SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Effects of ropinirole and rosiglitazone on action potential characteristics and ion currents in canine ventricular cells

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INTRODUCTION

1. Proarrhythmia syndromes

Proarrhythmia phenomenon, the idea that antiarrhythmic drug therapy can aggravate the cardiac dysrhythmia being treated, or induce completely new clinical arrhythmia syndromes, has been recognized for decades. Proarrhythmia can show itself in various syndromes, each with its specific clinical manifestations and underlying mechanisms. Among proarrhythmia syndromes, acquired long QT syndrome is of capital importance being a potentially fatal side effect of class I and class III antiarrhythmic agents and several other drugs (antihistamines, antipsychotics, antimicrobials, etc.). Drug-induced QT prolongation and torsades de pointes ventricular tachycardia (TdP) are the most common causes of drug withdrawal in the present decade. Physicians also have to face the proarrhythmic effects of antiarrhythmic and non-antiarrhythmic therapy in the clinical practice day by day. Acquired long QT syndrome is usually associated with drugs having a blocking effect on the rapidly activating component of cardiac delayed rectifier K^+ current (I_{Kr}). Prolongation of the repolarization can reactivate inward currents and facilitate the development of early afterdepolarizations. Early depolarization-induced premature ventricular beats can trigger reentry and TdP if increased dispersion of repolarization is present. The attacks may stop spontaneously, but sometimes they persist long enough to provoke syncope, or even sudden cardiac death if ventricular fibrillation evolves.

In the last three decades, our approach to the pathogenesis, diagnosis and treatment of dysrhythmias has fundamentally changed. High incidence of rhythm disturbances and their serious consequences including sudden cardiac death necessitates extensive clinical, cellular and molecular research. The coordination between basic research and clinical practice allows research results to be integrated into everyday practice very soon so they can play a determinative role in diagnosis and therapy of arrhythmias.

2. Parkinson's disease and ropinirole

2.1. Autonomic dysfunction in Parkinson's disease

Parkinson's disease is a progressive, degenerative disorder of the central nervous system that is more common in the elderly. The primary symptoms of Parkinson's disease result from greatly reduced amount of dopamine in the nigrostriatal pathway due to the death of dopamine-generating cells in the substantia nigra. The motor symptoms due to dysfunction of the extrapyramidal system include rest tremor, slowness of movement, rigidity, and postural instability. Moreover, patients with Parkinson's disease are known to suffer from various degrees of autonomic disturbances. The imbalance of cardiac autonomic tone results in reduced baroreflex sensitivity and heart rate variability.

Electrocardiogram recordings in individuals with Parkinson's disease are influenced by artifact related to muscle tremor. However, patients with Parkinson's disease have abnormally prolonged QT interval due to autonomic dysfunction. The risk of cardiac dysrhythmias and sudden cardiac death can be enhanced by administration of any QT interval prolonging drugs.

2.2. Cardiac electrophysiological effects of ropinirole

Ropinirole is a non-ergoline dopamine receptor agonist effectively used for treatment of Parkinson's disease. Ropinirole selectively stimulates central and peripheral D2 and D3 receptors without any interference with serotonergic mechanisms. In spite of its widespread clinical use, there is little information available on the cardiac electrophysiological effects of ropinirole. Recently, repetitive appearance of torsades de pointes type of ventricular tachycardia has been observed in a ropinirole-treated patient. The case was finally diagnosed as an acquired long QT syndrome likely caused by ropinirole.

Low concentrations (2.2–3.5 ng/ml, corresponding to 7–12 nM) of ropinirole were shown to lengthen QTc interval in conscious beagle dogs, while higher concentrations (2.5 and 25 μM) were required to lengthen action potential duration (APD) by 41 and 106 ms, respectively, in isolated canine Purkinje fiber preparations. No detailed voltage clamp analysis on the effects of ropinirole in native cardiac preparations has been published so far. The only

information in this regard was obtained by studying human ether-à-go-go-related gene (HERG) channels expressed in Chinese hamster ovary (CHO) cells, where 0.1–10 μM ropinirole exerted suppressive effect on HERG current with an IC50 value of 1.2 μM.

3. Type 2 diabetes mellitus and rosiglitazone

3.1. Type 2 diabetes mellitus

Type 2 diabetes mellitus is a metabolic disorder that is characterized with insulin resistance and relative insulin deficiency. An estimated 1-1.5 million people have diagnosed or undiagnosed diabetes in Hungary, and the number of people with prediabetes is nearly the same. Type 2 diabetes is associated with 2-4 times higher risk of cardiovascular disease, and the risk is nearly the same in prediabetes. Patients with diabetes mellitus exhibit a high incidence of diabetic cardiomyopathy, characterized by complex changes in the electrical and mechanical properties of the heart. The most prominent electrical alteration is the prolongation of the QTc interval and increased QTc dispersion. In the isolated cardiomyocytes of diabetic rats, significant prolongation of APD and reduction of K^+ currents were reported.

