UDC 577.352 : 615.214 : 576.89

Effects of mecamylamine on the electrophysiological properties of LCC-channels in rat cerebellar Purkinje neurons

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The release of Ca^{2+} from intracellular stores requires a compensatory countercurrent of K^+. However, the nature of the LCC-channels, previously proposed for creating this countercurrent, remains unknown. To test the hypothesis that LCC-channels are involved in Ca^{2+} release, it is required to find their specific blocking agent first. In this study, we aim to examine the effects of mecamylamine on LCC-channels to assess whether it can be considered as their effective blocker. Cells' nuclei were extracted from Purkinje neurons of 3 to 4-week-old Wistar rats. The rats' cerebellum was cut into thin slices, and roughly homogenized; then the nuclei-containing pellet was resuspended and transferred into the bath of an inverted microscope, where ion currents through the LCC-channels were recorded using a nucleus-attached configuration and voltage-clamp mode of the patch-clamp technique. It was found that mecamylamine (1 mmol/l), when applied to the intranuclear side of the LCC-channels, decreased the current amplitude at negative membrane potentials (by approximately 16-17% at -40 and -60 mV, respectively) and slightly increased it at positive ones. It also reduced the open probability (P_o) of the channels at the potential of -40 mV by 45%. Conversely, when mecamylamine was added to the perinuclear side of these channels, the current amplitude decreased at both positive and negative potentials, while no change of P_o was registered. The side-dependent effect may indicate differences in the molecular structure between intranuclear and perinuclear domains of LCC-channels. Additionally, it was discovered that mecamylamine induces channel flickering when applied to either side of the membrane.

Key words: ion channels; membrane conductivity; patch-clamp; mecamylamine; nicotinic acetylcholine receptor antagonists; calcium homeostasis; electrophysiology; modulation

INTRODUCTION

Ca^{2+}-mediated signaling pathways are crucial for numerous intracellular processes in excitable cells [1–3]. The onset of these processes is inextricably linked with the release of Ca^{2+} cations from the intracellular stores, such as the endoplasmic reticulum [4, 5], which is in turn interconnected with the nuclear envelope. Still, this release requires not only a chemical gradient of Ca^{2+} between the cytoplasm and the intrareticular space, but also an additional driving force of K^+ ions entering the reticulum to compensate for Ca^{2+} movement to the cytoplasm. Without it, Ca^{2+} concentrations on both sides of the endoplasmic membrane would rapidly equalize, and the release would cease [6]. Despite the importance of the K^+ counter flow for the proper functioning of the excitatory cells, the channel(s) responsible for its generation is/are still unknown. In 2007, TRIC channels were proposed for this role [7, 8]. Indeed, it was shown that the knockout of the TRIC channels' genes compromises Ca^{2+} release from the intracellular stores, suggesting their implication in this process.

However, earlier, in 2005, a different candidate was proposed by Marchenko et al. [9], namely, LCC-channels (Large Conductance Cation channels). It is known that the density of LCC-channels in the nuclear membrane correlates with the density of the nuclear IP3Rs [10], suggesting a possible functional

connection. The next logical step in testing the hypothesis of their involvement in Ca^{2+} release would be to analyze the changes imposed by the disablement of the abovementioned channels, either by the knockout of their corresponding genes (which are not described at the moment) or by chemical blockade. Still, finding a specific blocker requires screening and testing numerous substances. It was described that some antagonists of nicotinic acetylcholine receptors (nAChRs), such as pipecuronium bromide and rocuronium bromide [11], as well as neurotoxin II [12], have the ability to decrease the conductivity of LCC-channels. These results allow us to assume that modulators of nAChRs may possess some intrinsic features of chemical structure that mediate their influence both on nAChRs and on LCC-channels. To test this hypothesis, a comprehensive structural analysis of LCC-blockers is required. So far, only a limited number of nicotinic acetylcholine receptor modulators have been evaluated in the context of their capability to impact the electrophysiological properties of LCC-channels. In this study, we focused our efforts on researching mecamylamine, a known nAChR antagonist [13] to assess whether it can be considered an effective blocker of LCC-channels. Previously, mecamylamine - a bicyclic monoterpenoid that contains 2 fused rings - was broadly used in medical practice to treat hypertension [14]. It impairs the transmission at sympathetic ganglia, causing dilatation of the blood vessels, thus lowering blood pressure, meanwhile lowering its dosage to 2.5-5 mg twice per day prevents ganglionic side effects [15].

