Galantamine modulates the recovery from desensitization of nicotinic receptors in TE 671 cells

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Abstract

Galantamine, an anti-cholinesterasic drug, is also known to allosterically potentiate the activation of nicotinic receptors. In this study, we investigated whether galantamine may additionally influence the nicotinic receptor recovery from desensitization. Whole-cell patch clamp experiments were performed on TE 671 cells, which natively express muscle-type nicotinic receptors, and desensitization was assessed based on a repetitive stimulation protocol. The desensitization process was studied at different ACh concentrations (10 – 100 μM) alone or in the combination with galantamine (0.1 – 5 μM). Both in the absence and in the presence of galantamine, the decay of the peak current elicited by agonist application within each stimulation train was well described by one simple exponential function. A comparative analysis of the time constants and the values of peak steady-state current obtained for each experimental condition showed: (i) an apparent non-linear desensitization with increasing ACh concentrations and (ii) a modulatory effect of galantamine on desensitization with an apparent non-linear dependency on the drug concentration. The different desensitization patterns observed for the various combinations of ACh/galantamine concentrations can be explained by assuming the existence of at least two functional populations of nicotinic receptors differing in which regards the desensitization behavior and their affinity for ACh and galantamine.

Keywords: galantamine, nicotinic receptors, desensitization, patch-clamp

Introduction

Galantamine is an anticholinesterasic drug, currently approved for the treatment of Alzheimer's disease, which acts on the nicotinic pathogenic pathways by increasing the available amount of synaptic acetylcholine (ACh) in the central synapses (SRAMEK & al. [25]). Recent clinical studies have shown a mild improvement of the cognitive deficit in Alzheimer-like dementias when galantamine was used orally (16-24 mg/day; WILCOCK, [27]; CUMMINGS, [6]; ROCKWOOD & al. [21]). This result confirms previous studies on rabbits showing an increase of the animal's learning capacity in response to drug treatment (WOODRUFF-PACK & al., [28]).

Besides its anticholinesterasic action, galantamine is known to act directly on nicotinic receptors. Galantamine binding to nicotinic receptors has been demonstrated both *in vitro,* on the neuroblastoma derived cell line SH-SY5Y (DAJAS-BAILADOR & al. [7]) or in hippocampic neurons (SANTOS & al. [23]), and in vivo (in New Zealand White rabbits – WOODRUFF-PACK & al. [28]). Previous results by other groups (AKK & STEINBACH [1]; DAJAS-BAILADOR & al. [7]; LOPES & al. [16]) showed that galantamine has a bell-shaped modulatory effect on nicotinic receptor activation, by allosteric potentiation. Additionally, Samochocki and collaborators [22] showed that galantamine modulates the activity of three types of human nicotinic receptors (α3β4, α4β2, α6β4) expressed in HEK 293 cells. They report a bell-shaped effect of galantamine on 30 μM ACh induced currents, with a peak response at 1 μM galantamine. The bell-shaped effect has also been confirmed by other methods: (i) fluorescence detection of intracellular Ca^{2+} increase due to nicotinic stimulation in SH-SY5Y cells (DAJAS-BAILADOR & al. [7]), (ii) GABA-induced currents triggered by ACh release in rat hippocampus slices (SANTOS & al, [23]), and (iii) potentiation of 1 mM choline-induced currents in cultures of hippocampus neurons, at concentrations ranging from 1-10 μM galantamine, with an inhibitory effect at 100 μM (LOPES & al. [16]) Although galantamine can bind to the receptor in a noncompetitive fashion (AKK & STEINBACK [1]), its action has been shown to be inhibited by specific competitive nicotinic blockers as mecamylamine (DAJAS-BAILADOR & al. [7]; ARIAS & al. [4]). Recent results from Hansen and Taylor [12], using the soluble ACh-binding protein as a substitute model for both the analysis of ligand binding and X-Ray structural studies, suggest that galantamine could act at both competitive and non-competitive binding sites on the nicotinic ACh receptors. Galantamine shows no effects on muscarinic ACh receptors activity (SAMOCHOCKI & al. [22]). It is possible that at concentrations higher than 10 μM galantamine acts as an inhibitor (SAMOCHOCKI & al. [22]).

Moreover, studies by Fayuk and Yakel [10], working on hippocampal slices from 14- to 19-day- old rats, showed that acetylcholine-esterase inhibitors do not influence the current amplitude or kinetic of α7 nicotinic

receptors, but that they slow down the rate of recovery from desensitization by an indirect mechanism, increasing the available quantity of ACh by blocking the esterase. For non-α7 receptors, these inhibitors (including galantamine) significantly increased the ACh induced currents and slowed the decay phase of the nicotinic currents.

This study addresses the effects of galantamine on nicotinic receptor recovery from desensitization, by using a sequential stimulation protocol in order to discriminate between desensitization states of the nicotinic ACh receptors with different recovery times. The TE 671 cell line derived from human caucasian rhabdomyosarcoma, natively expressing muscular subtype of nicotinic receptors (OSWALD & al. [19]; SCHOEPFER & al. [24]), was used for electrophysiological measurements. Transient currents through the nicotinic receptors were recorded in the whole-cell configuration of the patch-clamp technique (HAMILL & al. [11]) after the application of ACh concentration jumps by means of fast perfusion methods. This paper reports the effects of different concentrations of galantamine on nicotinic receptor desensitization. For each ACh concentration studied we have observed a non-linear effect of galantamine concentration in desensitization. The results can be explained by assuming the co-existence of receptor subpopulations with different desensitization behavior and different affinities for both ACh and galantamine.

