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Possible mechanism of effect of the empagliflozin on cardiovascular mortality

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Abstract

Introduction. The development of heart failure is closely associated with the appearance of life threatening arrhythmias, which are often a terminal event for these patients. An analysis of randomized clinical trials of inhibitors of sodium-glucose cotransporter type 2 indicates the clinically significant potential of these drugs as agents with antiarrhythmic properties. However, at the moment the full mechanism by which this effect can be realized is still not fully understood.

Aim. To evaluate the effect of empagliflozin on the transmembrane calcium currents and the intracellular calcium transients on isolated ventricular cardiomyocytes of mice under conditions of normoglycemia.

Materials and methods. In the experiment, ventricular cardiomyocytes were isolated from 12 outbred male mice. 2 groups were formed: group № 1 – control ventricular cardiomyocytes; group № 2 – ventricular cardiomyocytes after two hours incubation with 5 μmol/L empagliflozin solution. Transmembrane calcium currents were recorded and intracellular calcium transients were assessed.

Results and discussion. Incubation of ventricular cardiomyocytes with empagliflozin significantly increased I_{Ca} current density and accelerated Ca^{2+} temporal dynamics. The amplitude of the Ca^{2+} wave and the rate of rise and decay were increased and the duration of the Ca^{2+} wave was shortened.

Conclusion. The result of the experiment indicates that empagliflozin is able to modulate Ca^{2+}-dependent mechanism of the excitation-contraction-coupling, enhancing and accelerating Ca^{2+} release into cytoplasm and reuptake. This presumably can optimize, namely reduce the time of systole and enhance it, which may be one of the important elements in the manifestation of empagliflozin antiarrhythmic properties.

Keywords: sodium-glucose co-transporter 2 Inhibitors, isolated ventricular cardiomyocytes, transmembrane calcium currents

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Alexey V. Karpushev, Dmitry Yu. Ivkin and Marina V. Krasnova – conception and design, data analysis and interpretation, manuscript writing. Alexey V. Karpushev, Valeria B. Mikhailova. and Ekaterina S. Klimenko – performing animal experiments, collection, and assembly of data. Sergey V. Okovityi and Alexander N. Kulikov – conception and design, administrative support. All authors participated in the discussion of the results. All authors have read and agreed to the published version of the manuscript.

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О возможном механизме влияния эмпаглифлозина на сердечно-сосудистую смертность

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Резюме

Введение. Развитие сердечной недостаточности тесно связано с появлением жизнеугрожающих аритмий, которые нередко являются терминальным событием для данных пациентов. Анализ рандомизированных клинических исследований ингибиторов натрий-глюкозного котранспортера 2 типа свидетельствует о клинически значимом потенциале данных препаратов как средств с антиаритмическими свойствами. Однако полный механизм, за счет которого может реализоваться данный эффект, в настоящий момент все еще остается до конца не изученным.

Цель. Оценка влияния эмпаглифлозина на трансмембранные токи кальция и внутриклеточные кальциевые переходные процессы на изолированных желудочковых кардиомиоцитах мышей в условиях нормогликемии.

Материалы и методы. В эксперименте проводили выделение желудочковых кардиомиоцитов от 12 аутбредных мышей самцов. Были сформированы 2 группы: группа № 1 – контрольные желудочковые кардиомиоциты; группа № 2 – желудочковые кардиомиоциты после двух часовой инкубации с 5 μмоль/л раствором эмпаглифлозина. Выполнялись запись трансмембранных токов кальция и оценка внутриклеточных кальциевых переходных процессов.

Результаты и обсуждение. Инкубация желудочковых кардиомиоцитов в присутствии эмпаглифлозина значительно увеличила плотность тока I_{Ca} и ускорила временную динамику Ca^{2+}. Амплитуда волны Ca^{2+} и скорость нарастания и затухания были увеличены, а продолжительность волны была сокращена.

Заключение. Результат эксперимента указывает на то, что эмпаглифлозин способен модулировать Ca^{2+}-зависимый механизм электромеханического сопряжения, усиливая и ускоряя выход Ca^{2+} в цитоплазму и обратный его захват. Это предположительно оптимизирует, а именно сокращает время систолы и усиливает ее, что может являться одним из важных элементов проявления антиаритмических свойств эмпаглифлозина.