3.2. General overview of thiazolidinediones

Rosiglitazone is a thiazolidinedione (TZD) oral hypoglycemic agent active in both diabetic animal models and patients with type 2 diabetes. TZDs, by increasing insulin sensitization, result in a strong and long lasting improvement of glycemic control which may be related to their potential β-cell preserving properties. TZDs are high affinity ligands for the peroxisome proliferator-activated receptor-γ, which regulates the expression of genes responsible for glucose and fat metabolism. These drugs improving the insulin-sensitivity in adipose tissue and skeletal muscle stimulate the expression and function of glucose transporters in the myocardium, resulting in improved glucose metabolism by the heart. Beyond improving glycemic control, rosiglitazone and pioglitazone have been shown to exert some cardiovascular benefits. Based on multiple effects (on glycemia, lipid profile, blood pressure, biomarkers) of TZDs it is assumed that these drugs reduce micro- and macrovascular complications of type 2 diabetes. Unfortunately, several large scale clinical studies reported that TZD therapy was associated with cardiovascular complications,

including their propensity to cause edema, weight gain, and subsequently symptoms of heart failure. Therefore, American Heart Association and American Diabetes Association recommended lower TZD doses at initiation of treatment and gradual dose escalation with observation to identify edema in patients with class I or II New York Heart Association (NYHA) heart failure. In patients with symptoms of NYHA class III or IV heart failure, complete avoidance of TZDs was recommended. Further analyses revealed elevated risk of myocardial infarction associated with rosiglitazone therapy, hence the US Food and Drug Administration (FDA) significantly restricted access to rosiglitazone, and the European Medicines Agency recommended the suspension of the drug.

3.3. Cardiac electrophysiological effects of thiazolidinediones

Rosiglitazone was shown to attenuate porcine action potential shortening induced both during ischemia and by the K_{ATP} channel opener levocromocalim. Moreover, rosiglitazone increased the propensity of ventricular fibrillation in ischemic pigs, which effect was attributed to inhibition of the cardiac ATP-sensitive K^+ channels. However, no direct evidence based on voltage clamp analysis is available to exclude the contribution of other ion currents.

AIM OF THE STUDIES

1. Studies on the cellular electrophysiologic effects of ropinirole

No detailed voltage clamp analysis on the effects of ropinirole in native cardiac preparations has been published so far. In the present work, we aimed to study the effects of ropinirole on action potential morphology and the underlying ion currents in isolated canine ventricular cardiomyocytes in a concentration-dependent manner. Although the therapeutic plasma levels of ropinirole were usually found between 25 and 80 nM, the studied concentration range was extended up to 300 μM in order to visualize potential side-effects threatening in case of overdose or intoxication. Canine ventricular cells were chosen because their electrophysiological properties are believed to be most similar to those of human regarding the distribution and kinetic properties of transmembrane ion currents.

2. Studies on the cellular electrophysiologic effects of rosiglitazone

The effects of rosiglitazone on glycemia and lipid profile were widely investigated. However, very limited data are available concerning the cardiac electrophysiologic effects of the drug. Rosiglitazone was shown to block a wide variety of non-cardiac ion channels. Moreover, rosiglitazone increased the propensity of ventricular fibrillation in pigs, which effect was attributed to inhibition of the cardiac ATP-sensitive K^+ channels. It remains to be elucidated, however, what is the explanation for the differences found between the effects of rosiglitazone and pioglitazone on cardiovascular outcomes despite their similar effects on glycemic control. In absence of relevant voltage clamp data on the effects of rosiglitazone in mammalian cardiac preparations, we aimed to study the concentration-dependent effects of rosiglitazone on action potential morphology and the underlying ion currents in isolated canine ventricular cardiomyocytes.

MATERIALS AND METHODS

1. Isolation of single canine ventricular myocytes

Adult beagle dogs (16 male, 48 female) were anesthetized with intravenous injections of 10 mg/kg of ketamine hydrochloride (Calypsol, Richter) +1 mg/kg of xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, The Netherlands). The hearts were quickly removed and placed in Tyrode solution. Single myocytes were obtained by enzymatic dispersion using the segment perfusion technique. Briefly, a wedge-shaped section of the ventricular wall supplied by the left anterior descending coronary artery was dissected, cannulated, and perfused with oxygenized Tyrode solution. Perfusion was maintained until the removal of blood from the coronary system and then switched to a nominally Ca^{2+}-free Joklik solution (Minimum Essential Medium Eagle, Joklik Modification, Sigma) for 5 min. This was followed by 30-min perfusion with Joklik solution supplemented with 1 mg/ml of collagenase (type II; Worthington, Chemical Co.) and 0.2% bovine serum albumin (fraction V., Sigma) containing 50 μM Ca^{2+}. Portions of the left ventricular wall were cut into small pieces, and the cell suspension obtained at the end of the procedure predominantly from the midmyocardial region of the left ventricle was washed with Joklik solution. Finally, the Ca^{2+} concentration was gradually restored to 2.5 mM. The cells were stored in Minimum Essential Medium Eagle until use.

