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Complete List of Authors:	Magyar, Tibor; University Szeged, Department of Pharmacology and Pharmacotherapy Árpádffy-Lovas, Tamás; University of Szeged, Pharmacology and Pharmacotherapy Pászti, Bence; University Szeged, Department of Pharmacology and Pharmacotherapy Tóth, Noémi; Szegedi Tudomanyegyetem, Department of Pharmacology and Pharmacotherapy Gyökeres, András; Szegedi Tudomanyegyetem, Department of Pharmacology and Pharmacotherapy Györe, Balázs; University of Szeged Gurabi, Zsolt ; Szegedi Tudomanyegyetem, Department of Pharmacology and Pharmacotherapy Nagy, Norbert; University of Szeged, MTA-SZTE Research Group of Cardiovascular Pharmacology, Hungarian Academy of Sciences Jost, Norbert; HUngarian Academy of Sciences, Division of Cardiovascular Pharmacology Virág, László; University of Szeged, Department of Pharmacology and Pharmacotherapy; HUngarian Academy of Sciences, Division of Cardiovascular Pharmacology Papp, Julius; Universiy of Szeged,, Department of Pharmacology and Pharmacotherapy Koncz, Istvan; Szegedi Tudomanyegyetem, Department of Pharmacology and Pharmacotherapy
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Muscarinic agonists inhibit the ATP-dependent potassium current and suppress the ventricle-Purkinje action potential dispersion

Tibor Magyar[a,§], Tamás Árpádffy-Lovas[a,§], Bence Pászti[a], Noémi Tóth[a], Jozefina Szlovák[a], Péter Gazdag[a], Zsófia Kohajda[b], András Gyökeres[a], Balázs Györe[d], Zsolt Gurabi[a], Norbert Jost[a,b,c], László Virág[a,c], Julius Gy. Papp[a,b], Norbert Nagy[a,b,#], István Koncz[a,*, #]

[a]Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, Szeged, Hungary;

[b]MTA-SZTE Research Group of Cardiovascular Pharmacology, Hungarian Academy of Sciences, Szeged, Hungary

[c]Department of Pharmacology and Pharmacotherapy, Interdisciplinary Excellence Centre, University of Szeged, Szeged, Hungary

[d]Faculty of Dentistry, University of Szeged, Hungary

[§] Shared first authorship

[#] Shared senior authorship

*Author for correspondence at:

István Koncz MD, PhD

Department of Pharmacology & Pharmacotherapy

Faculty of Medicine

University of Szeged

Dóm tér 12,

H-6720 Szeged, Hungary

E-mail: koncz.istvan@med.u-szeged.hu

Abstract

Introduction: Activation of the parasympathetic nervous system has been reported to have an antiarrhythmic role during ischemia-reperfusion injury by decreasing the arrhythmia triggers. Furthermore, it was reported that the parasympathetic neurotransmitter acetylcholine is able to modulate the ATP-dependent K-current ($I_{K\text{-}ATP}$), a crucial current activated during hypoxia. However, the possible significance of this current modulation in the antiarrhythmic mechanism is not fully clarified.

Methods: Action potentials were measured using the conventional microelectrode technique from canine left ventricular papillary muscle and free-running Purkinje fibers, under normal and hypoxic conditions. Ionic currents were measured using the whole-cell configuration of the patch clamp method.

Results: 5 μM acetylcholine did not influence the action potential duration (APD) either in Purkinje fibers or in papillary muscle preparations. In contrast, it significantly lengthened the APD and suppressed the Purkinje–ventricle APD dispersion when it was administered after 5 μM pinacidil application. 3 μM carbachol reduced the pinacidil-activated $I_{K\text{-}ATP}$ under voltage-clamp condition. Acetylcholine lengthened the ventricular action potential under simulated ischemia condition.

Conclusion: In this study we found that acetylcholine inhibits the $I_{K\text{-}ATP}$ and thus suppresses the ventricle-Purkinje APD dispersion. We conclude that parasympathetic tone may reduce the arrhythmogenic substrate exerting a complex antiarrhythmic mechanism during hypoxic conditions.

Key words: acetylcholine, Purkinje fibers, papillary muscles, hypoxia

Introduction

The parasympathetic nervous system has a crucial role in controlling the actual heart rate and impulse propagation via influencing the sinoatrial and atrioventricular nodes (Higgins et al., 1973). The parasympathetic nerve endings operate by releasing acetylcholine that acts on M_2-receptors, activating several intracellular signaling routes, and ultimately influencing the cardiac ion channels (Harvey and Belevych, 2003). Even though the parasympathetic nervous system primarily innervates the supraventricular areas of the heart, there are certain important ion channels in the ventricular muscle that are known to be influenced by the release of acetylcholine. It has been previously reported that the inward rectifier potassium current (I_{K1}; Koumi et al., 1995) and the slow component of the delayed rectifier (I_{Ks}; Pappano and Carmeliet, 1979) are inhibited, whereas $I_{K\text{-}ATP}$ and $I_{K\text{-}ACh}$ are activated by acetylcholine via G proteins (Terzic et al, 1994; Ito et al., 1994; Kim et al., 1997).

The importance of these effects of acetylcholine is underpinned by the fact that the activation of $I_{K\text{-}ATP}$ channels is well known during hypoxia/ischemia, in which situations the duration of the action potential is shortened (Weiss and Venkatesh, 1993). Furthermore, it was reported that vagal activation is also facilitated under ischemia–reperfusion (Recordati et al., 1971). This vagal activation during hypoxia could be antiarrhythmic, since it was reported that increased parasympathetic tone reduces the catecholaminerg-induced early and delayed afterdepolarizations (arrhythmia triggers) (Song et al., 1992), as well as the incidence of ventricular fibrillation (Zuanetti et al., 1987; Collins and Billman, 1989). However, the underlying mechanism of antiarrhythmic effect of M_2-receptor activation is not fully clarified. Arrhythmias may develop when an arrhythmogenic substrate (e. g., dispersion of repolarization) and arrhythmia triggers (e.g.: early and delayed afterdepolarizations) simultaneously exist in the heart. The arrhythmogenic substrate could be prominent at Purkinje–ventricle connection because of the relatively weak electrotonic coupling due to low number of gap junctions (Varró and Baczkó, 2010). As a consequence of the different

pharmacological susceptibility of Purkinje fiber and ventricular muscle (Baláti et al, 1998), the activation of $I_{K\text{-}ATP}$ may modulate the Purkinje and ventricular action potential duration (APD) to different extents, and the developed APD dispersion may contribute to the onset of arrhythmias.

The objective of this study was the investigation of the possible effect of acetylcholine on the $I_{K\text{-}ATP}$ and on the $I_{K\text{-}ATP}$-mediated action potential dispersion under normal and hypoxic conditions.

Methods

Human tissues

Non-diseased human hearts that were unusable for transplantation (based on logistical, not patient-related considerations) were obtained from organ donors. Before cardiac explanation, organ donor patients did not receive medication except dobutamine, furosemide and plasma expanders. The investigations conform to the principles outlined in the *Declaration of Helsinki* of the World Medical Association. All experimental protocols were approved by the Scientific and Research Ethical Committee of the Medical Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB), under ethical approval No 4991-0/2010-1018EKU (339/PI/010). Human cardiac tissue was stored in cardioplegic solution at 4°C for 4–8 hours.

Animals

All experiments using canine cardiac preparations were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication NO 85-23, revised 1996) and conformed to the Directive 2010/63/EU of the European Parliament. The protocols have been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-24-2017) and by the

Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/3331/2017).

Conventional microelectrode technique

Ventricular (papillary or trabecular) muscles were obtained from the right ventricle of canine hearts. Free-running Purkinje fibers were identified as false tendons and isolated from both ventricles of human and canine hearts. Canine hearts were removed through a right lateral thoracotomy from anesthetized (thiopental 30 mg/kg i.v.) mongrel dogs of either sex weighing 10–15 kg. At impalement, Purkinje fibers were observed under a surgical microscope (Zeiss OPMI PRO). The preparations were placed in Locke's solution and allowed to equilibrate for at least 2 hours while superfused (flow rate 4-5 ml/min) also with Locke's solution containing (in mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 22, and glucose 11. The pH of this solution was 7.40 to 7.45 when gassed with 95% O_2 and 5% CO_2 at 37 °C. In the experiments where the effects of tissue hypoxia were examined, we changed the gas mixture to 95% N_2 and 5% CO_2, pH remained at 7.40 to 7.45. All experiments were performed at 37 °C. During the equilibration period, preparations were stimulated at a basic cycle length of 500 ms. Electrical pulses of 0.5–2 ms in duration at twice the diastolic threshold in intensity (S_1) were delivered to the preparations through bipolar platinum electrodes. Transmembrane potentials were recorded using glass capillary microelectrodes filled with 3 M KCl (tip resistance: 5 to 15 MΩ). The microelectrodes were coupled through an Ag-AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier (Experimetria 2011). Intracellular recordings were displayed on a storage oscilloscope (Hitachi V-555) and led to a computer system (APES) designed for on-line determination of the following parameters: resting membrane potential, action potential amplitude, action potential duration at 10% to 90% repolarization and the maximum rate of rise of the action potential upstroke (V_{max}). Control recordings were obtained after equilibration period. The compounds used in all experiments were purchased from Sigma/Merck.