Thus, the aim of our study was to evaluate the changes of electrophysiological activity of LCC-channels of the nuclear membrane under the influence of mecamylamine.

METHODS

All the experiments were conducted according to the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and in compliance with the regulations of the Bioethics Committee of the Bogomolets Institute of Physiology (Protocol No. 4/25 from 16.04.2025).

The studies were performed on 3 to 4-week-old Wistar rats. The sample preparation technique described by Marchenko and Fedorenko [9, 10] was taken as a basis and modified to suit the needs of the present study better. Following the decapitation, the rat cerebellum was extracted and transferred into an ice-cold (4°C) NaCl-based solution (mmol/l): NaCl - 150, HEPES - 10, EDTA – 1; pH 7.4 for the subsequent manipulations.

At the next step, the cerebellum was cut into thin slices 0.3-0.5 mm thick and transferred into the Eppendorf tubes, which contained the homogenization solution (mmol/l): K-gluconate - 150, HEPES - 10, HEPES-K – 10; pH 7.2 with pre-added protease inhibitors, prepared following the manufacturer instructions («cOmplete Protease Inhibitor Cocktail tablets, Roche», Germany). The resulting concentration of the inhibitors was 1.6 mg/ml. Following the sample preparation, the brain fragments were frozen to prevent tissue degradation and stored for up to two days. On the day of the experiment, the samples were defrosted, then roughly homogenized with a syringe needle (0.84 mm) and centrifuged at 2000g for 5 min (miniSpin «EppendorfAG», Germany). All the mentioned procedures were conducted at low-temperature conditions to minimize autolytic processes.

After the centrifugation, the pellet, which contained Purkinje cells' nuclei, was resuspended in a KCl-based solution (mmol/l): KCl - 150, HEPES - 8, HEPES-K - 12, EGTA – 1; pH 7.2, and then transferred into the bath on the stage of the inverted microscope («Leica DM IRB», Germany) for the visual control of the following manipulations (Fig. 1). Three to four minutes were allocated for the nuclei to form a stable contact with the glass bottom of the bath; after that, the residual debris was washed away from the sample using the same KCl-based solution as the one used for the resuspension.

Ionic currents through the channels were recorded using the nucleus-attached configuration of the patch-clamp technique in voltage-clamp mode. The reference electrode was connected to the sample-containing bath of the microscope via an agar bridge. Meanwhile, the recording electrode was inserted into the patch micropipette, which contained the KCl-based solution of the previously mentioned composition, matching the bath solution as per the standard for the membrane-attached patch-clamp protocol.

After recording the activity of LCC-channels under control conditions in the KCl-based solution, 1 mmol/l mecamylamine solution (mmol/l): mecamylamine – 1, KCl – 150, HEPES – 8, HEPES-K – 12, EGTA – 1; pH 7.2 was applied via perfusion. Later, the test substance in the bath was again substituted with a control KCl-based solution in the same way to investigate the lasting effects of the investigated agent.

In the next stage of the experiment, we evaluated differences in the effects of mecamylamine depending on which side of the membrane it was applied to. For this purpose, a series of experiments was conducted with 1 mmol/l of mecamylamine solution of the same composition as described previously being added into the patch micropipette. Then, the bath solution was also substituted with the mecamylamine solution, allowing us to study the electrophysiological properties of the LCC-channels with the test substance interacting with both intra- and perinuclear sides of the protein.

