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**ORIGINAL PAPER**

# Effects of Quercetin on Muscle-Type Nicotinic Receptor Activation and Desensitization

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**ANCA POPESCU[A], ANA BICHO[B] AND CONSTANTA GANEA[A]**
[A] *Carol Davila University of Medicine and Pharmacy, Bucharest, Romania*
[B] *REQUIMTE, Departamento de Química, FCT-UNL, 2829-516 Caparica, Portugal*
***Corresponding author**: Constanta Ganea, Current address: Carol Davila University of Medicine and Pharmacy, Bucharest, Romania, Tel: +40 21 3180765, Fax: +40 21 3125955, Email address: cg@biofizica-umfcd.ro*

## Abstract

*We have studied, for the first time to our knowledge, the effects of Quercetin (QCT), a natural flavonoid, on the activation and desensitization behavior of the muscle-type nicotinic receptors natively expressed by the TE 671 cell line. The effect of QCT was assessed by means of the patch-clamp technique, in the whole-cell configuration. The application of QCT in the bath solution, at concentrations between 1 and 50 μM, influenced the whole-cell currents induced by 10 μM Ach in which regards peak current amplitude, activation and desensitization kinetic. The magnitude of the observed effects depended on the duration of exposure and on the QCT concentration in a non-linear fashion. Our data show that QCT influences the kinetic parameters related to the muscle-type nicotinic receptors activation and desensitization.*

**Key words**: quercetin, nicotinic receptors, patch-clamp, desensitization

## 1. Introduction

Quercetin (QCT) is a natural flavonoid, currently acknowledged for its antioxidant effects (ALLIANGANA [1], ALMAAS et al., [2]). Due to its planar structure, QCT attaches to the cellular membranes more than other flavonoids (VAN DIJK, [3]), which could result in interfering with cellular signaling pathways by non-specific, indirect effects on the membrane proteins. In vitro studies proved an antineoplastic effect of QCT on a very broad spectrum of tumors (LAROCCA et al., [4], XING et al., [5], CHAUHAN et al., [6], KUO et al., [7], NGUYEN et al., [8], VOLATE et al., [9]). On neural cells, QCT showed both neuroprotective and neurotoxic effects. At 10-50 μM, QCT facilitated the neuroprotective action of apigenin against the beta-amyloid induced toxicity (WANG et al., [10]), and diminished the intracellular acidification in some cytostatic drugs induced encephalopathy (CHATTON et al., [11]). Opposite effects have been noted on cultured cortical neurons exposed to QCT for 6 hours, where the flavonoid proved to be neurotoxic by interfering in several intracellular signaling pathways involving protein-kinases (SPENCER et al., [12]). On mice models, QCT acted as a neurotoxic drug by inhibiting lactate elimination from cells (BROWN et al., [13]).

Additionally, QCT modulates the activity of several membrane ionic channels and ionotropic receptors. It has been reported that QCT increases the transmembrane ionic flow through CFTR chloride channels (involved in the pathogeny of cystic fibrosis) (ILLEK & FISCHER,[14], SCHULTZ et al., [15]) and stimulates calcium release from sarcoplasmic reticulum deposits in the striate muscle by acting on ryanodinic calcium receptors (LEE et al., [16], BARAN et al., [17]). QCT is also an agonist on L-Type calcium channels, with reported EC50 of 5-7 μM (SAPONARA et al, [18], FUSI et al., [19], WU et al., [20]). QCT increases

the $K^+$ conductance in rat colonic crypt cells (CERMAK et al., [21]) and activates large conductance $Ca^{2+}$-activated $K^+$ channels (BKCa) in isolated myocytes from rat coronary arteries (COGOLLUDO et al., [22]), both effects reported at concentrations up to 10 μM. At a higher concentration (70 μM), QCT inhibited the $K^+$ channels in neuroblastoma cells (ROUZAIRE-DUBOIS et al., [23]). Some studies also show the influence of QCT on the activity of ionotropic receptors for GABA (GOUTMAN et al., [24], GOUTMAN & CALVO, [25]), Glycine (LEE et al., [26], SUN et al., [27]) and 5-hydroxytryptamine (LEE et al., [28]). In a recent work we have presented the results concerning the effect of galantamine, an anti-cholinesterasic drug, on nicotinic receptors desensitization (POPESCU et. al., [29]) and we have found different desensitization patterns for the various combinations of ACh/galantamine concentrations.

