Investigation of the Cardiac Electrophysiological Effects of Acetylcholine and Pharmacological Modulation of Early Repolarization Syndrome on Canine and Human Ventricular Preparations

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Summary of PhD thesis

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LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. Magyar T, Árpádffy-Lovas T, Pászti B, Tóth N, Szlovák J, Gazdag P, Kohajda Z, Gyökeres A, Györe B, Gurabi Z, Jost N, Virág L, Papp JG, Nagy N, Koncz I. Muscarinic agonists inhibit the ATP-dependent potassium current and suppress the ventricle-Purkinje action potential dispersion. *Can J Physiol Pharmacol*. 2021;99(2):247-53.
Impact factor: 2.245 (2021) **Scimago: Q2**

II. Koncz I, Verkerk A O, Nicastro M, Wilders R, Árpádffy-Lovas T, **Magyar T**, Tóth N, Nagy N, Madrid M, Lin Z, Efimov IR. Acetylcholine reduces I_{Kr} and prolongs action potentials in human ventricular cardiomyocytes. *Biomedicines* 2022; 10, 244.
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III. Verkerk, AO, Doszpod, IJ, Mengarelli, I, **Magyar, T**, Polyák, A, Pászti, B, Efimov, IR, Wilders, R, Koncz, I. Acetylcholine reduces L-type calcium current without major changes in repolarization of canine and human Purkinje and ventricular tissue. *Biomedicines* 2022, *10*, 2987.
Impact factor: 4,7 (2022) **Scimago: Q1**
Cumulative impact factor of publications related to the PhD thesis: 11,645

LIST OF OTHER PUBLICATIONS AND ABSTRACTS

I.Pászti B, Prorok J, **Magyar T**, Árpádffy-Lovas T, Györe B, Topal L, Gazdag P, Szlovák J, Naveed M, Jost N, Nagy

N, Varró A, Virág L, Koncz I. Cardiac electrophysiological effects of ibuprofen in dog and rabbit ventricular preparations: possible implication to enhanced proarrhythmic risk. *Can J Physiol Pharmacol*. 2021;99(1):102-9.
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II. Kohajda Z, Virág L, Hornyik T, Husti Z, Sztojkov-Ivanov A, Nagy N, Horváth A, Varga R, Prorok J, Szlovák J, Tóth N, Gazdag P, Topal L, Naveed M, Árpádffy-Lovas T, Pászti B, **Magyar T**, Koncz I, Déri S, Demeter-Haludka V, Aigner Z, Ördög B, Pátfalusi M, Tálosi L, Tiszlavicz L, Földesi I, Jost N, Baczkó I, Varró A. In vivo and cellular antiarrhythmic and cardiac electrophysiological effects of desethylamiodarone in dog cardiac preparations. *Br J Pharmacol*. 2022, 179(13):3382-3402.
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III. **Magyar Tibor,** Pászti Bence, Árpádffy-Lovas Tamás, Gyökeres András, Gurabi Zsolt, Jost Norbert, Varró András, Virág László, Antzelevitch Charles, Koncz István: Acetylcholine attenuates pinacidil-induced abbreviation of the action potential in canine cardiac Purkinje fibers and papillary muscles. EHRA International Congress, Lisbon, Portugal, 17-19 March 2019 (*congress abstract*).

IV. Árpádffy-Lovas Tamás, **Magyar Tibor,** Pászti Bence, Gurabi Zsolt, Jost Norbert, Charles Antzelevitch, Varró András, Virág László, Koncz István: Az acetilkolin mérsékli a pinacidil akciós potenciál időtartamot rövidítő hatását kutya Purkinje-rostokon és papillaris izmokon. Magyar Élettani Társaság Vándorgyűlése, Szeged, 2018. 06. 27-30 (*congress abstract*)

Cumulative impact factor of other publications: 10,984
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I. Introduction and Aims

1.1 Parasympathetic influence of the heart

Parasympathetic innervation can influence cardiac function in several aspects. As a result, cardiac automaticity decreases, conduction time of impulses increases, and the contractility of the cardiac muscle can either decrease (when the force of contraction was previously increased by compounds) or increase. Du et al found that acetylcholine (1 nM to 100 μM) evoked a positive inotropic effect on the control ventricular force of contraction in human ventricular preparations. Gilmour and Zipes described the positive inotropic effect of acetylcholine in canine Purkinje fibres. The activation of M_2 receptors by acetylcholine can modify directly or indirectly, through the modification of cAMP-dependent signaling pathway, the cardiac ion channels. Contrary to the previous idea numerous studies proved both anatomically and functionally the existence of rich parasympathetic innervation in the ventricle. Relatively little is known about the effect of acetylcholine on cardiac repolarization.