Materials and Methods

Cell culture

Cultures of Human Caucasian Rhabdomyosarcoma TE 671 cells (ECACC No. 89071904), were grown in DMEM medium (Dulbecco's modified eagle medium, with GlutaMAX^{TM}I and high glucose 4.5 g/l, and without sodium pyruvate, GIBCO-Invitrogen), supplemented with 10 % (v/v) bovine fetal serum and antibiotics (50 I.U/ml penicillin and 50 U.G/ml streptomycin). Cultures were kept at 37ºC and in a humidified atmosphere of 5% CO_2 (in air). Subculture was done by trypsinization every 4-5 days, when the cell cultures reached ~50-60% growth confluence.

Electrophysiology

Electrophysiological recordings were performed 24-72 hrs after cell subculture with an Axopatch 200A amplifier (Axon Instruments), under voltage clamp conditions in the whole-cell current recording configuration (HAMILL & al. [11]). Cell membrane was held at -70 mV for all measurements. Recording electrodes were pulled from borosilicate glass capillaries (Science products GB150T-8P) with a PB-7 vertical puller from Narishige and had typical open-tip resistances of 2–4 MΩ. Transient currents by the ACh receptor due to ACh concentration jumps were elicited by means of a fast perfusion system. Different reservoirs containing the test solutions were connected to delivery tubes (200 μm diameter) positioned ~300-500 μm away from the cell. The flow rate was controlled by positive pressure and it was adjusted prior to sealing by means of perfusing with a solution of a different refractive index (usually water) that can be visualized under the microscope. The delivery of solutions to the cell and its re-uptake (suction in between applications) were controlled by a system of piston pinch valves (General Valve Corporation, Fairfield, USA) commanded automatically (pClamp8 software, Axon Instruments, Foster City, CA, USA) by means of a computer connected to a trigger box (homemade). The whole-cell recordings of the transient currents were elicited by application of ACh alone or in combination with galantamine and/or the nicotinic receptor inhibitor metyllycaconitine, at different concentrations. The drug-containing solutions were applied to the cells in "stimulation trains". A stimulation train consisted in a sequence of concentration jumps with the duration of 1 second, 5 seconds apart. The number of drug applications in a stimulation train was variable for different cells, with application being repeated until steady-state desensitization was reached (when peak current did not change for two or three consecutive applications). To prevent accumulation of ACh in the bath solution in between pulses we adapted a peristaltic pump connected to a tube placed next to the cell.

Data were recorded with a digitizer board Digidata 1320A (Axon Instruments), which was controlled by pClamp 8 software (Axon Instruments), digitized with a sampling rate of 100 kHz and lowpass-filtered at 5 kHz. All experiments were performed at room temperature.

Solutions and Chemicals

Cell currents were measured in a bath solution containing (in mM): 135 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 HEPES (pH 7.4/NaOH). The pipette solution contained (in mM): 140 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 11 EGTA (pH 7.2/CsOH). Extracellular solutions containing ACh, galantamine and/or metyllycaconitine were prepared fresh before the experiment from 1 mM stock solutions (kept frozen). All drugs were purchased from Sigma.

Data analysis

Raw data analysis was performed with Clampfit 8.2 (pClamp8 software, Axon Instruments, Foster City, CA, USA). For each stimulation train, peak currents were measured and their values normalized to the first elicited peak current value. For each set of tested drug concentrations, data from different cells were averaged. When indicated,

the data shown are mean normalized current ± SE (n), where n is the number of cells used for a particular experiment. The trend of the averaged normalized peak current values for each experimental condition was fitted with a single exponential function that decayed with a characteristic time constant (τ) and reached a steady-state value I_{ss}. The statistical significance of the difference between the fits was determined with an F-test at 95% confidence level (*$p<0.05$). Statistical analysis and data fitting was performed with OriginPro 7.0 (Microcal, Northampton, MA).

Results

ACh – induced desensitization

In this work we investigated the possible effects of galantamine on nicotinic receptor desensitization. The occurrence of receptor desensitization with prolonged ACh stimulation has been first observed by Katz and Thesleff [13] at the frog neuromuscular junction, being followed by a slow recovery from desensitization once the stimulus is removed. Subsequent studies have shown that nicotinic receptor desensitization is a complex phenomenon, involving more than one component and molecular processes which occur at different time scales (e.g. ELENES & AUERBACH [9]; AUERBACH & AKK [3]). Here, an appropriate protocol was established in order to study the desensitization events occurring on the seconds time scale, by means of whole-cell current measurements. The duration of the ACh pulse and the period between pulses were the two important parameters that had to be adjusted in the stimulation protocol in order to achieve measurable effects. Repeated long ACh application periods (10 - 100 μM for 3 - 10 seconds) caused a strong reduction of the peak currents with repetitive stimulation, or even no response (complete desensitization). This result is consistent with desensitization occurring when receptors are subjected to prolonged agonist stimulation (CORRINGER & al. [5]). In addition, long intervals between ACh applications ($t > 20$ seconds) showed no obvious variations of peak current amplitude, and therefore no measurable desensitization. The best reproducible current response, in the particular context of our experimental setup, was obtained with 1 second ACh applications separated by 5 seconds intervals, for all the tested concentrations. A typical result is shown in Fig. 1A, where a decrease of peak current amplitude in response to repeated pulses of 10 μM ACh can be observed. Under these experimental conditions, the amplitude of the peak current eventually reaches a stable minimum value (indicated by the dotted line in the picture) and no further variation of the peak current is observed, due to reaching a dynamic balance between desensitization and recovery from desensitization. We will further refer to this condition as *steady-state desensitization*. The currents are inward, as expected for sodium influx through the open receptor, and were inhibited by metyllycaconitine (fig. 1B), a known competitive inhibitor for central (α7) nicotinic receptors (WARD & al. [26]), which also acts on some non-α7 nicotinic receptors (DEZAKI & al. [8], KIMURA & DEZAKI [15]; MOGG & al. [18]).