131 *2.3. Cell isolation*

132 Ventricular myocytes were enzymatically dissociated from the left ventricle of dog hearts.
133 Canine hearts were removed through a right lateral thoracotomy from anesthetized (thiopental
134 30 mg/kg i.v.) mongrel dogs of either sex weighing 10–15 kg. Cardiac myocytes were isolated
135 from the left ventricle, containing an arterial branch through which the segment was perfused
136 on a Langendorff apparatus with solutions in the following sequence: normal Tyrode's
137 solution (containing in mM: 144 mM NaCl, 0.4 mM NaH_2PO_4, 4 mM KCl, 0.53 mM $MgSO_4$,
138 1.8 mM $CaCl_2$, 5.5 mM Glucose, 5 mM HEPES, pH 7.4 adjusted with NaOH) for 10 min,
139 Ca^{2+}-free Tyrode solution for 10 min and Ca^{2+}-free Tyrode solution containing collagenase
140 (Worthington type II, 0.66 mg/mL). To the final perfusion solution protease (type XIV, 0.12
141 mg/mL) was added at the 15 and the 30 minutes for digestion.

142

143 *2.4. Measurement of ionic currents*

144 One drop of cell suspension was placed in a transparent recording chamber mounted on the
145 stage of an inverted microscope (Olympus IX51, Tokyo, Japan), and individual myocytes
146 were allowed to settle and adhere to the chamber bottom for at least 5–10 min before
147 superfusion was initiated and maintained by gravity. Only rod-shaped cells with clear
148 striations were used. HEPES-buffered Tyrode's solution (composition in mM: NaCl 144,
149 NaH_2PO_4 0.4, KCl 4.0, $CaCl_2$ 1.8, $MgSO_4$ 0.53, glucose 5.5 and HEPES 5.0, at pH of 7.4)
150 was used as the normal superfusate. During the measurement of $I_{K\text{-}ATP}$, 1 μM nisoldipine was
151 added to the bath solution to block $I_{CaL,}$ I_{Kr} was blocked by 0.1 μM dofetilide, and I_{Ks} was
152 blocked by 0.5 μM HMR-1556. Micropipettes were fabricated from borosilicate glass
153 capillaries (Science Products GmbH, Hofheim, Germany), using a P-97 Flaming/Brown
154 micropipette puller (Sutter Co, Novato, CA, USA), and had a resistance of 1.5–2.5 MΩ when
155 filled with pipette solution. The membrane currents were recorded with Axopatch-200B
156 amplifiers (Molecular Devices, Sunnyvale, CA, USA) by applying the whole-cell
157 configuration of the patch-clamp technique. The membrane currents were digitized with 250

kHz analogue to digital converters (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA) under software control (pClamp 8 and pClamp 10, Molecular Devices, Sunnyvale, CA, USA). The composition of the pipette solution (in mM) was the following: 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).

2.5 Statistical analysis

Results are expressed as mean ± S.E.M. Normality of distributions was verified using Shapiro-Wilk test, and homogeneity of variances was verified using Bartlett's test in each treatment group. Statistical comparisons were made using analysis of variance (ANOVA) for repeated measurements, followed by Bonferroni's post-hoc test. Differences were considered significant when $p < 0.05$.

Results

1. Acetylcholine lengthened the APD after pinacidil-mediated action potential shortening

Canine Purkinje fibers and ventricular papillary muscles were paced at 500 ms cycle length. In canine Purkinje fibers (PFs; n=15), acetylcholine (5 μM) did not affect the repolarization (233.6±4.7 to 231.7±4.6; Figures 1A and 1E). In contrast, in canine Purkinje fibers (n=8), the $I_{K\text{-}ATP}$ activator pinacidil, applied in 5 μM concentration, significantly abbreviated APD_{90} (207.7±7.0 ms vs 113.1±9.1 ms, $p<0.05$) values. After steady state was reached, acetylcholine was administered. Within 3 minutes, acetylcholine prolonged APD_{90} to 147.3±7.4 ms, partially reversing the effects of pinacidil (Figures 1B and 1E; $p<0.05$).

Similarly, as observed in Purkinje fibers, 5 μM acetylcholine alone failed to influence the APD of the ventricular muscle (APD_{90}: 172.6±5.7 ms vs 172.8±5.3 ms). Pinacidil (n=5; 5 μM) pretreatment significantly abbreviated the APD_{90} value (187.9±4.5 ms vs 163.7±6.4 ms, $p<0.05$), similarly to the effects observed in the case of PFs. After a period of

30 minutes, sufficient to reach a steady state, acetylcholine was added to the superfusate. Within 4 minutes, acetylcholine (5 μM) prolonged APD_{90} to 172.1±7.4 ms ($p<0.05$), thus partially reversing the effects of pinacidil (Figures 1D and 1E).

2. Acetylcholine decreased the calculated APD dispersion between PF and VM

The changes in the difference between the APD_{90} values of PF and VM can be used to infer the effects of pinacidil and acetylcholine on the dispersion between these cardiac tissue types (Figure 2). The control APD_{90} dispersion (9.5%, 20 ms) was significantly increased upon 5 μM pinacidil application (44.7%, 51 ms). On the other hand, subsequently applied 5 μM acetylcholine markedly decreased the repolarization heterogeneity (16.9%, 28 ms; $p<0.05$).

3. Carbachol decreased the pinacidil-induced current activation

During ionic current measurements, voltage ramps were used from a holding potential of -90 mV. Membrane potential was hyperpolarized to -120 mV, and then was slowly (over 36 s) depolarized to 60 mV. Ionic currents were analyzed and compared at 0 and +30 mV. We found that carbachol did not change the control current when it was applied *without* pinacidil (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). In contrast, when 5 μM pinacidil was applied first, subsequently employed carbachol significantly reduced 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 → 5 μM pinacidil: 3.17±0.3 pA/pF → 3 μM carbachol: 2.26±0.3 pA/pF, n=8, $p<0.05$).

These measurements were carried out with acetylcholine as well. However, we found carbachol to be more stable during the applied long voltage protocol.

4. Acetylcholine restored the APD after hypoxia-induced action potential shortening

Simulated hypoxia, achieved by gassing the solution with N_2 and CO_2 instead of O_2 and CO_2, resulted in a significant abbreviation of APD_{90} from 181.4±5.7 ms to 135.0±8.6 ms ($p<0.05$, Figures 4A and 4B), and a decrease in amplitude (103.7±2.8 mV vs 92±3.5 mV). The maximum rate of depolarization was also decreased (185.8±15.8 V/s vs 156.1±20.6 V/s). When applied during hypoxia, 5 µM acetylcholine caused a significant APD_{90} prolongation to 164.4±4.4 ms, partially reversing the effect of hypoxia on the repolarization. AMP returned to a normal range (102.1±1.6 mV), while V_{max} remained at 156.0±16.1 V/s.

5. Acetylcholine caused a slight abbreviation in human Purkinje fibers

In human PFs (n=2), acetylcholine in 5 µM concentration caused a slight abbreviation of APD_{90} from 269.0±28.4 to 251.6±42.85 ms and APD50 from 184.4±20.0 ms to 173.3±27.1 ms without affecting other characteristics of the action potential (Figure 5).

Discussion

In this study we investigated the electrophysiological effects of muscarinic agonists on the $I_{K\text{-}ATP}$ current. We found that (i) under normal conditions acetylcholine did not influence the action potential duration. (ii) In contrast, when $I_{K\text{-}ATP}$ was pharmacologically activated by pinacidil, subsequently applied acetylcholine lengthened the action potential duration as well as (iii) reduced the pinacidil-induced ventricle-Purkinje APD dispersion. (iv) In line with this, carbachol inhibited the $I_{K\text{-}ATP}$ that was previously activated by pinacidil. (v) Acetylcholine increased the APD after hypoxia-induced action potential shortening.

Acetylcholine inhibits the $I_{K\text{-}ATP}$ in canine ventricular myocytes

It is well known that acetylcholine shortens the atrial APD and has been implicated in atrial fibrillation (Nakayama et al, 1968). Acetylcholine directly affects the GIRK1/4 or Kir3.1/Kir3.4 channels (Nobles et al, 2018; Corey and Clapham, 1998), encoded by *KCNJ3*

238 and *KCNJ4* genes (Kurachi, 1995). These channels are largely expressed in atrial, SA and AV
239 nodal cells (Galindo et al, 2016; Navarro-Polanco et al, 2013). At the same time, previous
240 studies (Terzic et al, 1994; Ito et al., 1994) claimed that acetylcholine activates the $I_{K\text{-}ATP}$
241 channels, even though the physiological consequences of this effect on the action potential
242 were not clarified.
243
244 The $I_{K\text{-}ATP}$ ATP-sensitive potassium channels comprise hetero-octamers consisting of four
245 inward rectifying potassium channel pore-forming subunits (Kir6.1 or Kir6.2, encoded by
246 *KCNJ8* and *KCNJ11* genes, respectively) and four ATP-binding cassette protein
247 sulphonylurea receptors (SUR1 or SUR2, encoded by *ABCC8* and *ABCC9* genes,
248 respectively; Inagaki et al, 195). An important feature of the $I_{K\text{-}ATP}$ is its closed state under
249 physiological intracellular ATP levels (i. e., under normoxia) and its activation by metabolic
250 stress, when the ratio of ATP/ADP is decreased, e. g., during myocardial ischemia (Deutsch et
251 al., 1991).
252
253 Activation of the sarcolemmal $I_{K\text{-}ATP}$ during myocardial ischemia shortens the action potential
254 of various cardiac tissues to different extents, thus it may promote APD dispersion and re-
255 entry type arrhythmias (Janse and Wit, 1989). Accordingly, several investigations found $I_{K\text{-}}$
256 $_{ATP}$ activation to be pro-arrhythmic (Chi et al., 1990), suggesting that sarcolemmal $I_{K\text{-}ATP}$
257 inhibition may prevent arrhythmias induced by myocardial ischemia and ischemia/reperfusion
258 (Billman et al, 1998; Englert et al, 2003; Vajda et al, 2007).
259
260 In our experiments under normal conditions, we found no effect of carbachol on the
261 membrane current (Figure 3) and, similarly, acetylcholine failed to influence the ventricular
262 and Purkinje APDs (Figures 1A and 1C). The observed discrepancy between our and previous
263 results, where an activation of $I_{K\text{-}ATP}$ was described upon acetylcholine administration (Terzic

et al, 1994; Ito et al, 1994; Kim et al., 1997), could be the consequence of the species difference and the distinct experimental conditions.

