Current Research in Physiology 5 (2022) 1–7

Contents lists available at ScienceDirect

Current Research in Physiology

journal homepage: www.sciencedirect.com/journal/current-research-in-physiology

The role of M3 receptors in regulation of electrical activity deteriorates in the rat heart during ageing

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ARTICLE INFO

Keywords:
Heart
Rat
Action potential
Calcium current
Muscarinic receptors
Ageing
M3 receptors

ABSTRACT

Ageing is a complex process which affects all systems of the organism and therefore changes the environment where the heart is working. In this study we demonstrate the ageing-related changes in the mechanisms of parasympathetic regulation of mammalian heart. Electrophysiological effects produced by selective activation of M3-cholinoreceptors were compared in isolated cardiac preparations from young adult (4 months), adult (1 year) and ageing (2 years) rats using sharp glass microelectrode technique. M3-receptors were activated with muscarinic agonist pilocarpine (10^{-5}M) in the presence of selective M2 antagonist AQ-RA741 (10^{-7}M). In atrial and ventricular myocardium from young rats M3 stimulation induced shortening of action potentials(APs), while no significant effect was observed in both elder groups. The main mechanism of M3-induced AP shortening is inhibition of L-type Ca^{2+} current, estimated using whole-cell patch-clamp. It was negligible in atrial myocytes from ageing animals in comparison with young rats. The loss of sensitivity to stimulation of M3-receptors is due to decrease in M3 gene expression, shown by RT-PCR both in atrial and ventricular samples from ageing rats. Thus, in ageing rat heart M3-receptors are down-regulated and not involved in regulation of electrical activity.

1. Introduction

Autonomic nervous system regulates the mammalian heart function and maintains the adaptation of cardiovascular system to the changing environment. Parasympathetic autonomic modulation of cardiac activity is mediated mainly by acetylcholine (ACh). Released from post-ganglionic parasympathetic intramural neurons this neurotransmitter affects cardiac myocytes via membrane muscarinic receptors. Although the prevalent subtype of muscarinic receptors in the heart is M2 subtype (Dhein et al., 2001), M3 muscarinic receptors also take part in regulation and maintenance of cardiac function (see Wang et al., 2007 for review). Particularly, M3 receptors mediate cholinergic regulation of such bioelectrical parameters as action potential (AP) waveform and heart rate both under normal physiological conditions (Wang et al., 1999; Abramochkin et al., 2012) and during the initiation and progression of cardiac diseases (Liu et al., 2008; Wang et al., 2012).

Molecular mechanisms which mediate electrophysiological effects of M3 receptors activation in mammalian myocardium have been elucidated in a number of studies and depend on animal species. In guinea pigs ((Shi et al., 1999a) and dogs (Shi et al., 1999b) selective stimulation of M3 receptors leads to activation of delayed rectifying K^+ current I_{KM3} which facilitates membrane repolarization, i.e. shortens AP. In rats, M3 stimulation induces AP shortening as well, but via activation of phosphoinositol signaling pathway (Abramochkin et al., 2008) and subsequent suppression of L-type calcium current I_{CaL} (Filatova et al., 2017). The increase in K^+ conductance in rat working cardiomyocytes is attributed exclusively to activation of M2 receptors (Filatova et al., 2017).

Ageing is a complex process which affects all systems of the organism and therefore changes the environment where the heart is working. It is well-known that the number of patients struggling from cardiovascular diseases dramatically elevates with increasing age making the problem of cardiac ageing socially important, especially for the developed nations (Chiao and Rabinovitch, 2015). Particularly, the probability of heart rhythm disturbances is higher in elder people in comparison with those who are young. For example, the prevalence of atrial fibrillation in

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https://doi.org/10.1016/j.crphys.2021.12.001
Received 19 October 2021; Received in revised form 25 November 2021; Accepted 2 December 2021
Available online 8 December 2021

people aged of 60–70 years is about 4%, while in those over 80 years this parameter estimates 20% (Zoni-Berisso et al., 2014). Molecular mechanisms of cardiac ageing are complex and still not well understood. Recently, our group has demonstrated drastic down-regulation of M3 receptors expression and their functional significance in rat myocardium during early postnatal ontogenesis (Tapilina and Abramochkin, 2016). Moreover, a decrease in expression of M3 receptors has been shown in mouse model of cardiac ageing (Wang et al., 2018). Taking into account previously demonstrated antiarrhythmic effects of M3 stimulation in ventricular myocardium of rats (Wang et al., 2012) and guinea pigs (Liu et al., 2008), the ageing-related changes in M3-mediated regulation of mammalian cardiac electrical activity is of interest.

The present study aims to compare the effects of selective stimulation of M3 muscarinic receptors on atrial and ventricular AP waveform in young adult (4 months), adult (1 year) and ageing (2 years) rats and elucidate the mechanisms of M3-mediated age-related electrophysiological effects.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by Ethics Committee of NMRCC. Male Wistar rats were held in the animal house under a 12-h:12-h light:dark photoperiod at 22–24 °C in standard T4 cages and fed *ad libitum*. The animals were divided into 3 groups. The rats of the first group were delivered to the animal house at the age of 4 months and were involved in experiments within the next ten days. The rats of the other two groups were delivered at the same age and held in described conditions for the next 9–11 months (1 year old group) or 21–24 months (2 year old group).