So far 3 clinical trials have been paid attention to vagal nerve stimulation as a potential therapeutic option of chronic heart failure.

1.2 Cardiac electrophysiological effects of hypoxia and ischemia

During acute ischemia, numerous cardiac electrophysiological changes have been described such as the depolarization of resting membrane potential, shortening of action potential duration, augmentation of the dispersion of repolarization. In the background of these in addition to humoral factors, there might be parasympathetic modulation in the functioning of ion channels such as I_{KATP}.

1.3 The Early Repolarization Syndrome

In 2008 Michel Haissaguerre and his colleagues suggested a possible link between the early repolarization pattern and sudden cardiac death. Early Repolarization Syndrome defined as J-point elevation of more than 1 mm on the ECG in at least 2 lateral or inferior leads (terminal QRS slurring or notching) accompanied by idiopathic ventricular fibrillation. Besides genetic background increased parasympathetic tone, associated ischemic heart

disease, or hypothermia also play an important role in the pathogenesis.

1.4 Aims

In this dissertation I would like to present new data on the cardiac electrophysiological effects of acetylcholine and Early Repolarization Syndrome research.

II. Methods

2.1 Conventional microelectrode technique

Mongrel dogs of either sex weighing 10-15 kg were used. After appropriate intravenous anesthesia (thiopental 30 mg/kg i.v. or phenobarbital 60 mg/kg i.v.) canine hearts were rapidly removed by right lateral thoracotomy and placed in previously perfused Locke solution. Human hearts were acquired from organ donors that logistically were not suitable for transplantation and were kept in cardioplegic solution at 4°C for 4-8 hours. The investigations were carried out due to the Declaration of Helsinki of the World Medical Association. Right ventricular muscles and Purkinje fibres from both ventricles with a small ventricular muscle were isolated. Preparations were superfused (flow rate 4-5 ml/min) with

Locke's solution for at least 2 hours on pH between 7,40 to 7,45 by adding 95% O_2 and 5% CO_2 at 37,0°C. During hypoxia, mixture of 95% N_2 and 5% CO_2 were used at 37°C with unchanged pH. Stimulation was executed through platinum bipolar electrodes at basic cycle length 500 and 1000 ms with two times the diastolic threshold intensity (S_1) for 0,5-2 ms duration.

Glass capillary microelectrodes filled with 3 M KCl solution were used to measure the transmembrane potential (tip resistance: 5 to 15 MΩ). The microelectrodes were connected through an Ag–AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier (Experimetria Ltd. 2011, Budapest, Hungary). On a storage oscilloscope (Hitachi V-555) recorded action potentials were revealed then conducted to our computer system (APES) developed for analisation of measured action potentials.

2.2 The patch-clamp technique

Following previously described intravenous anaesthesia and cardiectomy left ventricle wall consisting of arterial branch was isolated. It was perfused through the

cannulated branch on a Langendorff apparatus with solutions in the following protocol: for 10 min normal Tyrode solution pH 7.4 adjusted with NaOH then Ca^{2+}-free Tyrode solution for 10 min and Ca^{2+}-free Tyrode solution containing collagenase (Worthington type II, 0.66 mg/mL). Finally solution protease (type XIV, 0.12 mg/mL) was administered at the 15 and 30 minutes for the enzimatic dissociation. After enzimatic reaction one drop of cell suspension was installed in a transparent recording chamber attached on the stage of an inverted microscope (Olypmus IX51, Tokyo, Japan). Single, rod shaped myocytes with clear striations were waited to settle and stick on the bottom for at least 5-10 minutes before HEPES-buffered Tyrode's superfusion at pH of 7.4 was started. Before the detection of I_{KATP} 1 μM nisoldipine was administered to the solution to antagonise the I_{Ca}, 0.1 μM dofetilide to block the I_{Kr} and 0.5 μM HMR-1556 to inhibit I_{Ks}. Patch pipettes were made from borosilicate glass capillaries (Science Products GmbH, Hofheim, Germany) by a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA) (tip resistance: 1.5–2.5 MΩ). Micropipettes were filled with the solution

containing (in mM): KOH 110, KCl 40, K_2ATP 5, $MgCl_2$ 5, EGTA 5, HEPES 10 and GTP 0.1 (pH was adjusted to 7.2 by aspartic acid).

The membrane currents were registered with Axopatch-200B amplifiers (Molecular Devices, Sunnyvale, CA, USA) by using the whole-cell configuration of the patch-clamp technique. Analogue to digital conversations were carried out with 250 kHz converter (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA) under software surveillance (pClamp 8 and pClamp 10, Molecular Devices, Sunnyvale, CA, USA).

