A physical model of nicotinic ACh receptor kinetics

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We present a new approach to nicotinic receptor kinetics and a new model explaining random variabilities in the duration of open events. The model gives new interpretation on brief and long receptor openings and predicts (for two identical binding sites) the presence of three components in the open time distribution: two brief and a long. We also present the physical model of the receptor block. This picture naturally and universally explains receptor desensitization, the phenomenon of central importance in cellular signaling. The model is based on single-channel experiments concerning the effects of hydrocortisone (HC) on the kinetics of control wild-type (WT) and mutated αD200Q mouse nicotinic acetylcholine receptors (nAChRs), expressed in HEK 293 cells. The appendix contains an original result from probability renewal theory: a derivation of the probability distribution function for the duration of a process performed by two independent servers.

I. INTRODUCTION

A *generally accepted kinetic model* (GAKM) of ACh receptor gating assumes that the receptor opens when one or two agonist molecules get bound to it and shuts before agonist(s) dissociation (reviewed by Hille (2001)). If only one molecule is bound to the receptor, it opens for a short time; two bound molecules result in a long opening of the receptor. It was however noted, that an excess of brief openings, that appeared in some recordings at high agonist concentrations, is not consistent with this interpretation (Colquchoun et al., 1985, Sine et al., 1987 , Hallermann et al., 2005). Thus, the question of how the binding sites contribute to channel gating is still open.

The GAKM assumes that the ACh-AChR binding is formed as a result of the interaction between the π-electron systems originating from the agonist-binding-sites-aromatic-amino-acids, and the cholinum nitrogen, native to the quaternary ammonium ligands (reviewed by Arias (2000)).What is usually not discussed is the fact that close to the ACh binding site there is an electrically noneutral amino acid, the negatively charged aspartic acid, which may have some influence on the agonist binding or tracking. To check the role of this amino acid for the ACh receptor's kinetics we performed experiments on the αD200Q receptor (Mukhtasimova et al., 2005) with the negatively charged aspartic acid removed and replaced by a polar amino acid (glutamine). Previous studies suggested that the amino acid αD200 played a role in the process of receptor activation, i.e. in the process that starts after a binding of the agonist (O'Leary et al., 1992, Akk et al., 1996). In our present studies we go a bit further: we examine the role of this amino acid in the process of the receptor block.

We studied the receptor's kinetics, in both wild and mutated versions, to better understand the limits of the validity of the GAKM discussed above. This model has very severe consequences for our understanding of the structure of the receptor. Applications of various blockers, such as the open-channel blockers and the use of GAKM predicts, for example, the amino acid content of an ion channel (Leonard et al., 1988). Validity of the models describing how the receptor is built is thus very strongly dependent on the model of the receptor's kinetics.

The classical open-channel blockers' theory is based on the observation that the voltage dependence in the blockers' action occurs only when the blockers are electrically charged (Neher et al., 1978, Akk et al., 2003). It turns out, however, that there exist electrically neutral molecules (such as HC (Bouzat et al., 1996, Nurowska et al., 2002) and physostigmine (Wachtel, 1993)), which block the ACh receptor with a very strong voltage dependence. They prove that the voltage dependency is quite a complex phenomenon, which is not only a consequence of the molecule charge.
Another doubt about the GAKM comes from the fact that the open-channel blockers action is very often dependent on the agonist concentration. The occurrence of this dependence is so common that some authors consider it as the main feature of the open-channel blockers (Buisson et al., 1998). This is in direct contradiction with the claim of the open-channel blockers' theory that the blocker enters into the ionic channel: once the blocker is in the channel, the agonist concentration should not influence the block.

In this paper we introduce a model of the receptor blocking which resolves the problems discussed above. Our model explains how an electrically neutral blocker (HC) induces a voltage dependent effect. It also explains the dependence of the blocker action on the agonist concentration. This model naturally and universally explains receptor desensitization. Molecular rearrange-

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ments causing receptor desensitization were, up to now, poorly understood. Our model of the receptor block requires a new kinetic model of the AChR gating. The formulation of this new kinetic model is also given and its consequences for the kinetic theory are discussed.

II. METHODS

Cell culture The human kidney cell line HEK 293 was routinely cultured in DMEM, supplemented with heat-inactivated foetal calf serum (10%), L-glutamine (4mM), penicillin (100 units/ml^{-1}) and streptomycin (100 μg/ml^{-1}). Cells were incubated in an atmosphere containing 5% CO_2 at 37 °C. One day before transfection, cells were seeded into culture dishes.

Transfection. Mouse nAChR subunit (α, β, δ, γ) cDNAs in the expression vector pRBG4 (Sine, 1994) and the mito-GFP construct were transiently expressed in HEK 293 cells using LipofectAmine Plus TM Reagent (Life Technologies, Inc). The WT subunits and the mutated α-subunit with the D200 (aspartic acid) to Q (glutamine) substitution were the generous gift of Prof. Steven Sine (Receptor Biology Lab., Mayo Foundation, Rochester). Mito-GFP was a kind gift from Prof. Adam Szewczyk (Nencki Institute, Warsaw). A total of 1 μg of cDNA per 35 mm culture dish in the ratio 2:1:1:1:1 (α:β:δ:γ:GFP) was used. The αD200Q contained the mutation on both α subunits.