2.2. Isolation of cardiac multicellular preparations and microelectrode recordings

Rats were anesthetized with intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. Heparin (1000 U/kg) was added to the anaesthetics solution to prevent blood coagulation in the coronary vessels of the excised heart. The chest was opened and the heart was rapidly excised and rinsed with Tyrode solution of the following composition (in mM): NaCl 118.0, KCl 2.7, NaH_2PO_4 2.2, $MgCl_2$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 25.0, glucose 11.0, bubbled with carbogen gas (95% O_2 + 5% CO_2), pH 7.4. The preparations of right ventricle free wall and right atrium (including the intercaval region) were isolated and pinned with the endocardial side up to the bottom of experimental chamber (3 ml) perfused with Tyrode solution (10 ml/min, 37.5 °C). Since the right atrial preparations contained the sinoatrial node they were beating spontaneously throughout the experiment. Right ventricular wall preparations were paced throughout the experiment with a pair of silver Teflon-coated electrodes (pacing rate – 5 Hz, pulse duration – 1 ms, pulse amplitude – 2 times threshold).

After an hour of equilibration in the perfusion chamber, transmembrane potentials were recorded from the endocardial surface of preparations with sharp glass microelectrodes (30–45 MΩ) filled with 3M KCl connected to a high input impedance amplifier Model 1600 (A-M Systems, Sequim, WA, USA). The signal was digitized and analysed using specific software (L-card, Russia; DI-Soft, Russia; Synaptosoft, USA). Stable impalements were maintained during the entire period of drugs application. Changes in the resting membrane potential, AP amplitude and AP duration at 90% of repolarization (APD90) were determined.

2.3. Selective stimulation of M3 cholinoreceptors in multicellular preparations and cardiomyocytes

To induce the selective activation of M3 cholinoreceptors we have used the protocol with muscarinic agonist pilocarpine validated by several earlier studies (Abramochkin et al., 2012; Wang et al., 2012; Filatova et al., 2017), although more selective and high-affine antagonists of M2 and M3 receptors were used. Muscarinic agonist pilocarpine (10^{-5}M) with slight selectivity to M1 and M3 receptors was applied to the experimental chamber alone or in the presence of highly selective M2 antagonist AQ-RA 741, 10^{-7}M (Doods et al., 1991, 1994; Ivanova et al., 2019) after prior exposure of preparations to AQ-RA 741 alone. M3 antagonist J-104129 with high selectivity for M3 over M2 receptors (Mitsuya et al., 2000) was used in special control experiments to ensure that cholinergic effects remaining in the presence of AQ-RA 741 are attributed to M3 receptor activation.

2.4. Isolated myocyte preparation

Isolated cardiac myocytes were obtained using the previously described technique (Isenberg and Klockner, 1982) with slight modifications (Abramochkin and Vornanen, 2014; Abramochkin et al., 2013). The rat hearts were briefly isolated as described above and mounted onto a Langendorff apparatus for retrograde perfusion with Ca^{2+}-free solution containing (in mM): NaCl 125, KCl 4, NaH_2PO_4 1.7, $NaHCO_3$ 25.2, $MgCl_2$ 0.55, Na-pyruvate 5, glucose 11, taurine 20, bovine serum albumin 1 g/ml; pH 7.4 buffered with carbogen gas (95% O_2, 5% CO_2) at 37C. After 5 min of perfusion with the Ca^{2+}-free solution the hearts were perfused for 45–60 min with the same solution provided with 0.7 mg ml^{-1} collagenase type II (Worthington, USA), 0.1 mg ml^{-1} protease type XIV (Sigma, USA) and 20 μM $CaCl_2$. After 45–50 min of perfusion the ventricles were separated, chopped and triturated to release the cells into Kraftbruhe medium containing (in mM): glutamic acid 50; HEPES 20; taurine 20; $MgSO_4$ 3; KCl 30; EGTA 0.5; KH_2PO_4 30; glucose 10; pH 7.2 at room temperature. The atria were perfused with enzymes for 55–60 min, and then were treated the same way as ventricles. The cells were stored in Kraftbruhe medium and used within 5–6 h.

2.5. Whole-cell patch clamp

The whole-cell voltage clamp recording of and Ca^{2+} currents was performed using an Axopatch 200B (Molecular Devices, CA, USA) amplifier. The myocytes were superfused in a small recording chamber (RC-26; Warner Instrument Corp, Brunswick, CT, USA; volume 150 μl) mounted on an inverted microscope with an external K^+ or Cs^+-based solution at room temperature (24 ± 0.5 °C). The first solution was used to obtain the whole-cell configuration and contained (in mmol·l^{-1}): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 10 Hepes, with pH adjusted to 7.4 at 20 °C with NaOH. Cs^+-based solution was used for Ca^{2+} current measurement and had the same composition excluding KCl substituted with equimolar CsCl.