In contrast, an important, and, to the best of our knowledge, previously not published result of our study is that carbachol is able to suppress the pinacidil-activated $I_{K\text{-}ATP}$. As a consequence, in parallel tissue action potential experiments, acetylcholine lengthened the APD as long as it was previously shortened by the application of $I_{K\text{-}ATP}$-activator pinacidil. Since $I_{K\text{-}ATP}$ activation could be arrhythmogenic (Chi et al., 1990) by causing an increase in the APD dispersion, this effect of acetylcholine raises the possibility of a novel antiarrhythmic mechanism of the previously described antiarrhythmic effect of parasympathetic activation during hypoxia (Song et al., 1992; Zuanetti et al., 1987; Collins and Billman, 1989).

Our experiments conducted under hypoxic conditions provided similar results (i. e., acetylcholine lengthened the hypoxia-induced shortened ventricular action potential; Figure 4). Even though tissue hypoxia is a complex phenomenon (Carmeliet, 1999), during which several factors change simultaneously (e. g., Ca^{2+}_i, Na^{+}_i, pH, conductance of gap junctions, membrane potential etc.), it is feasible that $I_{K\text{-}ATP}$ activation, as a response to ATP depletion, is an important factor in the observed action potential shortening. Since acetylcholine lengthened the action potential under hypoxic conditions, we suggest $I_{K\text{-}ATP}$ inhibition as a possible underlying mechanism.

Acetylcholine decreased the pinacidil-induced ventricle–Purkinje APD dispersion

Free-running Purkinje fibers connect to the ventricular muscle on a small surface area, providing a relatively large-resistance coupling (Tranum-Jensen et al., 1991), and a large sink for current flow that favors conduction blocks more than other parts of the healthy myocardium. Also, due to the weaker electrotonic coupling, the dispersion of repolarization here can be greater than in other areas (Martinez et al., 2018), causing the Purkinje–ventricle

APD ratio to have critical importance in arrhythmia generation. In our experiments, we found significantly greater shortening in Purkinje fibers caused by pinacidil that could be the consequence of the generally weaker repolarization reserve that makes the Purkinje action potential to be more susceptible to any pharmacological interventions (Varró et al, 2000; Baláti et al, 1998). Similarly, acetylcholine exerted larger lengthening in the Purkinje fiber probably by the same reason that ultimately led to reduced ventricle–Purkinje APD dispersion. The reduction of the ventricle–Purkinje fiber APD dispersion could suppress the arrhythmogenic substrate providing a narrower vulnerable period for a critically timed extrasystole to trigger a life-threatening arrhythmia under hypoxic conditions.

Proposed mechanism

Since inhibition of the K-ATP channels is possible by blocking various PKA-mediated pathways (Tinker et al, 2018.), we suggest that the decrease of cAMP levels caused by the activation of cardiac muscarinic receptors using acetylcholine/carbachol was the factor that decreased the density of the $I_{K\text{-}ATP}$ current in patch clamp measurements, leading to the subsequent prolongation observed in action potential durations.

Conclusions

We found that muscarinic agonists inhibit the $I_{K\text{-}ATP}$. Therefore, during $I_{K\text{-}ATP}$-mediated action potential shortening, acetylcholine causes asymmetrical action potential lengthening between ventricular muscle and Purkinje fiber that leads to reduced APD dispersion.

These results suggest that the parasympathetic tone beyond suppressing the catecholaminerg-induced arrhythmogenic triggers (Song et al., 1992) may be also able to reduce the arrhythmogenic substrate under hypoxic conditions.

Study Limitations

(i) In our experiments, the ventricular and Purkinje fiber action potentials were measured from electrically uncoupled tissue samples.

(ii) The presented effects were attributed to the M2 muscarinic receptor; nevertheless, the exact level of contribution of other receptor subtypes was not addressed. To achieve this, further studies are needed, utilizing specific agonist and antagonist drugs.

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455 **Figure Legends**

456 **Figure 1.** Representative traces of Purkinje fiber (A, B) and ventricular muscle preparations
457 (C, D); 5 μM acetylcholine (red dotted lines) alone caused no changes in either preparation
458 type (A, C), while it caused significant prolongation when applied cummulatively after 5 μM
459 pinacidil (B, D, pinacidil effect represented as blue dashed lines). Bars in panel E represent
460 the values of APD_{90} in each treatment group, from top to bottom corresponding to the traces
461 A to D. Abbreviations under bars: C, control; P, pinacidil, A, acetylcholine. The pacing cycle
462 length was 500 ms. Values are mean ± SEM; *,# $p<0.05$ RM-ANOVA followed by
463 Bonferroni's post-hoc test.

464

465 **Figure 2.** Pinacidil (5 μM) increased the action potential duration dispersion (indicated by
466 ΔAPD_{90} in percentages, and in ms above the bars) between Purkinje fiber and ventricular
467 muscle preparations, while acetylcholine (5 μM), when applied after pinacidil, decreased
468 dispersion. The pacing cycle length was 500 ms.

469

470 **Figure 3.** Effect of carbachol on $I_{K\text{-}ATP}$. Ionic currents were measured under a slow voltage
471 ramp protocol (panel A) between -120 mV and 60 mV. The currents were analysed at 0 and
472 30 mV. Panel B demonstrates original representative current traces (left) and bar graphs
473 (right) where 3 μM carbachol (dotted line) failed to influence the control current analysed at
474 0 mV. Inset shows identical current fractions between –3 mV and 45 mV (indicated by dashed
475 rectangle). Current traces in panel C as well as in the inset, illustrate large increase of the
476 membrane current after application of 5 μM pinacidil (blue dashed line) that was inhibited by
477 the subsequently applied 3 μM carbachol (red dotted line). In bar graphs (right), asterisk
478 denotes significant change between control (left column) and pinacidil (middle column),
479 while hash tag indicates significant change between pinacidil (middle column) and carbachol
480 (right column).

481

Figure 4. Representative action potential trace (A) showing that hypoxic conditions caused significant action potential duration abbreviation and decreased mean diastolic potential and amplitude in canine ventricular preparations (blue dashed line), while acetylcholine (5 μM) caused a significant prolongation in action potential duration (red dotted line). Values of APD_{90} are represented as bars (B). Abbreviations under bars: C, control; H, hypoxia, A, acetylcholine. The pacing cycle length was 500 ms. Values are mean ± SEM; *,#$p<0.05$, RM-ANOVA followed by Bonferroni's post-hoc test.

Figure 5. Representative action potential showing the effect of acetylcholine (5 μM, red dotted line) on a Purkinje fiber taken from a human donor heart (A). Values of APD_{90} are represented as bars (B). Abbreviations under bars: C, control; A, acetylcholine. The pacing cycle length was 500 ms. Values are mean ± SEM.

Muscarinic agonists inhibit the ATP-dependent potassium current and suppress the ventricle-Purkinje action potential dispersion

Tibor Magyar[a,§], Tamás Árpádffy-Lovas[a,§], Bence Pászti[a], Noémi Tóth[a], Jozefina Szlovák[a], Péter Gazdag[a], Zsófia Kohajda[b], András Gyökeres[a], Balázs Györe[d], Zsolt Gurabi[a], Norbert Jost[a,b,c], László Virág[a,c], Julius Gy. Papp[a,b], Norbert Nagy[a,b,#], István Koncz[a,*, #]

[a]Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, Szeged, Hungary;

[b]MTA-SZTE Research Group of Cardiovascular Pharmacology, Hungarian Academy of Sciences, Szeged, Hungary

[c]Department of Pharmacology and Pharmacotherapy, Interdisciplinary Excellence Centre, University of Szeged, Szeged, Hungary

[d]Faculty of Dentistry, University of Szeged, Hungary

[§] Shared first authorship

[#] Shared senior authorship

*Author for correspondence at:

István Koncz MD, PhD

Department of Pharmacology & Pharmacotherapy

Faculty of Medicine

University of Szeged

Dóm tér 12,

H-6720 Szeged, Hungary

E-mail: koncz.istvan@med.u-szeged.hu

Abstract

Introduction: Activation of the parasympathetic nervous system has been reported to have an antiarrhythmic role during ischemia-reperfusion injury by decreasing the arrhythmia triggers. Furthermore, it was reported that the parasympathetic neurotransmitter acetylcholine is able to modulate the ATP-dependent K-current ($I_{K\text{-}ATP}$), a crucial current activated during hypoxia. However, the possible significance of this current modulation in the antiarrhythmic mechanism is not fully clarified.