Ключевые слова: ингибиторы натрий-глюкозного котранспортера 2 типа, изолированные желудочковые кардиомиоциты, трансмембранный ток кальция

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. А. В. Карпушев, Д. Ю. Ивкин и М. В. Краснова – концепция и дизайн, анализ и интерпретация данных, написание рукописи. А. В. Карпушев, В. Б. Михайлова и Е. С. Клименко – проведение экспериментов на животных, сбор и обработка данных. С. В. Оковитый и А. Н. Куликов – концепция и дизайн, административная поддержка. Все авторы участвовали в обсуждении результатов, прочитали и согласились с опубликованной версией рукописи.

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INTRODUCTION

The course of chronic heart failure (CHF) in patients is often complicated by the occurrence of severe life threatening arrhythmias [1]. Sodium-glucose cotransporter type 2 inhibitors (SGLT2is) in a number of clinical trials (EMPEROR, EMBODY, DAPA–HF) has demonstrated a significant increase in protection against the occurrence of life threatening arrhythmias that can lead to sudden cardiac death (SCD) in patients. An extensive meta-analysis of 34 randomized clinical trials has demonstrated that using of SGLT2is is associated with a marked reduction in the risk of atrial arrhythmias and SCD [2].

Empagliflozin, one of the first representatives of SGLT2is, demonstrated its cardioprotective effect [3], has shown its antiarrhythmic potential in a number of preclinical studies. One of the first hypotheses was the possibility of inhibition the sodium-hydrogen exchanger isoform-1 (NHE–1) by the drug, which reduces the intake of Na^+ and Ca^{2+} into the cytoplasm of cardiomyocytes and increases the Ca^{2+} content in mitochondria, however, these data are contradictory [4–6]. In animals with CHF, the use of empagliflozin prevents the activation of late Na^+ current and related arrhythmogenic events [7]. It is assumed that this effect on voltage-dependent Na^+ channels activity is regulated by an indirect mechanism, which may include suppression of the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and prevention of the CaMKII-dependent Ca^{2+} leakage from the sarcoplasmic reticulum [8]. Ca^{2+} overload is considered as potential factor in the genesis of various serious arrhythmias [9]. However, it is currently unknown whether intracellular Ca^{2+} handling can be an empagliflozin target regardless of Ca^{2+} overload, as an important chain of excitation-contraction coupling in cardiac myocytes. Can empagliflozin have potential effects on contractile cardiac function through modulation of Ca^{2+} handling? Thus **the aim of this study** was to evaluate the effect of empagliflozin on transmembrane Ca^{2+} currents and Ca^{2+} transients in isolated ventricular cardiomyocytes of mice.

MATERIALS AND METHODS

The animal study protocol was written in full compliance with the principles of the Basel Declaration, the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European Treaty Service No. 123, 18 March 1986), and the Order of the Ministry of Health of the Russian Federation No. 199n (1 April 2016) "On the approval of the Rules of Good Laboratory Practice". Experimental protocols were approved by Almazov National Medical Research Centre Ethical Committee.

Experimental animals and study object

The weight of male white outbred mice ($n = 12$) after the adaptation period at the beginning of the experiment was 23.6 ± 3.4 g. The animals were kept under 12/12-hour light-dark conditions and received standard feed and drinking water ad libitum.

The following 2 groups were included in the experiment:

- Group №1 – ventricular cardiomyocytes of mice without empagliflozin incubation (Control);
- Group №2 – ventricular cardiomyocytes of mice after two-hour incubation with 5 μmol/L empagliflozin solution (Treatmen).

Isolation of Ventricular Cardiomyocytes

Intraperitoneal injection of heparin (1000 U/kg) was used to prevent blood coagulation in the coronary vessels of the heart. The mouse was euthanized by decapitation with the use of the rodent guillotine. The chest cavity was opened and the heart was rapidly exci-