Patch pipettes with mean (±SEM) resistance of 2.06 ± 0.82 MΩ (n = 64) were pulled from borosilicate glass (Sutter Instrument, CA, USA) using PIP-6 puller (HEKA Instruments, Germany). The pipettes were filled with Cs^+-based electrode solution containing (in mmol·l^{-1}): CsCl 130, tetraethylammonium chloride (TEA) 15, $MgCl_2$ 1, oxaloacetate 5, EGTA 5, MgATP 5, MgGTP 0.03 and HEPES 10 with pH adjusted to 7.2 with CsOH. 25 μM β-escin was added to Cs^+-based pipette solution as a perforating agent, only the freshly-prepared solution with β-escin was used throughout one experimental day, but not stored further. Perforated patch technique allowed to record I_{CaL} with minimal rundown (Fu et al., 2003; Filatova et al., 2017). The peak current density after 10 min of continuous recording was not less than 90% of control level. Pipette capacitance was compensated after obtaining the seal with a resistance >2 GΩ. The whole cell capacitance and access resistance were completely compensated using the amplifier manual controls after the

perforation of membrane. The mean cell capacitance was 52.6 ± 17.8 pF, the mean access resistance was decreasing during the gradual perforation of cell membrane by β-escin until stabilization at the level of 7.7 ± 1.9 MΩ (n = 34). The recording of I_{CaL} and drug testing was started only after access resistance stabilization. In order to obtain current densities the peak currents were normalized by cell capacitance.

I_{CaL} was elicited using protocol with 2-step square-pulse depolarization from the holding potential of −80 mV. The first 100 ms step of depolarization to −40 mV allowed to completely inactivate sodium current and I_{CaT}, therefore the following 400 ms depolarization to −30 - +40 mv with 10 mV steps induced only I_{CaL}.

2.6. Measurements of M3 receptor gene expression

For real time PCR was used isolated atria and ventricles tissues from 3 groups of animal. After preparation samples were dipped into RNA stabilization solution (IntactRNA, Evrogen, Russia) for 24 h at 4 °C and stored at 20 °C before used. RNA was extracted from samples with the usage of acid-guanidine-phenol-chloroform method (ExtractRNA, Evrogen, Russia). RNA was treated from genomic DNA with the usage of DNase I (2000 e.a./ml, NEB,USA) for 60 min at 42 °C. Total RNA (500 ng) was reverse-transcribed by MMLV RT kit this random primers (Evrogen, Russia) according to the protocol recommended by the manufacturer.

Quantitative RT-PCR was carried out in a total volume 25 μl containing qPCRmix-HS SYBR (Evrogen, Russia) containing intercalating fluorescent dye SYBR Green I. The assay was performed using PCR detection system BioRad CFX96. Melting was initiated at 95 °C during 5 min. The cycling profiles were consisted of denaturation of 1 min at 95 °C, annealing of 30 s at 60 °C, extension of 30 s at 72 °C, for 50 cycles and final step of 10 min at 72 °C.

Sequences for primers were as follows: M3-receptors - CAAGTGGTCTTCATTGCCTTCT (forward), GCCAGGCTTAAGAGGAAGTAGTT (reverse) and GAPDH - CAGCGATGCTTTACTTTCTGAA (forward), GATGGCAACAATGTCCACTTT (reverse). For standard curves for RT-PCR was used serially diluted genomic DNA from rat liver.

2.7. Drugs

All antagonists and agonist of muscarinic receptors were purchased from Tocris Bioscience (Bristol, UK). Selective activation of M3-receptors was performed by simultaneous application of nonspecific muscarinic agonist pilocarpine hydrochloride and high affinity and selective M2 antagonist 11-[[4-[4-(Diethylamino)butyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AQ-RA 741). Also we used selective M3 muscarinic receptor antagonist (<R)- < -Cyclopentyl- < -hydroxy-N-[1-(4-methyl-3-pentenyl)-4-piperidinyl] benzeneacetamide fumarate (J-104129). β-escin was purchased from Sigma (St. Louis, MO, USA). Collagenase type II was purchased from Worthington (Lakewood, NJ, USA).

2.8. Statistics

All data are presented as mean ± S.D. for n experiments. The significance of pilocarpine effect on AP parameters and amplitude of I_{CaL} was checked using two-way repeated measures ANOVA with further Tukey's post hoc multiple comparisons test. Similar non-repeated measures analysis was used to compare the AP parameters, density of I_{CaL} and the magnitude of effects of muscarinic agonist in preparations from 3 age groups of experimental animals. One-way ANOVA was used to compare level of expression of M3 receptor gene in myocardium samples from 3 animal groups. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of selective M3 stimulation on electrical activity in atrial and ventricular preparations from young and aged rats

In control conditions APD90, AP amplitude and RMP did not differ significantly between 3 groups of animals either in atrial or ventricular myocardium preparations. In atrial preparations control APD90 did not significantly change with age (54.1 ± 15.6 ms in 4 months-old rats (n = 8), 65.2 ± 19.5 ms in 1 year old (n = 7), 58.9 ± 18.1 ms in 2 year old (n = 6), Fig. 1, p > 0.05). In ventricular preparations (Fig. 2) APD90 was 42.9 ± 13.3 ms in 4 months-old rats (n = 8), 39.5 ± 12 ms in 1 year old (n = 7) and 51.8 ± 10.7 ms in 2 year old (n = 8) animals. In the latter group ventricular APs were significantly longer than in preparations from young adult rats (p < 0.05). AP amplitude and RMP did not differ significantly between 3 age groups (p > 0.05).