Electrophysiology. Single-channel currents were recorded from cell-attached patches 1-2 days after transfection (Hamill et al., 1981). The bath saline (NES) contained (mM): NaCl 140, KCl 2.8, $CaCl2_2$, $MgCl2_2$, glucose 10, HEPES buffer 10 (pH 7.4). The pipette contained NES and acetylcholine (ACh, Sigma-Aldrich Co Ltd): 50-500 nM ACh in experiments with the WT receptor and 10-100 μM ACh in experiments with αD200Q receptor. HC (Sigma-Aldrich Co Ltd, 0.02-1 mM) was incorporated in the pipette solution by dissolving it in NES (Loftsson et al., 2003): the suspension of HC was heated (35 °C for about 4 hours) and left under constant agitation for another 24 h at room temperature.
If not otherwise stated, results represent experiments performed at 60 mV of pipette potential.

Data Analysis ACh-induced currents were recorded using an Axopatch 200B (Axon Instruments, Foster City, CA, USA). The signals were filtered (2-3 kHz, -3dB) and transferred at 50 kHz, using the Digidata 1200 interface (Axon Instruments), to a hard disc. Single currents were analyzed using the pClamp 7 software (Axon Instruments). Transitions were detected using a threshold-crossing algorithm, with a threshold for the open and the closed states set at about 50% of the mean channel current level. Only recordings with rarely overlapping openings were analysed and only openings to the first level were included in the dwell-time distributions. In case of stable patches, after performing recordings at different membrane potentials, we performed the second registration at +60 mV of pipette potential starting about 15-20 min after the patch formation. The receptor kinetics recorded at +60 mV immediately after the patch formation and after 15 min were compared. We did not observe any significant changes neither in time constants nor in current amplitude. In most of experiments the dwell-time distributions were constructed from more than 1000 events, however, in few cases from more than 300 events. Open and blocked time constants τ_{open} and $\tau_{blocked}$ of the open and closed time distributions were fitted by the method of maximum likelihood using the PSTAT program (pClamp7) with a probability density function being the sum of n exponential terms, i.e.

$$f(t) = \sum_{i=1}^{n} a_i \frac{1}{\tau_i} \exp(-\frac{t}{\tau_i})$$

where a_i represents the area of the i-th component. The open time distribution of the WT receptor was fitted with 2 components ($n = 2$); τ_{open} corresponds to the long component of the open time distribution. The open time distribution of the mutant αD200Q receptor was fitted with 1 component only ($n = 1$). $\tau_{blocked}$ corresponds to the component that is present in the closed time distribution of the blocked receptor only, and such, its area increases with HC concentration. It should be also mentioned that due to the resolution of our single channel recording (0.110 -0.180 ms), very brief events were probably undetected. This, however, should not affect the estimation of the duration of the blocked events ($\tau_{blocked}$). They appear only within the bursts having at least two openings. It was shown previously (Nurowska et al., 2002) that in such bursts brief openings are absent. Since brief openings exist only as isolated openings, they can not affect the closed periods within a burst.

Statistical analysis. Data are given as mean ± SEM. The unpaired Student's t-test was used to determine the statistical significance. If not otherwise stated, results are pooled for different ACh concentrations and number n of samples is given in parentheses.

III. RESULTS

The inclusion of ACh, in the patch pipette, induced single-channel currents in both types of cells expressing WT receptors or mutated αD200Q nACh receptors (Fig. 1). However, the activity of the αD200Q receptor was visible only when the ACh concentration was approaching micromoles. Below this concentration of ACh the activity of the αD200Q receptor was negligible. For this reason, in experiments with the αD200Q receptor, we increased the agonist concentration up to 10-100 μM. This activated the receptor, but contrary to the standard behaviour of the receptor in high concentration of ACh, did not cause receptor desensitization (Fig. 1A). Actually only one component was present in the closed time distributions which time constant decreased with the agonist concentration increase. A second component in the

closed time distribution appeared only after adding the hormone.

The single-channel *I-V* relationship gave a slope conductance of 33.4 ± 0.8 pS ($n = 9$) for the WT receptor, and 32.0 ± 0.8 pS ($n = 17$) for the αD200Q receptor, suggesting similar pore conductance properties. The mean open-channel lifetime (i.e. the mean time constant of the main component, τ_{open}) of the WT receptor was 6.90 ± 0.78 ms (50-500 nM ACh) and did not depend on agonist concentration. The τ_{open} of the αD200Q receptor increased with the agonist concentration increase, being 0.81 ± 0.22 ms for 10 μM ACh and 1.57 ± 0.46 ms for 100 μM ACh ($P< 0.0001$, two-tailed *t*-test). Even so, τ_{open} of the mutated receptor was significantly smaller than τ_{open} of the WT receptor, in the presence of 50-500 nM ACh ($P< 0.001$, two-tailed *t*-test; Fig. 1B). The voltage-dependency of τ_{open} for the mutated receptor was, nevertheless, maintained (Fig.1B).

In the presence of 1 mM HC in the patch pipette, there was no significant effect on the single-channel *I-V* slope conductance, which was 32.0 ± 0.5 pS ($n = 10$) for the WT receptor and 31.6 ± 0.5 pS ($n = 12$) for the αD200Q receptor. In both types of cells, HC decreased τ_{open} in a concentration-dependent manner (Fig. 2), and produced a characteristic burst-opening effect. When HC blocked the WT receptor, τ_{open} did not change with change of the patch membrane potential, or changed very weakly (Fig. 3). However, when HC blocked the mutated receptor, τ_{open} increased with increasing membrane hyperpolarization (Fig. 3). Interestingly, the blocked events induced by HC were voltage-dependent in the WT receptor, but we did not notice any obvious voltage-dependency of the mean time constants of the blocked events ($\tau_{blocked}$) in the mutated receptor (Fig. 4). For all HC concentrations $\tau_{blocked}$ of the αD200Q receptor were significantly shorter than $\tau_{blocked}$ of the WT receptor (Fig. 5A; $P< 0.001$). This decrease in the $\tau_{blocked}$ for the mutated receptor was not induced by the high agonist concentration, since our experiments with different agonist concentrations and the αD200Q receptor excluded this possibility (Fig. 5B). In both types of receptors, $\tau_{blocked}$ depended on the HC concentration (Fig. 5A).

