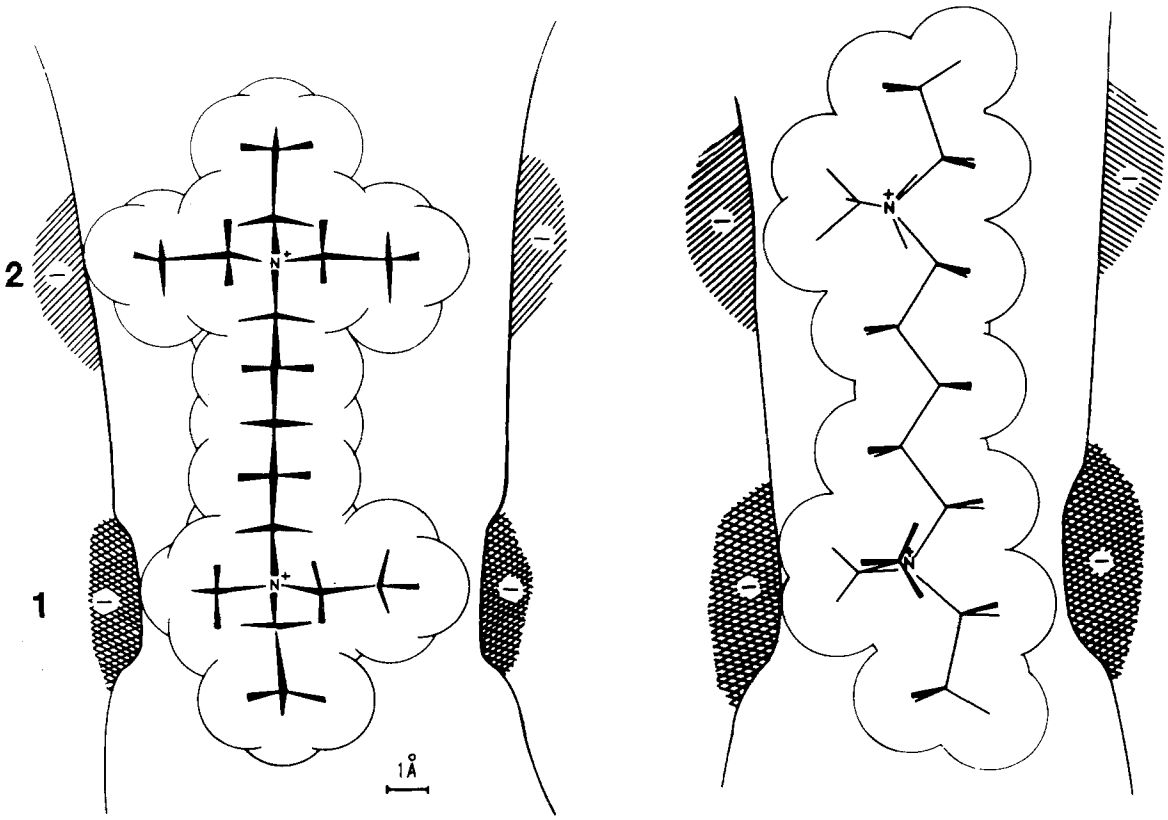


Fig. 8. The model of the narrow part of the AChR ion channel with the bound productive conformer tg^-t of the compound (II) shown as projections on two perpendicular planes. *1* and *2* are the regions where the first and the second anionic sites of the channel are located. Triethylammonium head of the blocker (in the upper part of the figure) is in the most preferable conformation

1989). This location is similar to that of local anesthetics binding site in the ionic channel of frog end-plate AChR (Adams, 1977). Low voltage dependence of the blocking effects in the majority of the drugs studied on frog end-plate (Table 3) indicates that their binding sites are located not far from the outer surface of the membrane. The only exception is the compound (III) with the critical size of triethylammonium cationic head. The change of the membrane potential from -80 to -120 mV is accompanied by marked increase of the activity. It should be noted that hyperpolarization causes an increase in association rather than in dissociation rate constants for binding of compound (III). In the sense of the proposed model, this fact could be interpreted as a result of some increase in the profile of the narrow spot due to membrane hyperpolarization, or, alternatively, as a result of a propulsion of the cation caused by electric field.

Estimates of the profile of muscle AChR ion channel were reported (Huang, Catterall & Ehrenstein, 1978; Dwyer et al., 1980). Comparing the sizes of permeable and nonpermeable cations Dwyer et al. (1980) obtained the channel profile as the truncated rectangle 6.5 × 6.5 Å. This profile was large enough for permeation of triethylmethylammonium but not tetraethylammonium ion. These two ions are likewise the characteristic moieties of the compounds (II) and (III) in our investigation. The channel profile in our experiments is larger (6.1 × 8.3 Å) than that in muscle AChR. The results obtained in this work as well as those reported by other authors shows that the ionic channel of muscle and neuronal AChRs exhibit markedly different affinity to the same series of channel-blocking molecules. The origin of this difference is not yet clear.

Now a variety of sophisticated approaches such as site-directed mutagenesis (Dani, 1989), combining of subunits of different origin (Imoto et al., 1988; Papke et al., 1989; Charnet et al., 1990), computer modeling (Furois-Corbin & Pullman, 1989), labeled ligand techniques (Changeux et al., 1987; Gui & Hucho, 1987; Herz, Johnson & Taylor, 1989) are used to elucidate the structure of AChR ionic channel and localize binding sites for deferent ligands. We believe that analysis of conformation-activity rela-

tionships of ligands should become more fruitful being used in combination with some of these approaches.

Besides the considered mechanism of action of the *bis*-ammonium compounds, other mechanisms of noncompetitive blocking action of these drugs are known (Gurney & Rang, 1984; Colquhoun, Ogden & Mathie, 1987; Skok, 1987). However, it is the assumption that the drugs interact with the open ion channel which best explains the structure-activity relationship for *bis*-cationic blocking drugs found in this work.

In conclusion, the analysis of conformation-activity relationship for *bis*-cationic cholinolytics has yielded a model of the binding site for these drugs in neuronal nicotinic AChR with the dimensions of the narrow profile of the ion channel in the region where the binding site is located corresponding to a rectangle 6.1 × 8.3 Å.

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