sed. The heart was attached to a Langendorff apparatus using aorta cannulation for retrograde perfusion with Ca^{2+}-free solution of the following composition in mmol/L: 116.27 NaCl, 4.03 KCl, 1.66 NaH_2PO_4, 25.24 $NaHCO_3$, 30 taurine, 4.91 Na-piruvate, 2.2 $MgCl_2$, 10 hepes, 11.11 glucose and 1 mg/ml bovine serum albumin, pH 7.2 adjusted with NaOH. After 10 min perfusion with the Ca^{2+}-free solution the heart was perfused for 18–20 min with the same solution containing 0.45 mg/ml type II collagenase (Worthington, Lakewood, NJ, USA) and 20 μmol/L $CaCl_2$. The solutions was continuously bubbled with carbogen containing 95 % O_2 and 5 % CO_2. The temperature was equilibrated at +37 °C. The atriums were removed and ventricular myocardium was destroyed mechanically (by cutting with surgical scissors and gently pipetting) to isolate individual cells. Cardiomyocytes were stored in the Kraftbrühe (KB) medium containing in mmol/L: 50 L-glutamic acid, 20 hepes, 20 taurine, 3 $MgSO_4 \cdot 7H_2O$, 30 KCl, 0.5 EGTA, 30 KH_2PO_4, 10 glucose, pH 7.2 adjusted with KOH. The cells were stored in the KB medium for 2 hours before actual experiments and for up to 5–6 hours.

Recording of Ionic Currents

The whole-cell voltage clamp recordings of Ca^{2+} channel currents ICa were performed in the freshly isolated ventricular myocytes at +37 °C. Data acquisition was performed with amplifier Axopatch 200B and Clampex software, version 10.3 (Molecular Devices, San Jose, CA, USA). The ionic currents were acquired at 20–50 kHz and low-pass filtered at 5 kHz using the analog-to-digital interface Digidata 1440A acquisition system (Molecular Devices, San Jose, CA, USA). Patch pipettes of 2.5–3.5 MΩ resistance were pulled from the borosilicate glass B150-110-10 (Sutter Instrument, Novato, CA, USA) with a puller P-1000 (Sutter Instrument, Novato, CA, USA). The pipette and cell capacities and access resistance were completely compensated. The series resistance was compensated by 85–90 %.

I_{Ca} was recorded in the bath solution contained in mM/L: 130 NaCl, 5 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Na-pyruvate, 10 hepes, 10 glucose, pH 7.4 adjusted with NaOH. The pipette solution contained in mM/L: 130 CsCl, 1 $MgCl_2$, 5 EGTA, 10 hepes, 5 MgATP, 15 tetraethylammonium-Cl, pH 7.2 adjusted with CsOH. I_{Ca} was elicited from the holding potential of –50 mV with 200 ms prepulse to –40 mV to inactivate voltage-dependent sodium current and 300 ms depolarizing voltage steps from –40 to +50 mV in 10 mV increment. All pulse protocols were applied more than 5 min after membrane rupture. The current density, I_{Ca} normalized to the cell membrane capacitance, was plotted against the voltage steps. The recorded current traces were analyzed using Clampfit software, version 10.3 (Molecular Devices, San Jose, CA, USA).

Recording of the Ca^{2+} Transients

The intracellular Ca^{2+} transients were recorded in the freshly isolated ventricular myocytes at +37 °C. Cells were loaded with 5 μmol/L Fura-2 AM by 20 min incubation the dark at room temperature in Tyrode solution contained in mmol/L: 140 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 hepes, pH 7.4 adjusted with NaOH. Fluorescence imaging was performed using a fluorescence photometry setup (IonOptix, Milton, MA, USA). The Ca^{2+} transients were detected with excitation at 340 or 380 nm and emission at 510 nm during a 1-Hz field-stimulation with 10-ms twice-threshold strength square-wave pulses. The fluorescent signal traces were recorded as the ratio of the emissions at the corresponding excitation wavelengths and were analyzed using IonWizard software, version 7.4.3.160 (IonOptix, Milton, MA, USA).

Statistical analysis

Statistical analysis was carried out in STATISTICA v10.0 (StatSoft, Tulsa, OK, USA). All data are presented as mean values ± standard-error-of-mean (SEM). Data were tested for normality using the Shapiro–Wilk test, then tested for significant differences using the unpaired Mann-Whitney test. The significance threshold was set at $p < 0.05$.

RESULTS

Effects of Empagliflozin on the Ca^{2+} Channel Current

We demonstrate that 2 hours incubation with 5 μmol/L empagliflozin significantly increased the current density of I_{Ca} in the wild-type mice (Group № 2) ventricular myocytes (figure 1, table 1). This result indicate that empagliflozin is able to modulate, namely enhance, the Ca^{2+} channel activity.