Nicotinic receptor currents were studied with 10 - 100 μM ACh. At these concentrations the total number of ACh receptors likely to be open was not yet reached, as indicated by increasing current values with greater ACh concentrations (Fig 1C). The currents values considered were the average whole-cell current peak amplitudes for the first agonist application in a stimulation train (therefore, before onset of receptor desensitization).

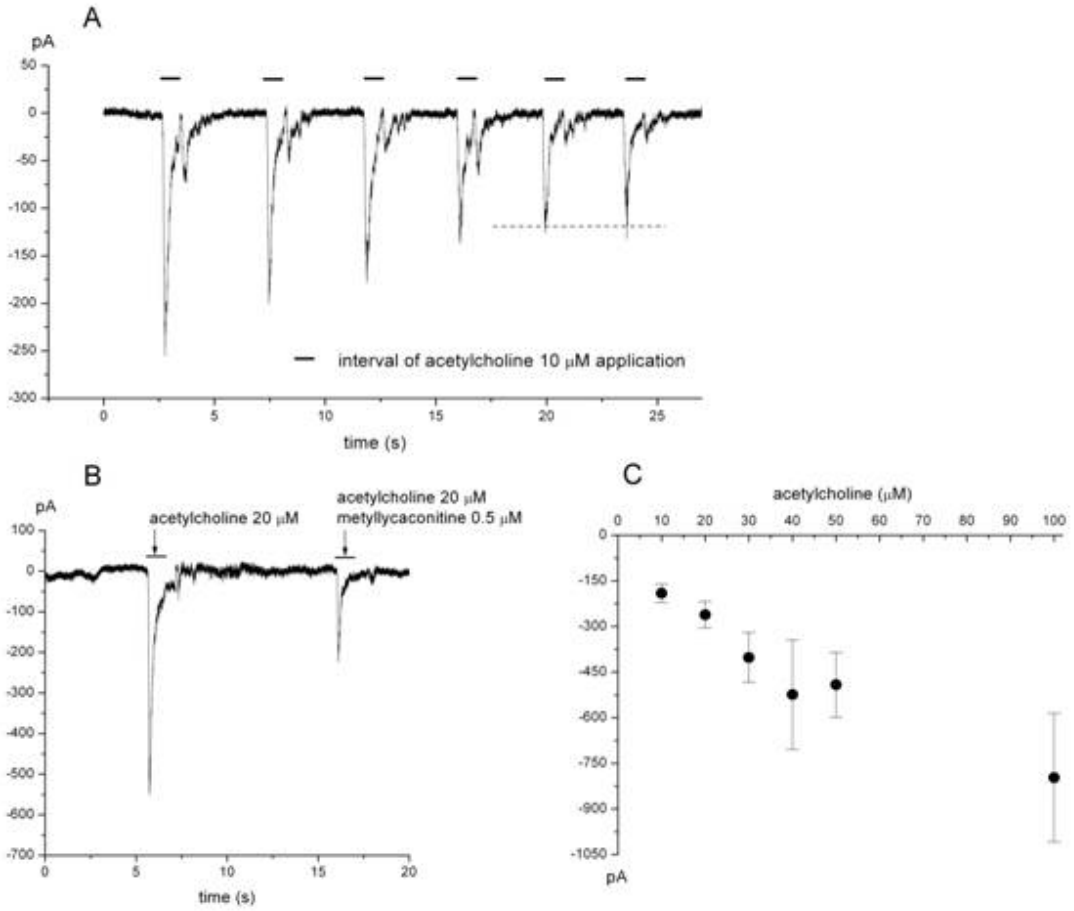

Figure 1. (A) Typical whole-cell currents recorded from a TE 671 cell in response to a 10 μM ACh stimulation train; the bar represents the duration of ACh application and the doted line indicates steady-state desensitization. (B) Inhibitory effect of 50 nM metyllycaconitine on the whole-cell nicotinic currents induced by 20 μM ACh. (C) Dependence of the average non-desensitized whole-cell current on the ACh concentration.

ACh induces receptor desensitization in a concentration-dependent manner (Fig. 2). Average peak current amplitude decreases exponentially over repeated application, and we observe a difference between both the exponentially decrease time constant (τ, s) and the steady-state desensitization values for the different concentrations of ACh. We observe no statistically significant differences between the desensitization curves determined at 10 μM and 20 μM ACh. As the ACh concentration increases, we notice higher desensitization, yet without a linear dependency on the ACh concentration. If for 30 μM and 40 μM, the degree of desensitization increases with the ACh concentration (compared to the desensitization observed with 10 μM ACh), at 50 μM ACh the steady-state value of the peak current is similar to the values observed for 10-20 μM and smaller than the steady-state value found with 30 μM. By further increasing the concentration to100 μM ACh, the degree of desensitization increases when compared to the results obtained with 50 μM, but without outgrowing the maximal value observed in our experiments with 40 μM ACh.