2. Recording of action potentials

All electrophysiological measurements were performed at 37°C. The rod-shaped viable cells showing clear striation were sedimented in a plexiglass chamber allowing continuous superfusion with oxygenized Tyrode solution. Transmembrane potentials were recorded using 3 M KCl-filled sharp glass microelectrodes having tip resistance between 20 and 40 MΩ. These electrodes were connected to the input of an Axoclamp-2B amplifier (Axon Instruments). The cells were paced through the recording electrode at steady cycle length of 1 s using 1-ms-wide rectangular current pulses with 120% threshold amplitude. Concentration-dependent effects of ropinirole were determined in a cumulative manner by applying increasing concentrations of the drug between 0.1 and 300 μM. Concentration-

dependent effects of rosiglitazone were analyzed by applying increasing concentrations between 1 and 100 μM. Each concentration was superfused for 3 min, and the washout lasted for 10 min. These incubation and washout periods were sufficient to develop steady-state drug effects and practically full reversion. Action potentials were digitized at 200 kHz using Digidata 1200 A/D card (Axon Instruments) and stored for later analysis.

3. Conventional voltage clamp

The cells were superfused with oxygenized Tyrode solution. Suction pipettes, fabricated from borosilicate glass, had tip resistance of 2 MΩ after filling with pipette solution containing K-aspartate, 100; KCl, 45 mM; MgCl2, 1 mM; HEPES, 5 mM; EGTA, 10 mM; and K-ATP, 3 mM, or alternatively, KCl, 110 mM; KOH, 40 mM; HEPES, 10 mM; EGTA, 10 mM; TEACl, 20 mM; and K-ATP, 3 mM, when measuring potassium or calcium currents, respectively (pH=7.2 in both cases). Membrane currents were recorded with the Axopatch-2B amplifier using the whole-cell configuration of the patch clamp technique. After establishing high-resistance seal (1–10 GΩ) by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction or by applying 1.5 V of electrical pulses for 1 ms. The series resistance was typically 4–8 MΩ before compensation (usually 50–80%). Experiments were discarded when the series resistance was high or substantially increasing during the measurement. Outputs from the clamp amplifier were digitized at 100 kHz under software control (pClamp 6.0, Axon Instruments). Ion currents were normalized to cell capacitance, determined in each cell using short hyperpolarizing pulses from −10 to −20 mV.

Concentration-dependent effects of ropinirole and rosiglitazone were determined in a cumulative manner by applying increasing concentrations of both drugs between 1 and 300 μM.

4. Action potential voltage clamp

After formation of the gigaseal, action potentials were recorded in current clamp mode from the myocytes superfused with Tyrode solution. The pipette solution was identical to that used for potassium current measurement under conventional voltage clamp conditions. The cells were continuously paced through the recording electrode at steady stimulation frequency of 1 Hz so as a 1–2-ms gap between the stimulus artifact and the upstroke of the action

potential could occur. Ten subsequent action potentials were recorded from each cell, which were on-line analyzed. One of these action potentials, having an APD_{90} value closest to the average of the ten recorded action potentials, was delivered to the same cell at the identical frequency as command voltage after switching the amplifier to voltage clamp mode. The current trace obtained under these conditions is a horizontal line positioned at the zero level except for the very short segment corresponding to the action potential upstroke. Ropinirole was applied at concentrations of 10 and 100 μM, and rosiglitazone was applied at concentrations of 1, 10 and 100 μM. The profile of the ion currents blocked by the drugs was determined by subtracting the pre-drug curve from the post-drug one. This procedure resulted composite current profiles containing three distinct current peaks after reverting its polarity: an early outward for I_{to}, an inward for I_{Ca}, and a late outward for I_{Kr}.

5. Statistics

Results are expressed as mean±SEM values. Statistical significance of differences was evaluated using one-way ANOVA followed by Student's t test. Differences were considered significant when P was less than 0.05. All animal care and experimental procedures conformed to 'Guide for the Care and Use of Laboratory Animals' (NIH publication No 85–23, revised 1996) and to the principles outlined in the Declaration of Helsinki and were approved by the local ethical committee.

RESULTS

1. Results of studies on the cellular electrophysiologic effects of ropinirole

1.1. Effect of ropinirole on action potential configuration

Ropinirole treatment caused concentration-dependent complex changes in action potential morphology in canine ventricular myocytes, paced at a constant frequency of 1 Hz, including lengthening or shortening of APD, reduction of the amplitude of early (phase-1) repolarization, decrease in maximum rate of depolarization (V_{max}), and depression of the plateau potential. Ropinirole displayed biphasic effect on action potential duration. Micromolar concentrations of ropinirole significantly increased APD (APD_{90} at concentrations≥1 μM and APD_{50} at concentrations≥3 μM). However, elevation of ropinirole concentration to 300 μM resulted in a shortening of APD_{50} values. Regarding the other actions of ropinirole, reduction of phase-1 amplitude was significant statistically at concentrations≥10 μM, while suppression of V_{max} and depression of the plateau potential were significant at concentrations≥30 μM. All these effects were readily reversible after superfusion with ropinirole-free Tyrode solution for 10 min. Ropinirole-induced changes in V_{max} and APD were frequency dependent. The V_{max} block was more prominent at fast driving rates, i.e., when the pacing cycle length was decreased below 1 s, while both lengthening of APD (by 30 μM ropinirole) and shortening of APD (by 300 μM ropinirole) were more pronounced at longer cycle lengths displaying features of reverse rate dependence.