Table 1. **I_{Ca} peak current density at 0 mV, pA/pF**

Group/Parameters	I_{Ca} peak current density at 0 mV
Group № 1 – control	13.1 ± 1.5
Group № 2 – treatment	18.0 ± 1.4
p-value	$p = 0.0301$

Effects of Empagliflozin on the Ca^{2+} transients

We evaluated parameters of the intracellular Ca^{2+} transients in mouse ventricular myocytes. 2 hours incubation with 5 μmol/L empagliflozin significantly accelerated the Ca^{2+} transient dynamics in the control mice ventricular myocytes. The Ca^{2+} wave amplitude and rate of rise and decay were increased and wave duration was shortened (figure 2, table 2).

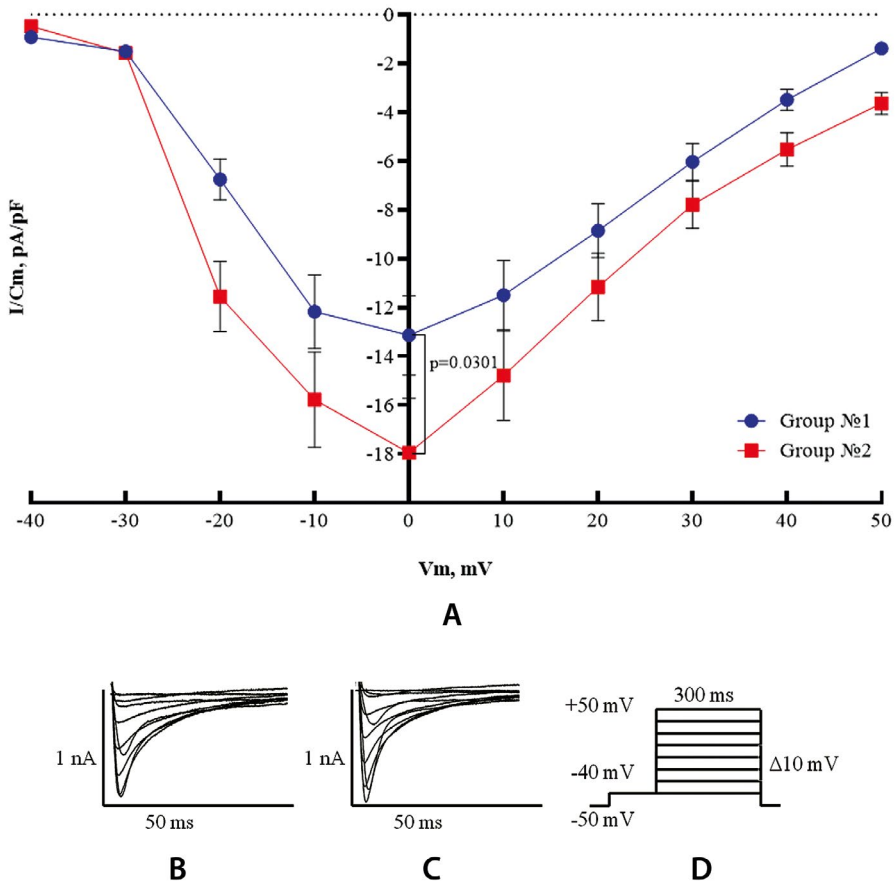

Figure 1. The I_{Ca} current density in the ventricular myocytes of wild-type mice:

A – The current density–voltage relationship of the I_{Ca} in mouse ventricular cardiomyocytes. Insert shows the voltage clamp protocol; B – Representative whole–cell current traces of I_{Ca} in ventricular cardiomyocytes in Group № 1; C – Representative whole–cell current traces of ICa in ventricular cardiomyocytes in Group № 2; D – Test Stimulus Delivery Protocol.

Note: Group № 1 – control; Group №2 – treatment

Taken together experimental data, we can conclude that empagliflozin has a modulating effect on the entire Ca^{2+}-dependent mechanism of the excitation-contraction-coupling.

Table 2. Parameters of the intracellular Ca^{2+} transients.