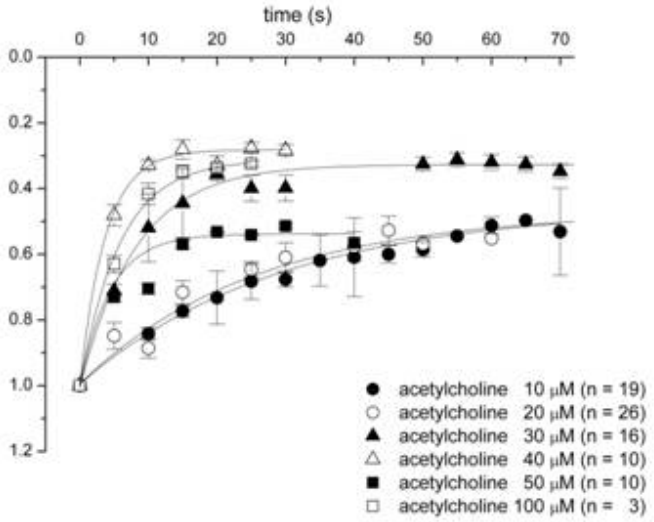

Figure 2. Desensitization trends (time-dependent variation of peak current amplitude with repeated drug applications) for different ACh concentrations. Values are means ± SE, and currents are normalized. The number of tested cells, for each concentration, is indicated between brackets.

Effect of galantamine on ACh-induced desensitization

The effect of galantamine on the receptors desensitization process was studied by analyzing the desensitization trends obtained with different combinations of galantamine and ACh concentrations (Fig. 3). Galantamine concentration jumps alone did not induce receptor currents (not shown). The results show different trends for the action of galantamine on currents induced by different concentrations of ACh. Figure 3A shows the results obtained with 10 μM ACh in the presence and absence of galantamine. There are no statistically significant differences between desensitization observed without galantamine and with 0.1 μM galantamine. With 0.5 μM galantamine, we observed no significant reduction of peak current amplitude between successive applications, which corresponds to no apparent desensitization or very fast recovery from desensitization. As the galantamine concentration increases, the receptor desensitization becomes more important, both in which regards the desensitization rate and the steady-state value. With 1 μM galantamine we still notice less desensitization compared to ACh control. At the highest concentration tested (5 μM), galantamine shows an opposite effect, by increasing the receptor desensitization.

Co-application of 0.1 μM galantamine and 20 μM ACh induces no differences from the control. All the other tested concentrations of galantamine prevented, to a degree, the ACh-induced desensitization (Fig. 3B). In particular, 5 μM galantamine induces a significant difference ($*p < 0.05$) of the desensitization trend, as compared to ACh applied alone. Moreover, we notice a reversed response pattern as compared with the results obtained with 10 μM ACh, since the protective effect seems to be higher with increasing concentrations of galantamine. We did not find a statistically significant difference between the fits for galantamine 0.5 and 1 μM and ACh alone.

Similarly to the results obtained with 20 μM ACh, increasing galantamine concentrations induced less receptor desensitization and/or faster recovery from desensitization when combined to 30 μM ACh (Fig. 3C). The highest effect, leading to very little apparent desensitization over the studied time period, is observed with 5 μM galantamine. There is only a small difference between the steady-state desensitization observed with 0.5 μM galantamine and 1 μM galantamine, but there is an increase of the time constant τ which shows a direct dependency on the galantamine concentration for all the tested concentrations. An interesting aspect is observed with 0.1 μM galantamine, which induces higher steady-state desensitization as compared to the control.

The results obtained with 40 μM ACh are shown in figure 3D. Galantamine exerts an effect on receptor desensitization with 5 μM. With 1 μM galantamine, the desensitization trend closely follows the response obtained with ACh 40 μM applied alone. With 50 μM ACh, 1 μM galantamine also fails to induce significant differences to the control (Fig. 3E), but contrarily to the former example, we noticed diminished desensitization with 5 μM galantamine.

In order to verify whether or not desensitization depends on the ratio between the ACh and the galantamine concentrations, we tested 2.5 μM galantamine with 50 μM ACh (Fig. 3E) and 5μM galantamine with 100 μM ACh (Fig. 3F). These experimental situations present a similar galantamine to ACh ratio as 1 μM galantamine and 20 μM ACh. We notice that the traces corresponding to the three situations differ in which regards the steady-state

desensitization. The results with 20 and 100 μM show less desensitization as compared to control, while those with 50 μM ACh show an opposite effect.

Influence of inhibition on galantamine-modulated desensitization

To gather more information on the mechanism by which galantamine acts on the nicotinic receptor, we performed a group of experiments in the presence of the competitive inhibitor metyllycaconitine. Besides the expected inhibitory effect on the whole-cell current amplitude, two major findings came out from this group of experiments (Fig. 4): (i) the co-application of metyllycaconitine changed the desensitization pattern of the currents elicited by 20 μM ACh pulses and, (ii) in the presence of 100 nM metyllycaconitine, 5 μM galantamine failed to prevent desensitization of the currents induced by 20 μM ACh.

Discussion

This study addresses the issue of modulation of nicotinic receptor recovery from desensitization. As a first step in our research, we established a stimulation protocol for the nicotinic receptor expressing cells which could allow us to detect and study the recovery from ACh-induced short-term receptor desensitization. It is known that the opening and closing time constants for nicotinic receptor channels (due to ACh binding) are in the millisecond range (KELESHIAN & al. [14]). In our protocol, the interval between successive applications was of 5 seconds, enough to assure that by the time of the next stimulation all the previously open receptors entered either a closed, responsive state or a desensitized, non-responsive state. The decrease of current amplitude with repeated ACh stimulation can be, therefore, attributed to progressively smaller numbers of available, responsive, closed receptors due to receptor desensitization. This is also supported by our observation that, when receptors are subjected to continuous ACh stimulation, the recorded whole-cell current returns to 0 (in less than 5 seconds) after an initial increase, independently of ACh persistence in the surrounding environment.