Methods: Action potentials were measured using the conventional microelectrode technique from canine left ventricular papillary muscle and free-running Purkinje fibers, under normal and hypoxic conditions. Ionic currents were measured using the whole-cell configuration of the patch clamp method.

Results: 5 μM acetylcholine did not influence the action potential duration (APD) either in Purkinje fibers or in papillary muscle preparations. In contrast, it significantly lengthened the APD and suppressed the Purkinje–ventricle APD dispersion when it was administered after 5 μM pinacidil application. 3 μM carbachol reduced the pinacidil-activated $I_{K\text{-}ATP}$ under voltage-clamp condition. Acetylcholine lengthened the ventricular action potential under simulated ischemia condition.

Conclusion: In this study we found that acetylcholine inhibits the $I_{K\text{-}ATP}$ and thus suppresses the ventricle-Purkinje APD dispersion. We conclude that parasympathetic tone may reduce the arrhythmogenic substrate exerting a complex antiarrhythmic mechanism during hypoxic conditions.

Key words: acetylcholine, Purkinje fibers, papillary muscles, hypoxia

Introduction

The parasympathetic nervous system has a crucial role in controlling the actual heart rate and impulse propagation via influencing the sinoatrial and atrioventricular nodes (Higgins et al., 1973). The parasympathetic nerve endings operate by releasing acetylcholine that acts on M_2-receptors, activating several intracellular signaling routes, and ultimately influencing the cardiac ion channels (Harvey and Belevych, 2003). Even though the parasympathetic nervous system primarily innervates the supraventricular areas of the heart, there are certain important ion channels in the ventricular muscle that are known to be influenced by the release of acetylcholine. It has been previously reported that the inward rectifier potassium current (I_{K1}; Koumi et al., 1995) and the slow component of the delayed rectifier (I_{Ks}; Pappano and Carmeliet, 1979) are inhibited, whereas $I_{K\text{-}ATP}$ and $I_{K\text{-}ACh}$ are activated by acetylcholine via G proteins (Terzic et al, 1994; Ito et al., 1994; Kim et al., 1997).

The importance of these effects of acetylcholine is underpinned by the fact that the activation of $I_{K\text{-}ATP}$ channels is well known during hypoxia/ischemia, in which situations the duration of the action potential is shortened (Weiss and Venkatesh, 1993). Furthermore, it was reported that vagal activation is also facilitated under ischemia–reperfusion (Recordati et al., 1971). This vagal activation during hypoxia could be antiarrhythmic, since it was reported that increased parasympathetic tone reduces the catecholaminerg-induced early and delayed afterdepolarizations (arrhythmia triggers) (Song et al., 1992), as well as the incidence of ventricular fibrillation (Zuanetti et al., 1987; Collins and Billman, 1989). However, the underlying mechanism of antiarrhythmic effect of M_2-receptor activation is not fully clarified. Arrhythmias may develop when an arrhythmogenic substrate (e. g., dispersion of repolarization) and arrhythmia triggers (e.g.: early and delayed afterdepolarizations) simultaneously exist in the heart. The arrhythmogenic substrate could be prominent at Purkinje–ventricle connection because of the relatively weak electrotonic coupling due to low number of gap junctions (Varró and Baczkó, 2010). As a consequence of the different

pharmacological susceptibility of Purkinje fiber and ventricular muscle (Baláti et al, 1998), the activation of $I_{K\text{-}ATP}$ may modulate the Purkinje and ventricular action potential duration (APD) to different extents, and the developed APD dispersion may contribute to the onset of arrhythmias.

The objective of this study was the investigation of the possible effect of acetylcholine on the $I_{K\text{-}ATP}$ and on the $I_{K\text{-}ATP}$-mediated action potential dispersion under normal and hypoxic conditions.

Methods

Human tissues

Non-diseased human hearts that were unusable for transplantation (based on logistical, not patient-related considerations) were obtained from organ donors. Before cardiac explanation, organ donor patients did not receive medication except dobutamine, furosemide and plasma expanders. The investigations conform to the principles outlined in the *Declaration of Helsinki* of the World Medical Association. All experimental protocols were approved by the Scientific and Research Ethical Committee of the Medical Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB), under ethical approval No 4991-0/2010-1018EKU (339/PI/010). Human cardiac tissue was stored in cardioplegic solution at 4°C for 4–8 hours.

Animals

All experiments using canine cardiac preparations were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication NO 85-23, revised 1996) and conformed to the Directive 2010/63/EU of the European Parliament. The protocols have been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-24-2017) and by the

104 Department of Animal Health and Food Control of the Ministry of Agriculture and Rural
105 Development (authority approval number XIII/3331/2017).
106
107 *Conventional microelectrode technique*
108 Ventricular (papillary or trabecular) muscles were obtained from the right ventricle of canine
109 hearts. Free-running Purkinje fibers were identified as false tendons and isolated from both
110 ventricles of human and canine hearts. Canine hearts were removed through a right lateral
111 thoracotomy from anesthetized (thiopental 30 mg/kg i.v.) mongrel dogs of either sex
112 weighing 10–15 kg. At impalement, Purkinje fibers were observed under a surgical
113 microscope (Zeiss OPMI PRO). The preparations were placed in Locke's solution and
114 allowed to equilibrate for at least 2 hours while superfused (flow rate 4-5 ml/min) also with
115 Locke's solution containing (in mM): NaCl 120, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 22, and
116 glucose 11. The pH of this solution was 7.40 to 7.45 when gassed with 95% O_2 and 5% CO_2
117 at 37 °C. In the experiments where the effects of tissue hypoxia were examined, we changed
118 the gas mixture to 95% N_2 and 5% CO_2, pH remained at 7.40 to 7.45. All experiments were
119 performed at 37 °C. During the equilibration period, preparations were stimulated at a basic
120 cycle length of 500 ms. Electrical pulses of 0.5–2 ms in duration at twice the diastolic
121 threshold in intensity (S_1) were delivered to the preparations through bipolar platinum
122 electrodes. Transmembrane potentials were recorded using glass capillary microelectrodes
123 filled with 3 M KCl (tip resistance: 5 to 15 MΩ). The microelectrodes were coupled through
124 an Ag-AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier
125 (Experimetria 2011). Intracellular recordings were displayed on a storage oscilloscope
126 (Hitachi V-555) and led to a computer system (APES) designed for on-line determination of
127 the following parameters: resting membrane potential, action potential amplitude, action
128 potential duration at 10% to 90% repolarization and the maximum rate of rise of the action
129 potential upstroke (V_{max}). Control recordings were obtained after equilibration period. The
130 compounds used in all experiments were purchased from Sigma/Merck.

131 *2.3. Cell isolation*

132 Ventricular myocytes were enzymatically dissociated from the left ventricle of dog hearts.
133 Canine hearts were removed through a right lateral thoracotomy from anesthetized (thiopental
134 30 mg/kg i.v.) mongrel dogs of either sex weighing 10–15 kg. Cardiac myocytes were isolated
135 from the left ventricle, containing an arterial branch through which the segment was perfused
136 on a Langendorff apparatus with solutions in the following sequence: normal Tyrode's
137 solution (containing in mM: 144 mM NaCl, 0.4 mM NaH_2PO_4, 4 mM KCl, 0.53 mM $MgSO_4$,
138 1.8 mM $CaCl_2$, 5.5 mM Glucose, 5 mM HEPES, pH 7.4 adjusted with NaOH) for 10 min,
139 Ca^{2+}-free Tyrode solution for 10 min and Ca^{2+}-free Tyrode solution containing collagenase
140 (Worthington type II, 0.66 mg/mL). To the final perfusion solution protease (type XIV, 0.12
141 mg/mL) was added at the 15 and the 30 minutes for digestion.

142

143 *2.4. Measurement of ionic currents*

144 One drop of cell suspension was placed in a transparent recording chamber mounted on the
145 stage of an inverted microscope (Olympus IX51, Tokyo, Japan), and individual myocytes
146 were allowed to settle and adhere to the chamber bottom for at least 5–10 min before
147 superfusion was initiated and maintained by gravity. Only rod-shaped cells with clear
148 striations were used. HEPES-buffered Tyrode's solution (composition in mM: NaCl 144,
149 NaH_2PO_4 0.4, KCl 4.0, $CaCl_2$ 1.8, $MgSO_4$ 0.53, glucose 5.5 and HEPES 5.0, at pH of 7.4)
150 was used as the normal superfusate. During the measurement of $I_{K\text{-}ATP}$, 1 μM nisoldipine was
151 added to the bath solution to block $I_{CaL,}$ I_{Kr} was blocked by 0.1 μM dofetilide, and I_{Ks} was
152 blocked by 0.5 μM HMR-1556. Micropipettes were fabricated from borosilicate glass
153 capillaries (Science Products GmbH, Hofheim, Germany), using a P-97 Flaming/Brown
154 micropipette puller (Sutter Co, Novato, CA, USA), and had a resistance of 1.5–2.5 MΩ when
155 filled with pipette solution. The membrane currents were recorded with Axopatch-200B
156 amplifiers (Molecular Devices, Sunnyvale, CA, USA) by applying the whole-cell
157 configuration of the patch-clamp technique. The membrane currents were digitized with 250

158 kHz analogue to digital converters (Digidata 1440A, Molecular Devices, Sunnyvale, CA,
159 USA) under software control (pClamp 8 and pClamp 10, Molecular Devices, Sunnyvale, CA,
160 USA). The composition of the pipette solution (in mM) was the following: KOH 110, KCl
161 40, K_2ATP 5, $MgCl_2$ 5, EGTA 5, HEPES 10 and GTP 0.1 (pH was adjusted to 7.2 by aspartic
162 acid).