Parameters/ Group	Group № 1 control	Group № 2 treatment	*p*-value
Ca^{2+} wave amplitude	0,096 ± 0,005	0,134 ± 0,007	$p < 0,0001$
Rate of rise	1,232 ± 0,072	1,875 ± 0,095	$p < 0,0001$
Rate of decay	0,555 ± 0,033	0,847 ± 0,047	$p < 0,0001$
Ca^{2+} wave duration at 10 % decay	0,173 ± 0,003	0,148 ± 0,002	$p < 0,0001$
Ca^{2+} wave duration at 50 % decay	0,256 ± 0,005	0,218 ± 0,004	$p < 0,0001$
Ca^{2+} wave duration at 90 % decay	0,519 ± 0,013	0,473 ± 0,012	$p = 0,0094$

DISCUSSION

SGLT2 inhibitors have attracted significant research interest due to a number of clinical trials and researches demonstrating the substantial benefits of these drugs in the therapy of heart failure by lowering the risk of cardiovascular events, hospitalization, and mortality [10–11]. Despite the fact that cardiac SGLT2 expression is negligible [12–14], SGLT2 inhibitors appear to have cardio-specific effects on pathophysiological molecular mechanisms in the cell. One of the empagliflozin targets in cardiomyocytes has been suggested the sodium-hydrogen exchanger [4–6; 15–17]. Next target for the SGLT2 inhibitors may be considered the late Na^{+} current [7; 17–19]. Another important mechanism, which may be clinically beneficial, is Ca^{2+} handling [20]. Intracellular Ca^{2+} is a critical initiator of the contractile cycle in cardiac myocytes [21]. Therefore, proper regulation of Ca^{2+} dynamics is significant for normal heart function.