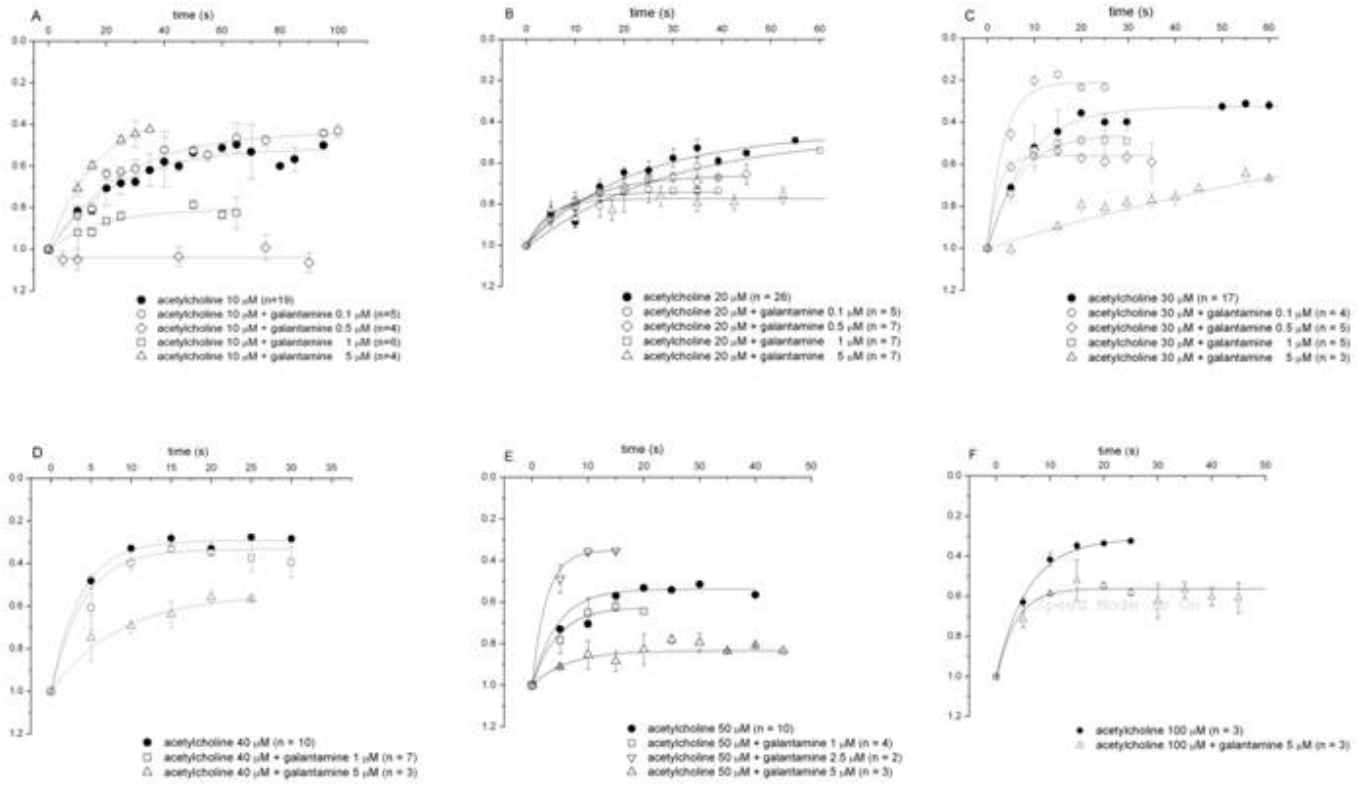

Figure 3. Desensitization trends for currents induced by 10 μM (A), 20 μM (B), 30 μM (C), 40 μM (D), 50 μM (E) and 100 μM (F) ACh, alone or in combination with different galantamine concentrations. The lines are best fits to each set of data with a single exponential function. Currents are normalized, and values are means ± SE. The number of tested cells, for each concentration, is indicated between brackets.

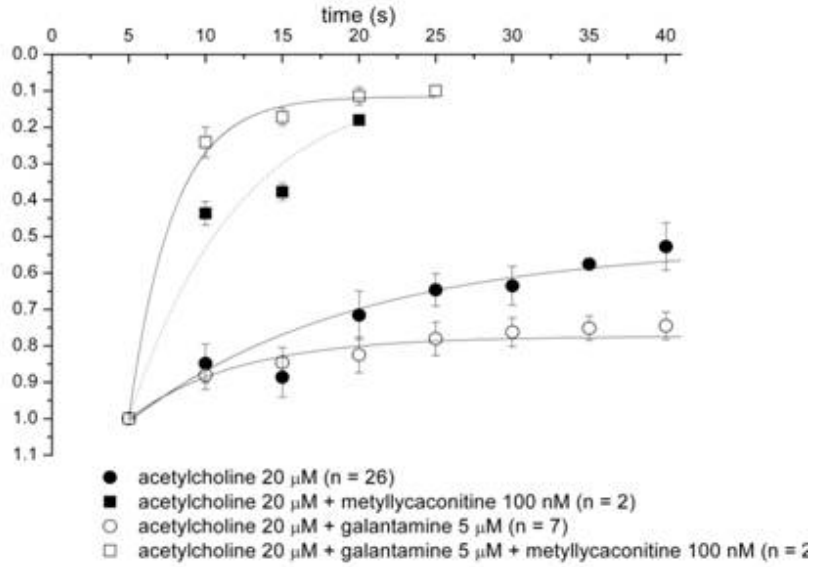

Figure 4. Effect of metyllycaconitine inhibition on 20 μM ACh-induced desensitization, in the presence or absence of 5 μM galantamine. The lines are best fits to each set of data with a single exponential function. Values are means ± SE. The number of tested cells, for each experimental situation, is indicated between brackets.