To our knowledge, very little is known about the QCT effects on the nicotinic receptors. The data reported so far indicate the involvement of QCT on the regulation of several membrane systems and raise the question of whether or not the neuroprotective or neurotoxic effects of QCT can also be explained by interference in nicotinic pathways. The aim of our study was to investigate by means of the whole-cell patch-clamp method the effects of QCT on muscle-type nicotinic receptors, natively expressed by the TE 671 cell line (STRATTON et al., [30]).

## 2. Materials and Methods

### *2.1. 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.

### *2.2. 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 et al., [31]). 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 means of a system of piston pinch valves (General Valve Corporation, Fairfield, USA) controlled 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 acetylcholine (ACh), in the presence of different concentrations of QCT applied in the bath solution. Recordings were made at 10 minutes interval, until seal was lost. The ACh-containing solutions were applied to the cells in "stimulation trains". A stimulation train consisted in a sequence of concentration jumps with

the duration of 1-2 second, 5 seconds apart or 20 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). The steady-state desensitization reflects a dynamic balance between actual desensitization and recovery from desensitization of the cellular receptors. 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 pCLAMp8 software (Axon Instruments), digitized with a sampling rate of 100 kHz and lowpass-filtered at 5 kHz. All experiments were performed at room temperature.

***2.3. 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 were prepared fresh before the experiment from 1 mM stock solutions (kept frozen). QCT was prepared as 10mM stock solution in DMSO and further diluted in bath solution; the concentration of DMSO in the bath solution was less than 1%. All drugs were purchased from Sigma.

***2.4. Data analysis***

Raw data analysis was performed with Clampfit 8.2 (pClamp8 software, Axon Instruments, Foster City, CA, USA), and the peak values of the currents as well as the whole-cell current decay kinetic (evaluated by fitting the current decay phase with a single or double exponential function) were determined. In order to study recovery from desensitization, for each stimulation train, peak current values were normalized to the first elicited peak current value, and 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 ($\tau_d$). 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 were performed with OriginPro 7.0 (Microcal, Northampton, MA).

## 3. Results

We have started by studying the effect of QCT on the peak current amplitude of the whole-cell currents elicited by applications of ACh 10 μM. QCT was applied at different concentrations in the cell bath solution, and the current amplitude was measured immediately after the application of QCT (t = 0) and afterwards at 10 and at 20 minutes of cell exposure to QCT. As a control, we performed the same experiments in the absence of QCT in the bath solution. The current amplitude was normalized to the value obtained at t = 0. Figure 1 presents the variation of peak current amplitude with the time of exposure to QCT (A) and the concentration of QCT (B). We notice, as a general tendency, an increased amplitude of the whole-cell currents at 10 minutes after the initial ACh application (immediately after breaking the membrane into whole-cell), both in the absence of QCT in the bath solution and in the presence of QCT up to 10 μM.