1.2. Effect of ropinirole on cardiac ion currents measured by conventional voltage clamp

In these experiments, performed under conventional voltage clamp conditions, cumulative concentration-dependent drug effects were studied between 0.1 and 300 μM, increasing the concentration of ropinirole in steps of 0.5 log units.

The rapid component of the delayed rectifier K^+ current (I_{Kr}) was activated by 1-s-long depolarizing pulses to +50 mV arising from the holding potential of −40 mV. I_{Kr} was assessed

as tail current amplitudes recorded following repolarization to the holding potential. I_{Ca} and I_{Ks} were suppressed by 5 μM nifedipine and 1 μM HMR-1556, respectively. The amplitudes of the I_{Kr} current tails were progressively decreased by increasing concentrations of ropinirole. The IC_{50} value and Hill coefficient were estimated to be 2.7±0.25 μM and 0.92±0.09, respectively, in the average of five myocytes. Voltage-dependence of activation of I_{Kr} was shifted towards more negative potentials by 3 μM ropinrole, which caused approximately a 50% inhibition on the current. The half-activation voltage ($V_{0.5}$) of I_{Kr} was shifted by ropinirole from −14.4±1.8 to −21.3± 1.1 mV (n=6, $P<0.05$). Due to this activation shift, the block apparently increased with increasing depolarizations to more positive voltages: It was relatively small when the current was activated at −20 mV, while it was represented close to half of control current amplitude at voltages more positive than +10 mV. In addition, ropinirole accelerated the activation of I_{Kr}. The time constant obtained for activation of IKr decreased from 34±4 ms to 14±1 ms by 3 μM ropinirole (n=6, $P<0.05$). According to these results, the ropinirole-induced I_{Kr} blockade developed in spite of acceleration of activation. No significant changes in the fast and slow deactivation time constants were observed with 3 μM ropinirole.

The transient outward current (I_{to}) was activated by depolarization to +50 mV arising from the holding potential of −80 mV and having duration of 200 ms. Before each test pulse, a short (5 ms) depolarization to −40 mV was applied in order to inactivate the fast Na^+ current, while I_{Ca} and I_{Kr} were blocked by 5 μM nifedipine and 1 μM E4031, respectively. The blocking effect of ropinirole on I_{to} was statistically significant at concentrations≥10 μM.

L-type calcium current (I_{Ca}) was recorded at +5 mV using 400-ms-long depolarizations arising from the holding potential of −40 mV. In these experiments, Tyrode solution was supplemented with 3 mM 4-aminopyridine, 1 μM E4031, and 1 μMHMR-1556 in order to block K^+ currents. Ropinirole blocked I_{Ca} in a concentration-dependent manner causing statistically significant block at concentrations≥30 μM.

The slow component of the delayed rectifier K+ current (I_{Ks}) was activated by 3-s-long depolarization to +50 mV, and the amplitude of tail current was determined at the holding potential of −40 mV after repolarization. I_{Ca} was inhibited by 5 μM nifedipine, and I_{Kr} was blocked by 1 μM E4031. The inward rectifier K+ current (I_{K1}) was studied by applying

hyperpolarizations to −135 mV from the holding potential of −40 mV. The steady-state current was determined after 400 ms. Neither I_{Ks} nor I_{K1} was significantly modified by ropinirole concentrations less than 300 μM.

1.3. Effect of ropinirole on the ion currents under action potential clamp conditions

The early outward current peak arises when I_{to} is suppressed, while the inward deflection indicates a blockade of I_{Ca}. The late outward current peak, coincident with terminal repolarization of the canine action potential, is a mixture of I_{Kr} plus I_{K1}. In our case, however, it is likely caused by pure I_{Kr} blockade, since—as we have previously shown— I_{K1} was not affected by 10 or 100 μM ropinirole. 10 μM ropirinole suppressed both I_{Kr} and I_{to}, while I_{Ca} was also suppressed by 100 μM ropinirole under action potential voltage clamp condition—in line with results of conventional voltage clamp experiments. All these effects were readily reversible upon washout.

2. Results of studies on the cellular electrophysiologic effects of rosiglitazone

2.1. Effect of rosiglitazone on action potential configuration

Rosiglitazone treatment caused complex, concentration-dependent changes in action potential morphology in canine ventricular myocytes (eight cells from four dogs), paced at a constant frequency of 1 Hz, including reduction of the amplitude of early (phase-1) repolarization, the maximum velocity of depolarization (V_{max}), and depression of the plateau potential. These effects of rosiglitazone were significant at concentrations of 10 μM and higher. Action potential duration was little affected by rosiglitazone; however, APD_{50} was significantly shortened by 30 μM, while APD_{90} was lengthened by 100 μM of rosiglitazone. In spite of the reduction of V_{max}, the amplitude of action potential was not decreased by rosiglitazone. In contrast, it was significantly increased in the presence of 100 μM rosiglitazone. All these effects of rosiglitazone developed rapidly (within 2–3 min) and were readily reversible after superfusion with rosiglitazone-free Tyrode solution for 10 min.