Patch pipettes with the resistance ranging from 7 to 15 MΩ for the study were premade before the experiment from the borosilicate glass microcapillaries using a PC-10 Vertical Puller («Narishige», Japan). A Visual-Patch 500 («Bio-Logic», France) amplifier was used for the signal amplification. Currents through the LCC-channels were recorded with a discretization time of 200 μs. Manual processing of the recordings was performed in Clampfit 10.7 («Axon Instruments», USA), statistical analysis and dependency graphs plotting were conducted in Origin 2018, 64-bit («OriginLab Corporation», USA). The main electrophysiological parameters of the LCC-channels that were researched in this study were the amplitude of the currents through the channels and the open-state probability (P_o). These parameters were evaluated at both positive (+40 mV; +60 mV) as well as negative (-40 mV; -60 mV) membrane potentials.

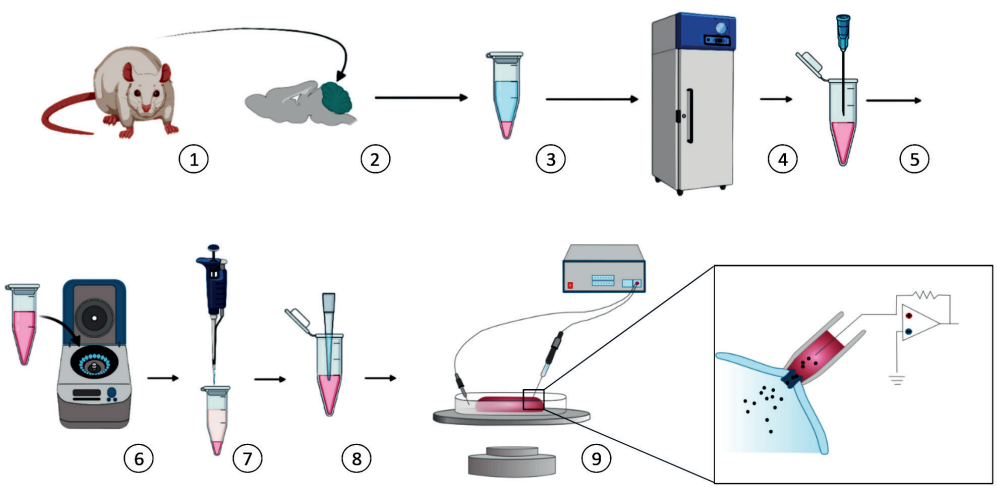

Fig. 1. General scheme of the experiment. 1-2 - extraction of the rat's cerebellum; 3 - transferring brain slices into Eppendorf microtubes; 4 - storing samples in a frozen state; 5 - defrosting and rough homogenization with a syringe needle; 6 - centrifugation at 2000g for 5 min; 7 - solution substitution; 8 - resuspension of the nuclei-containing pellet; 9 - recording of the ion currents using the patch-clamp technique

The current amplitude was calculated by the Clampfit 10.7 in-built tools as a difference in the average current values between closed and open states of the channels. Taking into account the tendency of some channels to close due to the quick change of conditions shortly after the attachment of the patch pipette to the patch site, the probability of the channels being in an open state was estimated after the number of channels within the registration stabilized. Another complication that influences the precision of P_o evaluation is a so-called channel flickering: repetitive and fast-paced switches of the channel between conductive and non-conductive states. To negate the flickering effect, a data reduction procedure was used with a reduction factor of 10 and the substitute average method, meaning that every 10 data points with certain values of the current amplitude were transformed into 1 data point, calculated as an average value between them. The resulting recordings with a stable number of channels and reduced flickering due to the data reduction were further used to estimate P_o. During this procedure, changes between open and closed states that lasted less than 10 ms and/or had an amplitude less than 50% of the established for currents through the LCC-channels at the given potential were ignored to prevent inflation of P_o values.

The final results were presented in M ± m format due to the normal data distribution. Comparisons between the control and test groups were conducted using a 2-tailed t-test of the first type, considering that the measurements were repeatedly performed on the same nuclei, but in consecutively changing conditions. However, in cases when data points were obtained from different sets of nuclei, the third type of 2-tailed t-test was used.