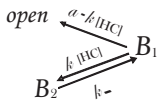
IV. DISCUSSION

The main conclusion of our experiments is that $1/\tau_{block}$ decreases linearly with the HC concentration growth (Fig. 5A). This means that the kinetic constant describing the passage from a *blocked* to an *open* state is given by $a - k[HC]$ where a and k are $[HC]$-independent constants. We thus have

$$open \overset{a-k[HC]}{\longleftarrow} block.$$

This suggests that the state *blocked* is a complex state consisting of two substates B_1 and B_2, between which there exists a passage $B_1 \rightarrow B_2$ in the direction opposite to the direction *blocked* → *open* with a kinetic constant equal to k[HC]:

(1)



Here k_- denotes the dissociation constant of the blocker. It follows that the passage $B_1 \rightarrow$ *open* presented by this scheme is not connected with the dissociation of the blocker; the blocker dissociates when passing from B_2 to B_1, thus the state B_1 is a state without a bound blocker.

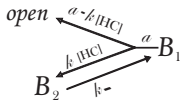
The complex scheme (1) has a kinetic constant corresponding to the passage *blocked* → *open* given by

$$k_{open} = \frac{a - k[HC]}{1 + \frac{k[HC]}{k_-}}. \tag{2}$$

Note that formula (2) becomes $a - k[HC]$ in the limit $k_- >> k[HC]$. This limit corresponds to the situation when the state B_2 is very much shorter in the dwell-time than the state B_1.

A consequence of the postulated kinetic model described by scheme (1) is that state B_1 is very particular: the receptor leaves state B_1 with the same frequency a independently of the presence of the blocker. Only after leaving this state the receptor 'decides' to pass to precisely one of the two possible states *open* and B_2. Thus, the two states *open* and B_2, which can not be occupied simultaneously, originate from the same event: the abandonment of state B_1 by the receptor. This means that the rough scheme (1) describing the kinetics of the receptor is more precisely represented by:

(3)



We further postulate that the state B_1 is unliganded. This in particular means that the opening of the receptor: $B_1 \rightarrow$ *open* is caused by a binding of the agonist to the binding (activating) site. It also means that, in an open receptor, the agonist is not permanently attached to the binding site. It switches between the state when it is bound, the states when it is off the binding site, again the state in which it is bound, and so on. The existence of two binding sites prevents the receptor from being shut: in moments when the agonist leaves one binding site the receptor will still be in an open state, provided that another agonist molecule is bound in the second binding site. The same is true when the second agonist molecule leaves the binding site: as only when the first agonist molecule is bound, will the receptor be

opened. We assume that after leaving the binding site the agonist eventually comes back, due to the attractive electric force associated with the negative charge of the close-to-the-binding-site-amino acid D200. This assumption was confirmed by our experiments: we performed a series of experiments with a mutated αD200Q receptor in which the amino acid D200 was replaced by a polar amino acid Q (with much smaller negative charge). As we expected, in such a mutated receptor the openings were much shorter than the openings of the wild type receptor (Fig. 1B).

These considerations enable us to formulate the following 'oscillating' model of the receptor opening:

- The receptor has two sites, say A and B, at which the agonist (also called the activator) can be bound. It opens immediately after one molecule of the activator is bound to A or B, and stays open as long as *at least one* of the binding sites A or B is occupied. The activators dissociate from A and B after some time, t_A and t_B, respectively. These times have the same exponential probability distribution with parameter $\lambda > 0$.

- Let us assume that the receptor opens because of an agonist binding at A. Then the agonist stays bound to the receptor at A for time t_A. We also assume that the second binding site binds the second agonist's molecule after some time (which depends on agonist concentration), say η, measured from the moment of the opening of the receptor. η is a random variable which, if it is greater than t_A, the opening time of the receptor is simply t_A.

- If $\eta \leq t_A$ the receptor is still opened, but now with *both* A and B occupied. In such situation each of the two agonists may dissociate independently after the respective times t_A and t_B. Each of them returns to its binding site after time t_x, provided that the other agonist is still bound. The time t_x is a random variable having the exponential distribution with the parameter $\mu > 0$, which is the same for both agonist molecules.

- The situation in which the molecules from A and B leave and return from and to their binding sites repeats, until the moment in which both binding sites are empty. In such situation the receptor shuts down.

The above assumptions about the receptor's kinetics suffice to derive an explicit formula for the probability $\mathbf{P}\{opentime > t\}$ of the receptor's open time. This original derivation is given in the Appendix.

Assuming that the shift time η is a random variable with probability distribution $F(x)$ not depending on t_A, t_B and μ, and denoting τ=opentime, we obtain the formula for $\mathbf{P}\{\tau > t\}$ given in the Theorem 1 in the Appendix (expression 7).

We emphasize that the *analytic* expression (7) for the open time distribution is in total agreement with Monte Carlo simulations of the open time, assuming that the receptor obeys the proposed model and has the shift time distribution $F(x) = 1 - \mathrm{e}^{-\nu x}$ (see Fig.6).