In cooperation with the Department of Pharmacology and Pharmacotherapy of Semmelweis University, rat and wild type and histidine decarboxylase knockout (HDC-KO) mice ventricular papillary muscles were also studied. Rosiglitazone exerts complex, very

diverse (sometimes similar, sometimes different) electrophysiological actions in small rodent ventricular papillary muscles and canine ventricular cardiomyocytes. Rosiglitazone exerted a concentration dependent depression of APA in rats, but an increase in APA was observed in wild type as well as in HDC-KO mice. Rosiglitazone caused a significant concentration-dependent reduction of V_{max}, an indirect indicator of Na^+ current (I_{Na}) density in wild type mice. However, this effect was not observed in HDC-KO mice. In rat ventricular papillary muscles, rosiglitazone exerted a significant concentration-dependent reduction of V_{max}, similarly to data of Kavak et al. Action potential duration, especially during the most terminal phase of repolarization was also affected. APD90 was shortened in mice, while it was prolonged in rat in a concentration-dependent manner.

2.2. Effect of rosiglitazone on cardiac ion currents measured by conventional voltage clamp

In these experiments, performed under conventional voltage clamp conditions, cumulative concentration-dependent drug effects were monitored between 1 and 300 μM, increasing the concentration of rosiglitazone usually in steps of 0.5 log units. Kinetic properties of the channel gating were studied at concentrations, which were close to the half effective blocking concentration of rosiglitazone on the given ion current.

I_{to} was activated by depolarizations to +50 mV arising from the holding potential of -80 mV and having duration of 200 ms. Before each test pulse, a short (5 ms) depolarization to -40 mV was applied in order to inactivate the fast Na^+ current (I_{Na}), while I_{Ca} and I_{Kr} were blocked by 5 μM nifedipine and 1 μM E4031 respectively. I_{to} was suppressed by rosiglitazone in a concentration-dependent manner in the five myocytes studied (each from a different animal). This effect of rosiglitazone was statistically significant at concentrations ≥10 μM, the EC_{50} value was 25.2 ± 2.7 μM, and the Hill coefficient 1.27 ± 0.19. The effect of rosiglitazone developed rapidly (within 2–3 min) and was fully reversible upon washout. Rosiglitazone altered the gating properties of I_{to}. The current inactivated as a sum of a faster and a slower component. The amplitude of both components was significantly decreased in the presence of 30 μM rosiglitazone. The fast time constant was not modified, while the slow time constant was doubled by rosiglitazone. Activation of I_{to} required larger depolarizations in the presence of rosiglitazone and the activation threshold was shifted from -20 to +20 mV, while no significant change was observed in the voltage-dependence of inactivation.

I_{Kr} was activated by 1 s long depolarizing pulses to +50 mV, from the holding potential of -40 mV. I_{Kr} was assessed as tail current amplitudes recorded following repolarization to the holding potential. I_{Ca} and the slow delayed rectifier K^+ current were suppressed by 5 μM nifedipine and 1 μM HMR-1556 respectively. The amplitudes of the I_{Kr} current tails were progressively decreased by increasing concentrations of rosiglitazone. The EC_{50} value and Hill coefficient were estimated to be 72.3 ± 9.3 μM and 0.91 ± 0.1, respectively, as the mean of five myocytes, each derived from a different animal. Similarly to results observed with I_{to}, suppression of the I_{Kr} tails developed rapidly and was fully reversible. Relaxation of I_{Kr} current tails (deactivation) followed biexponential kinetics. Although the time constants of both components decreased, this effect failed to reach the level of statistical significance ($P \geq 0.05$). In contrast, the amplitudes of both components were significantly reduced by 100 μM rosiglitazone. Voltage-dependence of activation of I_{Kr} was moderately shifted towards more negative potentials by 100 μM rosiglitazone: the half-activation voltage ($V_{0.5}$) of I_{Kr} was shifted from -4.8 ± 3.2 to -10.2 ± 1.3 mV (five cells, each from a different dog, $P < 0.05$). Due to this shift of activation, the block apparently increased with increasing depolarizations to more positive voltages, it was negligible at membrane potentials ≤-5 mV, while it represented close to half of control current amplitude at voltages more positive than +10 mV. No change in the monoexponential time constant of activation of I_{Kr}, determined by using the tail envelope test, was observed in the presence of 100 μM rosiglitazone.

I_{Ca} was recorded at +5 mV using 400 ms long depolarizations arising from the holding potential of -40 mV. In these experiments, Tyrode solution was supplemented with 3 mM 4-aminopyridine, 1 μM E4031 and 1 μM HMR-1556 in order to block K^+ currents. Rosiglitazone blocked I_{Ca} in a concentration-dependent manner causing statistically significant block at concentrations ≥10 μM. The EC_{50} value and Hill coefficient were 82.5 ± 9.4 μM and 0.82 ± 0.08, respectively, in the six myocytes obtained from five dogs. I_{Ca} was only partially reversed during the 10 min period of washout following rosiglitazone treatment. Rosiglitazone altered slightly the gating properties of I_{Ca} as well. This current inactivated as a sum of two exponential components and no significant changes in the time constants were observed. Although amplitudes of both the fast and slow components were decreased by 100 μM rosiglitazone, suppression of the slow component was more pronounced. Rosiglitazone had no effect on the current–voltage relationship obtained for I_{Ca} by plotting the amplitudes of the current against the respective test potentials. In contrast, steady-state inactivation of I_{Ca} was enhanced by 100 μM rosiglitazone. The half inactivation voltage ($V_{0.5}$) was significantly

shifted towards more negative potentials (from -12.9 ± 0.3 mV to -15.5 ± 0.3 mV, $P \leq 0.05$, five cells, each from a different animal).