RESULTS AND DISCUSSION

In our research, we discovered that mecamylamine (1 mmol/l) applied in the bath solution statistically significantly decreased the amplitude of the currents through the LCC-channels at all negative applied potentials, namely -60 and -40 mV. Representative fragments of the registrations are shown in Fig. 2A, and the amplitude diagrams based on these fragments are depicted in Fig. 2B. At the potential of -60 mV, the amplitude of the currents through the LCC-channels decreased from -11.72 ± 0.46 pA (n = 4) in control to -9.75 ± 0.51 pA (n = 5) in the mecamylamine solution ($P < 0.05$). Additionally, we found a significant difference ($P < 0.05$) between the amplitudes in control and after washing away the tested substance with the KCl-based solution (-9.90 ± 0.18 pA), which may indicate the lasting effect of mecamylamine and the strength of its binding with LCC-channels. The average amplitude changes between the mentioned stages are illustrated in Fig. 2C. Similar results were obtained when the potential of -40 mV was applied. The amplitude of the currents through the LCC-channels reduced from -7.88 ± 0.29 pA in control to -6.64 ± 0.45 pA when in the presence of mecamylamine ($P < 0.05$; n = 5). In the meantime, at +40 mV and +60 mV, as shown in Fig. 2C, the amplitude increased from 7.18 ± 0.34 pA to 7.62 ± 0.34 pA (+40 mV; $P < 0.05$; n = 5) and from 11.35 ± 0.53 pA to 11.72 ± 0.41 pA (+60 mV; $P < 0.05$; n = 4). This effect has not been described for either gallamine or nicotine, the other modulators of nicotinic acetylcholine receptor. The general tendency we can infer is that mecamylamine at a concentration of 1 mmol/l decreases the amplitude of the currents through the LCC-channels at negative membrane potential by 16-17%, and slightly, although statistically significantly increases it by 3-6% at positive ones. To understand such an effect, molecular studies of the LCC-channels are required. Additionally, as it is shown in Fig. 2A, we observed an increased channel flickering under the effect of mecamylamine. This process is characterized by rapid changes between the conductive and non-conductive states of the channel and reflects the mechanical blockade of the channel's pore. It can be assumed that mecamylamine is not capable of

forming stable enough bonds with the pore-forming domains or essential for modulation parts of LCC-channels to permanently eliminate the K^+ current.

It was also discovered that mecamylamine applied in the bath solution decreases the probability of LCC-channels being in an open state (P_o) at the potential of -40 mV, as can be seen in Fig. 2D. At this potential, we registered a statistically significant ($P < 0.05$; $n = 5$) decrease of P_o from 0.49 ± 0.04 in control to 0.27 ± 0.07 when mecamylamine was applied. It is relevant to point out that at the potential of -60 mV, we also observed a tendency of mecamylamine to decrease P_o of LCC-channels. However, further analysis proved it to be not statistically significant, as one out of four data points contradicted this trend. We can infer from this that mecamylamine is a relatively effective LCC-channels inhibitor, which can decrease both the amplitude and P_o of these channels. Still, the effect of this substance on the probability of LCC-channels being in an open state was pronounced only at the potential of -40 mV.

To evaluate whether there is a difference in the effects of mecamylamine depending on what side of the membrane it was applied to, in the next stage, the substance was applied through a patch micropipette, causing it to interact with the perinuclear side of LCC-channels, instead of the intranuclear one, as was the case when