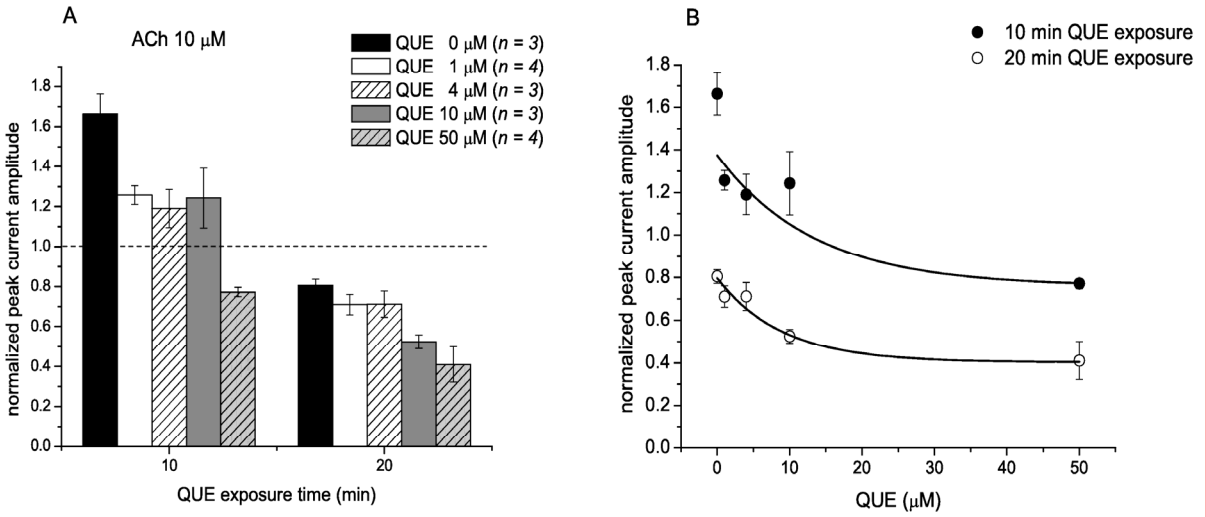


**Figure 1.** Influence of extracellular QCT application on the whole-cell peak current amplitude elicited by 10 μM ACh. A. Variation of whole-cell peak current amplitude with the time of exposure to QCT, in the absence (control) and in the presence of 1μM, 4μM, 10 μM and 50 μM QCT. Values are means ± SD, n values are indicated in the figure. Current values are normalized to the value at t = 0. B. Variation of the whole-cell peak current amplitude on the QCT concentration, for 10 min and 20 min of exposure to QCT. Values are means ± SD, n values correspond to the ones indicated in A. The continuous lines are general exponential trend-lines.

The current increase is more significant in the absence of QCT (1.66 of the initial value), compared to 1.2 observed for QCT concentrations of 1 μM, 4 μM and 10 μM (with no significant differences observed between the results for these 3 concentrations). However, at 50 μM QCT, the peak current value at 10 minutes of QCT exposure is only 0.77 of the initial value. At 20 minutes of exposure, a decrease in peak current amplitude is visible regardless of the QCT concentration, with a greater decrease observed for increasing QCT concentrations (Fig. 1A). Fig. 1B shows the dependency of the normalized whole-cell peak current value on the QCT extracellular concentration. Although the peak current values are decreased after 20 minutes of exposure as compared to those after 10 minutes, the decreasing profile is similar. The same experiments performed in the presence of only DMSO applied in the bath solution revealed no differences to the control (n = 3, results not shown), so the described effects can be entirely attributed to QCT.

We have further performed an analysis of the whole-cell current decay phase, in the absence of QCT (control) and for intervals of QCT exposure between 10 and 50 minutes (Fig. 2). In the absence of QCT, the current decay phase can be successfully fitted with a single exponential function $y = y_0 + y_{\max} e^{-t/\tau}$, where y is the current value (pA), $y_0$ the value of the base-line current (pA), $y_{max}$ is the peak current value (pA), t is the time (ms) and τ the time constant of the current decay phase (ms). In the presence of QCT, the current decay phase is best fitted with a sum of two exponentials $y = y_0 + y_{\max 1} e^{-t/\tau_1} + y_{\max 2} e^{-t/\tau_2}$ (Fig. 2A). The dependency of the current decay time constants on the time of exposure to QCT 1 μM is presented in figure 2B. For this concentration, we observe a general tendency for the time constants $\tau_1$ and $\tau_2$ to increase with increasing time of exposure to QCT, with a slight maximal value noted at 40 min of exposure.

Figure 2C shows the dependency of the current decay time constants on the QCT concentration, for a 20 minutes exposure time. We noticed a maximum value of the time constants reached at 10 μM QCT, for 20 min in the bath solution (light grey trace). The third aspect that we approached was the effect of QCT on nicotinic receptor desensitization.