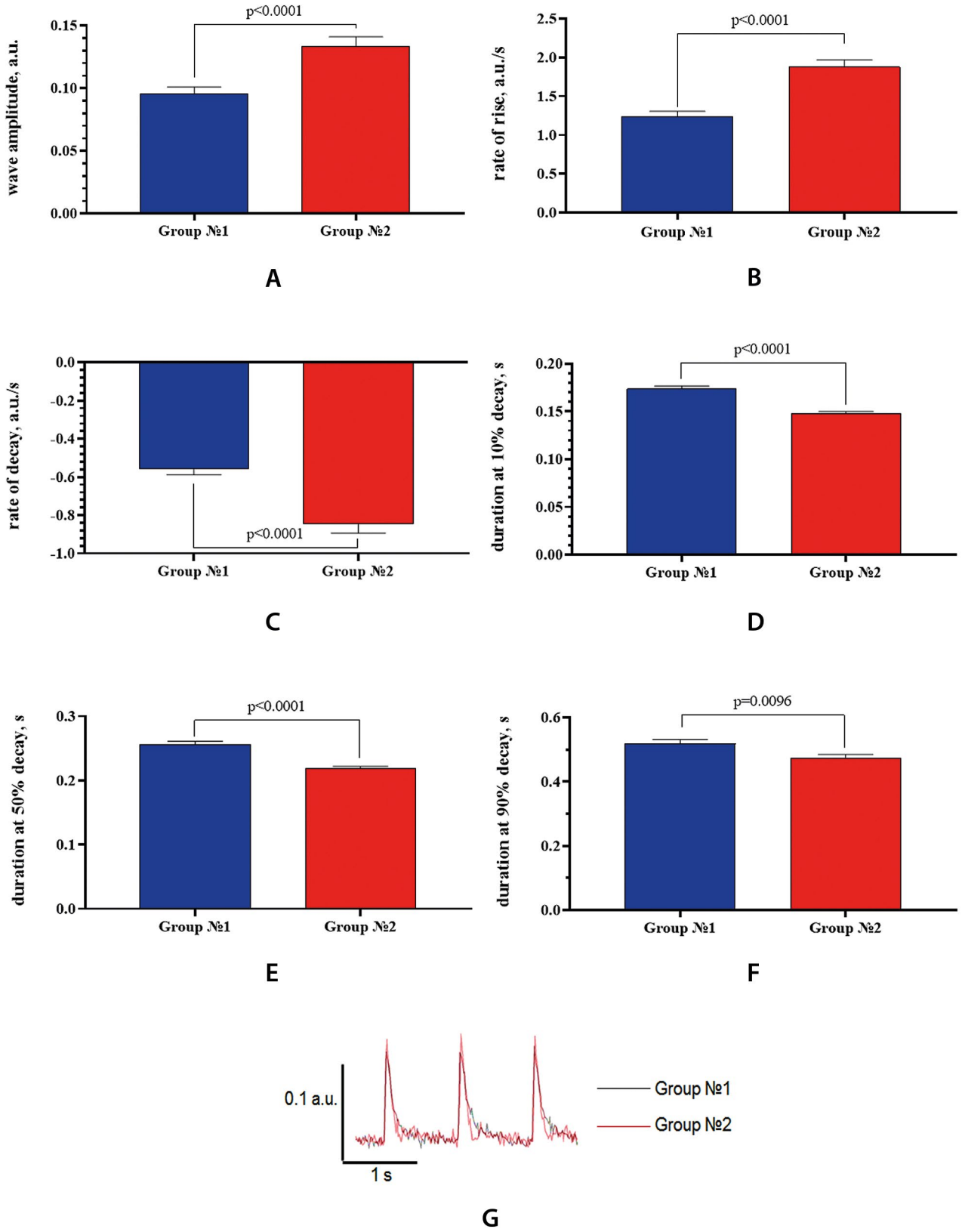

Figure 2. **Parametrs of Ca^{2+} wave:**

A – Ca^{2+} wave amplitude; B – Ca^{2+} wave rate of rise; C – Ca^{2+} wave rate of decay; D – Ca^{2+} wave duration at 10 % decay; E – Ca^{2+} wave duration at 50 % decay, F – Ca^{2+} wave duration at 90 % decay; G – Representative Ca^{2+} wave traces

Note: Group № 1 – control; Group № 2 – treatment

The present study was designed to determine the potential effects of empagliflozin on the intracellular Ca^{2+} kinetics and handling. For this purpose we focused in estimating the effects of the SGLT2 inhibitor on the Ca^{2+} channel current and the Ca^{2+} transients [22]. Our experiments have shown that empagliflozin increased the current density of I_{Ca}, accelerated the Ca^{2+} transient dynamics, and increased the Ca^{2+} transient amplitude in cardiac myocytes of healthy outbred mice. The data from our experiments are consistent with the data from previous study, which showed that 24 hrs of empagliflozin exposure significantly increased the amplitude and reduced the half-time for the recovery of the Ca^{2+} transients [23]. Additionally, it should be noted, that empagliflozin normalizes impaired kinetics of the Ca^{2+} transients and restores reduced current density of I_{Ca} in ventricular cardiomyocytes from animal models of diabetes mellitus [17] and diabetic cardiomyopathy [24]. However, Pabel and colleagues (2020) failed to observe empagliflozin effects on the Ca^{2+} transient amplitude and kinetics, diastolic Ca^{2+} levels, and sarcoplasmic Ca^{2+} load in human cardiomyocytes derived from induced pluripotent stem cells after chronic treatment with empagliflozin [25]. It is also noteworthy that another representative of the class of SGLT2 inhibitors dapagliflozin was shown to reduce the amplitude of the Ca^{2+} transients and I_{Ca} in rat ventricular myocytes [26].

The cardiac beneficial effects of SGLT2 inhibitors treatment in terms of Ca^{2+} handling are thought to include the prevention of intracellular Ca^{2+} overload [28]. This is thought to result from the inhibition of ryanodine receptor 2 phosphorylation, which prevents Ca^{2+} leakage, and from increased reuptake of Ca^{2+} into the sarcoplasmic reticulum due to enhanced SERCA2a function [17; 24, 28, 29]. The latter explains the decrease in the decay time of the Ca^{2+} transients. The assessment provided in this study allows speculating that the enhanced and accelerated intracellular Ca^{2+} dynamics might reflect empagliflozin's ability to optimize the force and timing of the cardiac contractile cycle, to reduce the time of systole. Presumably, this may be one of the important elements in the manifestation of empagliflozin antiarrhythmic properties. Thus we hypothesize the presence of an additional cardioprotective effect of empagliflozin associated with contractile function. This hypothesis is supported by the results of a recent large meta-analysis of studies in humans, which convincingly confirmed the ability of NGLT2-inhibitors to increase the left ventricular ejection fraction [30].

CONCLUSION

The result of the experiment indicates that empagliflozin is able to modulate Ca^{2+}-dependent mechanism of the excitation-contraction-coupling, enhancing and accelerating Ca^{2+} release into cytoplasm and reuptake. This presumably can optimize, namely reduce the time of systole and enhance it, which may be one of the important elements in the manifestation of empagliflozin antiarrhythmic properties.

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