When pilocarpine was applied alone, the marked significant shortening of APs at 90% repolarization level (p < 0.05) was observed in atrial (Fig. 1) and ventricular (Fig. 2) preparations from each group of rats. AP amplitude and RMP were not affected by pilocarpine. Effects of pilocarpine were completely reversible – AP duration similar to the control was restored after 10 min washout. Noteworthy, in atrial preparations from 2 year old rats the effects of pilocarpine were less prominent than in young rats (p < 0.05, Fig. 1), while no age-dependency was observed for magnitude of pilocarpine-induced AP shortening in ventricular myocardium (Fig. 2).

When M2 receptors were blocked with 10^{-7}M AQ-RA 741, pilocarpine induced AP shortening only in young adult rats (Fig. 1a,d, Fig. 2). This effect was attributed to stimulation of M3 receptors since it was completely abolished in the presence of J-104129 (Figs. 1d and 2). In two elder groups selective activation of M3 receptors failed to alter significantly AP parameters both in atrial (Fig. 1b and c) and ventricular (Fig. 2) preparations. In atrial preparations effect of selective M3 stimulation in young adults was significantly stronger than in 1 year and 2 year old rats (p < 0.05, Fig. 1d). Thus, ageing decreases the changes in AP morphology induced by selective stimulation of M3 receptors in rat myocardium.

3.2. Effects of selective M3 stimulation on L-type Ca^{2+} current in cardiac myocytes from young and aged rats

In rat atrial cardiac myocytes the basal values of I_{CaL} amplitude increased in aged animals group. In 2-year old animals the current at 0 mV was −5.02 ± 1.86 pA/pF, which was significantly larger than −3.5 ± 0.85 pA/pF in 4 month-old rats (p < 0.05, Fig. 3a–f). The current amplitude in myocytes from 1 year old rats was between these values (−4.71 ± 1.75 pA/pF) and did not differ significantly from current in either young or old rats (p > 0.05).

In the absence of muscarinic antagonists, 10^{-5}M pilocarpine produced marked reduction of peak I_{CaL} (Fig. 3g) in atrial myocytes from 4 month-old and 1 year old rats (p < 0.05), while in the cells from 2 year old animals this effect was below the significance level (p > 0.05). When M2 receptors were blocked with 10^{-7}M AQ-RA 741, application of pilocarpine led to significant attenuation of I_{CaL} only in atrial cells from the youngest group of animals (p < 0.05, Fig. 3a–c,g). Stimulation of M3 receptors did not affect the shape of I–V curve, the maximal current was always elicited by depolarization to 0 mV (Fig. 3 d-f). The double-block of M2 and M3 receptors with 10^{-7}M AQ-RA 741 and 5 × 10^{-8}M J-104129, respectively, completely prevented the effect of pilocarpine. Noteworthy, neither AQ-RA 741, nor the mixture of both blockers applied prior to pilocarpine changed the amplitude of I_{CaL}.

Thus, selective stimulation of M3 cholinoreceptors in atrial myocytes from young rats leads to effective suppression of I_{CaL}. During ageing, sensitivity of I_{CaL} to stimulation of M3 receptors strongly decreases.