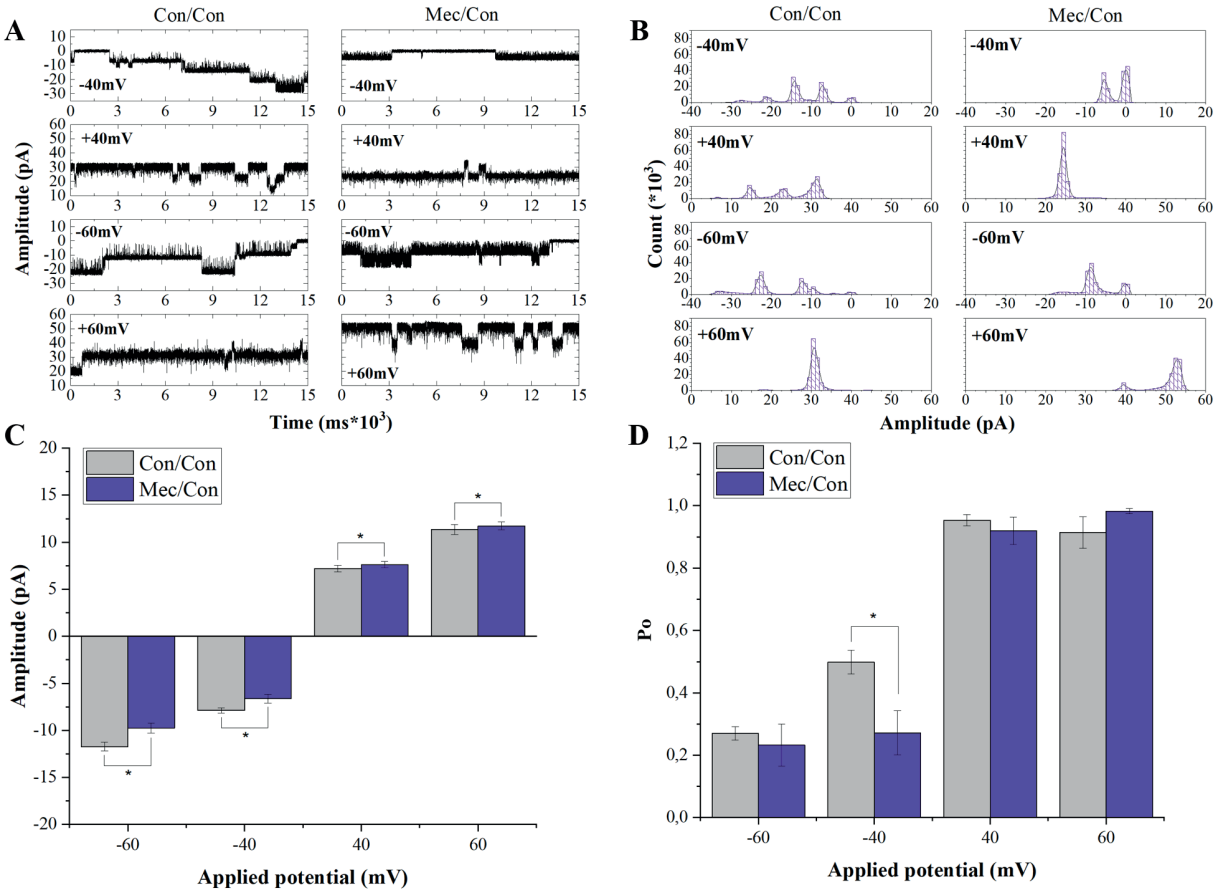

Fig. 2. Effects of mecamylamine in the concentration of 1 mmol/l applied to the bath solution on electrophysiological properties of LCC-channels. Con/Con - no active substance was applied; Mec/Con - mecamylamine solution was added to the bath solution, but not into the patch micropipette. A - typical fragments of registrations. B - corresponding to the registration fragments' amplitude diagrams. C - diagram of average amplitude changes caused by mecamylamine. D - influence of the mecamylamine on the average P_o of LCC-channels. *$P < 0.05$ compared to control