The formula (7) is a superposition of three exponential functions. The first one, which has the time constant -$1/s_1$, we attribute to the distribution of long openings. The brief openings consist of two closely spaced components with the corresponding time constants equal to: -$1/s_2$ and $1/(\nu+\lambda)$. Note, that neither of them has a time constant equal to the mean time a single agonist molecule stays in the activating site, contrary to what is often assumed for brief openings. Note also that three components in the open time distribution were already observed in high resolution (Hallermann et al., 2005, Parzefall et al., 1998) and classical (Hatton et al., 2003) single channel recordings. The formula (7) fitted by the minimum χ^2 method was in perfect agreement with the open time distributions measured for WT and αD200Q receptors in all 38 recordings. Examples of the comparison of the theoretical distribution (7) with experimentally obtained distributions of the open events for both receptors are shown on Fig.6. The mean values of the fitting parameters λ and μ did not depend on agonist concentration and were: $\lambda = 1.03\pm0.19$ ms^{-1}, $\mu = 19.0\pm4.6$ ms^{-1} for the WT receptor ($n = 17$) and $\lambda = 1.35\pm0.11$ ms^{-1}, $\mu = 3.30\pm0.76$ ms^{-1} for the αD200Q receptor ($n = 21$). Similar values of the λ parameter suggest that the times the agonist stays in the activating site (t_A and t_B) are not affected by the presence of the amino acid D. However, since the μ parameter obtained for the WT receptor differs significantly (P <0.0001) from the same parameter obtained for the mutated receptor, we suggest that when closed to the activating site, the agonist stays in the electric field of the amino acid D. This refers to the open receptor. Mean parameters ν obtained for both receptors do not differ significantly (for the WT receptor $\nu = 3.64\pm1.20$ ms^{-1} and for the αD200Q receptor $\nu = 1.64\pm0.47$ ms^{-1}), despite the different agonist concentration used in the experiments. Thus, ν parameter depends not only on the agonist concentration, but also on the presence of the amino acid D.

We now pass to the discussion of the receptors's blocking mechanism.

We assume that the unblocked receptor acts according to the kinetic model presented above. This model admits a shortening of the opening time in the situation when one of the binding sites is unoccupied. In such situation the returning-to-its-binding-site-agonist may encounter a molecule that blocks its entrance to the site (without really binding to this site), resulting in turn in the closure of the receptor. We postulate that the molecule responsible for such a block is HC.

To present the detailed blocking mechanism we first explain what happens in the blocked receptor with the agonist molecules which activated it. The observed existence of bursts suggests that these molecules do not

return to the extracellular solution. Indeed, if the receptor in the B_1 state was activated by binding the agonist molecules from the extracellular solution, then $\tau_{blocked}$ would *decrease* with the agonist concentration. Since this is not observed (Fig. 5B), we conclude that the agonist needed for the transition $B_1 \rightarrow open$ comes from the region which is not accessible to other molecules of the activator. Since it is widely accepted (Miyazawa et al., 1999) that the receptor's activating sites are located in cavities accessed through narrow tunnels, we conclude that even after the dissociation the activating molecules remain in their tunnel-cavities. Since these cavities are of the correct size and shape to accommodate one ACh molecule (Miyazawa et al., 1999) no other agonist molecule could bind to its binding site until the ACh molecule leaves the tunnel-cavity. Note that the agonist staying in the cavity-tunnel experiences the electrostatic repulsion from other agonist molecules trying to enter the tunnel. It forces the agonist to stay at the bottom of the tunnel. If the agonist binding site is not located there, then $\tau_{blocked}$ should *increase* with the ACh concentration. Such a behavior of $\tau_{blocked}$ was observed in our experiments with receptor αD200Q (P <0.001, Fig. 5B). We thus postulate that the agonist binding site is situated in another place than the end of the tunnel (Fig. 7).

The main difference between state B_1 and the closed state is that in state B_1, both tunnels associated with the activating sites are occupied by the corresponding agonist molecules, which at every moment may be bound to the activating sites (recall that state B_1 is a state without a blocker). Surprisingly state B_1 (without a bound blocker) lasts longer than the blocked state B_2. This is again caused by the repulsive action of the outside agonists molecules which are close to the entrance to the tunnel. They prevent the agonist in the tunnel from reaching the binding site. Since these molecules are not present at the entrance to the tunnel all of the time, there are moments when the entrance to the tunnel is free. This occurs with frequency a. At such moments the agonist within the tunnel can approach the entrance and bind to the site, opening the receptor again. Moments when the entrance to the tunnel is not occupied by the outside agonist molecules can be also used by the blocker to enter the tunnel. The blocker, as electrically neutral, does not feel the repulsive potential of the agonist within the tunnel. Entering the tunnel, it can freely penetrate it up to the agonist binding site. Once there, it prevents the inside agonist from binding. Note that the presence of the blocker does not decrease the duration of the B_1 state: the blocker has a chance to enter the tunnel only when an agonist may return to the binding site, thus only when the receptor leaves state B_1. This agrees with scheme (3).

Since external molecules repulse the inside tunnel-cavity agonist from the activating site, it is clear that τ_{block} is longer in a receptor in which the entrance to the tunnel is more frequently occupied by the agonist. The measured $\tau_{blocked}$ (Fig. 5A) indicates that such situation occurs in the WT receptor (despite of the lower concentration of the agonist!), which thus must have agonist's molecules at the entrance to the tunnel more often than the αD200Q receptor. We believe that the reason for this difference is the negative charge of the amino acid $D200$, which most probably attracts positively charged molecules of the activator towards the entrance of the tunnel in the WT receptor. This also explains why the probability of the opening of the WT receptor is higher than the probability of the opening of the αD200Q receptor.