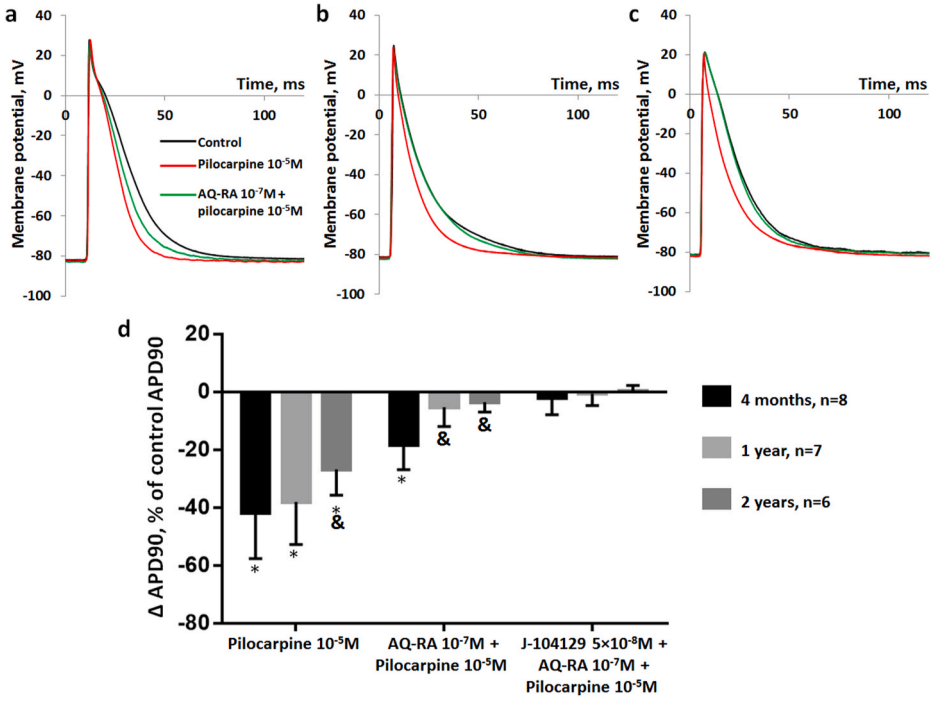

Fig. 1. Effects of pilocarpine on AP waveform in rat right atrial preparations. (**a,b,c**) Original traces of APs recorded under the effect of 10^{-5}M pilocarpine alone and in the presence of 10^{-7}M AQ-RA 741 (selective stimulation of M3 receptors) in right atrial preparations from young (**a**), adult (**b**) and ageing (**c**) rats. AP traces are superimposed on respective control traces, which were recorded right before pilocarpine application. (**d**) Relative changes of AP duration at 90% repolarization level induced by 10^{-5}M pilocarpine alone or in the presence of M2 and M3 blockers, expressed in % of control AP duration. * - significant reduction of APD, $p < 0.05$, paired *t*-test. & - significant difference from 4 months old rats, $p < 0.05$, two-way ANOVA with further Tukey's post hoc multiple comparisons.

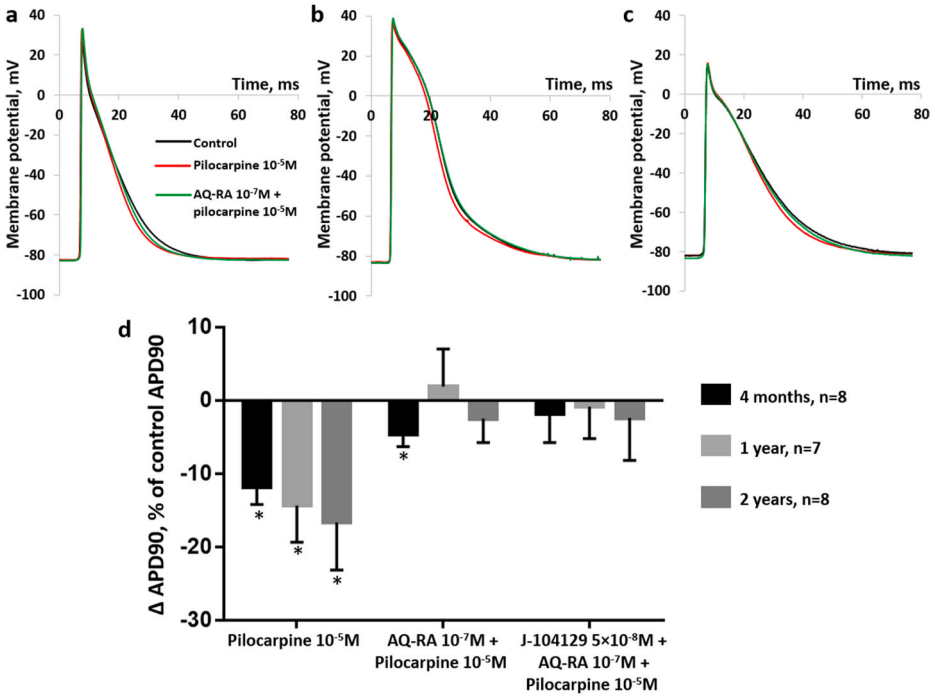

Fig. 2. Effects of pilocarpine on AP duration in rat right ventricular wall preparations. (**a,b,c**) Original traces of APs recorded under the effect of 10^{-5}M pilocarpine alone and in the presence of 10^{-7}M AQ-RA 741 (selective stimulation of M3 receptors) in right ventricular wall preparations from young (**a**), adult (**b**) and ageing (**c**) rats. AP traces are superimposed with respective control traces, which were recorded right before pilocarpine application. (**d**) Relative changes of AP duration at 90% repolarization level induced by 10^{-5}M pilocarpine alone or in the presence of M2 and M3 blockers, expressed in % of control AP duration. * - significant reduction of APD, $p < 0.05$, paired *t*-test. No significant differences between 3 age groups of rats were detected by two-way ANOVA with further Tukey's post hoc multiple comparisons.

3.3. Expression of M3 receptor gene in atrial and ventricular myocardium of young and aged rats

According to the results of RT-PCR, M3 receptor mRNA is present in the myocardium of rats of all age groups. However, the expression level of the M3 receptor gene decreases both in the atrial (Fig. 4a) and ventricular (Fig. 4b) myocardium with ageing of animals. The age-dependence is clearer in atrial myocardium, although ventricular myocardium of 2 year old animals has significantly lower level of M3 receptor mRNA content than the samples from 4 month-old animals.