mecamylamine was added directly into the bath as described earlier. Representative fragments of the registrations obtained in this stage are presented in Fig. 3A. Amplitude diagrams for the corresponding fragments are shown in Fig. 3B. When mecamylamine was applied via the patch pipette at the same concentration of 1 mmol/l, a statistically significant decrease in the amplitude of currents through LCC-channels was observed at both positive and negative membrane potentials. At the potential of -60 mV, the amplitude decreased from -11.56 ± 0.46 pA to -9.98 ± 0.08 pA ($P < 0.05$; $n = 7$), and then further decreased to -7.99 ± 0.13 pA ($P < 0.001$ compared to control; $n = 2$) when mecamylamine was applied on both sides of the nuclear membrane. At the potential of -40 mV, we saw a similar picture: the decrease of the amplitude from -7.97 ± 0.18 pA to -7.22 ± 0.10 pA ($P < 0.01$; $n = 9$) with mecamylamine on the one side (in patch micropipette) and to -6.13 ± 0.06 pA ($P < 0.001$ compared to control; $n = 2$) when the tested substance was applied to both sides (in a bath and patch micropipette). The dependence of the average amplitude changes on the membrane potential and mecamylamine concentration is depicted in Fig. 3C. The decrease in the current amplitude when mecamylamine was applied to the perinuclear side was significant at the positive membrane potentials as well. Thus, at the potential of +40 mV, we registered the amplitude decrease from 7.51 ± 0.37 pA to 6.36 ± 0.20 pA ($P < 0.05$; $n = 6$) and at the potential of +60 mV from 11.39 ± 0.41 pA to 9.67 ± 0.23 pA ($P < 0.01$; $n = 5$). It is worth noting that the effect of the gradual decrease of the amplitude, reaching its maximum when mecamylamine was applied to both sides, was observed only at negative membrane potentials and was absent at positive ones. On the contrary, when the test substance was added into the bath as well, at positive membrane potentials we saw an increase in the currents' amplitude to 7.02 ± 0.02 at +40 mV ($P < 0.05$ compared to mecamylamine being only in the pipette; $n = 2$) and 10.82 ± 0.16 at +60 mV ($P < 0.01$ compared to mecamylamine being only in the pipette; $n = 2$), with its reversion almost to the control values. These results correspond to what we observed in the previous stage of the experiment, when adding mecamylamine into the bath increased the amplitude of the currents through the LCC-channels at positive potentials and decreased them at negative ones. As in the case of mecamylamine being added to the bath, we also registered channel flickering when the test substance was added via the patch pipette. This effect is represented in Fig. 3A and may indicate that the mechanical blockade of the LCC-channel's pore is non-specific to the side of the channel.

Opposite to the results obtained when mecamylamine was added to the bath solution, no effect on the LCC-channels' probability of being in an open state was registered when the tested substance interacted with their perinuclear side, as shown in Fig. 3D.

The acquired results support at least two key conclusions. First, mecamylamine can decrease the amplitude of the currents through the LCC-channels at negative membrane potentials when applied on either side of the nuclear membrane. Second, the nature of this inhibition might be different, based on the fact that the amplitude-increasing effect observed at positive potentials when mecamylamine was added to the bath and interacted with the intranuclear side of the membrane was absent when the reagent was applied through the patch micropipette and acted on its perinuclear side. Different effects of the same substance might be attributed to the different domain structure of the LCC-channels, depending on the side of the membrane.

According to the analysis of the average amplitude changes at different membrane potentials, mecamylamine, as an LCC-channels blocker, is less effective compared to neurotoxin II, nicotine, pipecuronium bromide, and rocuronium bromide, described in our previous research [11, 12], but its effects on LCC-channels were accompanied by a higher degree of currents' amplitude decrease when compared