The inward rectifier K+ current (I_{K1}) was studied by applying hyperpolarizations to -135 mV from the holding potential of -40 mV. The steady-state current was determined 400 ms after the beginning of the pulse. I_{K1} was not significantly modified by rosiglitazone up to the concentration of 100 μM in the four cells challenged, each obtained from a different dog. Above this concentration (at 300 μM) a small but fully reversible suppression of I_{K1} was observed.

2.3. Effect of rosiglitazone on ion currents under action potential clamp conditions

The profile of an ion current may be markedly different when comparing under conventional voltage clamp and action potential clamp conditions. An advantage of the action potential clamp technique is that the effect of any drug on the net membrane current can be recorded allowing thus to monitor drug effects simultaneously on more than one ion current. Furthermore, this technique enables us to record true current profiles flowing during an actual cardiac action potential. Of course, in the case of a drug acting on more than one ion current, such as rosiglitazone, a series of peaks can be detected on the current trace, each of them corresponding to the fingerprint of an individual ion current. Accordingly, the early outward current peak arises when I_{to} is suppressed, while the inward deflection indicates a blockade of I_{Ca}. The late outward current peak, coincident with terminal repolarization of the canine action potential, is a mixture of I_{Kr} plus I_{K1}. In our case, however, it is likely caused by pure I_{Kr} blockade, because – as we have previously shown – I_{K1} was not affected by 100 μM rosiglitazone. Rosiglitazone suppressed I_{to}, I_{Kr} and I_{Ca} under action potential voltage clamp conditions in a concentration-dependent and largely reversible manner – in line with results of conventional voltage clamp experiments.

DISCUSSION

1. Discussion of studies on the cellular electrophysiologic effects of ropinirole

1. 1. Effects of ropinirole on action potential morphology are in line with voltage clamp data

Effects of ropinirole on native cardiac ion currents were first analyzed in this study. The results revealed that ropinirole suppressed several ion currents in a concentration-dependent manner with the concomitant alterations of action potential morphology. These changes observed in the configuration of the action potential can be deduced from suppression of the various ion currents. For instance, micromolar concentrations of ropinirole inhibited I_{Kr}, and as a consequence, APD was lengthened. Lengthening of APD_{90} was significant at concentrations ≥1 μM, which is in a good agreement with the IC_{50} of 2.7 μM obtained with ropinirole for I_{Kr} blockade. The ropinirole-induced I_{Kr} block cannot be explained by shifts in channel gating, since the observed acceleration of activation tends to increase, rather than decrease, the current. On the other hand, the blockade was voltage dependent, as it was augmented with increasing depolarizations. This feature may be a consequence of use-dependent interaction between ropinirole and the channel. The Hill coefficient of close to unity is congruent with the involvement of a single binding site.

At higher concentrations, other ion currents, like I_{to} and I_{Ca}, were also inhibited by ropinirole. These effects were significant at concentrations≥10 and 30 μM ropinirole, respectively, resulting in reduction of phase-1 repolarization and depression of the plateau potential. Shortening of APD_{50} in the presence of very high concentrations (300 μM) of ropinirole may also be explained by the I_{Ca} blockade. Reduction of V_{max}, evident at concentrations≥30 μM, is due to inhibition of I_{Na}, since V_{max} is believed to be a good indicator of Na^+ current density. The rate-dependent component of V_{max} block showed relatively fast kinetics, since the magnitude of block sharply increased when the cycle length was reduced below 1 s. This suggests that the dissociation time constant of ropinirole from the Na^+ channel may also be shorter than 1 s.

1.2. Clinical implications

Ropinirole is considered to be a relatively safe drug in terms of acute cardiovascular complications. The lowest concentration of ropinirole that caused statistically significant changes in our study was much higher than those peak plasma levels obtained in patients or in animals under in vivo conditions. Peak plasma concentration of 20–24 ng/ml (corresponding to 65–80 nM) is typical in patients receiving 24 mg of ropinirole daily. After a single dose of 12 mg, 33 ng/ml (corresponding to 110 nM) of peak plasma concentration was measured in human subjects. Considering a 40% plasma protein binding, it is not likely that normally dosed ropinirole can alter cardiac electrogenesis in healthy individuals, although a case of sinus node dysfunction has already been reported with ropinirole. In case of intoxication, ropinirole concentration in the plasma may reach the critical micromolar range with the concomitantly increased risk of torsades de pointes. Similarly, the normal application of ropinirole to patients with inherited or acquired long QT syndrome may be dangerous for the same reason. Indeed, repeated appearance of torsades de pointes has been observed by the authors in a ropinirole-treated patient. Therefore, although ropinirole seems to be a relatively safe drug, in patients with Parkinson's disease having also cardiac disorders, ECG control may be suggested during ropinirole therapy. Moreover, coadministration of ropinirole with drugs that can prolong repolarization should be preferably avoided.