4. Discussion

Aging of the organism leads to marked changes in all organ systems, including cardiovascular system. Thereby, the microenvironment of cardiac myocytes alters with age and thus stimulates appropriate adaptations and/or leads to disadaptations which have negative impact on cardiac function. For example, it is widely accepted that pacemaker function of mammalian sinoatrial node deteriorates with aging resulting in age-associated sinus bradycardia (reviewed by Dun and Boyden, 2009). Thereby, all working myocytes of the aged heart will excite in a

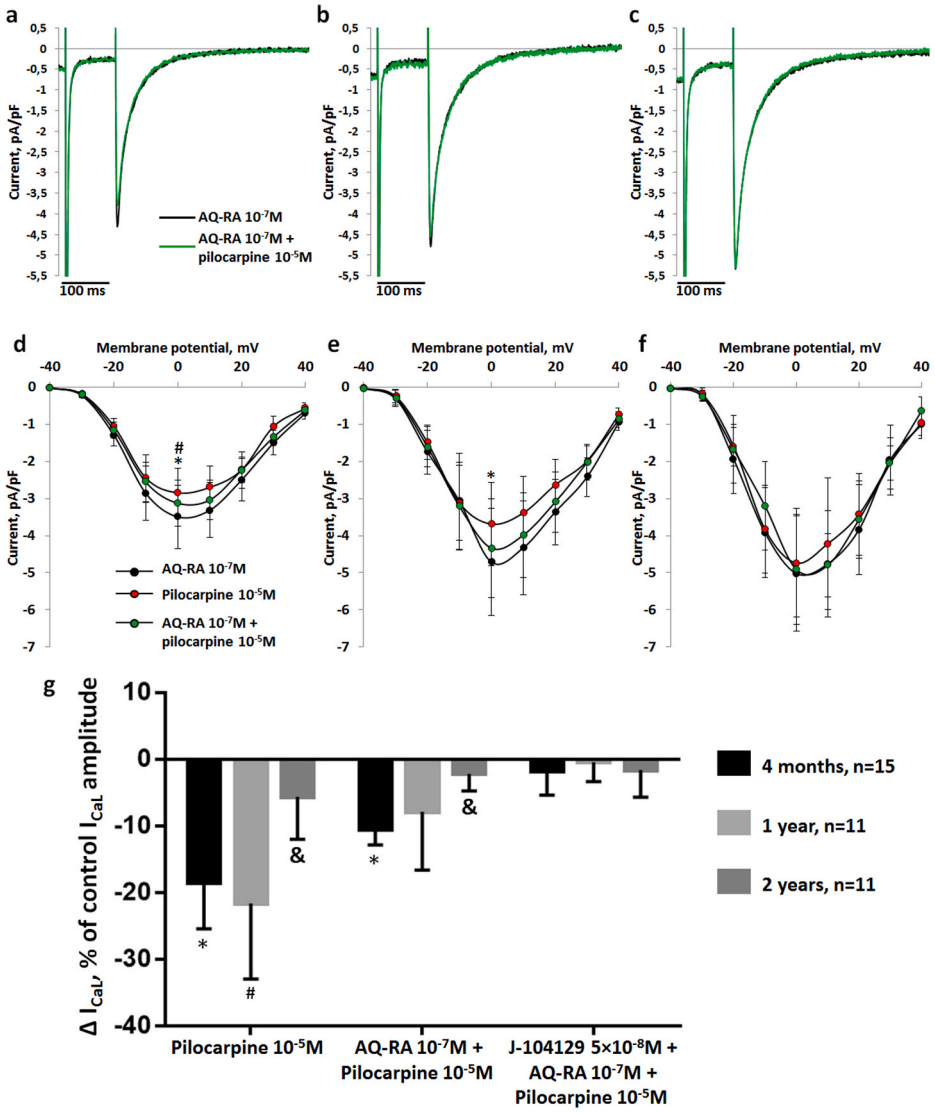

Fig. 3. Effects of selective M3-stimulation on the L-type calcium current (I_{CaL}) in rat atrial myocytes. (**a-c**) Original tracings of I_{CaL} induced by depolarization to 0 mV, from 3 different representative experiments recorded in the presence of M2 blocker AQ-RA 741 before and during the application of 10^{-5}M pilocarpine. (**d-f**) Comparison of I–V curves of I_{CaL} recorded in atrial myocytes from young (d), adult (e) and ageing (f) rats before and during the application of pilocarpine in the presence of AQ-RA 741. I_{CaL} I–V curves recorded under the effect of pilocarpine without M2 blocker are also provided. (**g**) relative reduction of peak I_{CaL} amplitude at 0 mV in myocytes from 3 age groups of rats, induced by 10^{-5}M pilocarpine alone, in the presence of M2 blocker or combination of M2 and M3 blockers. The results are presented as means ± S.E.M. of 11–15 myocytes (n stated on the figure) from 4 rats. The current was elicited using protocol with 2-step square-pulse depolarization from the holding potential of −80 mV to −40 mV (first step for inactivation of I_{Na}) and further to −30 - +40 mV with 10 mV increment. The current amplitude was calculated as the difference between the peak I_{CaL} and the current in the end of depolarizing pulse. * - significant effect of pilocarpine at 0 mV, $p < 0.05$, paired *t*-test. # - significant effect of pilocarpine in the presence of AQ-RA 741 at 0 mV, $p < 0.05$, paired *t*-test. & - significant difference from 4 months old rats, $p < 0.05$, two-way ANOVA with further Tukey's post hoc multiple comparisons.

slower rhythm, which may be considered as altered factor of their environment leading to changes in ionic channels expression. Moreover, in such altered conditions, the potential sources of ectopic activity might induce tachyarrhythmias with greater ease (Dun and Boyden, 2009; Wongchareon et al., 2007). The autonomic regulation of cardiac electrical activity affects various ionic currents and may also undergo age-related changes increasing or decreasing the effectiveness of adaptation to altered conditions.