This justifies our next postulate, that the location of amino acid $\alpha 200$ is different in an open and a closed receptor: if the receptor is open, the location of amino acid $\alpha 200$ is close to the activating site *inside* the cavity-tunnel; if the receptor is closed, $\alpha 200$ is located *outside* the cavity-tunnel, but still close to its entrance.

Summing up: it is the HC molecule that initiates the blockage of the receptor. At the initial stage of the block ACh molecules do not act as blockers, since the open receptor does not attract them from the extracellular region toward the tunnel-cavity where the block occurs. Once the receptor is in the blocked state the situation changes: the closed receptor exposes the negative charge to the outside of the tunnel, starting the process of attracting the extracellular molecules towards the tunnel-cavity. Thus, in the process of blocking, both ACh and HC participate. On the one hand the ACh molecule, located at the entrance to the tunnel-cavity, repulses the agonist trapped in the tunnel from the activating site, on the other hand the HC molecule, when the tunnel entrance is empty, enters into it, preventing other molecules from reaching the activating site. If the location of the amino acid D was changing with the membrane potential, then the action of HC would be unaffected (HC is an uncharged molecule). But it would alter the action of ACh. We postulate that the amino acid D location *is* voltage dependent. This implies the voltage dependency of τ_{block} in the WT receptor. In αD200Q the situation is different. The frequency of the ACh approaches to the tunnel cavity mainly depends on the ACh concentration, and not on the attraction of the amino acid $\alpha 200$, which in the case of the amino acid Q is weak. Thus in the mutated receptor $\tau_{blocked}$ has no significant voltage dependence (Fig. 4).

Finally we emphasize, that although the state B_1 is very peculiar, we believe that it occurs very commonly. For this state to exist the blocker is not needed. It is a closed state, which differs from the usual closed state by the presence of unbound agonist molecules in the tunnel-cavities in both subunits. If such a state randomly emerges (e.g. in high concentration of the agonist) then, if the agonist concentration remains high, exiting from this state would be very difficult: the highly concentrated activator molecules will be present at the entrance to the tunnel-cavity repulsing the agonist from the binding site. We associate our state B_1 with a particular closed state of the receptor which is reported to be observed only in

high agonist concentration and to be characterized by a very long duration. Thus, state B_1 corresponds to the desensitization of the receptor. That in the process of this desensitization amino acid $D200$ plays a role is confirmed by our experiments, in which such desensitization was not present in the receptor αD200Q, even if we went to agonist's concentration as high as 0.1 mM.

Experiments with other blockers and αD200 mutants are needed to confirm the role of αD200 amino acid in mechanisms of the receptor block and desensitization. A presence of charged amino acids close to the agonist binding pockets may be in this family of receptors (shared by the nicotinic, the $GABA_A$ and the 5-HT_3 receptors) the common feature (Schreiter et al., 2003, Hartvig et al., 2000), making the proposed model of the receptor desensitization universal for other ligand-gated ion channels.

V. APPENDIX

Let us consider a system formed by two identical servers (say A and B) working independently and let ξ_1, ξ_2 stand for the time of their trouble-free operation. It is supposed that $\mathbf{P}\{\xi_i > t\} = e^{-\lambda t},\ \lambda > 0,\ t \geq 0,\ i = 1, 2$. At the initial time $t = 0$ both servers are in working condition and, say, server A starts to work. The second server starts to work with some delay $\zeta \geq 0$ having a distribution $\mathbf{P}\{\zeta < t\} = F(t),\ t \geq 0$. In the case of a breakdown of any server its repairing starts immediately and it lasts η_1, η_2 time-unites respectively. It is supposed that $\mathbf{P}\{\eta_i > t\} = e^{-\mu t},\ \mu > 0,\ t \geq 0 i = 1, 2$. After the repair is finished the server starts to work again. The above mentioned random variables are all supposed to be independent.

The breakdown of the whole system occurs if both servers are not in working conditions (i.e., they are being repaired).

Let τ denote the time at which the system breaks down for the first time. We are interested in finding $\mathbf{P}\{\tau > t\}$. This is given in the following theorem.

Theorem 1

$$\mathbf{P}\{\tau > t\} = \int_0^t e^{-\lambda u}\Psi(t-u)dF(u) + (1 - F(t))e^{-\lambda t}, \tag{4}$$

where

$$\Psi(t) = \frac{(s_2 + \mu + 3\lambda)e^{s_2 t} - (s_1 + \mu + 3\lambda)e^{s_1 t}}{s_2 - s_1}, \tag{5}$$

$$s_{1,2} = \frac{-\mu - 3\lambda \pm \sqrt{\mu^2 + 6\mu\lambda + \lambda^2}}{2}. \tag{6}$$

And if $F(t) = 1 - e^{-\nu t},\ \nu > 0,\ t \geq 0$, then

$$\mathbf{P}\{\tau > t\} = \frac{\lambda(\nu - \mu)}{(\lambda + \nu)(\nu - \mu - 2\lambda) + 2\lambda^2} e^{-(\nu+\lambda)t} + \frac{\nu}{s_2 - s_1}\Big(\frac{s_2 + \mu + 3\lambda}{\lambda + \nu + s_2}e^{s_2 t} - \frac{s_1 + \mu + 3\lambda}{\lambda + \nu + s_1}e^{s_1 t}\Big). \tag{7}$$

Proof.
Consider first the simple case when only one server is present and let $P(t)$ denote the probability of its being in working condition at time t. The memoryless property of an exponential distribution allows us to write down the following equation

$$P(t) = e^{-\lambda t} + \lambda\mu \int_0^t e^{-\lambda u} \int_0^{t-u} e^{-\mu v} P(t - u - v)dvdu, \quad t > 0$$

which implies

$$P(t) = \frac{1}{\mu + \lambda}\Big(\mu + \lambda e^{-(\lambda+\mu)t}\Big), \quad t \geq 0. \tag{8}$$