2. Discussion of studies on the cellular electrophysiologic effects of rosiglitazone

2.1. Effects of rosiglitazone on action potential morphology are in line with effects on the underlying ion currents

The results revealed that rosiglitazone suppressed several ion currents in a concentration-dependent manner with the concomitant alterations in the configuration of the action potential. For instance, the rosiglitazone-induced decrease in phase-1 repolarization may be due to reduction of I_{to}. Similarly, the depression of the plateau may be a consequence of inhibition of Ca^{2+} and Na^{+} currents, while the observed suppression of V_{max} is believed to be a good indicator of I_{Na} blockade. In spite of the multiple actions of rosiglitazone on cardiac ion channels, action potential duration was little affected by rosiglitazone, except for the moderate reduction of APD_{50} at 30 μM and lengthening of APD_{90} at 100 μM concentration. The lack of marked effects of rosiglitazone on action potential duration suggests that

inhibition of inward (window I_{Na} and I_{Ca}) and outward (I_{Kr} and I_{to}) currents are relatively well compensated. Also, suppression of V_{max} is usually accompanied with reduction of action potential amplitude. This effect was not observed with rosiglitazone – in contrast – action potential amplitude was significantly increased by the highest rosiglitazone concentration applied (100 μM). This effect is likely to be due to the simultaneous blockade of I_{Na} and I_{to}, with the former effect tending to decrease, and the latter tending to increase, the amplitude of action potentials. Finally, the lack of depolarization is compatible with the inability of rosiglitazone to alter I_{K1} at concentrations up to 100 μM.

2.2. Comparison with troglitazone

Our present results allow some comparison between the cellular cardiac electrophysiological effects of rosiglitazone and troglitazone. Troglitazone blocked I_{Ca} with IC_{50} values close to 10 μM in rat, rabbit and guinea pig myocytes. This value is significantly smaller than the IC_{50} of 92 μM obtained with rosiglitazone in canine ventricular cells. The inhibitory effect of troglitazone on I_{Na} is also much stronger than that of rosiglitazone. In contrast to our results, where 100 μM rosiglitazone caused less than 50% reduction of V_{max}, 1 μM of troglitazone induced 50% V_{max} blockade, while 10 μM of the compound fully eliminated action potentials in rabbit ventricular myocytes. Thus the difference between the inhibiting potency of rosiglitazone and troglitazone seems to be at least one order of magnitude.

2.3. Effects of rosiglitazone on action potential morphology in different species

The effects of rosiglitazone on APD_{90} and APA show apparently interspecies differences. These differences can probably be explained by differences in action potential morphology and in the kinetic properties of the rat, murine and canine K^+ currents (over-exposed I_{to}, absence of plateau phase in rats and mice). Another possible explanation for the differences seen with the HDC-KO group might be the fact that these animals are more susceptible to autoimmune diabetes. Histidine decarboxylase knockout mice lack endogenous histamine, and they are characterized by impaired glucose tolerance. Furthermore, they have autoantibodies reactive to glutamic acid decarboxylase, a possible target antigen of the diabetogenic autoimmune process. Previous data showed that electrophysiological changes relevant to diabetes (i.e. prolongation of repolarization and depression of V_{max}) developed in

these animals without any diabetes induction. These characteristics can be observed in the present study, where in HDC-KO control group APD_{90} was lengthened and V_{max} was depressed comparing to the control values of the wild type animals. Whereas direct ionic current measurements in the case of rats and mice were not performed, the results suggest that rosiglitazone can alter the activity of some cardiac ion channels. This suggestion was supported by data from murine diabetic model showing that chronic treatment of rosiglitazone could modify expression of genes for K^+ channel/channel interacting proteins.

2.4. Clinical implications

The lowest concentration of rosiglitazone that caused statistically significant changes in our study was much higher than the peak plasma levels obtained in patients. Peak plasma concentration of 0.8 μg·mL-1 (corresponding to 2 μM) is typical in patients after receiving a single dose of 8 mg rosiglitazone. Therefore, it is not likely that rosiglitazone, in normal doses, would alter cardiac electrogenesis in healthy individuals. In line with this, no case of sudden death has been reported in association with rosiglitazone therapy. However, it is very important to emphasize that the present results with rosiglitazone were obtained in healthy mammalian hearts, while rosiglitazone is usually used in diabetic patients. Considering that diabetes is known to induce marked remodelling in the set of cardiac ion currents in all studied mammalian species, further studies in diabetic animal models should be performed. The probable proarrhythmic side effects of rosiglitazone could be not fully excluded in elderly diabetic patients having cumulated cardiovascular risk factors. Indeed, rosiglitazone was shown to increase propensity for ventricular fibrillation in animal models of ischemic heart disease. Moreover, in case of decreased elimination or overdose, rosiglitazone concentrations in the plasma are likely to reach a much higher level, probably several tens of micromoles, where a variety of cardiac ion channels could be suppressed thus favouring the development of cardiac arrhythmias.