163

164 *2.5 Statistical analysis*

165 Results are expressed as mean ± S.E.M. Normality of distributions was verified using
166 Shapiro-Wilk test, and homogeneity of variances was verified using Bartlett's test in each
167 treatment group. Statistical comparisons were made using analysis of variance (ANOVA) for
168 repeated measurements, followed by Bonferroni's post-hoc test. Differences were considered
169 significant when $p < 0.05$.

170

171 **Results**

172 *1. Acetylcholine lengthened the APD after pinacidil-mediated action potential shortening*

173 Canine Purkinje fibers and ventricular papillary muscles were paced at 500 ms cycle length.
174 In canine Purkinje fibers (PFs; n=15), acetylcholine (5 μM) did not affect the repolarization
175 (233.6±4.7 to 231.7±4.6; Figures 1A and 1E). In contrast, in canine Purkinje fibers (n=8), the
176 $I_{K\text{-}ATP}$ activator pinacidil, applied in 5 μM concentration, significantly abbreviated APD_{90}
177 (207.7±7.0 ms vs 113.1±9.1 ms, $p<0.05$) values. After steady state was reached, acetylcholine
178 was administered. Within 3 minutes, acetylcholine prolonged APD_{90} to 147.3±7.4 ms,
179 partially reversing the effects of pinacidil (Figures 1B and 1E; $p<0.05$).

180

181 Similarly, as observed in Purkinje fibers, 5 μM acetylcholine alone failed to influence the
182 APD of the ventricular muscle (APD_{90}: 172.6±5.7 ms vs 172.8±5.3 ms). Pinacidil (n=5;
183 5 μM) pretreatment significantly abbreviated the APD_{90} value (187.9±4.5 ms vs
184 163.7±6.4 ms, $p<0.05$), similarly to the effects observed in the case of PFs. After a period of

30 minutes, sufficient to reach a steady state, acetylcholine was added to the superfusate. Within 4 minutes, acetylcholine (5 μM) prolonged APD_{90} to 172.1±7.4 ms ($p<0.05$), thus partially reversing the effects of pinacidil (Figures 1D and 1E).

2. Acetylcholine decreased the calculated APD dispersion between PF and VM

The changes in the difference between the APD_{90} values of PF and VM can be used to infer the effects of pinacidil and acetylcholine on the dispersion between these cardiac tissue types (Figure 2). The control APD_{90} dispersion (9.5%, 20 ms) was significantly increased upon 5 μM pinacidil application (44.7%, 51 ms). On the other hand, subsequently applied 5 μM acetylcholine markedly decreased the repolarization heterogeneity (16.9%, 28 ms; $p<0.05$).

3. Carbachol decreased the pinacidil-induced current activation

During ionic current measurements, voltage ramps were used from a holding potential of -90 mV. Membrane potential was hyperpolarized to -120 mV, and then was slowly (over 36 s) depolarized to 60 mV. Ionic currents were analyzed and compared at 0 and +30 mV. We found that carbachol did not change the control current when it was applied *without* pinacidil (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). In contrast, when 5 μM pinacidil was applied first, subsequently employed carbachol significantly reduced 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 → 5 μM pinacidil: 3.17±0.3 pA/pF → 3 μM carbachol: 2.26±0.3 pA/pF, n=8, $p<0.05$).

These measurements were carried out with acetylcholine as well. However, we found carbachol to be more stable during the applied long voltage protocol.

211 *4. Acetylcholine restored the APD after hypoxia-induced action potential shortening*

212 Simulated hypoxia, achieved by gassing the solution with N_2 and CO_2 instead of O_2 and CO_2,
213 resulted in a significant abbreviation of APD_{90} from 181.4±5.7 ms to 135.0±8.6 ms ($p<0.05$,
214 Figures 4A and 4B), and a decrease in amplitude (103.7±2.8 mV vs 92±3.5 mV). The
215 maximum rate of depolarization was also decreased (185.8±15.8 V/s vs 156.1±20.6 V/s).
216 When applied during hypoxia, 5 µM acetylcholine caused a significant APD_{90} prolongation to
217 164.4±4.4 ms, partially reversing the effect of hypoxia on the repolarization. AMP returned to
218 a normal range (102.1±1.6 mV), while V_{max} remained at 156.0±16.1 V/s.

219

220 *5. Acetylcholine caused a slight abbreviation in human Purkinje fibers*

221 In human PFs (n=2), acetylcholine in 5 µM concentration caused a slight abbreviation of
222 APD_{90} from 269.0±28.4 to 251.6±42.85 ms and APD50 from 184.4±20.0 ms to
223 173.3±27.1 ms without affecting other characteristics of the action potential (Figure 5).

224

225 **Discussion**

226 In this study we investigated the electrophysiological effects of muscarinic agonists on the
227 $I_{K\text{-}ATP}$ current. We found that (i) under normal conditions acetylcholine did not influence the
228 action potential duration. (ii) In contrast, when $I_{K\text{-}ATP}$ was pharmacologically activated by
229 pinacidil, subsequently applied acetylcholine lengthened the action potential duration as well
230 as (iii) reduced the pinacidil-induced ventricle-Purkinje APD dispersion. (iv) In line with this,
231 carbachol inhibited the $I_{K\text{-}ATP}$ that was previously activated by pinacidil. (v) Acetylcholine
232 increased the APD after hypoxia-induced action potential shortening.

233

234 *Acetylcholine inhibits the $I_{K\text{-}ATP}$ in canine ventricular myocytes*

235 It is well known that acetylcholine shortens the atrial APD and has been implicated in atrial
236 fibrillation (Nakayama et al, 1968). Acetylcholine directly affects the GIRK1/4 or
237 Kir3.1/Kir3.4 channels (Nobles et al, 2018; Corey and Clapham, 1998), encoded by *KCNJ3*

238 and *KCNJ4* genes (Kurachi, 1995). These channels are largely expressed in atrial, SA and AV
239 nodal cells (Galindo et al, 2016; Navarro-Polanco et al, 2013). At the same time, previous
240 studies (Terzic et al, 1994; Ito et al., 1994) claimed that acetylcholine activates the $I_{K\text{-}ATP}$
241 channels, even though the physiological consequences of this effect on the action potential
242 were not clarified.
243
244 The $I_{K\text{-}ATP}$ ATP-sensitive potassium channels comprise hetero-octamers consisting of four
245 inward rectifying potassium channel pore-forming subunits (Kir6.1 or Kir6.2, encoded by
246 *KCNJ8* and *KCNJ11* genes, respectively) and four ATP-binding cassette protein
247 sulphonylurea receptors (SUR1 or SUR2, encoded by *ABCC8* and *ABCC9* genes,
248 respectively; Inagaki et al, 195). An important feature of the $I_{K\text{-}ATP}$ is its closed state under
249 physiological intracellular ATP levels (i. e., under normoxia) and its activation by metabolic
250 stress, when the ratio of ATP/ADP is decreased, e. g., during myocardial ischemia (Deutsch et
251 al., 1991).
252
253 Activation of the sarcolemmal $I_{K\text{-}ATP}$ during myocardial ischemia shortens the action potential
254 of various cardiac tissues to different extents, thus it may promote APD dispersion and re-
255 entry type arrhythmias (Janse and Wit, 1989). Accordingly, several investigations found $I_{K\text{-}}$
256 $_{ATP}$ activation to be pro-arrhythmic (Chi et al., 1990), suggesting that sarcolemmal $I_{K\text{-}ATP}$
257 inhibition may prevent arrhythmias induced by myocardial ischemia and ischemia/reperfusion
258 (Billman et al, 1998; Englert et al, 2003; Vajda et al, 2007).
259
260 In our experiments under normal conditions, we found no effect of carbachol on the
261 membrane current (Figure 3) and, similarly, acetylcholine failed to influence the ventricular
262 and Purkinje APDs (Figures 1A and 1C). The observed discrepancy between our and previous
263 results, where an activation of $I_{K\text{-}ATP}$ was described upon acetylcholine administration (Terzic

et al, 1994; Ito et al, 1994; Kim et al., 1997), could be the consequence of the species difference and the distinct experimental conditions.