Now we consider the original system but without delay, i.e., $\zeta \equiv 0$, and let $\Psi(t)$ denote the probability that such a system will be work without breakage up to time t. Using the memoryless property of exponential distributions of the random variables ξ_i, η_i, $i = 1, 2$, and the formula (8), we get

$$\Psi(t) = e^{-\lambda t} + \frac{\lambda\mu}{\lambda + \mu} \times \tag{9}$$
$$\int_0^t e^{-\lambda u}(\mu + \lambda e^{-u(\lambda+\mu)}) \int_0^{t-u} e^{-(\mu+\lambda)v}\Psi(t - u - v)dvdu$$
$$+ \frac{\lambda}{\lambda + \mu}\int_0^t e^{-\lambda u}(\mu + \lambda e^{-u(\lambda+\mu)})e^{-(\mu+\lambda)(t-u)}du, \quad t > 0.$$

To find the function $\Psi(t)$ we use the method of Laplace transform. If we denote

$$\psi(s) = \int_0^\infty e^{-st}\Psi(t)dt, \quad s > 0,$$

and apply to (9) the Laplace transform with a parameter $s > 0$, we get

$$\psi(s) = \frac{1}{s + \lambda} + \frac{\lambda\mu}{\mu + \lambda}\Big(\frac{\mu}{s + \lambda} + \frac{\lambda}{s + \mu + 2\lambda}\Big)\frac{1}{s + \mu + \lambda}\psi(s) +$$
$$+ \frac{\lambda}{\mu + \lambda}\frac{1}{s + \mu + \lambda}\Big(\frac{\mu}{s + \lambda} + \frac{\lambda}{s + \mu + 2\lambda}\Big),$$

which gives

$$\psi(s) = \frac{s + \mu + 3\lambda}{s^2 + s(\mu + 3\lambda) + 2\lambda^2} = $$
$$= \frac{1}{s_2 - s_1}\Big(\frac{s_2 + \mu + 3\lambda}{s - s_2} - \frac{s_1 + \mu + 3\lambda}{s - s_1}\Big), \tag{10}$$

where the numbers $s_{1,2}$ are defined in (6). Taking the inverse Laplace transform, we obtain (5). If now a delay is present and its distribution is $\mathbf{P}\{\zeta < t\} = F(t)$, then we obtain (4). **End of Proof.**

VI. ACKNOWLEDGMENTS

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[1] Akk, G., Sine, S., Auerbach, A., 1996. Binding sites contribute unequally to the gating of mouse nicotinic D200N acetylcholine receptors. J. Physiol 496, 185-196.

[2] Akk, G., Steinbach, J.H., 2003. Activation and block of mouse muscle-type nicotinic receptors by tetraethylammonium. J Physiol 555, 155-168.

[3] Arias, H.R., 2000. Localization of agonist and competitive antagonist binding sites on nicotinic acetylcholine receptors. Neurochem Int 36, 595-645.

[4] Bouzat, C., Barrantes, F.J., 1996. Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone. Possible allosteric mechanism of channel blockade. J Biol Chem 271, 25835-25841.

[5] Buisson, B., Bertrand, D., 1998. Open-channel blockers at the human alpha4beta2 neuronal nicotinic acetylcholine receptor. Mol Pharmacol53, 555-5 63.

[6] Colquhoun, D., Sakmann, B., 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. J Physiol 369, 501-557.

[7] Hallermann, S., Heckmann, S., Dudel, J., Heckmann, M., 2005. Short openings in high resolution single channel recordings of mouse nicotinic receptors. J Physiol 563, 645-662.

[8] Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391, 85-100.

[9] Hartvig, L., Lukensmejer., B., Liljefors, T., Dekermendjian, K., 2000. Two conserved arginines in the extracellular N-terminal domain of the $GABA_A$ receptor α_5 subunit are crucial for receptor function. J Neurochem 75, 1746-53.

[10] Hatton, C.J., Shelley, C., Brydson, M., Beeson, D., Colquhoun, D., 2003. Properties of the human muscle nicotinic receptor, and of the slow-channel myasthenic syndrome mutant eL221F, inferred from maximum likelihood fits. J Physiol, 547, 729-760.

[11] Hille, B. 2001 Ion Channels of Excitable Membranes. Sinauer Associates, Massachusetts.

[12] Leonard, R.J., Labarca, C.G., Charnet, P., Davidson, N., Lester, H.A., 1988. Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. Science 242, 1578-1581.

[13] Loftsson, T., Matthiasson., K., Masson, M., 2003. The effects of organic salts on the cyclodextrin solubilization of drugs. Int J Pharm 262, 101-107.

[14] Miyazawa, A., Fujiyoshi, Y., Stowell, M., Unwin, N., 1999. Nicotinic Acetylcholine Receptor at 4.6 A resolution: Transverse Tunnels in the Channel Wall. J Mol Biol 228, 765-786.

[15] Mukhtasimova, N., Free, C., Sine, S.M., 2005. Initial coupling of binding to gating mediated by conserved residues in the muscle nicotinic receptor. J Gen Physiol 126, 23-39.

[16] Neher, E., Steinbach, J.H., 1978. Local anesthetics transiently block currents through single acetylcholine-receptor channels. J. Physiol 277, 153-176.

[17] Nurowska, E., Ruzzier, F., 2002. Modulation of acetylcholine receptor channel kinetics by hydrocortisone. Biochim Biophys Acta 1564, 14-20.