Apparent non-linearity of ACh-induced desensitization

The first feature emerging from our experiments is the apparent non-linearity of the ACh-induced desensitization. Using a different stimulation protocol which involves continuous application of agonist, Reitstetter, Lukas and Gruener [20] observed a direct correlation between the degree of desensitization of the nicotinic receptor and the concentration of agonist and duration of exposure. Based on their results, they propose the existence of more then one desensitized states for the nicotinic receptor. Our results confirm the straightforward correlation between steady-state desensitization and agonist concentration until 40 μM, while at higher concentrations (50 μM and 100 μM) a surprisingly lower desensitization of the receptors is observed. This particular variation of the response can be explained by the existence of several desensitized states of the receptor. The existence of at least 5 desensitization states of the nicotinic receptor was also confirmed by Elenes and Auerbach [9] by means of single-channel analysis on mouse nicotinic receptors expressed in HEK cells.

The sequential stimulation protocol that we used allowed us to gather extra information regarding the possible kinetics of receptor transitions among desensitized states and also the affinity of the modulatory sites for ACh. As resulting from our experiments, it is highly probable that, at lower concentrations, ACh binds to a first population of receptors, which possesses high-affinity binding sites and shows a longer recovery-from-desensitization time. The effects are proportional to the concentration of ACh, up to a point where a concentration threshold is reached and ACh also binds to a second population of receptors, with low-affinity binding sites but faster recovery from desensitization (Fig. 5.). With continuous stimulation, one can measure the total amount of receptors which enter the two desensitized states and therefore the observed effect is proportional to the concentration of ACh. With sequential stimulation, as it is the case with our protocol, the ACh free interval between successive applications is long enough to allow the recovery from desensitization of the receptors from the second population (low-affinity, short desensitization). Therefore, the measured effects on desensitization in our case concern only the first population of receptors, characterized by high affinity and long-time desensitization.

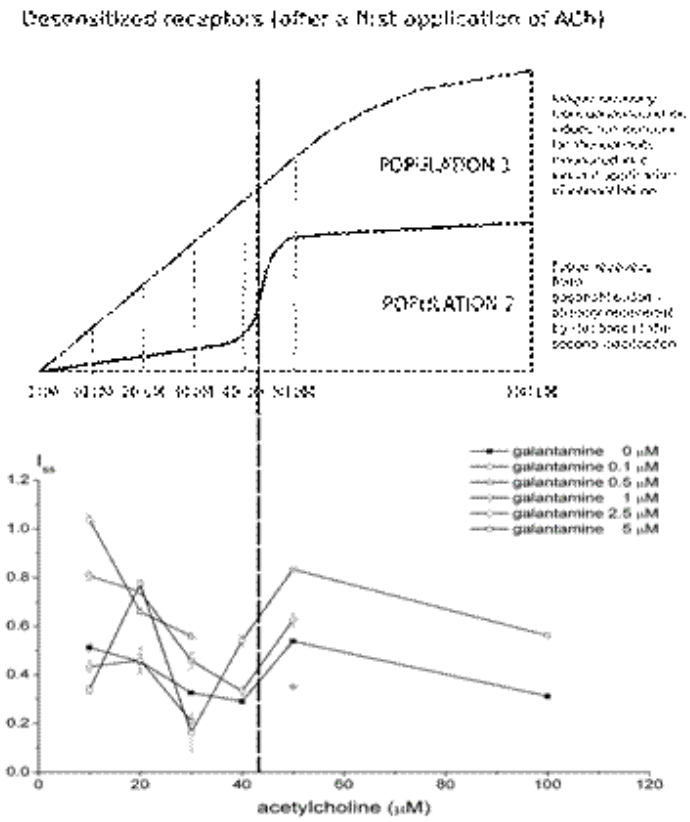

Figure 5. Explicative diagram (qualitative) depicting the involvement of two different nicotinic receptor populations from TE 671 cells in the whole-cell current evoked by ACh applications. Population 1 has a higher affinity to ACh and slower recovery from desensitization, while population 2 has a lower affinity to ACh, but recovers faster from the desensitized state. The total number of receptors desensitized by a first application of ACh within a stimulation train consists of receptors from both populations, but at the moment of a second application only the receptors belonging to the first population are still desensitized (therefore non-responsive). While at concentrations lower than 40 μM, ACh binds predominantly to population 1, at higher concentrations the involvement of the second population of receptors increases (upper half of the diagram). This proposed mechanism explains our experimental data (lower half of the diagram), where we measured less desensitization in steady-state (correlated to higher peak current I_{ss}) for 50 μM ACh compared to 40 μM ACh.