In contrast, an important, and, to the best of our knowledge, previously not published result of our study is that carbachol is able to suppress the pinacidil-activated $I_{K\text{-}ATP}$. As a consequence, in parallel tissue action potential experiments, acetylcholine lengthened the APD as long as it was previously shortened by the application of $I_{K\text{-}ATP}$-activator pinacidil. Since $I_{K\text{-}ATP}$ activation could be arrhythmogenic (Chi et al., 1990) by causing an increase in the APD dispersion, this effect of acetylcholine raises the possibility of a novel antiarrhythmic mechanism of the previously described antiarrhythmic effect of parasympathetic activation during hypoxia (Song et al., 1992; Zuanetti et al., 1987; Collins and Billman, 1989).

Our experiments conducted under hypoxic conditions provided similar results (i. e., acetylcholine lengthened the hypoxia-induced shortened ventricular action potential; Figure 4). Even though tissue hypoxia is a complex phenomenon (Carmeliet, 1999), during which several factors change simultaneously (e. g., Ca^{2+}_i, Na^+_i, pH, conductance of gap junctions, membrane potential etc.), it is feasible that $I_{K\text{-}ATP}$ activation, as a response to ATP depletion, is an important factor in the observed action potential shortening. Since acetylcholine lengthened the action potential under hypoxic conditions, we suggest $I_{K\text{-}ATP}$ inhibition as a possible underlying mechanism.

Acetylcholine decreased the pinacidil-induced ventricle–Purkinje APD dispersion

Free-running Purkinje fibers connect to the ventricular muscle on a small surface area, providing a relatively large-resistance coupling (Tranum-Jensen et al., 1991), and a large sink for current flow that favors conduction blocks more than other parts of the healthy myocardium. Also, due to the weaker electrotonic coupling, the dispersion of repolarization here can be greater than in other areas (Martinez et al., 2018), causing the Purkinje–ventricle

APD ratio to have critical importance in arrhythmia generation. In our experiments, we found significantly greater shortening in Purkinje fibers caused by pinacidil that could be the consequence of the generally weaker repolarization reserve that makes the Purkinje action potential to be more susceptible to any pharmacological interventions (Varró et al, 2000; Baláti et al, 1998). Similarly, acetylcholine exerted larger lengthening in the Purkinje fiber probably by the same reason that ultimately led to reduced ventricle–Purkinje APD dispersion. The reduction of the ventricle–Purkinje fiber APD dispersion could suppress the arrhythmogenic substrate providing a narrower vulnerable period for a critically timed extrasystole to trigger a life-threatening arrhythmia under hypoxic conditions.

Proposed mechanism

Since inhibition of the K-ATP channels is possible by blocking various PKA-mediated pathways (Tinker et al, 2018.), we suggest that the decrease of cAMP levels caused by the activation of cardiac muscarinic receptors using acetylcholine/carbachol was the factor that decreased the density of the $I_{K\text{-}ATP}$ current in patch clamp measurements, leading to the subsequent prolongation observed in action potential durations.

Conclusions

We found that muscarinic agonists inhibit the $I_{K\text{-}ATP}$. Therefore, during $I_{K\text{-}ATP}$-mediated action potential shortening, acetylcholine causes asymmetrical action potential lengthening between ventricular muscle and Purkinje fiber that leads to reduced APD dispersion.

These results suggest that the parasympathetic tone beyond suppressing the catecholaminerg-induced arrhythmogenic triggers (Song et al., 1992) may be also able to reduce the arrhythmogenic substrate under hypoxic conditions.

Study Limitations

(i) In our experiments, the ventricular and Purkinje fiber action potentials were measured from electrically uncoupled tissue samples.

(ii) The presented effects were attributed to the M2 muscarinic receptor; nevertheless, the exact level of contribution of other receptor subtypes was not addressed. To achieve this, further studies are needed, utilizing specific agonist and antagonist drugs.

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455 **Figure Legends**

456 **Figure 1.** Representative traces of Purkinje fiber (A, B) and ventricular muscle preparations
457 (C, D); 5 μM acetylcholine (red dotted lines) alone caused no changes in either preparation
458 type (A, C), while it caused significant prolongation when applied cummulatively after 5 μM
459 pinacidil (B, D, pinacidil effect represented as blue dashed lines). Bars in panel E represent
460 the values of APD_{90} in each treatment group, from top to bottom corresponding to the traces
461 A to D. Abbreviations under bars: C, control; P, pinacidil, A, acetylcholine. The pacing cycle
462 length was 500 ms. Values are mean ± SEM; *,# $p<0.05$ RM-ANOVA followed by
463 Bonferroni's post-hoc test.

464

465 **Figure 2.** Pinacidil (5 μM) increased the action potential duration dispersion (indicated by
466 ΔAPD_{90} in percentages, and in ms above the bars) between Purkinje fiber and ventricular
467 muscle preparations, while acetylcholine (5 μM), when applied after pinacidil, decreased
468 dispersion. The pacing cycle length was 500 ms.

469

470 **Figure 3.** Effect of carbachol on $I_{K\text{-}ATP}$. Ionic currents were measured under a slow voltage
471 ramp protocol (panel A) between -120 mV and 60 mV. The currents were analysed at 0 and
472 30 mV. Panel B demonstrates original representative current traces (left) and bar graphs
473 (right) where 3 μM carbachol (dotted line) failed to influence the control current analysed at
474 0 mV. Inset shows identical current fractions between –3 mV and 45 mV (indicated by dashed
475 rectangle). Current traces in panel C as well as in the inset, illustrate large increase of the
476 membrane current after application of 5 μM pinacidil (blue dashed line) that was inhibited by
477 the subsequently applied 3 μM carbachol (red dotted line). In bar graphs (right), asterisk
478 denotes significant change between control (left column) and pinacidil (middle column),
479 while hash tag indicates significant change between pinacidil (middle column) and carbachol
480 (right column).

481

482 **Figure 4.** Representative action potential trace (A) showing that hypoxic conditions caused
483 significant action potential duration abbreviation and decreased mean diastolic potential and
484 amplitude in canine ventricular preparations (blue dashed line), while acetylcholine (5 μM)
485 caused a significant prolongation in action potential duration (red dotted line). Values of
486 APD_{90} are represented as bars (B). Abbreviations under bars: C, control; H, hypoxia, A,
487 acetylcholine. The pacing cycle length was 500 ms. Values are mean ± SEM; *,#$p<0.05$,
488 RM-ANOVA followed by Bonferroni's post-hoc test.
489
490 **Figure 5.** Representative action potential showing the effect of acetylcholine (5 μM, red
491 dotted line) on a Purkinje fiber taken from a human donor heart (A). Values of APD_{90} are
492 represented as bars (B). Abbreviations under bars: C, control; A, acetylcholine. The pacing
493 cycle length was 500 ms. Values are mean ± SEM.

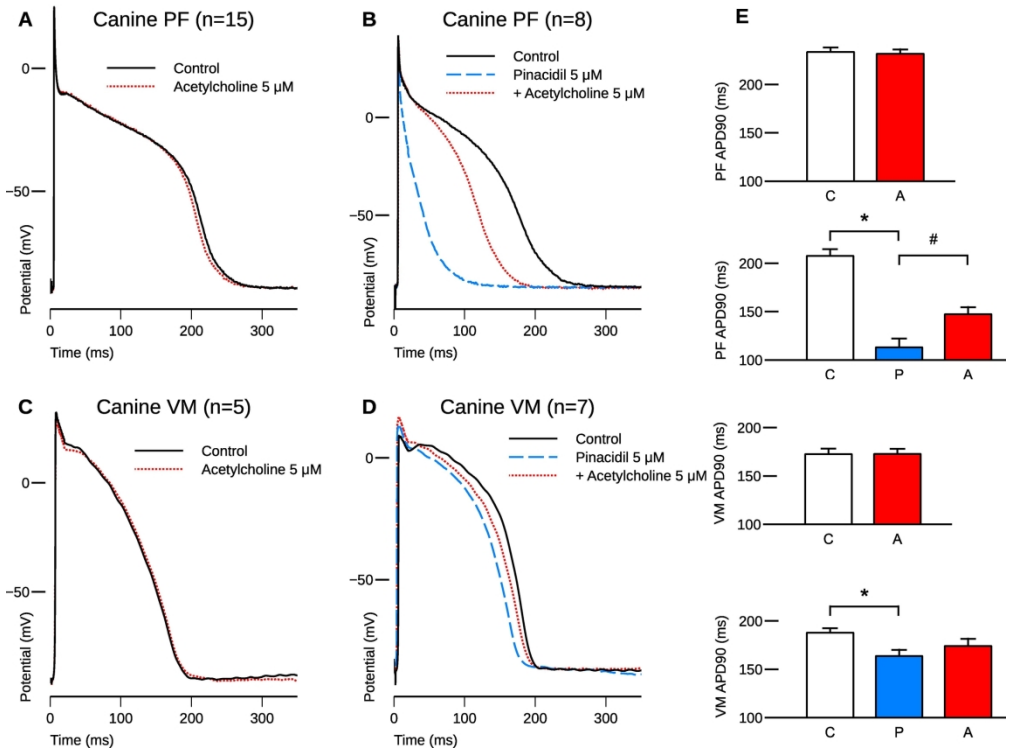

Figure 1. Representative traces of Purkinje fiber (A, B) and ventricular muscle preparations (C, D); 5 μM acetylcholine (red dotted lines) alone caused no changes in either preparation type (A, C), while it caused significant prolongation when applied cummulatively after 5 μM pinacidil (B, D, pinacidil effect represented as blue dashed lines). Bars in panel E represent the values of APD90 in each treatment group, from top to bottom corresponding to the traces A to D. Abbreviations under bars: C, control; P, pinacidil, A, acetylcholine. The pacing cycle length was 500 ms. Values are mean ± SEM; *,# $p<0.05$ RM-ANOVA followed by Bonferroni's post-hoc test.