[18] O'Leary, M.E., White, M.M., 1992. Mutational analysis of ligand-indduced activation of the Torpedo acetylcholine receptor. J Biol Chem 267, 8360-8365.

[19] Parzefall, F., Wilhelm, R., Heckmann, M., Dudel, J., 1998. Single channel currents at six microsecond resolution elicited by acetylcholine in mouse myoballs. J Physiol 512, 181-188.

[20] Schreiter, C., Hovius, R., Costioli, M., Pick, H., Kellenberger, S., Schild, L., Vogel, H., 2003. Characterization of the ligand-binding site of the serotonin 5-HT3 receptor: the role of glutamate residues 97, 224, and 235. J Biol Chem 278, 22709-16.

[21] Sine, S.M., 1994. Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of residues that determine curare selectivity. PNAS 90, 9436-9440.

[22] Sine, S.M. Steinbach, J.H. 1987. Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by high concentrations of agonist. J Physiol 385, 325-359.

[23] Wachtel, R.E. 1993. Physostigmine block of ion channels activated by acetylcholine in BC3H1 cells. Mol Pharmacol 44, 1051-1055.

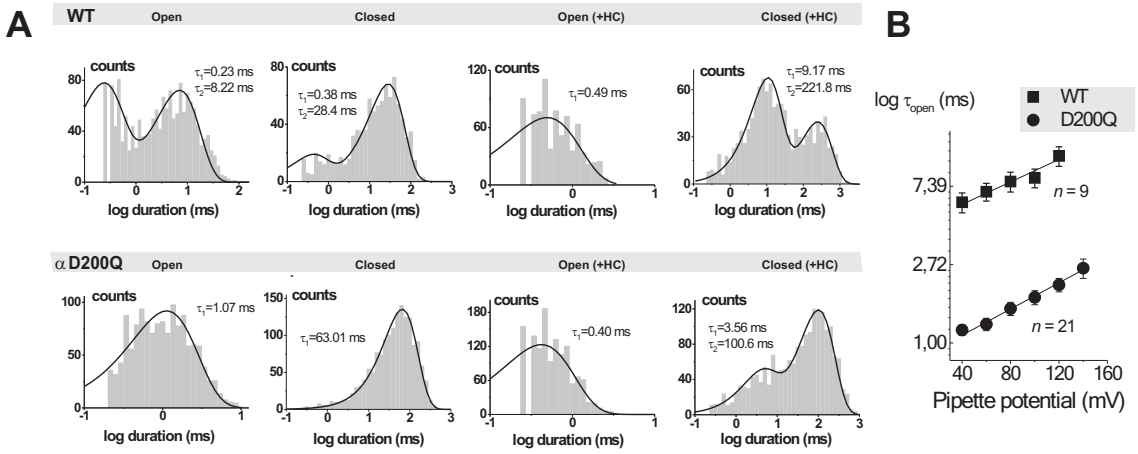

FIG. 1: (A)Dwell-time histograms obtained from single-channel recordings from WT and αD200Q receptors at +60 mV of pipette potential in control conditions and in the presence of 0.5 mM HC fitted with a single or a double exponential function with time constants τ_i; ACh concentration was 500 nM in recordings from WT and 100 μM from αD200Q receptors. (B) Voltage dependency of the τ_{open} of the WT and αD200Q receptors. Data points represent the mean $\pm$ SEM from n membrane patches. Points are plotted on a logarithmic scale and fitted with a function $f = A\exp((V-60)/b)$ where V is a pipette potential, $A = 6.93$ ms, $b = 139$ mV for the WT receptor and $A = 0.8$ ms, $b = 119$ mV for αD200Q receptor; note the similar slope (similar voltage-dependency) of both plots.

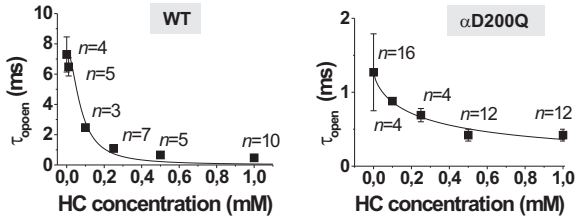

FIG. 2: HC decreased the open-channel lifetime. Plots show the mean τ_{open} $\pm$ SEM from n patches at +60 mV of pipette potential; ACh concentration was 500 nM in recordings from WT and 100 μM from αD200Q receptors. Solid line points are fitted with a function $f = 1/(\alpha + k_{+b}[x]^h)$ where x is HC concentration; $\alpha = 0.13$ ms^{-}1, k_{+b} =15.54 mM^{-h} ms^{-1}, $h = 1.84$; in WT receptor; $\alpha = 0.78$ ms^{-1}, k_{+b} =1.98 mM^{-h}ms^{-1}, $h = 0.7$ in αD200Q receptor.

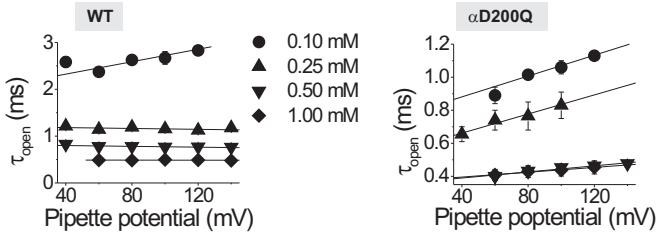

FIG. 3: The mean τ_{open} $\pm$ SEM at different membrane potentials and different HC concentrations. Solid lines are determined by a linear regression; n - from 2 to 7;

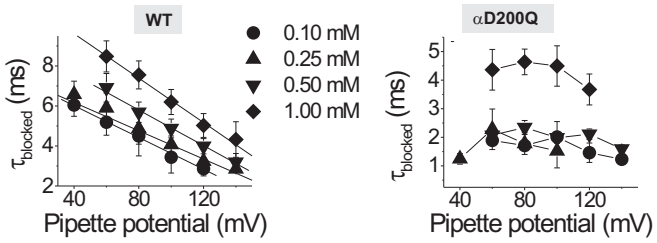

FIG. 4: The mean $\tau_{blocked}$ ± SEM at different membrane potentials and different HC concentrations; n - from 2 to 7; WT receptor: each solid line determined by a linear regression; the slope significantly differs from 0 for all HC concentrations (P<0.01); αD200Q receptor: for [HC]< 1 mM any clear relationship of the mean $\tau_{blocked}$ with membrane potential increase is not present.