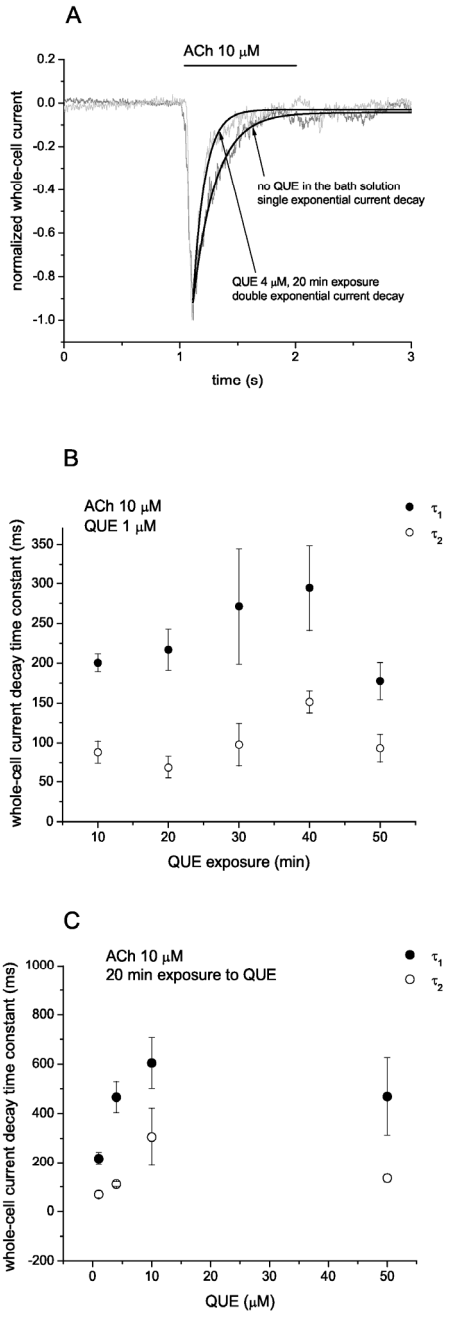


**Figure 2.** Influence of extracellular QCT application on the whole-cell current decay phase. A. Whole cell current recorded in the absence of QCT (dark grey trace), and in the presence of 4 μM QCT for 20 min in the bath solution (light grey trace). The continuous lines are theoretical function obtained by fitting the traces with a single exponential (for the control trace) and with a double exponential (for the trace in the presence of QCT). B. Dependency of the whole-cell current decay time constants on the time of exposure to 1 μM QCT, determined by fitting the experimental data with a double exponential function. Values are means ± SD, n = 4. C. Dependency of the current decay time constants on the QCT concentration, determined by fitting the experimental data with a double exponential function, for 20 minutes of exposure. Values are means ± SD, n = 3-4 for each data point.

As described within Methods, desensitization was assessed based on the peak current decrease profile within a stimulation train, which was fitted with a single exponential function, $I(t) = I_{SS} + I_{\max} e^{-t/\tau_d}$, where *I(t)* represents the peak current amplitude (within a stimulation train), $I_{ss}$ the peak current amplitude corresponding to steady-state desensitization, $I_{max}$ the difference between the peak amplitude of the first current in a stimulation train and $I_{ss}$, $t$ is the time and $\tau_d$ is an apparent desensitization time constant, reflecting the rate of current decrease within a stimulation train.

**Table 1.** Apparent desensitization time constants $\tau_d$ and steady-state desensitization $I_{ss}$ values

| **ACh / QCT concentration (μM)** | | **$\tau_d$ (s)** | **$I_{ss}$ (normalized values)** |
|---|---|---|---|
| *Protocol 1* – 5 s interval between successive applications | Ach 10 μM QCT 0 μM | 5.14 ± 2.53 | 0.79 ± 0.02 |
| | Ach 10 μM QCT 1 μM | 9.50 ± 2.49 | 0.50 ± 0.02 |
| | Ach 10 μM QCT 4 μM | 4.37 ± 1.08 | 0.66 ± 0.01 |
| *Protocol 2* – 20 s interval between successive applications | Ach 10 μM QCT 0 μM | 50.71 ± 9.20 | 0.44 ± 0.03 |
| | Ach 10 μM QCT 10 μM | 32.54 ± 7.15 | 0.12 ± 0.06 |
| | Ach 10 μM QCT 50 μM | 47.49 ± 9.50 | 0.05 ± 0.05 |

The current decrease within a stimulation train depends on both the actual desensitization rate and the rate of recovery from desensitization. The results for different stimulation protocols and different concentrations of QCT are presented in figure 3 and in table 1. We noticed an increased receptor steady-state desensitization in the presence of QCT, for all the tested concentrations, in comparison to the control trace. Interestingly, we noticed no statistically significant differences between the desensitization traces in the presence of a constant concentration of QCT at different exposure times (10 min and 20 min). Desensitization did not, however, increase in direct relationship with the QCT concentration – in fact, we noticed less steady-state desensitization with 4 μM QCT ($I_{ss}$ = 0.66 ± 0.01 pA) than with 1 μM QCT ($I_{ss}$ = 0.50 ± 0.02 pA), while the desensitization time constant is smaller at 4 μM QCT ($\tau_d$ = 4.37 ± 1.08 ms) as compared to 1 μM QCT ($\tau_d$ = 9.50 ± 2.49 ms).