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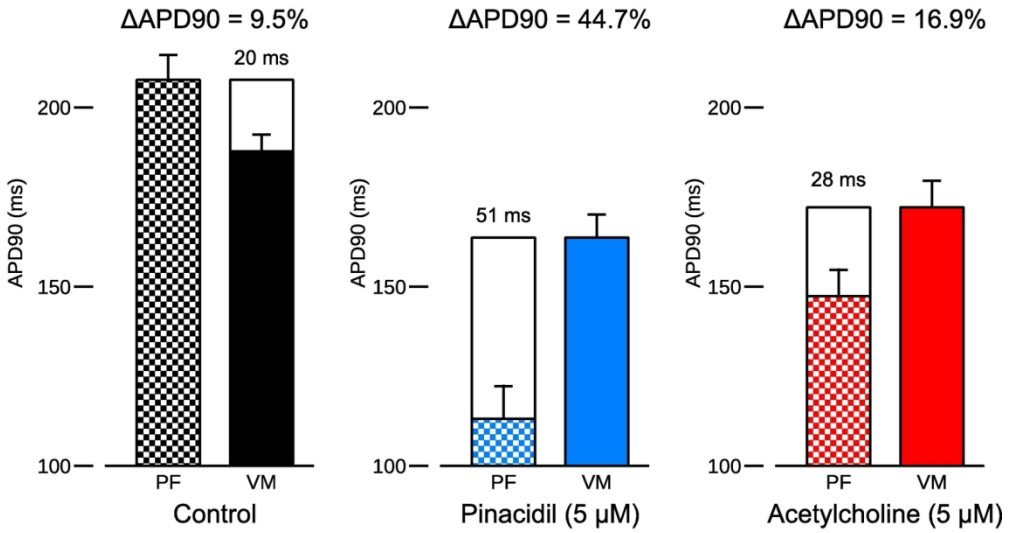

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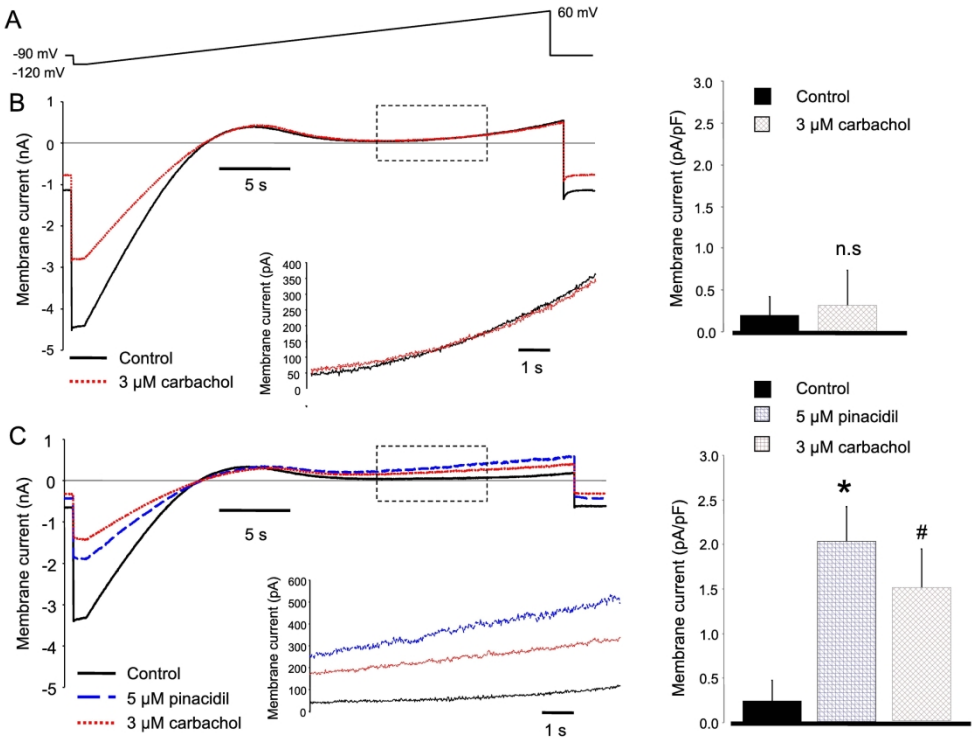

Figure 3. Effect of carbachol on IK-ATP. Ionic currents were measured under a slow voltage ramp protocol (panel A) between -120 mV and 60 mV. The currents were analysed at 0 and 30 mV. Panel B demonstrates original representative current traces (left) and bar graphs (right) where 3 μM carbachol (dotted line) failed to influence the control current analysed at 0 mV. Inset shows identical current fractions between –3 mV and 45 mV (indicated by dashed rectangle). Current traces in panel C as well as in the inset, illustrate large increase of the membrane current after application of 5 μM pinacidil (blue dashed line) that was inhibited by the subsequently applied 3 μM carbachol (red dotted line). In bar graphs (right), asterisk denotes significant change between control (left column) and pinacidil (middle column), while hash tag indicates significant change between pinacidil (middle column) and carbachol (right column).

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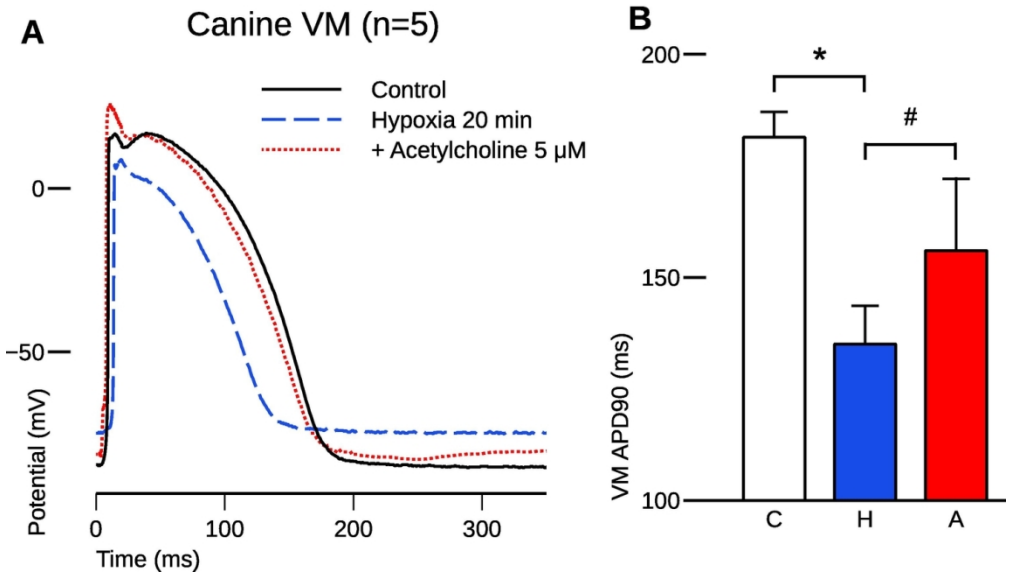

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126x74mm (300 x 300 DPI)

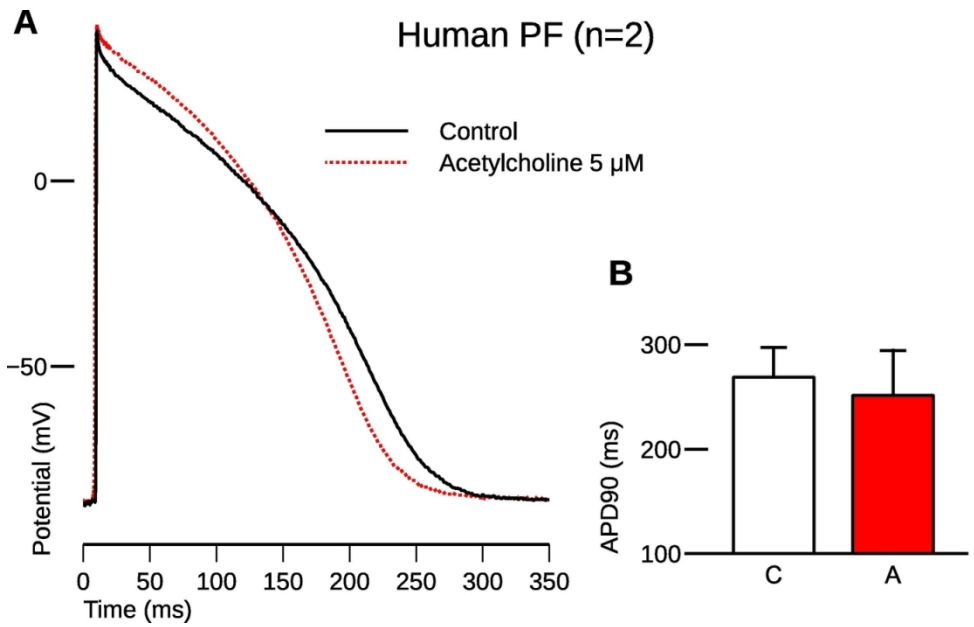

Figure 5. Representative action potential showing the effect of acetylcholine (5 μM, red dotted line) on a Purkinje fiber taken from a human donor heart (A). Values of APD90 are represented as bars (B). Abbreviations under bars: C, control; A, acetylcholine. The pacing cycle length was 500 ms. Values are mean ± SEM.