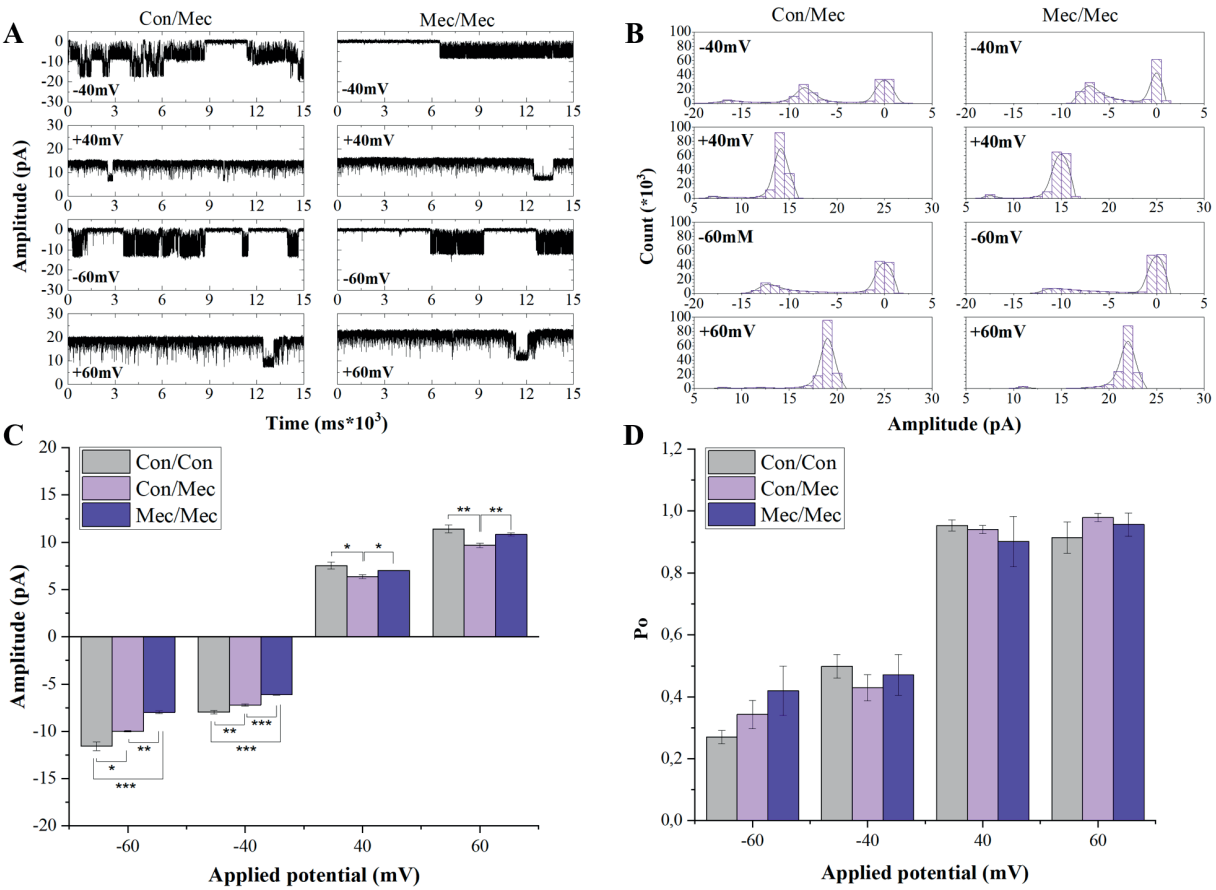

Fig. 3. Effects of mecamylamine at the concentration of 1 mmol/l applied via a patch micropipette on electrophysiological properties of LCC-channels. Con/Mec - mecamylamine was added into the patch pipette but not into the bath; Mec/Mec - mecamylamine was applied both in the patch pipette as well as the bath. A - representative fragments of registrations in the presence of mecamylamine in the patch pipette. B - amplitude diagrams, corresponding to the registrations, depicted in A. C - graphs of the average amplitude changes caused by the test substance. D - impact of the mecamylamine on the average probability of LCC-channels being in an open state. *P < 0.05; **P < 0.01; ***P < 0.001 compared to groups with different mecamylamine concentrations applied

to α-cobratoxin. Based on the potency of the blocking effect, mecamylamine ranks among the rest of the studied nicotinic acetylcholine receptor modulators as follows: neurotoxin II > nicotine >> pipecuronium bromide > rocuronium bromide > mecamylamine > α-cobratoxin. Compared to other classes of biologically active molecules, mecamylamine proved to be a more potent blocker than mydocalm and diprofol [12], despite the latter two also being able to decrease the probability of LCC-channels residing in an open state. Taking this into account, nicotine and neurotoxin II remain promising candidates for use in further research aimed at testing the hypothesis of LCC-channels' involvement in the generation of K^+ countercurrent, as these substances provide the most effective chemical blockade of the described channels.

Differences in the strength of the blocking activity are likely to be caused by the differences in the chemical structure of the described substances. Detection and further examination of the structural features responsible for the inhibiting effects on LCC-channels may lead to a better understanding of the structure of LCC-channels themselves, considering that a modulator is usually complementary to the regulatory site of the modulated protein, such as an ion channel.