In the second group of experiments, with ACh applications at 20 s intervals, we noticed that the time constant $\tau_d$ increased with the concentration of QCT for 10 μM QCT (32.56 ± 7.15 ms) and 50 μM QCT (47.49 ± 9.50 ms), reflecting faster recovery from desensitization and therefore longer time required to reach steady-state desensitization with 50 μM. A significant difference from the control (50.71 ± 9.20 ms) was noted only for 10 μM QCT (32.54 ± 7.15 ms). The steady-state desensitization was higher with 50 μM QCT (reflected by a smaller $I_{ss}$ value), and for both tested concentration the values for $I_{ss}$ were significantly lower compared to the control.

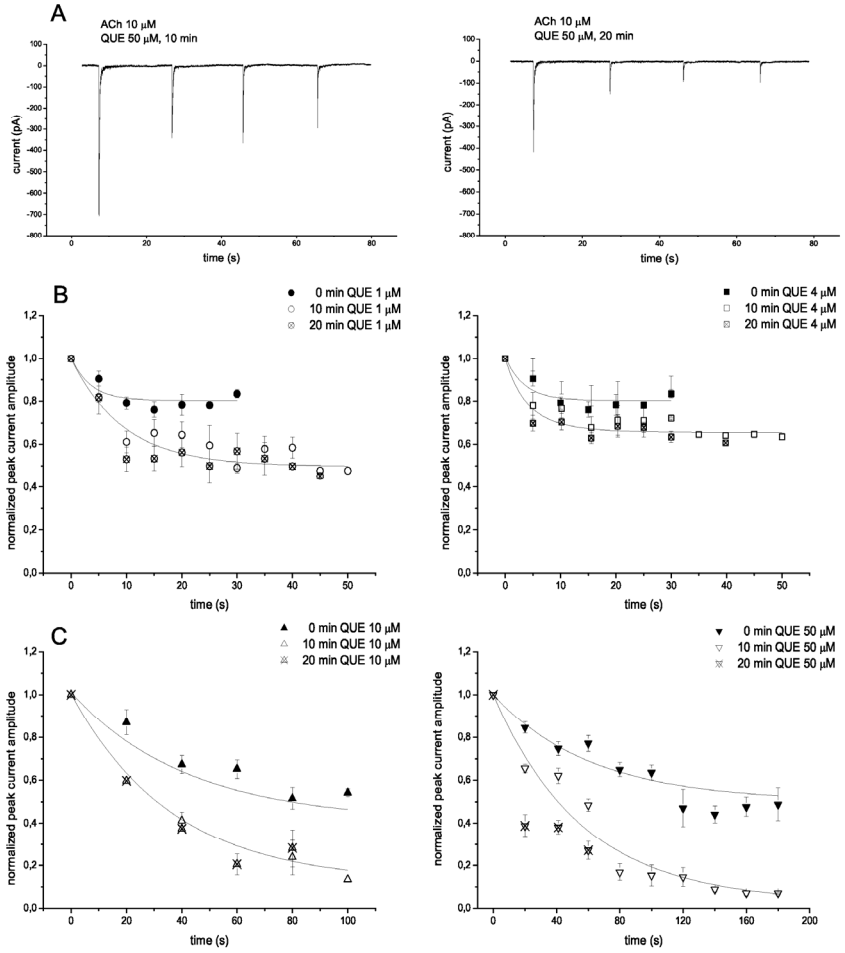


**Figure 3.** Influence of QCT on the nicotinic receptor desensitization, induced by repeated applications of 10 μM ACh in stimulation trains. A. Whole-cell current responses to an ACh stimulation train, at 20 seconds intervals between successive applications, for 10 minutes of extracellular exposure to QCT 50 μM (left) and 20 minutes of extracellular exposure to QCT 50 μM (right). B. Variation of whole-cell peak current amplitude within an ACh stimulation train (at 5 seconds intervals between successive applications of ACh), for extracellular exposure to QCT 1 μM (left) and 4 μM (right). No significant differences were found between data groups obtained at 10 min and 20 min of exposure, regardless of the concentration of QCT. The continuous lines represent single-exponential fitting functions. Each data point represents the mean value ± SD for 3-4 different experiments. C. Variation of whole-cell peak current amplitude within an ACh stimulation train (at 20 seconds intervals between successive applications of ACh), for extracellular exposure to QCT 10 μM (left) and 50 μM (right). No significant differences were found between data groups obtained at 10 min and 20 min of exposure, regardless of the concentration of QCT. The continuous lines represent single-exponential fitting functions. Each data point represents the mean value ± SD for 3-4 different experiments.

## 4. Discussion

In this work we tested whether QCT may influence the activity of the nicotinic receptors of TE 671 cells. Our results suggest that QCT modulates the activity of the muscle-type nicotinic receptors by influencing both the activation / inactivation kinetic of the nAChR and the nicotinic receptor desensitization / recovery from desensitization balance.