In the present study we demonstrate the marked changes in receptor mechanism of muscarinic agonist effects on rat cardiac electrophysiology associated with ageing. Previously, age-related decrease in contribution of M3 receptors to the mediation of cholinergic changes in AP morphology was shown in hearts of rats during early postnatal ontogenesis (Tapilina and Abramochkin, 2016). The same study demonstrates a decrease in M3 gene expression in rat atrial and ventricular myocardium associated with maturation. However, the relative impact of M3 receptor activation on cardiac electrical activity has never been estimated in old animals.

AP shortening induced by the activation of M3 cholinoreceptors has been previously observed in right atrium and ventricle from mice (Abramochkin et al., 2012) and rats (Filatova et al., 2017), as well as in guinea pig atrial myocardium (Wang et al., 1999). The results of our sharp microelectrode experiments clearly indicate that the ability of selective M3 stimulation to shorten APs deteriorates with ageing either in atrial (Fig. 1) or ventricular (Fig. 2) rat myocardium. The lack of sensitivity to the selective stimulation of M3 cholinoreceptors can be obviously explained by results of our quantitative RT-PCR measurements that show decrease of M3 receptor gene expression in 1 year old and, especially in 2 year old rats (Fig. 4). Our findings are in complete agreement with recent results of RT-PCR assays and Western blotting done in myocardium of aged mice. Both mRNA and protein expression levels of M3 receptor are decreased in the hearts of 18–20 months old mice if compared to 8–12 weeks old mice (Wang et al., 2018).

In rat cardiac myocytes, activation of M3 receptors leads to suppression of L-type Ca^{2+} current I_{CaL} either in normal conditions (Filatova et al., 2017) or during extracellular acidosis leading to enhancement of that current (Wang et al., 2012). In guinea pig (Shi et al., 1999a) and dog (Shi et al., 1999b), activation of specific I_{KM3} potassium current has been considered to be the major mechanism of M3-induced acceleration of repolarization, although this concept is not supported by experiments done in feline and rabbit cardiac myocytes (Moreno-Galindo et al., 2011; Rodriguez-Martinez et al., 2011). In rat atrial myocytes, pilocarpine fails to affect any of K^+ currents if M2 receptors are blocked with their selective antagonist methoctramine (Filatova et al., 2017). Thus, reduction of I_{CaL} seems to be the main, if not the only, ionic mechanism of M3-induced AP shortening in rat cardiac myocytes, while activation of

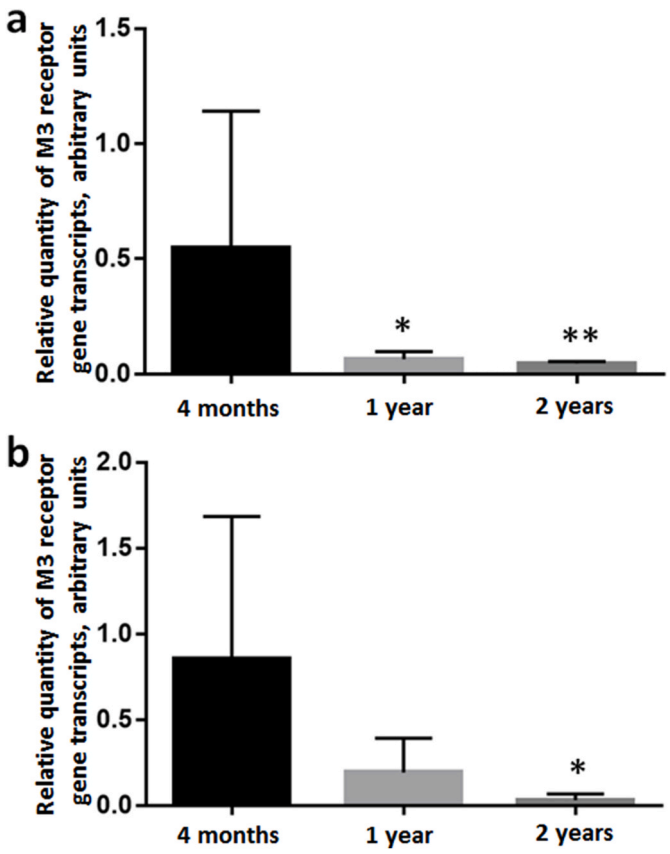

Fig. 4. Relative transcription level of M3 receptor gene in atrial (**a**) and ventricular (**b**) myocardium of rats from 3 age groups (n = 6 in each group). The y-axis shows the relative amount of mRNA in the samples, calculated from the results of RT-PCR. Results are normalized to the level of transcription of GAPDH gene. The results are shown in arbitrary units. * and ** - significant difference from young rats, one-way ANOVA, $p < 0.05$ and $p < 0.01$, respectively.

M2 receptors induces inward rectifier current I_{KACh} (Kim, 1991; Mizuno et al., 2008). Thereby, in the present study we focused on comparison of I_{CaL} sensitivity to M3 stimulation in myocytes from aged and young rats.