CONCLUSIONS

1. Mecamylamine at a concentration of 1 mmol/l is an LCC-channels inhibitor of average efficiency, which can decrease the amplitude of the K^+ currents through these channels at negative membrane potentials (by approximately 16-17% at -40 and -60 mV, respectively) when applied in bath solution, although the blocking effect of mecamylamine in this case is weaker compared to nicotine or pipecuronium bromide. The effect of mecamylamine differs depending on the membrane potential when applied to a bath solution, thus interacting with the intranuclear side of LCC-channels. In contrast to the effect observed at negative potentials, this substance slightly increases the current amplitude through LCC channels at positive potentials.

2. The effect of mecamylamine (1 mmol/l) varies depending on the side of the membrane to which it is applied. When administered via the patch pipette and acting on the perinuclear side of the LCC-channels, it is shown to decrease the currents' amplitude at both positive and negative membrane potentials.

3. Mecamylamine is shown to decrease the open probability of LCC-channels by 45%, but only when interacting with the intranuclear side of the channels and at the membrane potential of -40 mV. This substance is also capable of partial mechanical blockade of LCC-channels and causes channel flickering when applied on either side of the membrane.

Acknowledgments: This study was inspired by and based upon the ideas of S. Marchenko, who proposed LCC-channels for the role of the channels responsible for creating K^+ countercurrent during the release of Ca^{2+} cations from the intracellular stores.

Additionally, this research was partially supported by a grant for research projects of young scientists from the National Academy of Sciences of Ukraine (2021-2022), the project "Pharmacological sensitivity and expression of high-conductance cation channels in nuclei of various cell types" (State registration number: 0121U112012).

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВПЛИВ МЕКАМІЛАМІНУ НА ЕЛЕКТРОФІЗІОЛОГІЧНІ ВЛАСТИВОСТІ LCC-КАНАЛІВ НЕЙРОНІВ ПУРКІНЬЄ МОЗОЧКА ЩУРІВ

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Вивільнення Ca^{2+} із внутрішньоклітинних депо потребує наявності компенсаторного протитоку K^+, однак наразі залишається невідомою будова LCC-каналів, які можуть його забезпечувати. Для перевірки гіпотези про залученість цих каналів у вивільнення Ca^{2+}, насамперед має бути знайдений їх специфічний блокатор. Метою нашої роботи було дослідження впливу мекаміламіну на LCC-канали ,щоб встановити, чи може ця сполука розглядатися як їх ефективний блокатор. Ядра клітин були виділені з нейронів Пуркіньє щурів лінії Вістар віком 3-4 тиж. Мозочок щурів нарізали на тонкі зрізи та гомогенізували; преципітат, що містив ядра, було ресуспендовано і перенесено до ванночки інвертованого мікроскопа, де реєстрували іонні струми крізь LCC-канали методом patch-clamp з використанням конфігурації nucleus-attached у режимі voltage-clamp. Встановлено, що мекаміламін у концентрації 1 ммоль/л при аплікації з внутрішньоядерного боку LCC-каналів знижував амплітуду струмів при негативних потенціалах (на 16-17% при –40 та –60 мВ відповідно) та незначно збільшував її при позитивних. Також ця речовина на 45% зменшувала ймовірність перебування каналів у відкритому стані (P_o) при потенціалі –40 мВ. На противагу цьому, коли мекаміламін діяв з перинуклеарного боку LCC-каналів, амплітуда струмів знижувалася як при позитивному, так і при негативному потенціалі, тоді як значення P_o не зазнавали змін. Залежність ефекту речовини від того, з якого боку ядерної мембрани її було застосовано, може вказувати на наявність відмінностей між внутрішньоядерними та перинуклеарними доменами LCC-каналів. Також встановлено, що мекаміламін зумовлює явище миготіння каналів при аплікації з будь-якого боку мембрани.

Ключові слова: іонні канали; мембранна провідність; patch-clamp; мекаміламін; антагоністи нікотинових ацетилхолінових рецепторів, кальцієвий гомеостаз; електрофізіологія; модуляція.

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Received 07.05.2025