Two different mechanisms may be responsible for the decreased amplitude of whole-cell peak currents induced by 10 μM ACh applications. A first mechanism, non-specific and QCT-independent, accounts for the current decrease observed inclusively in the control experiment, and is probably related to the experimental protocols. The measured whole-cell currents through the open receptor are $Na^+$ currents, and their amplitude is highly dependent

on the $Na^+$ electrochemical gradient existing between the outside of the cell (bath solution) and the inside (pipette solution). Most likely, by maintaining the patched cell in the bath solution for a longer period of time (at least 20 minutes), the $Na^+$ gradient decreases due to passive diffusion of $Na^+$ into the cell, resulting in decreased amplitude of whole-cell currents. The passive $Na^+$ diffusion towards the interior of the cell is possibly accelerated by the pore-forming action reported for QCT on lipid bilayers, although this mechanism is probably not preponderant since pore formation is mainly observed after longer (> 1 hour) exposure times (MOVILEANU et al., [32]). Therefore, the decrease in current amplitude does not necessarily reflect a lower number of activated receptors. This mechanism explains the decrease of current amplitude in the control experiment (no QCT in the bath solution). Additionally, we cannot exclude the possibility of occurrence of desensitization or internalization of the protein occurring on a longer time scale (minutes).

The dependency of peak current decrease on the concentration of QCT suggests, however, also a specific modulatory effect of QCT on the nicotinic receptors. The experimental method does not allow us to positively identify the exact mechanisms of action. QCT binding saturation occurs most likely before 10 minutes of exposure, which explains the similarity of the peak-current amplitude to QCT concentration profiles observed for 10 and 20 minutes of exposure.

Our data also indicate that, in the presence of QCT applied in the bath solution, the decaying phase of the current can be fitted by a double exponential function, which suggests that at least 2 transitions of the receptor protein are involved in the process. It is currently accepted (as reviewed in (KARLIN, 2002) that, although there is a very small probability of channel opening for a nicotinic receptor unbound by ACh or bound to a single ACh molecule, the receptor opening probability is maximal for the receptor bound by two ACh molecules, meaning that the double-bound state activation and desensitization kinetic determines the behaviour of the measured whole-cell currents. Therefore, the deactivation phase of the whole-cell current can be fitted by a single exponential, as encountered in our control data in the absence of QCT. A possible explanation for the double-exponential current decay phase is that the drug may slow down one or more reactions involved in the process making it possible to discriminate between steps that presented similar kinetic parameters in the absence of QCT. It is also possible that the QCT increases the opening probability of a different state of the nicotinic receptor (for example, the monoligated receptor), which has a different activation / desensitization kinetic. As a result, the measured whole-cell current could reflect two different populations of receptors. Our experimental method did not allow us to determine precisely the conformational state stabilized by QCT, nor the exact binding site for QCT on the nicotinic receptor. We can not exclude that the effects observed in the presence of QCT were also due to documented non-specific binding of the drug to the lipid bilayer of the membrane (VAN DIJK et al., [3], IONESCU et al., [34], DRAGUSIN et al., [35]), indirectly influencing the conformational transitions of the receptor protein by modifying the physical properties of the membrane environment. Goutman *et al* (GOUTMAN et al., [24]), in their study focused on GABA receptors, observed similar modulatory effects of QCT on $\alpha4\beta2$ nicotinic receptors, serotonin 5-HT3A and glutamate AMPA/kainate receptors, which were interpreted as an indication for a non-specific effect of QCT on ionotropic receptors. However, our kinetic data support the hypothesis of direct QCT binding to a modulator site the nicotinic receptor. The binding stoechiometry cannot be determined from our data, since both the variation of peak currents amplitude and current decay time constants with the QCT concentration are indirect indicators of the QCT binding process. Additionally to the observed influence of QCT on the decaying phase of the ACh induced currents, we also observed a clear influence of QCT on the peak current trends within the stimulation trains. These experiments show that the drug also influences the balance of desensitized molecules and those which already recovered from

desensitization. The nonlinearity of the dependency of all the measured parameter (peak current amplitude, receptor deactivation and desensitisation) on the QCT concentration represents by itself an argument for a complex interfering mechanism of QCT in the nicotinic neurotransmission, at multiple levels, and possibly with simultaneous antagonist effects by influencing different regulatory links.

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