2.3 Statistical analysis

Results are presented as mean ± S.E.M. Shapiro-Wilk test was used to verify the normality of distribution and Bartlett's test to confirm homogeneity of variances in each group. Analysis of variance (ANOVA) was used to make statistical comparisons for the repeated measurements followed by Bonferroni's post-hoc test. $P < 0.05$ was considered statistically significant.

III. Results

3.1 Effect of acetylcholine on canine preparations with standard microelectrode technique

Acetylcholine (5 μM) alone to canine Purkinje fibres (BCL: 500 ms) did not prolong the repolarization of the action potential (233.6 ± 4.7 ms to 231.7 ± 4.6 ms, n=15). However, after proadministration of I_{KATP} activator pinacidil (5 μM) (n=8) which caused a significant and large APD_{90} reduction (from 207.7 ± 7 ms to 113.1 ± 9.1 ms, $p < 0.05$), the cumulative addition of acetylcholine (5 μM) resulted in significant APD_{90} lengthening as early as 3 minutes (from 113.1 ± 9.1 ms to 147.3 ± 7.4 ms, $p < 0.05$). Similarly in canine ventricular papillary muscle acetylcholine alone resulted no significant changes in action potential duration (172.6 ± 5.7 ms versus 172.8 ± 5.3 ms , n=5). But after pinacidil induced APD shortening (187.9 ± 4.5 ms vs 163.7 ± 6.4 ms, $p < 0.05$) acetylcholine (5 μM) was able to oppose incompletely the effect of pinacidil (from 163.7 ± 6.4 ms to 172.1 ± 7.4 ms, n=7).

The alterations in the distinction between the APD_{90} parameters of Purkinje fibres and ventricular muscles are utilized to evaluate the impacts of pinacidil and

acetylcholine on the APD dispersion between these tissues. The dispersion of control APD_{90} (9.5%, 20 ms, n=8 PF, n=7 VM) was significantly increased by the administration of pinacidil (APD_{90} dispersion: 44.7%, 51 ms, n=8 PF, n=7 VM). However, the cumulative addition of acetylcholine resulted in significant reduction of this dispersion (16.9%, 28 ms; n=8 PF, n=7 VM, *$p<0.05$).

3.2. Canine patch clamp measurements

Carbachol alone did not change the control current (0 mV control: 0.20 ± 0.2 pA/pF vs 3 μM carbachol: 0.32 ± 0.2 pA/pF, n=6 and +30 mV - control: 0.55 ± 0.4 pA/pF vs 3 μM carbachol: 0.74 ± 0.3 pA/pF, n=6) . Nevertheless, in case of prior 5 μM pinacidil application, carbachol administration resulted a significant attenuation of the current at both voltages (0 mV – control: 0.24 ± 0.2 pA/pF → 5 μM pinacidil: 2.03 ± 0.3 pA/pF → 3 μM carbachol: 1.51 ± 0.4 pA/pF, n=8, $p < 0.05$. +30 mV - control: 0.78 ± 0.6 pA/pF → 5μM pinacidil: 3.17 ± 0.3 pA/pF → 3 μM carbachol: 2.26 ± 0.3 pA/pF, n=8, $p < 0.05$).

3.3 The effect of acetylcholine during hypoxia

Hypoxic condition resulted in shortening of APD_{90} (181.4 ± 5.7 ms to 135.0 ± 8.6 ms) (n=5, $p<0.05$), reduction of

amplitude (103.7 ± 2.8 mV vs 92 ± 3.5 mV, n=5) and decreasion of the maximum rate of depolarization (185.8 ± 15.8 V/s vs 156.1 ± 20.6 V/s, n=5). After 5 μM acetylcholine application significant APD_{90} prolongation (from 135.0 ± 8.6 ms to 164.4 ± 4.4 ms) and AMP recovery to a normal range (102.1 ± 1.6 mV, n=5) were seen, while V_{max} remained at the same value (156.0 ± 16.1 V/s, n=5).

3.4 The effect of acetylcholine in human preparations

5 μM acetylcholine in non-diseased human Purkinje fibres (n=2) caused moderate shortening of APD_{90} (from 269.0 ± 28.4 to 251.6 ± 42.85 ms) and APD_{50} (from 184.4 ± 20.0 ms to 173.3 ± 27.1 ms).