The cardiovascular safety profile of rosiglitazone is still an open question, because of the conflicting data on its risk/benefit ratio. The ambiguous and contradictory clinical results lead to different reactions from medicines agencies. The US Food and Drug Administration (FDA) significantly restricted access to rosiglitazone, the European Medicines Agency recommended the suspension of the drug. The safety profile of rosiglitazone could be determined by additional multicenter studies but, considering ethical and financial aspects, it is very unlikely that the drug will now be tested in new clinical trials.

The US Senate Committee on Finance released a committee report in 2010 based on a twoyear inquiry of rosiglitazone. The senators also asked the FDA to describe what steps the agency has taken to protect patients in an ongoing clinical trial, and why the study is allowed to continue, given that the FDA itself estimated that the drug caused approximately 83.000 excess heart attacks between 1999 and 2007. The report concluded that the manufacturer, GlaxoSmithKline was aware of the possible cardiac risks years before such evidence became public.

To separate the desirable, beneficial and adverse negative side effects of the currently available PPARγ agonists, several drug discovery programs have attempted to identify PPARγ partial agonists having appropriate antidiabetic efficacy with less adverse actions. Balaglitazone is a selective partial agonist of PPARγ, with similar antihyperglycemic efficacy to that of rosiglitazone, but less pronounced body fluid retention properties than rosiglitazone. Compound 50 and MK-0533 are partial PPARγ agonists being in preclinical phase of development, both exerting marked antidiabetic activity with less adverse effects. However, adverse side effects led to a decline in overall TZD utilization and a shift toward greater use of other glucose-lowering drugs. The fate of TZDs seems to be sealed as the newer drugs of the incretin principle including incretin mimetics and dipeptidyl peptidase-4 inhibitors gain ground. According to the results of studies received so far, incretin-based therapy has a favourable safety profile, but long-term data especially on cardiovascular outcomes are needed before widespread use of these new agents.

SUMMARY

The cellular cardiac electrophysiological effects of ropinirole and rosiglitazone were investigated in isolated canine cardiomyocytes. The concentration-dependent effects of both drugs on action potential morphology and the underlying ion currents were studied in enzymatically dispersed canine ventricular cardiomyocytes using standard microelectrode, conventional whole-cell patch clamp, and action potential voltage clamp techniques.

Ropinirole, a dopamine receptor agonist increased action potential duration (APD_{90}) and suppressed the rapid delayed rectifier K^+ current (I_{Kr}) with an IC_{50} value of 2.7±0.25 μM and Hill coefficient of 0.92±0.09 at concentrations≥1 μM. At higher concentrations, ropinirole decreased the amplitude of early repolarization (at concentrations≥10 μM), reduced the maximum rate of depolarization and caused depression of the plateau (at concentrations≥30 μM), and shortened APD_{50} (at 300 μM) indicating a concentration-dependent inhibition of I_{to}, I_{Na}, and I_{Ca}. I_{Ks} and I_{K1} were not influenced significantly by ropinirole at concentrations less than 300 μM. Suppression of I_{Kr}, I_{to}, and I_{Ca} has been confirmed under conventional patch clamp and action potential voltage clamp conditions. The results indicate that ropinirole treatment may carry proarrhythmic risk for patients with inherited or acquired long QT syndrome due to inhibition of I_{Kr} - especially in cases of accidental overdose or intoxication.

Recent large clinical trials found an association between the antidiabetic drug rosiglitazone therapy and increased risk of cardiovascular adverse events. Because there is little information on the cellular cardiac effects of rosiglitazone, we investigated the cardiac electrophysiological properties of rosiglitazone on isolated rat and murine ventricular papillary muscle cells and canine ventricular myocytes using conventional microelectrode, whole cell voltage clamp, and action potential voltage clamp techniques.

At concentrations≥10 μM rosiglitazone decreased the amplitude of phase-1 repolarization, reduced the maximum velocity of depolarization and caused depression of the plateau potential in canine ventricular myocytes. In histidine-decarboxylase knockout mice as well as in their wild types rosiglitazone (1-30 μM) shortened APD_{90} and increased the action potential amplitude in a concentration-dependent manner. In rat ventricular papillary muscle cells rosiglitazone (1-30 μM) caused a significant reduction of action potential amplitude and maximum velocity of depolarization which was accompanied by lengthening of APD_{90}.

Rosiglitazone suppressed several ion currents in a concentration-dependent manner under conventional voltage clamp conditions in canine ventricular myocytes. The EC_{50} value

for this inhibition was 25.2±2.7 μM for the transient outward K^+ current (I_{to}), 72.3±9.3 μM for the rapid delayed rectifier K^+ current (I_{Kr}), and 82.5±9.4 μM for the L-type Ca^{2+} current (I_{Ca}) with Hill coefficients close to unity. The inward rectifier K^+ current (I_{K1}) was not affected by rosiglitazone up to concentrations of 100 μM. Suppression of I_{to}, I_{Kr}, and I_{Ca} has been confirmed under action potential voltage clamp conditions as well.

Alterations in the densities and kinetic properties of ion currents may carry serious proarrhythmic risk in case of overdose with rosiglitazone, especially in patients having multiple cardiovascular risk factors, like elderly diabetic patients.

Keywords: ropinirole, rosiglitazone, canine ventricular myocytes, action potential, ion currents

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