121x74mm (300 x 300 DPI)

Appendix

Introduction

Acetylcholine has been previously shown to augment J-point elevation and to induce phase-2 reentry, thus precipitating polymorphic ventricular tachycardia in preparations pretreated with agents designed to pharmacologically mimic the genetic defects previously shown to be associated with the early repolarization syndrome (ERS). Previously, Haïssaguerre et al. (2008) have described that extrasystolic activity arising from the Purkinje network is able to precipitate ventricular tachyarrhythmias in the setting of ERS. We examined Purkinje fibers under conditions pharmacologically mimicking the ion channel changes caused by the genetic defects previously reported to be associated with ERS, including gain of function in $I_{K\text{-}ATP}$ (*KCNJ8* and *ABCC9*) or I_{to} (*SCN1Bb and KCND3*) (Hu et al., 2014b; Barajas-Martínez et al., 2014; Haïssaguerre et al., 2009) or loss of function in I_{Ca} (*CACNA1C, CACNB2* and *CACNA2D1*) (Burashnikov et al., 2010; Napolitano and Antzelevitch, 2011) or I_{Na} (*SCN5A* and *SCN10A*) (Watanabe et al., 2011; Hu et al., 2014a), and applied an antiarrhytmic drug successfully used to treat ventricular tachyarrhythmias in ERS: cilostazol (Iguchi et al., 2013; Shinohara et al., 2014;).

Methods

Conventional microelectrode technique

All experiments were performed on canine Purkinje fibers using the conventional microelectrode technique. The preparations were placed in Locke's solution and allowed to equilibrate for at least 2 hours while superfused (flow rate 4-5 ml/min) also with Locke's solution containing (in mM): NaCl 120, KCl 4, CaCl2 2, MgCl2 1, NaHCO3 22, and glucose 11. The pH of this solution was 7.40 to 7.45 when gassed with 95% O2 and 5% CO2 at 37 °C. All experiments were performed at 37 °C. Electrical pulses of 0.5–2 ms in duration at twice the diastolic threshold in intensity (S1) were delivered to the preparations through bipolar platinum electrodes at a basic cycle length of 500 ms. Transmembrane potentials were recorded using glass capillary microelectrodes filled with 3 M KCl (tip resistance: 5 to 15 MΩ). The microelectrodes were coupled through an Ag-AgCl junction to the input of a

high-impedance, capacitance-neutralizing amplifier (Experimetria 2011). Intracellular recordings were displayed on a storage oscilloscope (Hitachi V-555) and led to a computer system.

Pharmacological models

Our pharmacological models of the early repolarization syndrome in Purkinje fibers were based on previous experiments (Koncz et al., 2014; Gurabi et al., 2014). We pharmacologically mimicked the ion channel changes caused by the genetic defects associated with ERS: pinacidil (5 μM; $I_{K\text{-}ATP}$ gain of function), NS5806 (7 μM; I_{to} gain of function), nisoldipine (1 μM; I_{Ca} loss of function), mexiletine (20 μM; I_{Na} loss of function). The more efficacious enantiomer of mexiletine, R-mexiletine was used (Gurabi et al., 2017); the concentration corresponds to a peak therapeutic plasma concentration (Varró and Lathrop, 1990). The application of each compound was followed by an equilibration period, enabling the tissue to reach steady-state, then the next compound was administered in a cumulative manner. Acetylcholine (5 μM) was used to simulate increased parasympathetic tone. Cilostazol (10 μM) was applied after acetylcholine.

Results

Model 1: Pinacidil + acetylcholine + cilostazol (n=6)

The effects of pinacidil and acetylcholine were described in the main article. Cilostazol caused a notable plateau elevation without changing repolarization (Figure A1-A).

Model 2: NS5806 + pinacidil + acetylcholine + cilostazol (n=5)

Cilostazol significantly increased action potential duration (APD) when applied after NS5806, pinacidil and acetylcholine (Figure A1-B).

Model 3: Mexiletine + NS5806 + cilostazol (n=4)

After inhibition of I_{Na} by mexiletine, followed by the activation I_{to} by NS5806 and the administration of acetylcholine, cilostazol caused a slight prolongation of the APD (Figure A1-C).

Model 4: Nisoldipine + NS5806 + acetylcholine + cilostazol (n=4)

Cilostazol was also applied after nisoldipine, NS5806 and acetylcholine, causing a slight plateau elevation and slight APD prolongation (Figure A1-D).

Conclusion

Since most conventional antiarrhythmic drugs, including beta-blockers, verapamil, lidocaine or amiodarone, are not capable of suppressing tachyarrhythmic episodes in the early repolarization syndrome, cilostazol should remain a prominent candidate in clinical trials related to early repolarization. Formerly, we found I_{to} blocking ability of cilostazol (Patocskai et al., 2016) next to its ability to augment I_{Ca} (Matsui et al., 1999). The above detailed normalization of the repolarization defect might carry a possible therapeutic value of cilostazol in early repolarization (ER), when the origin of arrhythmic activity is localized to the Purkinje system.

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149 **Appendix figure legend**

150 **Figure A1.** Representative action potential traces from canine Purkinje fibers showing the effects of
151 10 μM cilostazol (continuous lines) in the following models of the early repolarization syndrome (ERS,
152 dotted lines): pinacidil 5 μM + acetylcholine 5 μM (Model 1; A), NS5806 7 μM + pinacidil 5 μM +
153 acetylcholine 5 μM (Model 2; B), mexiletine 20 μM + NS5806 7 μM (Model 3; C), and nisoldipine
154 1 μM + NS5806 7 μM + acetylcholine 5 μM (Model 4; D).

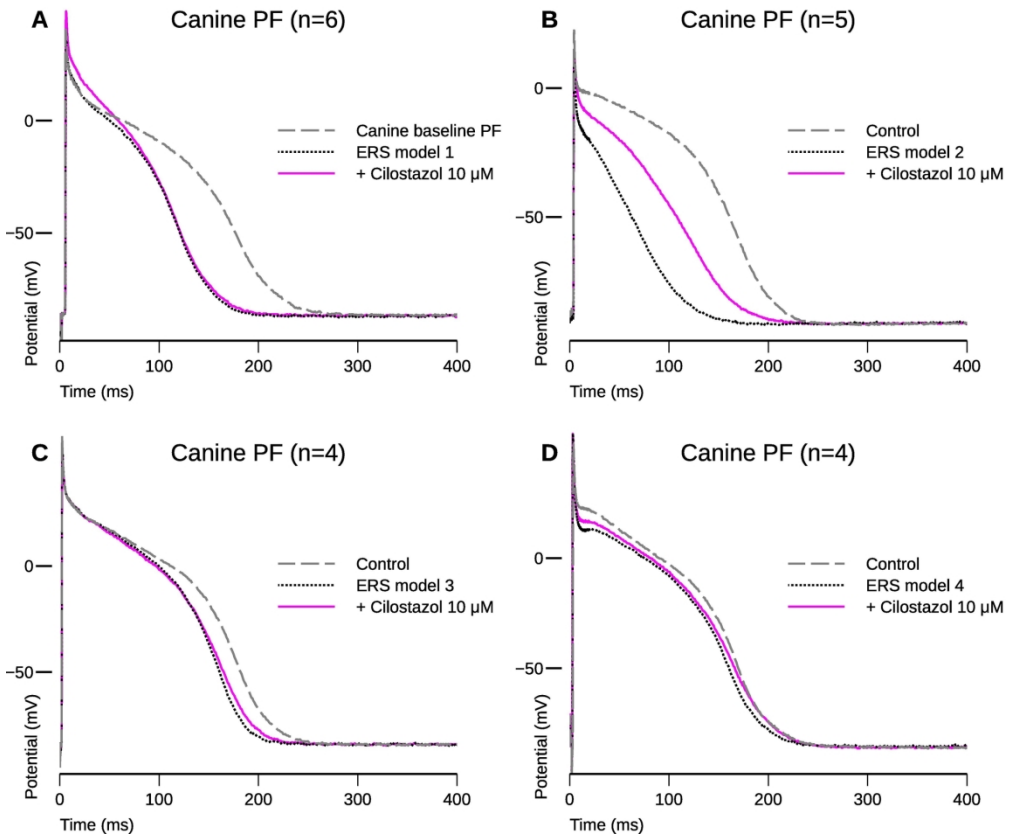

Figure A1. Representative action potential traces from canine Purkinje fibers showing the effects of 10 μM cilostazol (continuous lines) in the following models of the early repolarization syndrome (ERS, dotted lines): pinacidil 5 μM + acetylcholine 5 μM (Model 1; A), NS5806 7 μM + pinacidil 5 μM + acetylcholine 5 μM (Model 2; B), mexiletine 20 μM + NS5806 7 μM (Model 3; C), and nisoldipine 1 μM + NS5806 7 μM + acetylcholine 5 μM (Model 4; D).

177x149mm (300 x 300 DPI)