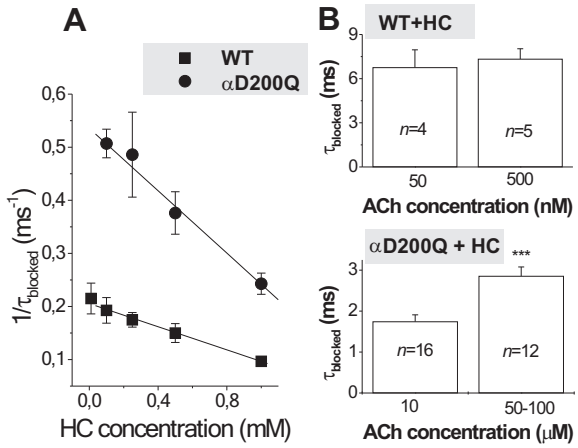

FIG. 5: (A) The reciprocal of the mean $\tau_{blocked}$ ± SEM at +60 mV of pipette potential at different HC concentrations; n - the same as in Fig. 2; ACh concentration was 500 nM in recordings from WT and 100 μM from αD200Q receptors; each line determined by a linear regression. (B) The mean $\tau_{blocked}$ ± SEM in the presence of 0.5 mM HC (n - number of cells); *** P <0.001.

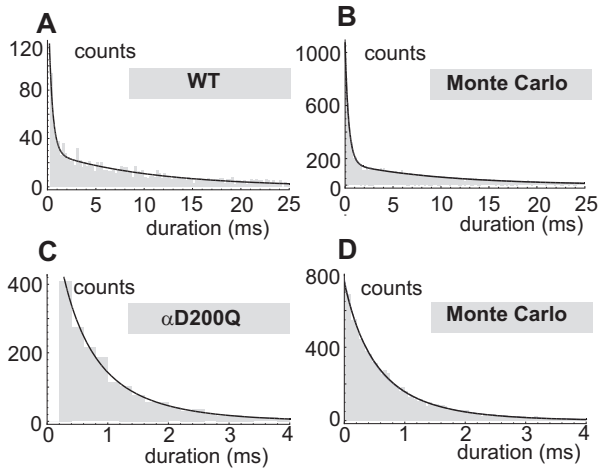

FIG. 6: (A) The example of the experimentally obtained open time distribution of WT receptor fitted with the derivative of the function (7). Fitting parameters are: $\lambda = 0.529$ ms^{-1}, $\mu = 4.343$ ms^{-1}, $\nu = 1.986$ ms^{-1}. The function is: $355.8(0.39\mathrm{e}^{-2.5t} + 0.19(0.30\mathrm{e}^{-5.84t} + 0.41\mathrm{e}^{-0.09t}))$. (B) The example of the distribution obtained by Monte Carlo simulation of the 'oscillating' model presented in the paper with the same parameters as in A fitted with the derivative of the function (7); simulation performed for 10000 events; fitting parameters are: $\lambda = 0.540$ ms^{-1}, $\mu = 4.409$ ms^{-1} and $\nu = 1.903$ ms^{-1}. (C) The example of the experimentally obtained open time distribution of αD200Q receptor fitted with the derivative of the function (7). Fitting parameters are: $\lambda = 1.562$ ms^{-1}, $\mu = 1.624$ ms^{-1}, $\nu = 0.648$ ms^{-1}. The function is: $464.8(0.81\mathrm{e}^{-2.2t} + 0.70(0.31\mathrm{e}^{-5.4t} + 0.77\mathrm{e}^{-0.90t}))$. (D) The example of the distribution obtained by Monte Carlo simulation of the 'oscillating' model presented in the paper with the same parameters as in C fitted with the derivative of the function (7); simulation performed for 10000 events; fitting parameters are: $\lambda = 1.510$ ms^{-1}, $\mu = 1.305$ ms^{-1} and $\nu = 0.473$ ms^{-1}.

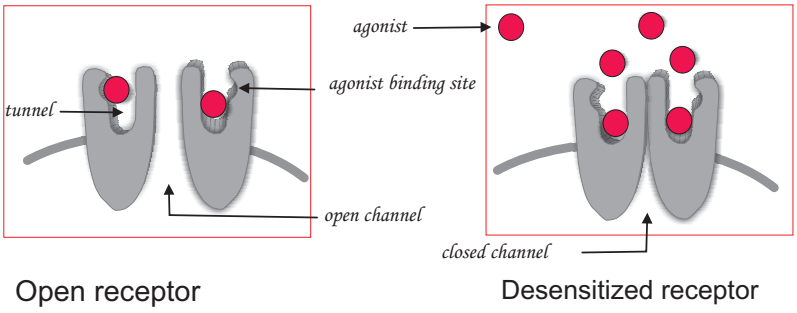

FIG. 7: Model of nACh receptor in two configurations: open and desensitized