Because ACh distributes among the two populations of receptors, it is possible that at a specific concentration (50 μM, in our case) the measured steady-state desensitization is actually less than at an immediately lower concentration, not because the total amount of desensitized receptors (induced by a first application of ACh) is lower, but because the high-affinity slow-recovery from desensitization bound receptors contribute less. The values obtained for τ (which give an indication of the rate of desensitization for each experimental situation) also change non-linearly with the tested ACh concentrations which constitutes a supplementary clue on involvement of several molecular mechanisms in nicotinic receptor desensitization.

Modulatory effect of galantamine

Our experimental data show that the presence of galantamine modulates the recovery from ACh-induced desensitization, an effect which is concentration dependent and non-linear. In our experimental conditions, galantamine alone did not induce nicotinic receptor currents (results not shown), which shows that the potentiating effect of galantamine requires ACh binding to the receptor. Moreover, the presence of the nicotinic receptor competitive inhibitor metyllycaconitine also inhibits the effect of galantamine.

Our results cannot be explained by assuming only a positive or a negative modulatory effect of galantamine, by allosteric binding at one or several receptor sites. Such a mechanism would imply that only the affinity of the receptor for ACh changes for different concentrations of galantamine. We would expect that the relative relationship between the desensitization curves obtained for different ACh concentrations, at a fixed galantamine concentration, should be the same as in the absence of galantamine, regardless of its concentration, but no such effect is observed, neither for the desensitization rate nor for the steady-state desensitization.

Figure 6 shows the dependency on the ACh and galantamine concentrations of the desensitization parameters (τ, I_{SS}) resulted by the fitting procedure applied to the experimental data. The rate of desensitization (reflected by τ)

shows a consistent tendency to increase with the ACh concentrations (Fig. 6.A). We notice differences between the τ values obtained for different galantamine concentrations and ACh between 10 μM and 30 μM, but at ACh concentrations higher than 40 μM the rate of desensitization reaches a plateau. The plateau seems not to be influenced by the concentrations of neither one of the applied drugs. If we look at the dependency of τ to the galantamine concentration (Fig. 6.B), we notice significant variations until 1 μM galantamine and no further major changes at higher galantamine concentrations regardless of the ACh concentration.

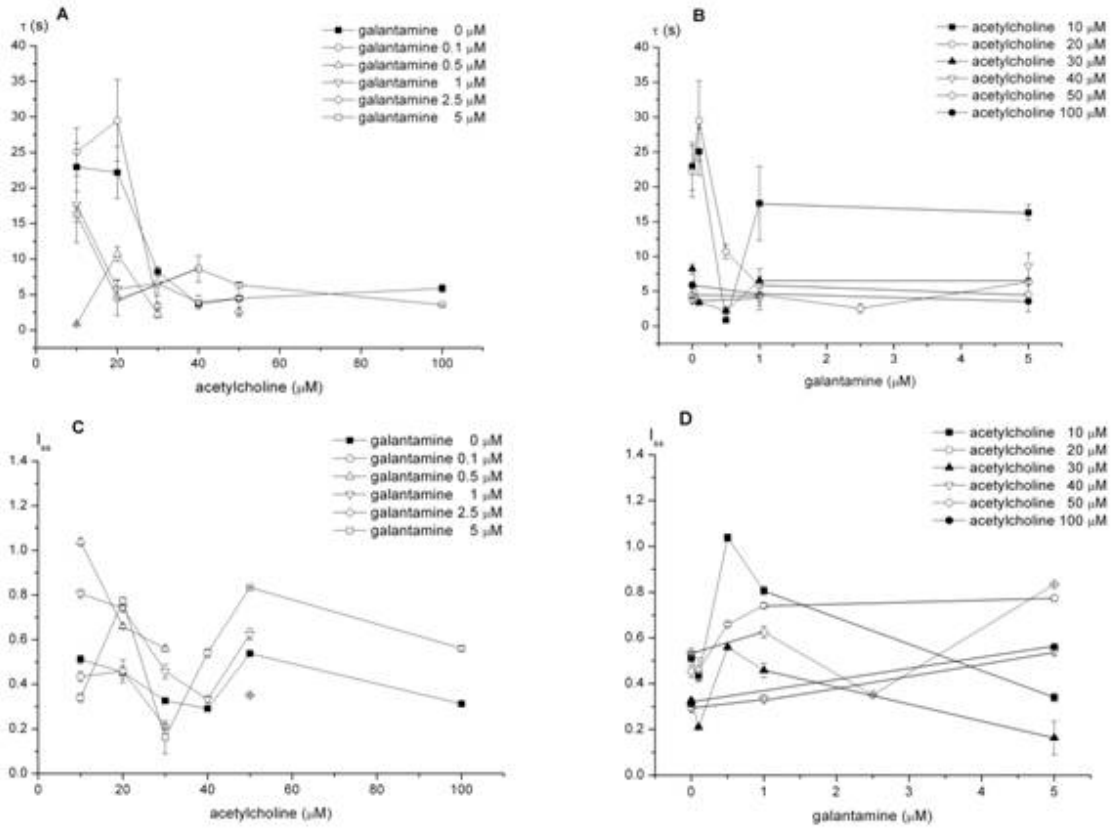

Figure 6. Dependency of the time constant τ (s) and steady-state desensitization whole-cell current I_{SS} (normalized values) on the concentration of applied ACh (A, C) and galantamine (B, D). τ and I_{SS} have been determined by fitting the experimental peak current decrease within a stimulation train with a mono-exponential function. Values are means ± SE.