Acetylcholine (5 μM) slightly lengthened the action potential duration in a human papillary muscle (BCL:1000 ms) and in a human Purkinje fibre (BCL: 500 ms) taken from a human donor heart with chronic heart failure. Besides that it can slightly shorten the action potential duration and caused plateau depression in a human subepicardial cell of a heart slice (BCL:1000 ms) and could elevate the AP plateau and slightly lengthened the action potential duration in a human midmyocardial cell of a heart slice (BCL:500 ms).

3.5 The effect of cilostazol on different ERS modells

During the first ERS model experiment 5 μM pinacidil and then 5 μM ACh were applied to Purkinje fibres (n=6). After that 10 μM cilostazol caused a notable plateau increase, but did not change the action potential duration.

In the second series 10 μM cilostazol was used after administration of 7 μM I_{to} agonist (NS5806), 5 μM I_{KATP} activator pinacidil and 5 μM acetylcholine cumulatively. Cilostazol resulted in significant APD_{90} lengthening (151.6 ± 9.9 to 175.6 ± 7.4 ms, n=5).

In the third model after nisoldipine (1μM), NS5806 (7 μM) and acetylcholine (5 μM), the same dose of cilostazol (10 μM) produced a small, but not significant plateau elevation and mild APD prolongation.

In the fourth model we administered 20 μM of Na^+ channel blocker R-mexiletine, than the I_{to} activator NS5806 (7 μM), and finally ACh (5 μM) were applied. In this case cilostazol slightly increased the action potential duration.

IV. Discussion

4.1 Investigation of the possible antiarrhythmic effect of acetylcholine

In dog ventricular preparations (Purkinje fibers and papillary muscles) acetylcholine (5 μM) alone did not influence significantly the action potential duration. Carbachol was capable of inhibiting the pinacidil-elicited ATP-sensitive K^+ current. In the corresponding tissular action potential recordings, acetylcholine prolonged the APD in case it was formerly abbreviated by the administration of pinacidil.

Hypoxia induced action potential duration shortening was substantially antagonized after administration of acetylcholine, we propose ATP-sensitive K^+ current block as a potential effect.

We found that Purkinje fibres were more sensitive to the pharmacological activation of I_{KATP} than the ventricular myocytes thus the APD dispersion continued to increase. After acetylcholine administration we saw the reduction of pharmacologically generated arrhythmogenic ADP dispersion elevation.

Effects of acetylcholine on different types of human ventricular preparations:

in case of a human midmyocardial preparation taken from a non-diseased heart, a mild APD prolongation and in case of a human epicardial preparation taken from another non-diseased heart a slight abbreviation of APD have been registered under the influence of acetylcholine.

Prolongation of the duration of the action potential was observed in a human papillary muscle and a Purkinje fibre extracted from reduced ejection fraction human heart (EF=29%) under the influence of Ach.

Finally, it is mentionable that during healthy, human, donor Purkinje fibre measurements, APD abbreviation was observed in response to 5 μM acetylcholine. The investigation of human Purkinje fibre preparations in the future with patch clamp technique is certainly important.

4.2 Investigation of pharmacological models of Early Repolarization Syndrome

We used several different pharmacological protocols to model ion channel mutations and acetylcholine effect to mimic increased parasympathetic tone on canine Purkinje fibres. Significant results were found in few cases, but in

all cases a minimal antagonizing effect of cilostazol (prolongation of APD, elevation of the action potential plateau) was detected. This raises the possibility that in case of Early Repolarization Syndrome, cilostazol may play a role in eliminating the arrhythmias. However, it should be mentioned that in connection with this series of measurements, other parts of the heart were not examined with present models.

V. Conclusions

The most important findings of this PhD thesis of the followings:

1. Acetylcholine was able to partially antagonize the APD shortening caused by the previously used I_{KATP} activator (5 μM pinacidil) and decreased the pinacidil induced APD dispersion elevation between ventricular papillary muscle and Purkinje fibre. Reproducing standard microelectrode measurements with patch clamp technique, we achieved similar result when we applied pinacidil and carbachol consecutively. After the generation of hypoxic environment, the use of

acetylcholine was also able to offset hypoxia-induced APD_{90} shortening, and decrease in amplitude.

2. Application of cilostazol on pharmacologically generated Early Repolarization Syndrome models resulted in partial normalization. Based on these, we confirmed the antiarrhythmic effect of cilostazol in Early Repolarization Syndrome.

3. In healthy Purkinje fibres with standard microelectrode technique, a slight APD shortening was observed, on the other hand, APD prolongation was found in a Purkinje fibre and a papillary muscle obtained from a heart failure human heart. Acetylcholine elicited mild APD prolongation in a human midmyocardial cell and evoked slight APD abbreviation in a human epicardial cell. Considering the small number of human preparations, further investigation with acetylcholine is necessary.

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