The results of patch-clamp experiments indicate that I_{CaL} density in rat atrial myocytes increases during ageing. To our knowledge, such relation has never been described in atrial cells, although it is known that I_{CaL} increases during early postnatal development in murine sinoatrial myocytes (Adachi et al., 2013). In rat ventricular myocytes, ageing is associated with AP prolongation (Janczewski et al., 2002) which is due to an increased single L-type Ca^{2+} channel activity (Josephson et al., 2002) and slowing of I_{CaL} inactivation (Walker et al., 1993).

We have found that selective stimulation of M3 receptors induces significant decrease of I_{CaL} only in atrial cells from 4 month-old rats (Fig. 3). This explains the inability of M3 stimulation to shorten APs in preparations of atrial myocardium from 1 year old and 2 year old rats. Noteworthy, in the absence of M2 blocker, pilocarpine suppressed I_{CaL} in myocytes from 1 year old rats indicating that in the case of moderate ageing the current can still be regulated via activation of M2 receptors leading to inhibition of protein kinase A and decrease in the extent of phosphorylation of L-type channels (Treinys et al., 2016).

The present study does not avoid several important limitations. First, possible non-specific actions of the subtype-selective muscarinic blockers should be taken into account. This concern is of greater importance since we used only one concentration of each muscarinic antagonist. However, the concentration was selected basing on the data about subtype-specificity of the used blockers. AQ-RA 741 has especially high affinity for M2 receptors ($pK_i = 8.3$) if compared to M3 receptors ($pK_i = 6.82$) (Doods et al., 1991). Moreover, in rats, cats and guinea-pigs AQ-RA 741 preferentially inhibits the vagally or agonist-induced bradycardia with -log ID50 = 7.24–7.53 i.v. Thereby, we proposed that 10^{-7}M AQ-RA 741 will be enough for almost complete block of M2 receptors in isolated myocytes and myocardial preparations. This assumption was confirmed by the fact that 5×10^{-8}M J-104129, which has 120-fold selectivity for M_3 receptors (Ki = 4.2 nM) over M2 receptors (Ki = 490 nM), completely abolished the effects of pilocarpine both in isolated tissues and isolated cells. Therefore, we suppose that the observed effects of pilocarpine in the presence of AQ-RA 741 are mediated by selective M3 activation.

Second, we did not check the possible involvement of K^+ currents in M3-mediated changes of AP waveform in myocardium from aged rats. However, it was shown that in young rats M3 stimulation does not induce any increase in K^+ conductance (Filatova et al., 2017). Since the magnitude of M3-mediated electrophysiological effects declines with age, it is very unlikely that an additional ionic mechanism of M3-induced AP shortening appears in the hearts of aged animals. Third, we failed to observe any effects of pilocarpine on I_{CaL} in ventricular myocytes from either young or aged rats. Therefore, the ionic mechanism of M3-mediated AP shortening is confirmed only in atrial myocytes. It is likely that muscarinic receptors in ventricular myocytes are damaged during the enzymatic isolation. Since their expression in ventricular myocardium is lower than in atrial (Hellgre et al., 2000; Tapilina and Abramochkin, 2016), it is not surprising that the effect of M3 stimulation disappears after isolation of single myocytes.

In conclusion, our findings demonstrate that ageing in rats is associated with a decrease of M3 receptor gene expression leading to the loss of mechanism of I_{CaL} down-regulation by muscarinic agonists. As a consequence, in aged rats cholinergic regulation of cardiac AP morphology is mediated exclusively via M2, but not M3 cholinoreceptors. The relationship between M3 receptors and cardiac diseases, including arrhythmias, pathological cardiac hypertrophy and heart failure has been confirmed in a number of studies (see Hang et al., 2013 for review). It is well-known that the occurrence of such diseases dramatically increases with age. Very recently, Wang et al. (2018) first demonstrated age-related down-regulation of M3 receptors in the mouse heart and proposed that activation of M3 receptors delays cardiac ageing by inhibiting the caspase-1/IL-1β signaling pathway. Down-regulation of M3 receptors leads to a stronger activation of this proinflammatory pathway which accelerates cardiac ageing (Salminen et al., 2012). Activation of M3-receptors was shown to protect the heart from ventricular arrhythmias (Wang et al., 2012; Liu et al., 2011). We suggest that the impairment of M3-mediated regulation of cardiac electrical activity, demonstrated in the present study, is an additional proarrhythmic factor at least in the ventricles of an aged heart. Further research should find out whether activation of remaining M3 receptors may protect the heart from ventricular arrhythmias during ageing.

Funding

This study was supported by the grant from the Russian Science Foundation (17-75-10181) to S.V.T. and by the Interdisciplinary Scientific and Educational School of Moscow University “Molecular Technologies of the Living Systems and Synthetic Biology” to D.V.A.

CRediT authorship contribution statement

Svetlana V. Tapilina: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Alexandra D. Ivanova:** Formal analysis, Investigation, Writing – review & editing. **Tatiana S. Filatova:** Formal analysis, Investigation, Writing – review & editing. **Pavel A. Galenko-Yaroshevsky:** Resources, Writing – review & editing, Supervision, Project administration. **Denis V. Abramochkin:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration,

Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Sergey Ulyanov for invaluable help in organizing molecular biology experiments.

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