A more interesting feature results from studying the steady-state desensitization profiles (I_{SS} variation with ACh and galantamine concentration). Both the dependency on ACh concentration and the dependency on galantamine concentration are non-liniar, suggesting a certain periodicity of nicotinic receptor desensitization. The periodical response is more obvious when we look at the dependency on ACh of the steady-state desensitization. The concentration of galantamine seems to modulate this behavior. For the lower ACh concentrations tested, a maximum value of Iss (corresponding to lower steady-state desensitization) is observed with 0.5-1 μM galantamine. Differently, with 40, 50 and 100 μM ACh, the profile of steady-state desensitization current increases with the tested galantamine concentrations.

In order to understand these data, we have to consider the particular stimulation protocol that we used. As we have already shown in the discussions regarding desensitization in the absence of galantamine, our data may not reflect the behavior of the entire population of ACh receptors, but only of those populations which remain in a desensitized state long enough to be still unresponsive after a stimulation-free interval (with no agonist available in the cell bath). The data obtained in the absence of galantamine point towards the existence of at least two different populations of desensitized receptors: one population with a higher affinity for ACh and longer life-span in the desensitized state, and other population with a lower affinity for ACh and a shorter desensitization life-time. Based on this hypothesis, the effects of galantamine resulting from our experiments can be explained by a modulatory action of galantamine predominantly on the low-affinity to ACh population of receptors (which will consequently show a higher affinity to galantamine). Subsequent to galantamine binding and in agreement to its allosteric potentiating effect, this second population of receptors will increase its affinity to ACh (in direct relationship to the applied galantamine concentration). At a very small concentration of galantamine (0.1 μM), the increase in affinity of the second population is not high enough to account for significant differences between steady-state

desensitization with and without galantamine (as shown in Fig. 6.A and C). When the galantamine concentration increases to 0.5μM – 1 μM, ACh binding to the second population of receptors increases due to its increased affinity for ACh. The effects are more evident at 10 μM – 30 μM ACh, where in the absence of galantamine the second population of receptors is not activated due to its natively low affinity for ACh. Therefore, with galantamine 0.5 μM – 1 μM our stimulation protocol leads to recording of apparently less desensitization at these particular combinations of concentrations. At higher concentrations of ACh (50 μM, 100 μM), the differences induced by galantamine are less obvious, since the second population of receptors was stimulated even in the absence of galantamine. A further increase of galantamine concentration could possibly be correlated to galantamine binding also to the first population of receptors, leading to a slight increase of its affinity to ACh. This effect is only visible at lower concentrations of ACh (10 – 20 – 30 μM), where it is recorded as apparently higher receptor desensitization.

The hereby proposed mechanism explains all our data sets, with a single exception: the data recorded for ACh 20 μM and galantamine 0.5 μM. One possible reason for this discrepancy could be that the actual number of receptor populations, showing different functional properties, is much higher, and the key to a more accurate data interpretation would reside in the balance between ACh and galantamine affinities and desensitization patterns for all these populations.

If we correlate the data regarding steady-state desensitization to the desensitization rate, the observed tendency of desensitization rate stabilization at a plateau value suggests that at higher concentrations of both ACh and galantamine a relative balance between the different populations of receptors is reached, in agreement to the proposed mechanism of action for galantamine.

Our explanation of the effects of galantamine on nicotinic receptors desensitization is therefore based on the idea of the co-existence of several populations of nicotinic receptors, with different affinities for agonists and modulators and different desensitization behavior, as a consequence of a particular initial state. From our data alone we cannot establish the precise location of the galantamine binding site on the nicotinic receptor and we cannot discriminate to which specific form of the receptor (open / closed / desensitized) galantamine binds. Nevertheless, we suggest that the binding of galantamine may modulate the relative contribution of the different possible subpopulations of ACh-bound open receptors or even influence their transition into the desensitized states.

Assuming that metyllycaconitine acts exclusively as a competitive inhibitor, we would expect that the number of open channels at a given time would be smaller, but that the presence of the inhibitor would not change the response pattern of the receptor to galantamine. Such an effect is not observed; our data indicate a significant difference between the desensitization patterns obtained with ACh alone and ACh + metyllycaconitine. Moreover, the desensitization curves recorded in the presence of metyllycaconitine show a similar tendency of stabilization at the same steady-state value. Although our recording time was not sufficiently long to reach a steady state, it is possible that the attainable value would be similar to both experimental situations in the presence of metyllycaconitine (irrespective of the presence of galantamine). If this is the case, we hypothesize that metyllycaconitine may not inhibit a sub-population of receptor proteins form TE 671 cells, that may have a distinct desensitization behavior. A second explanation of the observed effect could be related to a direct effect of metyllycaconitine on receptor desensitization by acting competitively with galantamine on one or more modulatory sites of the nicotinic receptors with higher affinity to metyllycaconitine (therefore accounting for the very small difference between the curves observed with metyllycaconitine +/- galantamine). Auerbach and Akk [3] proposed a model for mouse nicotinic receptor channels in which the receptor activation and desensitization reflect the activity of two separate, but interrelated, gates in the ion permeation pathway. Under this perspective, the location of the proposed modulatory situs for metyllycaconitine (and possibly also galantamine) binding on the nicotinic receptor could be on one or both of the activation gates. More recent studies (AUERBACH [2]; MITRA & al. [17]) by means of REFERs (analysis of the rate-equilibrium free-energy relationships) point towards an even more complex mechanism of activation of nicotinic receptors, taking place on a very broad energy landscape. We cannot rule out other possible effects of galantamine on different processes or kinetic steps leading to desensitization.

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